Chapter 8

Determination of chemical structures, biological activity and cytotoxicity of isolated compounds

8.1 Introduction

The elucidation of chemical structures involves using a combination of different techniques including nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), ultraviolet (UV) and infrared (IR) spectrometry. Two key points for solving complex problems are that no single spectrum will entirely solve a problem and that all information must be used simultaneously. It is preferable to get key structural facts from each spectrum and then assemble the pieces.

The best starting point is to obtain a molecular formula from the high resolution mass spectrum, or from the molecular ion mass and the number of signals seen in the proton and carbon NMR spectra. Second, determine the number of double bonds and/or rings present by calculating the degree of unsaturation. Third, determine functional groups and other molecular fragments present from the ^{1}H and ^{13}C NMR spectra. Fourth, assemble the pieces in all reasonable combinations. Fifth, eliminate wrong structures and verify the correct structure by reanalyzing the NMR spectra against the proposed structures.

8.2 Structure elucidation

For structural elucidation purposes, the compounds isolated were subjected to instrumental analysis. The NMR (^{1}H and ^{13}C) was determined at the HKI using DRX-500, DNMR, DRX-500 (Bruker, Germany), and TMS as internal standard. Isolated compounds were dried in a freeze-drier, weighed and dissolved in a suitable solvent e.g. deuterated solvent for NMR. Methanol, chloroform and dimethyl sulfoxide were used depending on the solubility of the compounds. The solutions were pipetted into NMR tubes and analyzed for ^{1}H, ^{13}C, distortionless polarization transfer (DEPT), correlated spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC). Mass spectrometry (MS) analysis was performed on the samples using a quadruple Mass Spectrometer.

8.3 Biological activity and cytotoxicity

The isolated compounds were tested for antiviral, antibacterial and antifungal activities as described in sections 3.9.2, 3.10.1 and 3.12 respectively. Cytotoxicity was also determined using the cell line MTT assay (section 3.9.1.4).

8.4 Results and Discussion

8.4.1 Identification of isolated compounds

Compound 1

Compound 1 was isolated as a white amorphous powder. The molecular formula was $C_{27}H_{46}O$, based on ESI-MS m/z: 387.5 $[M+H]^+$ together with ^1H and ^{13}C NMR data, corresponding to 5° unsaturation in the molecule. The ^{13}C together with DEPT spectrum exhibited 27 carbon signals: δ 140.8 (s), 121.7 (d), 71.8 (d), 56.8 (d), 56.1 (d), 50.2 (d), 45.9 (d), 42.3 (t), 39.8 (t), 37.2 (t), 36.5 (s), 36.1 (d), 33.9 (t), 31.9 (d), 31.7 (t), 29.2 (d), 28.2 (t), 26.2 (t), 24.3 (t), 23.1 (t), 21.1 (t), 19.8 (q), 19.4 (q), 19.0 (q), 18.7 (q), 11.9 (q), 11.8 (q). The ^1H NMR spectrum exhibited the signal of olefine protons δ 5.34, m, ^1H one oxide methine group δ 3.50, m, ^1H, six methyl groups δ 1.02, 0.95, 0.92, 0.90, 0.85, 0.66. Compound 1 showed characteristic steroid signals in δ 0.66 and δ 3.50. After comparing spectral data with literature, the structure was deduced as cholest-5-en-3-ol (Fig 8.1) (Aldrich Library, 1992).

Cholest-5-en-3-ol is a characteristic sterol of higher animals. It occurs either free or as esters, of fish liver oils, egg yolk, bile, bran, and gallstones. It is a constituent of the scent material of cotton-top tamarin monkeys (*Saguinus oedipus*), and of *Macoma balthica*. It is also used as a pharmaceutical aid (emulsifying agent). Exposure to very high doses has teratogenic effects (Dictionary of Natural Products, 2006). The compound is also found in virtually all plant oils, for example rapeseed oil (*Brassica napa*), soybean oil (*Glycine max*) and wheatgerm oil (*Triticum* spp.).

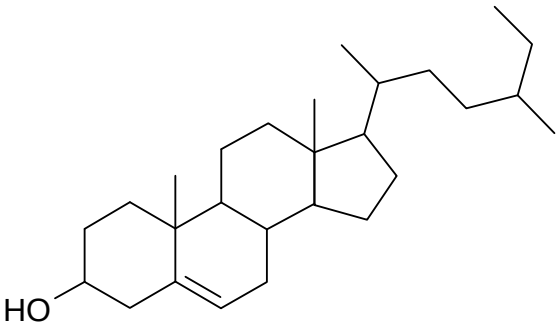

Fig 8.1. Structure of cholest-5-en-3-ol

Compound 2

Compound 2 was obtained as a colourless oil, with a molecular formula of $C_{20}H_{20}O$. From ESI-MS m/z: 319.24 $[M+ Na]^+$, 615.5 $[2M+ Na]^+$ together with ^1H and ^{13}C NMR spectral data, corresponding to 1° of unsaturation in the molecule. The ^{13}C NMR together with DEPT spectrum exhibited 20 carbon signals: δ 140.1 (s), 123.1 (d), 59.3 (t), 39.8 (t), 39.3 (t), 37.4 (t), 37.2 (t), 37.0 (t), 36.6 (t), 32.9 (d), 32.6 (d), 27.9 (q), 25.1 (t), 24.7 (t), 24.2 (t), 22.6 (q), 22.5 (q), 19.7 (q), 19.6 (q), 16.1 (q). The ^1H NMR spectra exhibited the signal of an olefine with protons δ 5.40, td, 1H, and oxide methylyne group δ 4.10, d, J=6.9, 2H, methylene group δ 1.95, d, J= 7.7, 2H, 5 methyl group δ 1,65, 0.86, 0.84, 0.83, 0.80. According to the data above and the degree of unsaturation in the molecule, it should be a long chain compound containing a double bond. After comparing the spectra with literature, the structure was deduced as 2-phyten-1-ol (Fig 8.2) (3, 7, 11, 15- tetramethyl-2-hexadecen-1-ol) (Goodman *et al.,* 1973; Skilleter and Kekwick, 1970).

