Chapter 8

Structure elucidation

8.1. Introduction

8.1.1. Nuclear Magnetic Resonance (NMR)

NMR is a valuable structure elucidation tool for organic and biological molecules. Besides qualitative information, NMR can provide valuable quantitative information about a sample. A normal liquid state 1D ^{1}H NMR spectrum is commonly recognized as a reliable method for quantification. Other nuclei have also been utilized with one-dimensional experiments, both in liquids and solid state (Martin *et al*., 1980 and Harris 1985).

The major limitation of NMR spectroscopy is the rather low detection sensitivity, rendering the experiments time-consuming compared to other methods used for molecular structure determination or verification such as X-ray crystallography or mass spectroscopy. This is because the sensitivity of the NMR signal depends on the small difference in the populations of the Zeeman energy levels. The separations between the nuclear spin states are small, corresponding to energies in the radiofrequency range. The population difference is given by the Boltzman distribution. For ^{1}H nuclei at room temperature and magnetic field of 10 T the difference in the population is in the order of 1 in 10^{5} which means that most of the nuclei do not contribute to the NMR signal. This is in contrast to optical spectroscopic methods such as, for instance, infrared (IR) spectroscopy where basically a single photon can be detected.

For a high-resolution NMR investigation using a conventional probe operating at ambient temperature the required amount of substance is often milligrams. In many applications the available amount of sample is limited, or the inherent solubility of the substance of interest may be low, or a dilute solution is required because the sample may tend to aggregate at higher concentrations. In such cases, the cryogenic probe technology moves the lower limit of the feasible sample concentration to the microgram and micromolar range. For biological macromolecules, the change in the sample requirement from the millimolar to the micromolar sample concentration range greatly increases the number of compounds that can be studied by NMR.

The strength of NMR spectroscopy is given by its multifarious applications, which range from statistical analysis of mixtures to the determination of three-dimensional structures for molecules of biological interest. The information content of NMR at the atomic level is both comprehensive and diverse. Thus, to improve the sensitivity has always been an important

development goal from the point of methodology and engineering. In an NMR experiment, the signal-to-noise ratio is usually augmented through computer averaging of accumulated transients. In the signal averaging, however, the signal-to-noise ratio is proportional to the square root of the number of transients. Consequently, a 3–4-fold sensitivity increase as provided by cryogenic probes, entails a 9–16-fold reduction in measurement time (Kovacs *et al*., in Press).

8.1.2. Mass spectrometry (MS)

Mass spectrometry is the most sensitive and selective method for molecular analysis and can yield information on the molecular weight as well as the structure of the molecule. Combining chromatography with mass spectrometry provides the advantage of both chromatography as a separation method and mass spectrometry as an identification method. In mass spectrometry, there is a range of methods to ionize compounds and then separate the ions (Gong *et al*., 2001a). Common methods of ionization used in conjunction with gas chromatography are electron impact (EI) and electron capture ionization (ECI). EI is primarily configured to select positive ions, whereas ECI is usually configured for negative ions (ECNI). EI is particularly useful for routine analysis and provides reproducible mass spectra with structural information, which allows library searching. GC–MS was the first successful online combination of chromatography with mass spectrometry, and is widely used in the analysis of essential oil in herbal medicines (Guetens *et al*., 2002).

With the GC–MS, people could produce not only a chromatographic fingerprint of the essential oil of the herbal medicine but also the information related to its most qualitative and relative quantitative composition (Li *et al*., 2001). Used in the analysis of the herbal medicines, there are at least two significant advantages for GC–MS, that is: (1) with the capillary column, GC–MS has in general very good separation ability, which can produce a chemical fingerprint of high quality; (2) with the coupled mass spectroscopy and the corresponding mass spectral database, the qualitative and relatively quantitative composition information of the herb investigated could be provided by GC–MS, which will be extremely useful for the further research for elucidating the relationship between chemical constituents in herbal medicine and its pharmacology in further research (Gong *et al*., 2001b).