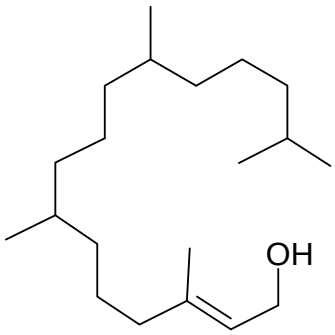

Fig 8.2. Structure of 2-phyten-1-ol

The compound is a constituent of nettles, *Leucas volkensii,* alga, *Perilla* spp, *Solidago virga-aurea, Tetragonia tetragonoides* (New Zealand spinach), *Garcilaria andersoniana, Megaceros flagellaris* and

other plants. It is an anticancer agent (colon and gastric cancer) and used in the preparation of vitamin E and K. The lethal dose 50 (LD_{50}) (rat, oral) is > 5000 mg/kg (Dictionary of Natural Products, 2006).

Fig 8.3 shows the TLC analysis of 2-phyten-1-ol. The R_f of 2-phyten-1-ol is 0.43 in hexane:chloroform (4:1).

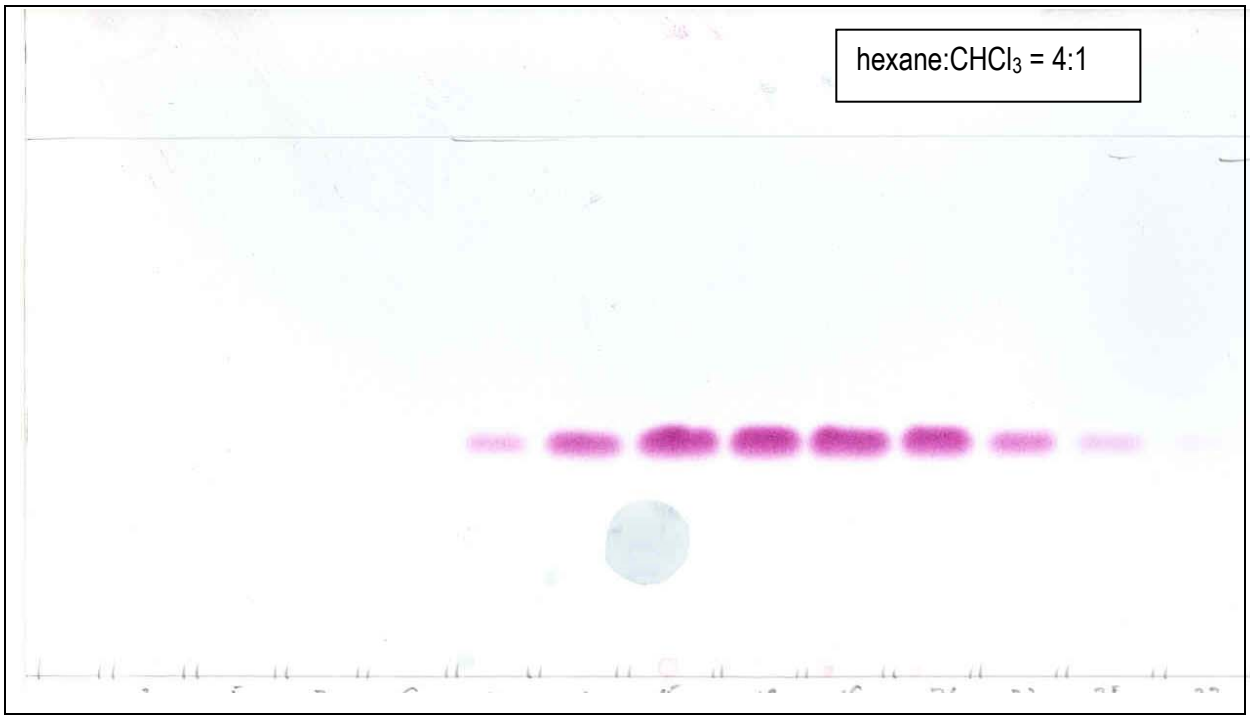

Fig 8.3. TLC of 2-phyten-1-ol

Compound 3

Compound 3 was obtained as a yellow amorphous powder, with a molecular formula of $C_{21}H_{20}O_{12}$, from ESI-MS m/z 464.9 $[M+H]^+$, 950.8 $[2M+Na]^+$ together with ^1H and ^{13}C spectra data, corresponding to 7° of unsaturation in the molecule. The ^{13}C NMR and DEPT spectrum exhibited 21 carbon signals: δ 179.4 (s), 165.9 (s), 162.9 (s), 159.0 (s), 158.4 (s), 149.8 (s), 135.6 (s), 123.2 (d), 122.9 (s), 117.6 (d), 115.9 (d), 105.6 (s), 104.4 (d), 99.8 (d), 94.7 (d), 78.3 (d), 78.1 (d), 75.7 (d), 71.2 (d), 62.5 (t). The ^1H NMR spectra aromatic proton signals δ 7.55, 6.88, 6.36, 6.17, pyranglucose signals in δ 5.23, 3.31-3.75. Since compound 3 has the characteristic signal δ 179.4, 162.9 as position 2 and 4 of isoflavonoids, the structure was assigned as an isoflavonoid. From 2D-NMR studies, the structure was determined as 3-glucopyranosyloxy-3', 4, '5, 7- tetrahydroxyflavone (quercetin-3-glucopyranoside, Fig 8.4).

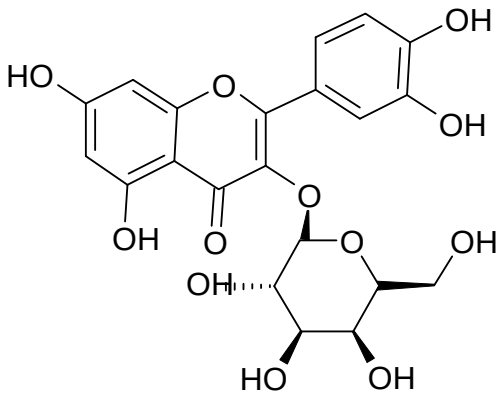

Fig 8.4. Structure of quercetin-3-glucopyranoside

Quercetin-3-glucopyranoside occurs widely in plants. It is used as a diuretic, antioxidant, and has antifungal activity. This compound has been isolated from *Gaultheria miqueliana* and many other plant species. Quercetin-3-glucopyranoside is present in red wine (Dictionary of Natural Products, 2006).

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). The addition of a 3-hydroxyl group yields a flavonol (Fessenden and Fessenden, 1982). Flavonoids are also hydroxylated phenolic substances but occur as a C_6 - C_3 unit linked to an aromatic ring. They are known to be synthesized by plants in response to microbial infection (Dixon *et al.*, 1983); it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms (Cowan, 1999). Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described for quinones. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al*., 1994). Catechol and pyrogallol, both hydroxylated phenols, have been shown to be toxic to microorganisms (Cowan, 1999). Catechol has two –OH groups, and pyrogallol has three. The site(s) and number of –OH groups on the phenol group are thought to be related to their relative toxicity to microorganisms with evidence that increased hydroxylation results in increased toxicity (Geissman, 1963). In addition, some authors have found out that more highly oxidized phenols are more inhibitory (Scalbert, 1991; Urs and Dunleavy, 1975).

Flavonoid compounds exhibit inhibitory effects against multiple viruses. Numerous studies have documented the effectiveness of flavonoids such as swertifranchoside (Pensuparp *et al*., 1994), glycyrrhizin from licorice (Watanbe *et al.*, 1996), and chrysin (Critchfield *et al*., 1996) against HIV. More than one study has found that flavone derivatives are inhibitory to respiratory syncytial virus (RSV) (Barnard *et al*., 1993; Kaul *et al*., 1985). Kaul *et al.* (1985) provided a summary of the activities and

modes of action of quercetin, naringin, herperetin, and catechin in *in vitro* cell culture monolayers. While naringin was not inhibitory to herpes simplex virus type 1 (HSV-1), polio virus type 1, parainfluenza virus type 3, or RSV, the other 3 flavonoids were effective in various ways (Cowan, 1999). An isoflavone found in a West African legume, alpinumisoflavone, prevents schistosomal infection when applied topically (Perrett *et al*., 1995).

Flavonoids lacking hydroxyl groups on their β-rings are more active against microorganisms than those with the –OH groups (Chabot *et al*., 1992). This finding supports the idea that the microbial target is the membrane. Lipophilic compounds would be more disruptive of this structure. However, several authors have also found the opposite effect, i.e. the more hydroxylation, the greater the antimicrobial activity (Sato *et al*., 1996).

Compound 4

Compound 4 was obtained as a brown amorphous powder, with molecular formula $C_9H_8O_3$. From ESI-MS m/z: 162.9 $[M-H]^+$ together with ^1H and ^{13}C NMR spectra data. The ^{13}C NMR and DEPT spectrum exhibited 9 carbon signals: δ 171.2 (s), 161.0 (s), 146.4 (s), 131.0 (d, CHx2), 127.3 (s), 116.9 (d, CHx2), 115.9 (d). The ^1H NMR spectra showed aromatic protons δ 7.60 (d, J= 15.0, 1H), 7.44 (d, J= 9.0, 2H) 6.81 (d, J= 9.0, 2H), 6.29 (d, J= 15.0, 1H). Proton NMR data showed a Z- form double bond and a benzo- group. Carbon-13 NMR showed the structure also contains a carboxyl acid group. From the analysis of molecular formula, NMR data and literature, the structure was deduced as 3-(4-hydroxyphenyl)-2- propenoic acid, or p-coumaric acid (Fig 8.5) (Aldrich Library, 1992).

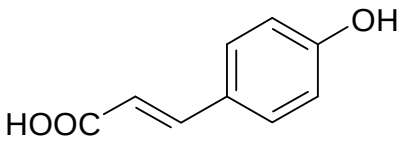

Fig 8.5. Structure of p-coumaric acid

p-Coumaric acid is widespread in plants, e.g. peel of black cherry (*Prunus serotina)*, lentil seeds, red clover (*Trifolium pretense*) and *Daviesia latifolia.* It is also found in *Larix sibirica* bark and *Miscanthus floridulu.* It shows cytostatic activity, and is an immunoactive agent, and inhibitor of stilbene oxidase (Dictionary of Natural Products, 2006).

Coumarins are phenolic substances made of fused benzene and α-pyrone rings (O' Kennedy *et al.*, 1997). Coumarins are responsible for the characteristic odour of hay. As of 1996, at least 1300 had been identified (Hoult and Paya, 1996). Their fame has come mainly from their antithrombic (Thastrup *et al*., 1985), anti-inflammatory (Piller, 1975), and vasodilatory (Namba *et al*., 1988) activities. Warfarin is a particularly well-known coumarin which is used both as an oral anticoagulant and as a rodenticide (Keating and O'Kennedy, 1997). It may also have antiviral effects (Berkada, 1978). Coumarins are known to be highly toxic to rodents (US Department of Health and Human Services, 1992) and therefore are treated with caution by the medical community. It appears that toxic coumarin derivatives may be safely excreted in the urine in humans (Weinmann, 1997).

Several other coumarins have antimicrobial properties. Thornes, while working at the Boston Hospital in 1954, sought an agent to treat vaginal candidiasis in pregnant patients and coumarin was found *in vitro* to inhibit *Candida albicans* (Thornes, 1997). During subsequent *in vivo* tests on rabbits, the coumarin-spiked water supply was inadvertently given to all the animals in the research facility and was discovered to be a potent contraceptive agent when breeding programmes started to fail (Thornes, 1997). Its estrogenic effects were later described (Soine, 1964). As a group, coumarins have been found to stimulate macrophages (Casley-Smith, 1997), which could have an indirect negative effect on infections. More specifically, coumarin has been used to prevent recurrences of cold sores caused by HSV-1 in humans (Berkada, 1978) but was found ineffective against leprosy (Thornes, 1997). Hydrocinnamic acids, related to coumarins, seem to be inhibitory to Gram-positive bacteria (Fernandez *et al*., 1996). Also, phytoalexins, which are hydroxylated derivatives of coumarins, are produced in carrots in response to fungal infection and can be presumed to have antifungal activity (Hoult and Paya, 1996).

General antimicrobial activity has been documented in woodruff (*Galium odorantum)* extracts (Thomson, 1978). All in all, data about specific antibiotic properties of coumarins are scarce, although many reports give reason to believe that some utility may reside in these phytochemicals (Bose, 1958; Hamburger and Hostettmann, 1991; Scheel, 1972).

Compound 5

Compound 5 was obtained as a pale yellow amorphous powder, with molecular formula $C_{17}H_{12}O_8$, from ESI-MS m/z: 345.1 $[M-H]^+$. Three methoxyl groups δ 4.38 (s, 3H), 4.21 (s, 3H), 4.01 (s, 3H), and two aromatic protons δ 7.66 (s, 1H), 7.74 (s, 1H) were found in proton NMR spectra. Comparing the

spectrum with literature, the structure was determined to be 2, 3, 8-tri-O-methylellagic acid (Fig 8.6) (Skilleter and Kekwick, 1970).