To fully elucidate a molecular structure, distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple bond correlation (HMBC), heteronuclear multiple quantum coherence (HMQC) and correlation spectroscopy (COSY) are also done.

8.1.3. Distortionless enhancement by polarization transfer (DEPT)

DEPT is a technique that gives information about the number of protons bonded to each carbon.

8.1.4. Heteronuclear multiple bond correlation (HMBC)

HMBC gives information about weak proton-carbon J-coupling. A weak proton-carbon J-coupling indicates that the proton is two, three, or four bonds away from the carbon. This experiment gives information about which protons are near to (but not directly bonded to) different carbons. HMBC can give an enormous amount of information about molecular structure, since the long-range proton-carbon correlations can include quaternary carbons, in addition to protonated carbons.

8.1.5. Heteronuclear multiple quantum coherence (HMQC)

HMQC gives information about strong proton-carbon J-couplings. A strong proton carbon J-coupling indicates that the proton is directly bonded to the carbon. HMQC is selective for direct C-H coupling

8.1.6. Correlation Spectroscopy (COSY)

Correlation Spectroscopy (COSY) gives information about pairs of protons that are J-coupled. This usually indicates that the protons are on adjacent carbons, e.g. 3-bonds away (though protons further apart may in some cases be J-coupled).

8.2. Materials and Methods

8.2.1. Nuclear Magnetic Resonance (NMR)

After column chromatography, precipitation of some fractions began to take place. These precipitates were collected, cleaned using various solvent systems starting with non-polar solvents e.g. hexane and then introducing ethanol, methanol, ethyl acetate, chloroform and acetone. The samples were passed through a Pasteur pipette plugged with cotton wool to facilitate the removal of impurities. The clean samples were weighed and dissolved in maximum 2 ml deuterated solvents used for NMR (Merck). In these studies, acetone was used as the solvent of choice, although other solvents were also attempted, because of its ability to dissolve a wide range of compounds. The samples were then pipetted into tubes

(Milmad, economy) with the aid of a Pasteur pipette and send to Mr Mathebula of the Chemistry department, University of Limpopo, MEDUNSA campus. ^{1}H NMR was run at either 300 or 400 MHz and ^{13}C at 75 MHz using the solvent signal as the reference. Structures were elucidated by Dr Mdee (Phytomedicine Programme).

8.2.2. Mass spectrometry (MS)

High Resolution Electron Impact Mass Spectroscopy (HREIMS) was performed on samples sent for analysis using a MASPEC II system (II32/A002) at University of Johannesburg (UJ). Mr Vorster also performed DEPT, HMBC and HMQC at UJ. Dr Mdee of University of Pretoria, Phytomedicine Programme performed the analysis.

8.2. Results

From **Chapter 7**, compound D and compound B were combined and labeled **compound I** (81 mg). Compound C was then labeled **compound II**. Due to the small amount isolated of compound A, it was not further studied. NMR analysis of compound I are shown in **figures 8.1** to **8. 7**.