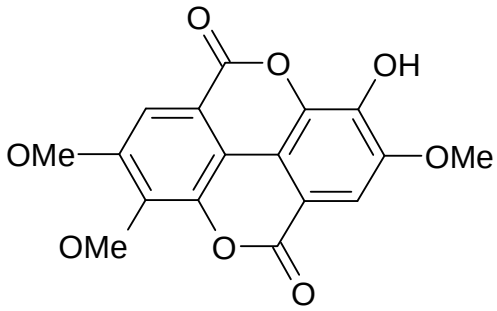

Fig 8.6. Structure of 2, 3, 8-tri-O-methylellagic acid

This compound is a constituent of the haemolymph of *Nasutitermes exitiosus,* and *Eugenia maire* (Dictionary of Natural Products, 2006).

Tannin is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency (Cowan, 1999). Their molecular weights range from 500 to 3000 (Haslam, 1996), and they are found in almost every plant part: bark, wood, leaves, fruits, and roots (Scalbert, 1991). They are divided into two groups, hydrolysable and condensed tannins. Hydrolysable tannins are based on gallic acid, usually as multiple esters with D-glucose; while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers. Tannins may be formed by polymerization of quinone units (Geissman, 1963). This group of compounds has received a great deal of attention in recent years, since it was suggested that the consumption of tannin-containing beverages, especially green teas and red wines, can cure or prevent a variety of ills (Serafini *et al*., 1994).

Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumour activity, and a wide range of anti-infective actions, have been assigned to tannins (Haslam, 1996). One of their molecular actions is to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Haslam, 1996; Stern *et al*., 1996). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins, etc. Tannins also complex with polysaccharides (Ya *et al*., 1988). The antimicrobial significance of this particular activity has not been explored. There is also evidence for direct inactivation of microorganisms: low tannin concentrations

modify the morphology of germ tubes of *Crinipellis perniciosa* (Brownlee *et al*., 1990). Tannins in plants inhibit insect growth (Schultz, 1988) and disrupt digestive events in ruminal animals (Butler, 1988).

Scalbert (1991) reviewed the antimicrobial properties of the tannins, listing 33 documenting the inhibitory activities of tannins. According to these studies, tannins can be toxic to filamentous fungi, yeasts, and bacteria. Condensed tannins have been determined to bind cell walls of ruminal bacteria, preventing growth and protease activity (Jones *et al*., 1994). Although this is still speculative, tannins are considered to be at least partially responsible for the antibiotic activity of methanolic extracts of the bark of *Terminalia alata* found in Nepal (Taylor *et al*., 1996). This activity was enhanced by UV light activation (320 to 400 nm at 5 W/m^2 for 2h). At least two studies have shown tannins to be inhibitory to viral reverse transcriptase (Kaul *et al*., 1985; Nonaka *et al*., 1990).

Tannins may also directly affect the metabolism of microorganisms, as suggested by modification of the morphology of the germ tube of *Crinipellis perniciosa* at low tannin concentrations (0.063 g/l) (Akpata and Akinrimisi, 1977). A drastic change in the morphology and growth pattern of bacteria was observed when they were grown in the presence of sub-inhibitory concentrations (0.6 g/l) of tannic acid or carob pod extract (Casley- Smith, Casley-Smith, 1997). *Pseudomonas fluorescens, Escherichia coli* or *Cellvibrio fulcus* formed chains of filaments whereas most cells were single when grown in the absence of tannins; the morphology of other bacterial species, although also subject to tannin inhibition, was not affected (Scalbert, 1991).

Some moulds develop easily on the surface of tannin-rich woods such as quebracho (Mcunier and Vancy, 1903) or European oak (Scalbert, 1991). Moulds such as *Aspergillus niger,* or *Penicillium glaucum* grow on the surface of the liquid of tannery pits (Mcunier and Vancy, 1903).

Compound 6

Compound 6 was obtained as a white powder, and its molecular formula was established as $C_{28}H_{51}O$. It is also known as (3 beta)-stigmast-5-en-3-ol; 22:23-dihydrostigmasterol; 24beta-ethyl-delta-5-cholesten-3beta-ol. Beta-sitosterol (Fig 8.7) is extremely insoluble in aqueous media and poorly soluble in lipid media. It is found in nature in ester and glycoside forms, both of which forms are more soluble than beta-sitosterol itself.

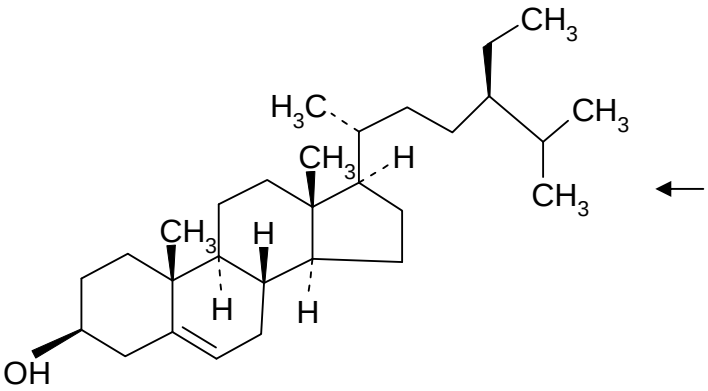

Fig 8.7. Structure of beta-sitosterol

Compound 6 is the most abundant phytosterol in the diet. It is also widely distributed in the plant kingdom and found in such botanicals as *Serenoa repens* (saw palmetto), *Curcurbita pepo (*pumpkin seed) and *Pygeum africanum* (Anonymous, 2005b).Chemically, beta-sitosterol is a very close relative of cholesterol. It differs from cholesterol by the presence of an ethyl group at the 24th carbon position of the side chain. Beta-sitosterol has possible activity in promoting prostate health. It also has cholesterol-lowering activity (Anonymous, 2005b).

Compound 7

Compound 7 was obtained as a yellow amorphous powder with molecular formula $C_{15}H_{14}O_7$. The ^{13}C-NMR and DEPT spectrum exhibited 15 carbons, δ 28.5, 69.2, 83.3, 95.9, 96.7, 101.2, 107.6, 132.0, 134.4, 147.3 (2 carbons), 157.2, 158.0, 158.2 (2 carbons). The proton chemical shifts are: 3.97 (m, H3), 4.55 (d, J= 7.2 Hz, H2), 5.88 (d, J= 2.2 Hz, H8), 5.94 (d, J= 2.2 Hz, H6), 6.4 (s, H2", H6'').

This compound, gallocatechin (Fig 8.8), was isolated by reverse phase chromatography on preparative HPLC eluting with water and acetonitrile. Hussein *et al*. (1999) reported this compound to be active against HIV-1 with an IC_{50} greater than 100 µg/ml. Mahmood *et al.* (1993) reported a 97% HIV-1 inhibition at 1 µg/ml. The ELISA antiviral assay method was used for the test. It is widespread in plants, occurring in broad beans, green tea, redcurrants and gooseberries. Gallocatechin possesses antiscorbutic, and antioxidant properties (Dictionary of Natural Products, 2006).

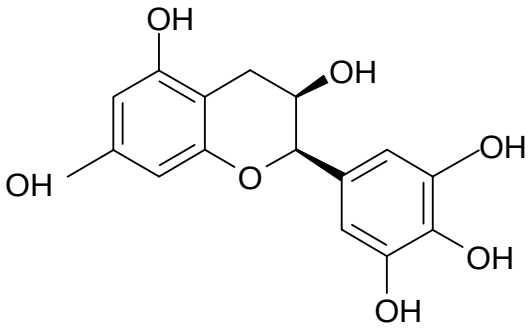

Fig 8.8. Structure of gallocatechin

Compound 8

Compound 8 was obtained as yellow needles and its molecular formula was determined to be $C_{15}H_{10}O_5$ and molecular weight 270. The ^{13}C- NMR and DEPT spectrum led to the conclusion that this compound had 15 carbons with the following chemical shifts: C-2 (163.8), C-3 (102.8), C-4 (181.8), C-5 (161.9), C-6 (98.8), C-7 (164.1), C-8 (94.0), C-9 (157.3), C-10 (103.7), C-1'(121.3), C-2' (128.4), C-3' (116.0), C-4' (161.5), C-5' (116.0), C-6' (128.4). ^1H- NMR 6.2 (d, H 6), 6.5 (d, H8), 6.8 (s, H3), 6.9 (d, H3', H6'), 7.9 (d, H2', H6'), 10.4 (s, O−H, 4'), 10.9 (s, O−H, 7), 13 (O−H, 5). This compound, apigenin (Fig 8.9) is found free or as glycosides in the stems, roots, leaves, seeds or fruits of a very wide range of plant species and has been found also in some fossil leaf tissues. It is used as an ethanol solution for photometric determination of aluminum, rare earth elements, beryllium, zirconium and cadmium. It shows antineoplastic activity *in vitro*, antispasmolytic agent and anti-inflammatory activity. It is also an enzyme inhibitor and superoxide scavenger (Dictionary of Natural Products, 2006).

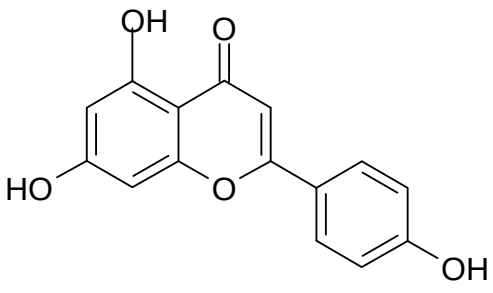

Fig 8.9. Structure of apigenin

Compound 9

Compound 9 (cosmosiin, Fig 8.10) was isolated as a yellow powder with formula $C_{21}H_{20}O_{10}$ and molecular weight 432. The ^{13}C, DEPT and ^{1}H NMR led to the conclusion that this compound had 21 carbons with the following chemical shifts: C-4 (181.7), C-2 (164.3), C-7 (162.8), C-5 (161.5), C-4' (161.0), C-9 (156.8), C-2', 6'(128.3), C-1' (120.8), C-3', 5' (116.0), C-10 (105.4), C-3 (102.9), C-!" (100.2), C-6 (99.5), C-8 (94.9), C-5" (77.1), C-3" (76.5), C-2'' (73.1), C-4'' (69.8), C-6'' (60.8). The ^{1}H NMR spectra had the following shifts: 6.2 (1H, d), 6.5 (1H, d), 6.8 (1H, s), 6.9 (2H, d), 7.9 (2H, d), 10.4 (1H, s) O-H (4'), 10.9 (1H, s) O-H (7), 13 O-H (5).

The compound is a constituent of flowers of *Cosmos bipinnatus, Zinnia elegans* and other plant species. It is an anti-HIV agent (Dictionary of Natural Products, 2006). Apigenin-7-O-glucoside has been shown to exhibit a moderate antiamoebic activity with IC50 value of 22.3 ± 3.2 µg/ml (Cimanga *et al.,* 2006).

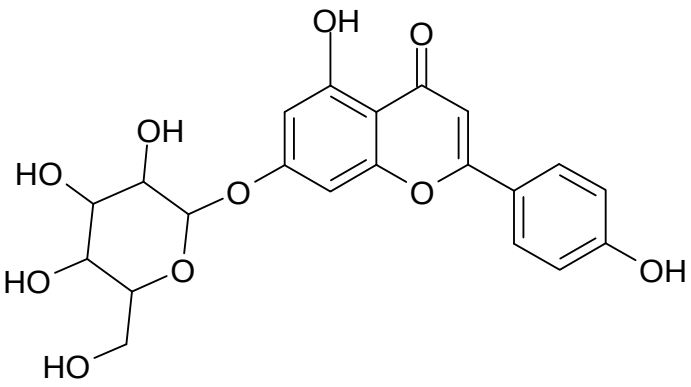

Fig 8.10. Structure of cosmosiin

Representative NMR spectra of the isolated compounds are attached in Appendix 1.

8.4.2 Biological activity of isolated compounds

8.4.2.1 Antiviral activity

The antiviral activity of extracts of *C. paniculatum* was determined using feline herpesvirus type 1 (an enveloped virus). The best result for the extracts was a 3.6 $\log_{10}$ reduction for the stem bark. It is very

interesting that water and acetone extracts had a similar antiviral activity. The acetone and water extract of the leaves all had a 3 log_{10} reduction of virus titre. The water extract of the root bark had a 3.4 log_{10} reduction value.

All the isolated compounds were tested for antiviral activity against Coxsackie virus B3 (CVB3), influenza virus A, and Herpes simplex virus type 1 (HSV1) and there was no activity.
It is surprising that no antiviral activity was found for compound 7 (gallocatechin) because Hussein *et al.* (1999) found this compound to be active against HIV-1 with an IC_{50} greater than 100 µg/ml.

It was a disappointment that there was no good activity with any of the isolated compounds despite the promising results obtained for the water and acetone extracts of the leaves against the sensitive feline herpesvirus. The isolated compounds did not show as significant a reduction in virus titre as that obtained for the water and acetone extracts of the leaves. This could be due to synergistic effects of various constituents of the plant material. The acetone extract of the leaves has previously been shown to inhibit HIV-2 replication with an EC_{50} of 3.0 µg/ml and selectivity index of 32 (Asres *et al.,* 2001). The acetone extract of the leaves inhibited feline herpesvirus with an EC_{50} of 2.8 µg/ml.