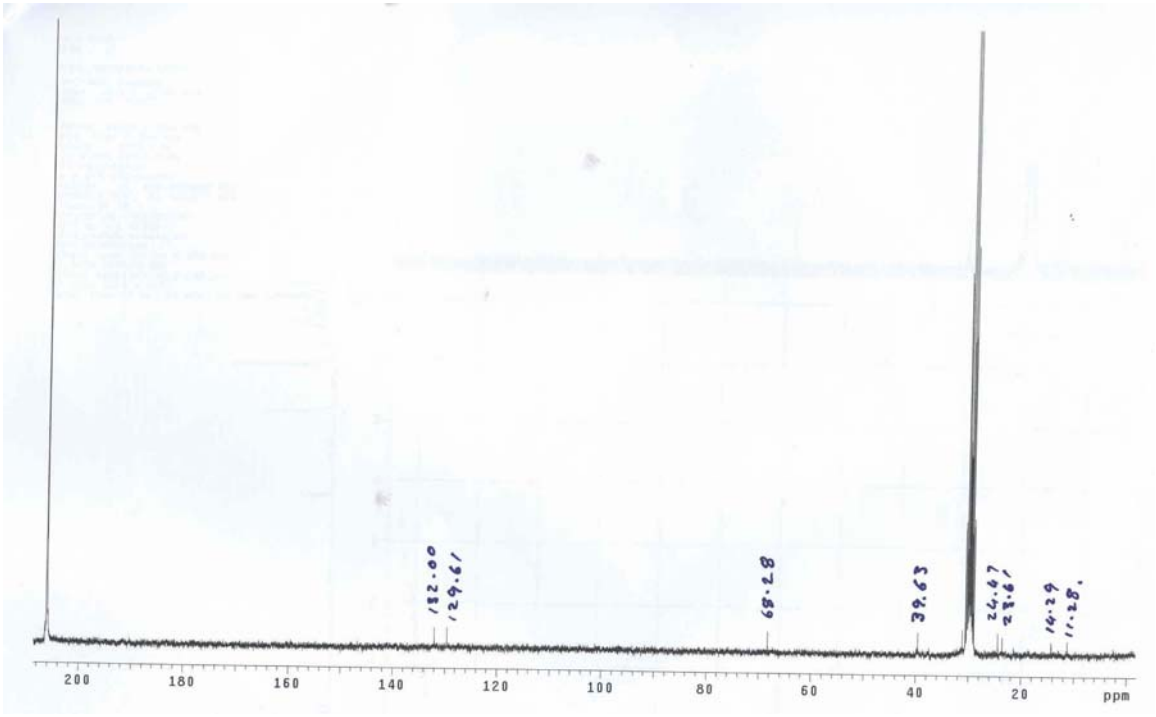


Figure 8.1. ^{13} C NMR spectrum of **Compound I**

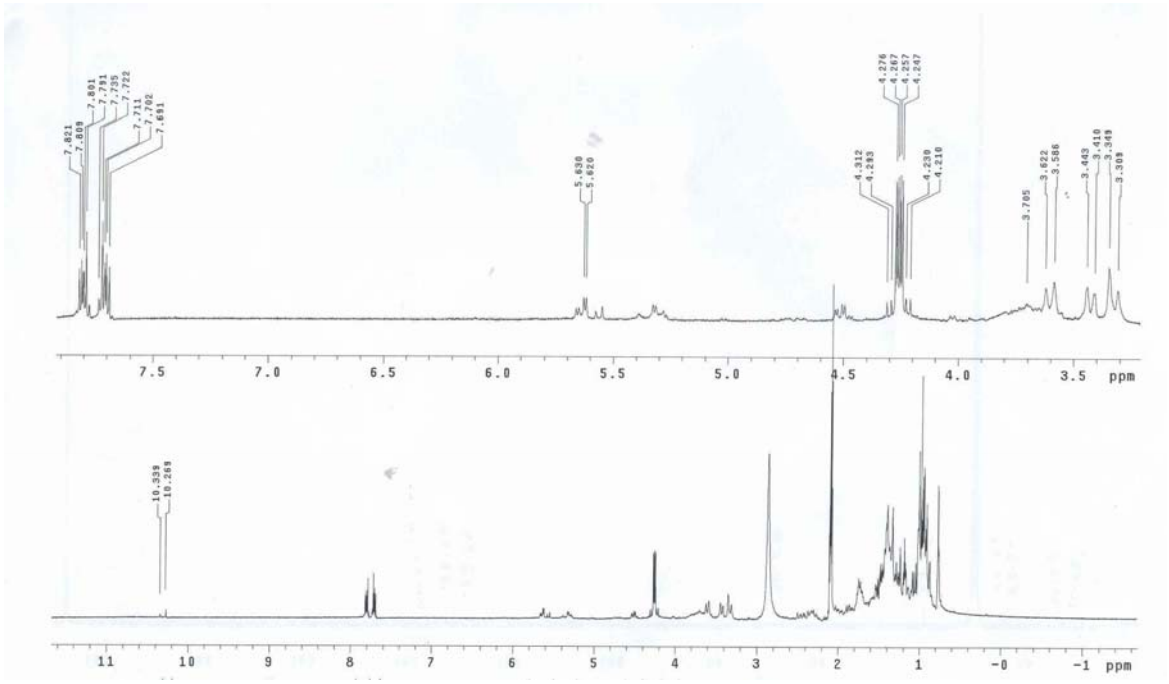


Figure 8.2. ^{1}H NMR spectrum of **Compound I**

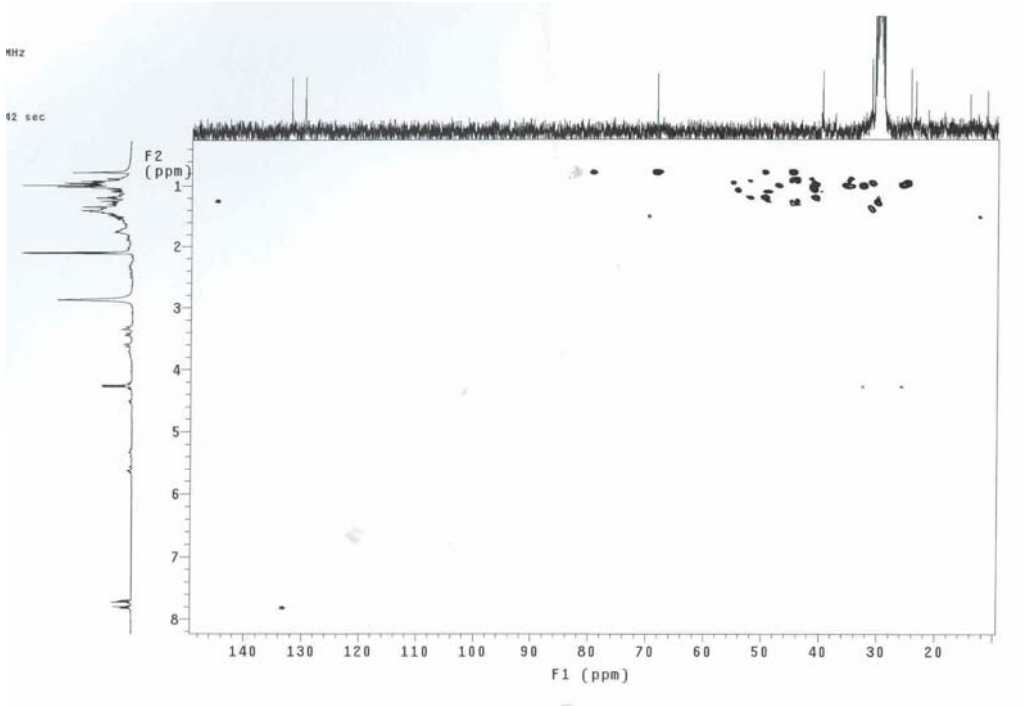


Figure 8.3. HMBC NMR spectrum of **Compound I**

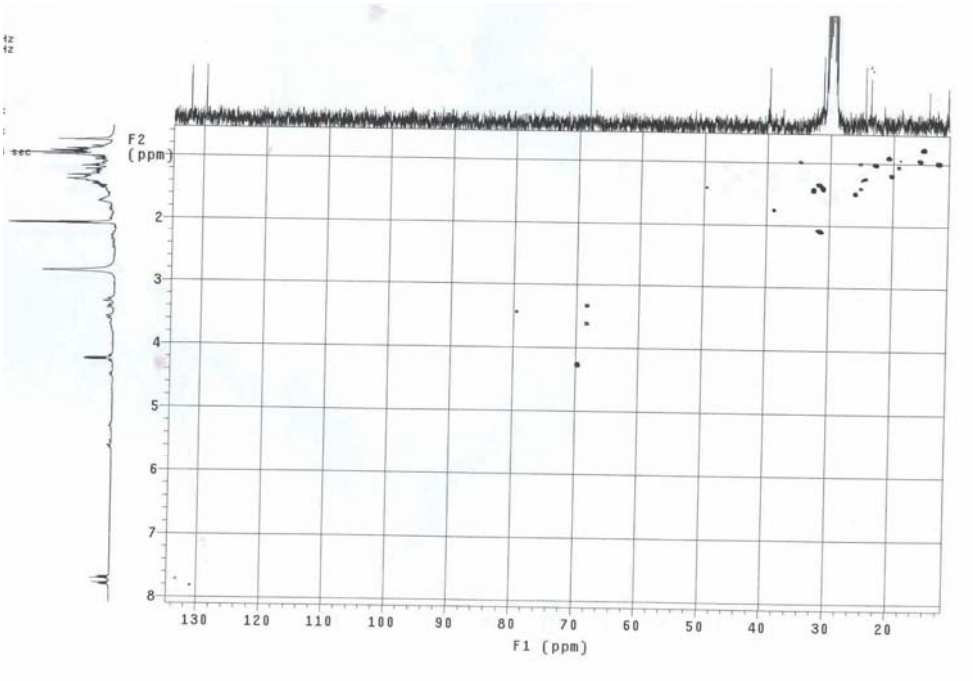


Figure 8.4. HSQC NMR spectrum of **Compound I**

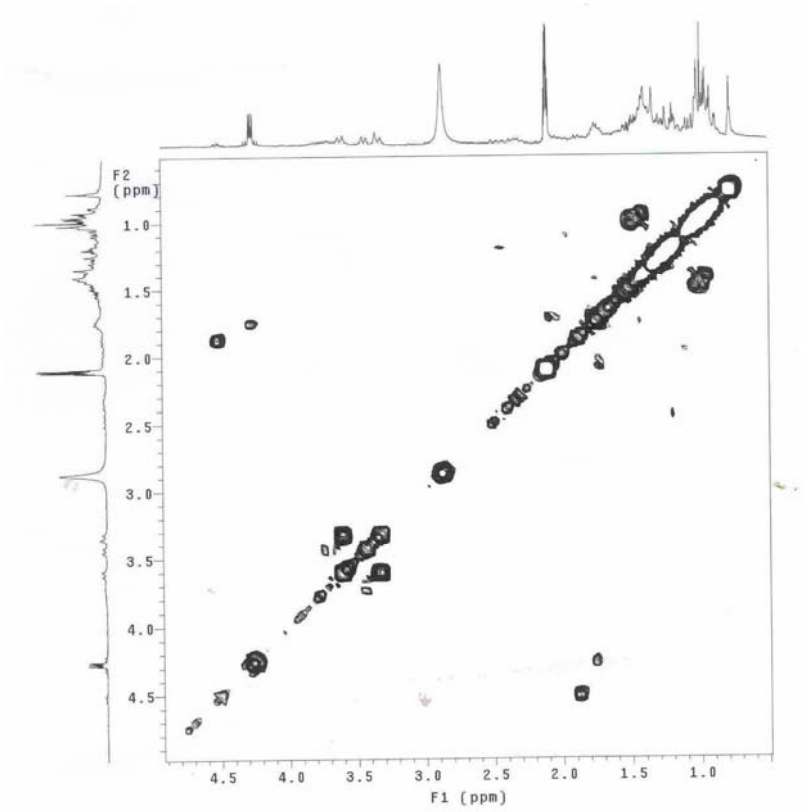


Figure 8.5. gCOSY NMR spectrum of **Compound I**