8.4.2.2 Antibacterial and antifungal activities

MIC values for some of the isolated compounds are presented in Table 8.1.

Table 8.1. MIC values (µg/ml) of some isolated compounds

Compound	*S. aureus*	*E. coli*	*E. faecalis*	*P. aeruginosa*
Methylellagic acid	>250	62.5	125	125
Isoquercitin	62.5	31.25	62.5	62.5
Gallocatechin	62.5	62.5	31.25	125
p-coumaric acid	>250	>250	>250	>250
Gentamicin	0.8	0.4	0.4	1.6

The MIC values of the compounds tested against the four species of bacteria ranged between 31.25 µg/ml to >250 µg/ml.

The agar diffusion method (described in section 3.12) was used in a separate group of experiments for the determination of antibacterial and antifungal activities against the microorganisms listed in Table 8.2.

A standard quantity of 50 µg of isolated compounds was placed in each agar well and the inhibition zones in the bacterial or fungal lawn measured after overnight incubation.

Table 8.2. Organisms tested for activity against isolated compounds

Organisms	Code
Bacteria	
Bacillus subtilis ATCC 6633 (IMET 10880)	Bs
Staphylococcus aureus (IMET 10760) SG 511	Sa
Escherichia coli SG 458	Ec
Pseudomonas aeruginosa K799/61	Pa
Mycobacterium vaccae IMET 10670	Mv
Fungi	
Sporobolomyces salmonicolor SBUG 549	Ss
Candida albicans BMSY 212	Ca
Penicillium notatum JP 36	Pn

Table 8.3. Results of antimicrobial activity on isolated compounds (diameter of inhibition zone in mm)

Compounds	Bs	Sa	Ec	Pa	Mv	Ss	Ca	Pn
Cholest-5-en-3-ol	10	0	15p	0	18p	23	0	13p
2-phyten-1-ol	10	0	14p	0	33p	22	0	13p
Gallocatechin	0	0	16p	0	16p	24	0	13p
Apigenin	0	0	14p	0	18p	27	0	13p

Diameter of inhibition zone

0-15 mm = No activity

16-20 mm = Moderate activity

21-25 mm = Good activity

>25 mm = Strong activity

p = Few colonies present in the inhibition zone (moderate activity)

Cholest-5-en-3-ol had good activity against *Sporobolomyces salmonicolor* (23 mm) and moderate activity against *Mycobacterium* vaccae (18p). The compound 2-phyten-1-ol had a strong activity against *Mycobacterium vaccae* (33p) and good activity against *Sporobolomyces salmonicolor* (22 mm). p-Coumaric acid has been found to be active against *Escherichia coli* and *Staphylococcus aureus* at pH

5.0, 6.0 and 7.0 and *Bacillus cereus* at pH 6.0, 6.5 and 7.0. p-Coumaric acid was generally the most effective inhibitor tested causing more than 99.9% inhibition of *E. coli* at 1000 µg/ml (pH 5.0, 48 hr), *S. aureus* at 500 µg/ml (pH 5.0, 48 hr), *B. cereus* at 500 µg/ml (pH 5.0, 48 hr), and B. cereus at 500 µg/ml (pH 7.0, 9 hr) (Herald and Davidson, 1983). Inhibition increased as pH decreased with *E. coli* and *S. aureus* but not in the case of *B. cereus. Bacillus cereus* appeared to be the most susceptible strain with 1000 µg/ml of the compounds tested causing > 99% inhibition at all three pH's (Herald and Davidson, 1983). It has been shown that extracts of some *Combretum* species (*C. glutinosum, C. hispidium, C. molle* and *C. nigricans*) have antifungal effects against dermatophytes as well as *Candida albicans* (Baba-Moussa *et al*., 1999). Baba-Moussa *et al.* (1999) proposed that tannins and saponins might be responsible for this activity and this might explain the good activity of cholest-5-en-3-ol and 2-phyten-1-ol.

Compound 7 (gallocatechin) had a strong activity against *Sporobolomyces salmonicolor* (24 mm) and a moderate activity against *Mycobacterium vaccae* (16p) and *E. coli* (16p).
Compound 8 (apigenin) had a strong activity against *Sporobolomyces salmonicolor* (27 mm) and moderate activity against *Mycobacterium vaccae* (18p). Yoichi *et al.* (2000) suggested that apigenin and the related flavonoids are potentially useful for the development of therapeutic treatments of MRSA infections. They also found that apigenin tested against the following organisms: *S. aureus, Bacillus cereus, E. faecalis, Acinetobacter calcoacetics, Citrobacter freundii, Enterobacter cloacae, E. coli, Proteus mirabilis, P. vulgaris* and *Salmonella typhimurium* resulted in MICs greater than 250 µg/ml. For both MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA) strains, the MIC for apigenin ranged between 3.9 to 15.6 µg/ml (Yoichi *et al.,* 2000).

Compound 9 (apigenin-7-O-glucoside) had a strong activity against *Sporobolomyces salmonicolor* (27 mm) and a moderate activity against *Mycobacterium vaccae* (18 p).

8.4.2.3 Cytotoxic activity of isolated compounds

The cytotoxic effects of the compounds were tested *in vitro* against HeLa, MDCK (Madin-Darby Canine Kidney) and GMK (Green Monkey Kidney) cell lines. The results are presented in Table 8.4.

Table 8.4. Cytotoxicity of isolated compounds

Compounds	HeLa	MDCK	GMK
Cholest-5-en-3-ol	25	>50	>50
2-phyten-1-ol	12.5	>50	>50
Isoquercitin	12.5	>50	>50
P- coumaric acid	25	>50	>50
Methylellagic acid	12.5	>50	>50
Beta-sitosterol	25	>50	>50
Gallocatechin	12.5	>50	>50
Apigenin	25	>50	12.5

Toxicity Categories:

CC_{10} > 100 µg/ml = Slight cytotoxicity

CC_{10} 10- 100 µg/ml = Moderate cytotoxicity

CC_{10} 1-10 µg/ml = Strong cytotoxicity

CC_{10} < 1 µg/ml = Extreme cytotoxicity

The compounds were more toxic to HeLa cell lines than MDCK and GMK cells. The GMK cell lines were the least affected with most CC_{10} values greater than 50 µg/ml. With the isolated compounds, the cytotoxicity was in the moderate toxicity range (CC_{10} = 12 µg/ml to > 50 µg/ml) compared to some fractions. The compound 2-phyten-1-ol is known to have anticancer activity (colon and gastric cancer) (Dictionary of Natural Products, 2006). It is also used in the preparation of Vitamins E and K (Dictionary of Natural Products, 2006). This is probably the reason this compound has strong antiproliferative activity.

There are very few reports on the antiproliferative and cytotoxic effects of the constituents of the Combretaceae. This study indicates that some members of the Combretaceae have antiproliferative and cytotoxic components, justifying further research.

8.5 Conclusion

There was very little antiviral activity observed for the two isolated compounds tested against feline herpesvirus. The extracts were more active compared to these two compounds, implying synergism. The hypothesis that antibacterial compounds isolated from *C. paniculatum* will have antiviral activity

could not be substantiated because not one of the isolated antibacterial compounds had good antiviral activity. It is much easier to use antibacterial activity in bioassay-guided fractionation. Since the hypothesis has been proven wrong, in future it will better to carry out antiviral activity at each stage of the isolation, though it is painstakingly slow. The aqueous extracts may contain antiviral compounds and are worth investigating further.

In the Phytomedicine Programme, we have frequently found that the biological activity of known compounds isolated earlier is not known. Consequently several biological activities of isolated compounds were determined where sufficient material was available.

Nine compounds were isolated and their structures determined by the aid of instrumental analysis. Our hypothesis that one can isolate potential antiviral compounds using antibacterial activity since it is easier to test for antibacterial activity was not substantiated because none of the isolated compounds had good antiviral activity. However, the compounds had some antibacterial and antifungal activity. A broad spectrum of antibacterial activity against Gram-positive and Gram-negative pathogens was shown.

The compounds were tested for antiviral activity against three viruses, namely Coxsackie virus (CVB3), Influenza virus A and herpes simplex virus type 1 (HSV1) but there was no activity. As the compounds isolated from the leaves of *Combretum paniculatum* in this study did not have antiviral activity, this implies that there could be a synergistic effect, or that the compound(s) with antiviral activity were not isolated. It is recommended that further research be carried out with a much larger quantity of plant material so that the antiviral compounds that were potentially not isolated owing to presence in small quantities may be isolated using antiviral assays to guide the fractionation process.

Chapter 9

General Conclusions

9.1 Introduction

The development of new antiviral and antibacterial drugs is challenging, taking into account the poor selective toxicity and fast selection of resistant variants with existing drugs. Virus infection is a common problem worldwide. Herpes simplex virus is found in over 60 million people in the US, most of whom are of child-bearing age. It is therefore necessary to find alternative active compounds. Pure compounds of plant origin have been shown to exhibit antiviral and antibacterial activities.

According to ethnobotanical literature, the genus *Combretum* is used widely for a variety of conditions in African traditional medicine. Members of this genus have the following biological activities: antifungal, anti-inflammatory, antibacterial, diuretic and molluscidal (Hutchings *et al*., 1996).

Asres *et al*. (2001) reported the antiviral activity of the leaf extract of *Combretum paniculatum* against HIV-2. The acetone extract of the leaf showed a high degree of antiviral activity against HIV-2 with an EC_{50} of 3 µg/ml and selectivity index of 32. Following from this, the aim of the present work was to isolate compounds, characterize them and evaluate them for antiviral and other biological activities.

The objectives were to:
Select the best extractant for the plant material in terms of quantity and antimicrobial activity
To determine antiviral, antibacterial and cytotoxic activities of extracts
To isolate and chemically characterize compounds
To determine the antiviral and other biological activities of isolated compounds.

9.2 Selection of the best extractant for the plant material

Acetone was selected as the best extractant based on the number of compounds extracted from the plant after TLC analysis and the number of bioactive compounds on bioautography. The number of compounds in the extracts was ranked in the following order by TLC analysis: acetone (7), DCM (5), carbon tetrachloride (4), ethanol (4), hexane (2) and THF (1), where the number of compounds

visualized on TLC plates are indicated in brackets. Water and methanol extracts did not separate with the TLC solvent systems used.

Antiviral activity of the acetone and water extracts of the leaves against feline herpesvirus produced a promising result with a 3 $\log_{10}$ reduction of virus titre. Bioautography of the acetone extract was used to determine the effectiveness of acetone as a solvent for extraction of antibacterial compounds. Many more compounds had antibacterial activity in the acetone extract after separating the extracts on a column, compared to the crude acetone extract. Eloff (1999) discovered that leaves of 27 southern African members of the Combretaceae had antibacterial activity when extracted with acetone and tested against *E. coli, S. aureus, P. aeruginosa* and *E. faecalis.* Acetone was therefore selected as extractant for isolating antibacterial compounds. Water extracted a good quantity of plant material so a mixture of acetone and water (70:30) was chosen for the bulk extraction process.

The amount of material extracted from the leaves of *C. paniculatum* per gram using different solvents ranged from 15 mg to 246 mg. These values are different to those obtained by Kotze and Eloff (2002) while working on *C. microphyllum* (26 to 174 mg), a plant closely related to *C. paniculatum*. Some authorities consider *C. paniculatum* to be synonymous with *C. microphyllum* (Germishuizen and Meyer, 2003) but others (Palgrave, 2002; Carr, 1988) recognize both species. The quantities of material extracted by water and DCM for the two species were significantly different. For *C. paniculatum,* water extracted the most (246 mg) followed by methanol (194 mg) while for *C. microphyllum*, methanol extracted 174 mg followed by DCM (106 mg). The differences shown in the TLC fingerprints and the amount of material extracted from the two plants supports recognition of the two species.

9.3 Determination of the antimicrobial, cytotoxic and antioxidant activities of extracts

Antiviral activity was determined for the leaf, stem bark and root bark extracts of *C. paniculatum.* The results were determined by noting cytopathic effect (CPE) and also by using a colorimetric MTT assay. The cells were examined for CPE and virus titre was calculated using the Karber formula. The antiviral activity of the stem bark was 3.6 $\log_{10,}$ that is, 3.6 $\log_{10}$ reduction (close to 4 000 fold) of virus titre, while the root bark displayed a 3.4 $\log_{10}$ reduction. The acetone extract of the leaves displayed a 1000 fold reduction. The EC_{50} of the acetone extract of the leaves was 2.8 µg/ml against feline herpesvirus type 1. Asres *et al*. (2001) reported that the acetone extract of *C. paniculatum* leaves collected in East Africa

inhibited HIV-2 replication with an EC_{50} of 3.0 µg/ml. This confirms that the acetone extract of the leaves has antiviral activity.