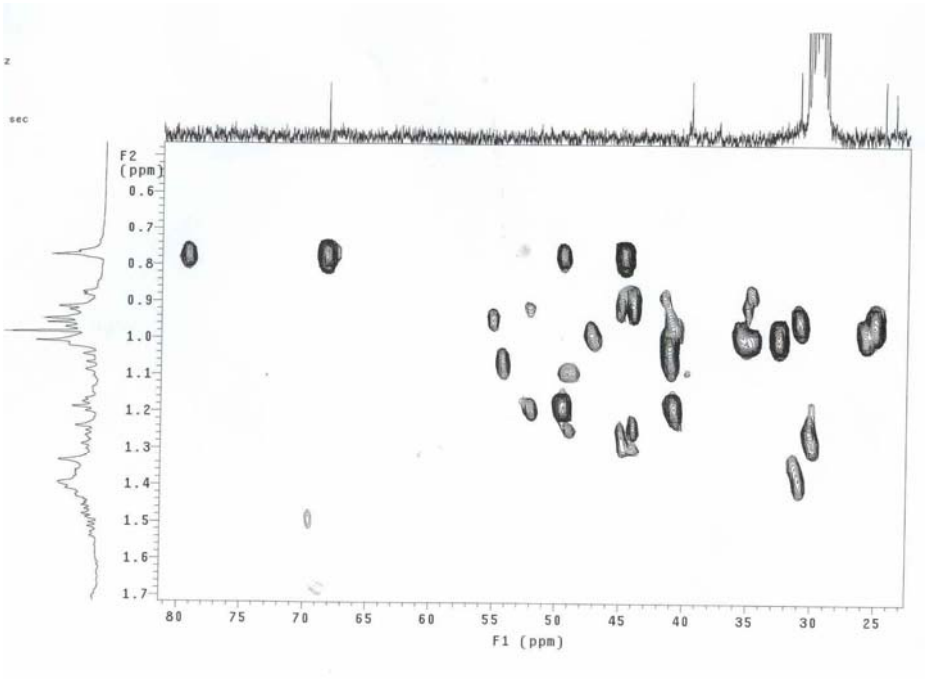


Figure 8.6. gHMBC NMR spectrum of **Compound I**

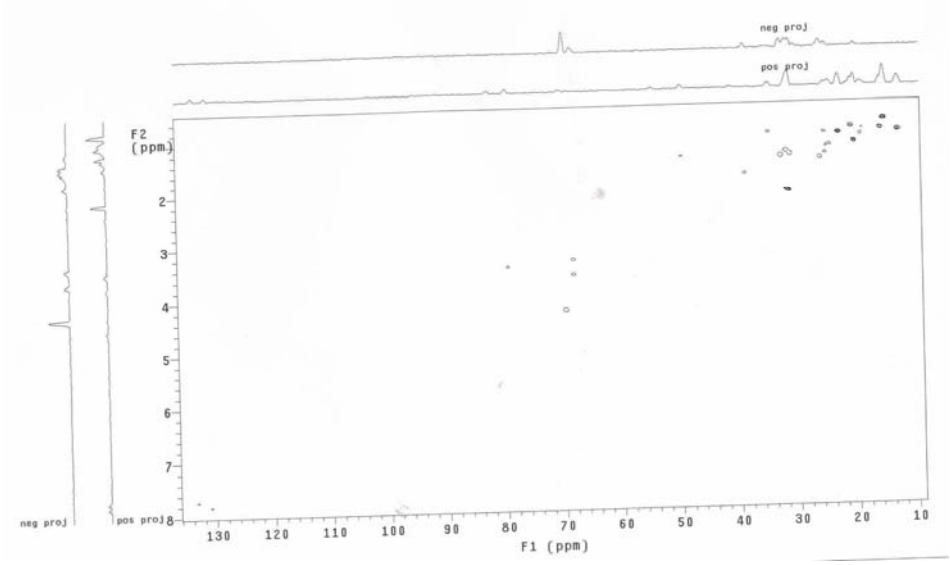


Figure 8.7. gHSQC NMR spectrum of **Compound I**

The bioassay-guided fractionation of the acetone extract by column chromatography lead to **compound II** with *m/z* 503 $(M-H)^-C_{30}H_{48}O_6$ and *m/z* 649 $(M-H)^-C_{36}H_{58}O_{10}$. The presence of one $C_6H(\delta_C 68.7, \delta_H 5.11)$ in compound II and the absence of $C_6H_2(\delta_C 18, \delta_H 1.41)$ in the ^1H, ^{13}C and DEPT spectra led to the identification of Compound II as terminolic acid.

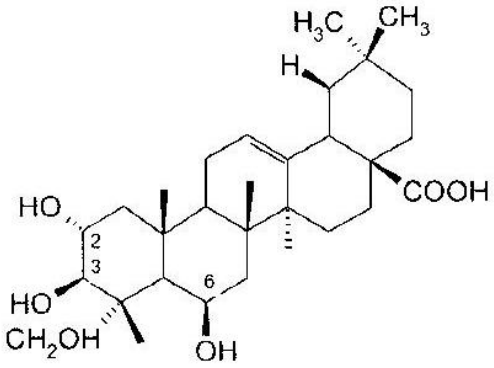
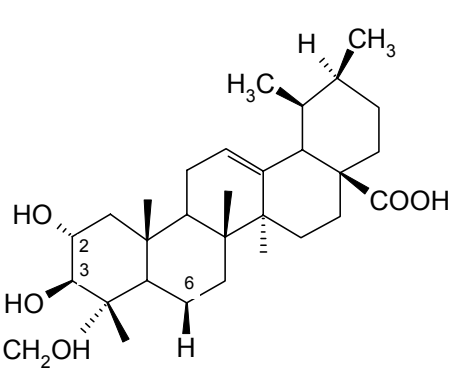
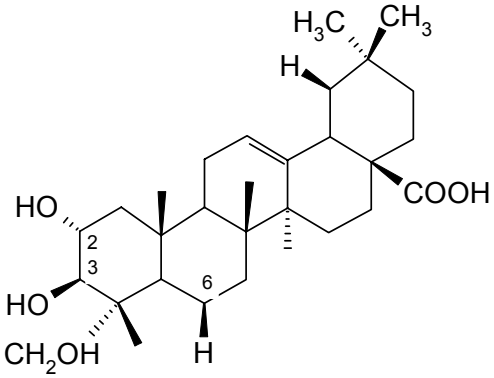


Figure 8.8. Terminolic acid

Compound I was a mixture of two inseparable compounds



Asiatic acid



Arjunolic acid

Figure 8.9. Compound **1,** a mixture of two inseparable compounds, which were asiatic acid (**1b**) and arjunolic acid (**1a**)

8.3. Discussion and Conclusion

It was the first time **compound II** (Terminolic acid) was isolated from *C. nelsonii*. It was previously isolated from *Terminalia macroptera* (Conrad *et al*., 1998) and they have studied its biological activities (*Bacillus subtilis* (5 μg/ml), *Pseudomonas fluorescens* (2.5 μg/ml) and *Cladosporium cucumerinum* (20 μg/ml)). It was also isolated from *Syzygium guineense* (Djoukeng et al., 2005) and they have found that it is active against *Escherichia coli* (6 μg/ml) and *Bacillus subtilis* (3 μg/ml). Due to lack of sufficient material, the antifungal activities of this compound were not determined and it was not further studied because it was not compound of interest. Compound 1 will be dealt with as a published paper in **chapter 10**. The title is: "**Biological activity of two related triterpenes isolated from *Combretum nelsonii* (Combretaceae) leaves".**