The MIC values against the selected bacteria obtained for the leaf extract were lower than those obtained for other members of the Combretaceae family (Eloff, 1999). The average MIC values ranged between 0.28 to 0.86 mg/ml. Eloff (1999) found that 27 members of the Combretaceae inhibited bacterial growth with MIC values between 0.1 to 6 mg/ml and an average of 2.01 mg/ml. Gram-positive bacterial strains were slightly more sensitive with an average MIC of 1.8 mg/ml while the Gram-negative strains had a higher average MIC value of 2.22 mg/ml. Bioautography of the extracts showed that there was good bacterial growth inhibition by compounds in acetone and ethyl acetate extracts.

Antioxidant activity was determined qualitatively by spraying developed TLC plates with 0.2% DPPH. Almost all the fractions possessed antioxidant compounds. Acetone and ethyl acetate extracts had more antioxidant compounds than the other extracts. Cytotoxicity was observed only at the highest concentration of extracts tested (0.28 mg/ml).

9.4 Preliminary isolation study

A preliminary fractionation was carried out to simplify the complex crude extracts and verify their antibacterial activity. The acetone extract of the leaves was separated in a silica column and the collected fractions pooled after TLC analysis. The pooled fractions were used to determine antibacterial activity. Nine of the 20 fractions had compounds with antibacterial activity against *S. aureus, E. coli* and *P. aeruginosa*.

The antibacterial activity of the root bark extract was also investigated as this may be a source of bioactive compounds. The antibacterial activity was determined after solvent/solvent partitioning of the acetone extract of the root bark. The chloroform, carbon tetrachloride and butanol fractions were separated into many compounds by TLC. Bioautography of the fractions from solvent/solvent fractionation separated three compounds with antibacterial activity against *S. aureus* in the chloroform fraction. The chloroform fraction was the most active.

The MIC of the root bark solvent/solvent fractions was determined against four bacterial strains and the average MIC values ranged from 0.42 to 1.04 mg/ml. The fractions were tested against 5 fungal strains and the average MIC values ranged from 0.47 to 1.19 mg/ml. According to Masoko *et al.* (2006), a

crude acetone leaf extract of *C. paniculatum* against five fungal strains was more active than the root bark fractions, with MIC values in the range 0.02 to 2.5 mg/ml. Because exactly the same procedures were used for the fractions and the crude acetone extract, the results may indicate synergistic effects in crude extracts.

From bioautography analysis, it appeared that similar bioactive compounds occurred in the root bark and leaves. Due to the small quantity of root bark available, and because using roots of the plant can kill the plant, only leaves were used for large scale extraction and isolation.

9.5 Isolation of antibacterial compounds

The separation of plant constituents was carried out using a combination of chromatographic techniques, namely column chromatography, thin layer chromatography and HPLC. The bulk leaf extraction was performed with acetone and water (70:30) because acetone extracted the most antibacterial compounds and water extracted the most material from the plant. During the isolation process, any compound that crystallized was chemically and biologically characterized.

At the HKI, biological assays are carried out in another facility, slowing the isolation process considerably and the period for research at the Institute was short. Nine compounds were isolated, not strictly by bioassay-guided fractionation, and subjected to instrumental analysis for structure elucidation. The quantities of the isolated compounds ranged from 5 to 10 mg. For future work, a larger initial amount of plant material is recommended to enable the isolation of more compounds present in small quantities.

9.6 Determination of chemical structures, biological assays and cytotoxicity of compounds

Experiments using 1D NMR, 2D NMR, ^{1}H, ^{13}C, HMBC, HMQC and COSY were used for structure elucidation. Elucidation of chemical structures involved a combination of different techniques including NMR and mass spectroscopy (MS). The structures of nine compounds were determined with the aid of instrumental analysis and literature. The compounds were: cholest-5-en-3-ol, 2-phyten-1-ol, isoquercitrin, p-coumaric acid, 2, 3, 8- tri-O-methylellagic acid, beta-sitosterol, gallocatechin, apigenin and apigenin-7-glucoside.

Biological activities were determined for each isolated compound where sufficient material was available. All the compounds were tested for antiviral activity against CVB3, influenzavirus A and herpes simplex virus type 1 and there was hardly any activity. The hypothesis that antibacterial compounds isolated from *C. paniculatum* may have antiviral activity was not substantiated because not one of the isolated antibacterial compounds had any antiviral activity. It is much easier to use antibacterial activity in bioassay-guided isolation. Since the hypothesis has been proven wrong, in future it will be better to carry out antiviral activity testing at each stage of the isolation. It may also be worthwhile to concentrate on aqueous extracts based on results reported in Table 5.1.

Antibacterial and antifungal activities were determined for the compounds, and 2-phyten-1-ol showed the best activity of all the compounds. It had a strong activity against *Mycobacterium vaccae* and good activity against *Sporobolomyces salmonicolor*. 2-Phyten-1-ol is a skin irritant and an anticancer agent. Apigenin had a strong activity against *S. salmonicolor* and moderate activity against *M. vaccae*. Gallocatechin had a good activity against *S. salmonicolor* and moderate activity against *E. coli* and *M. vaccae*. Cholest-5-en-3-ol had a good activity against *S. salmonicolor* and a moderate activity against *M. vaccae*.

Regarding cytotoxicity, the compounds were generally more toxic to the HeLa cell lines than the other cells, with CC_{10} values ranging from 12.5 to 25 µg/ml. The latter values fall in the moderate toxicity category. The compounds generally were less toxic to the GMK and MDCK cell lines with toxicity values all greater than 50 µg/ml which was of moderate toxicity. Some of the compounds have known toxicity. Cholest- 5-en-3-ol at high doses has teratogenic effects. Compound 2 (2-phyten-1-ol) is a skin irritant and has a low LD_{50} (rat, oral) greater than 5000 mg/kg (Dictionary of Natural Products, 2006). Isoquercitrin is a diuretic, antifungal and antioxidant agent. The LD_{50} for p- coumaric acid has been reported to be 657 mg/kg (muscle, ipr) (Dictionary of Natural Products, 2006).

The results obtained confirm the ethnobotanical use of many *Combretum* species for antibacterial infections. In future work, many compounds that are present in low concentrations could be isolated from *C. paniculatum* by starting with a large quantity of plant material. Synergistic effects on biological activity, particularly antiviral activity, of isolated compounds could be investigated. It is also possible that the antiviral compounds were not isolated and further work should be carried out making use of antiviral assay-guided isolation.