# ISOLATION OF SECONDARY METABOLITES FROM *Teclea nobilis* AND *Rapanea melanophloeos* ACTIVE AGAINST *Schistosoma mansoni*

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**EGERTON UNIVERSITY**

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## DECLARATION AND RECOMMENDATION

### DECLARATION

I, Njogu Mark Kimani, declare that this research thesis is my original work and has not been submitted wholly or in part for any award in any institution.

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### RECOMMENDATION

We wish to confirm that this research thesis has been prepared under our supervision and is presented for examination as per the Egerton University regulations with our approval.

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## DEDICATION

To

My dear dad; Isaiah Wang'endo, who always supports and believes in me. You are my inspiration and source of strength.

And

My lovely mom; Dorcas Njambi, whose love and prayers are immeasurable.

## COPYRIGHT

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## ABSTRACT

Helminthic infections have severe consequences on the health of millions of people worldwide and cause serious economic losses. Synthetic drugs have been effective wormicides, however, they are expensive, show side effects and develop resistance. This has generated interest in the use of plant based anti-helmintics, which seem to offer a reliable, cheap and cost effective method. In this research, the anti-helmintic activity of secondary metabolites from *Teclea nobilis* and *Rapanea melanophloeos*, which are used ethnomedically as dewormers, against *Schistosoma mansoni* are reported. The *Teclea nobilis* essential oil was extracted using hydro-distillation in a modified Clevenger apparatus. The essential oil was analyzed using gas chromatography-mass spectrometry and determined to be majorly constituted of monoterpenes and sesquiterpenes which had concentrations of 42.21 and 33.09%, respectively. The major monoterpenes were β-Ocimene (10.15%) and γ-Terpinene (6.11%) while major sesquiterpenes were β-Cadinene (4.98%) and 1,6-Germacradien-5-ol (4.38%). The essential oil showed lethal effects against *Schistosoma mansoni* miracidia with $LC_{50}$ and $LC_{90}$ values of 196.29 and 367.24 ppm, respectively. The non-volatiles of both *Teclea nobilis* and *Rapanea melanophloeos* were also active against *Schistosoma mansoni* miracidia. Fractionation of *Teclea nobilis* and *Rapanea melanophloeos* leaf ethyl acetate extracts over silica gel column chromatography yielded six compounds which were successfully identified through analysis of their 1D and 2D nuclear magnetic resonance spectroscopy and mass spectrometry data as well as comparison with literature data. Out of the six compounds, *Teclea nobilis* had four furoquinoline alkaloids; Tecleoxine **10**, Methylnkolbisine **13,** Kokusagine **21** and Nkolbisine **22** while the *Rapanea melanophloeos* had two benzoic acid derivatives; Myrsinoic acid B **23** and Myrsinoic acid C **24**. All these compounds were active against *S. mansoni* miracidia. Compound **23** which recorded $LC_{50}$ and $LC_{90}$ mortality values of 98.06 and 236.51 ppm, respectively was the most potent followed by compound **24** which registered $LC_{50}$ and $LC_{90}$ values of 139.89 and 314.23 ppm, respectively. The compounds **10**, **13** and **21,** which due to their small amounts could not be separated thus tested as a mixture, registered $LC_{50}$ and $LC_{90}$ values of 270.18 and 690.93 ppm, respectively and compound **22** which recorded $LC_{50}$ and $LC_{90}$ values of 287.97 and 631.73 ppm, respectively was the least potent. These findings show that these compounds can be used as lead compounds in the development of new, biodegradable, environmentally friendly and more potent miracidiacides or anti-helmintics.

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## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| APT | Attached proton test |
| COSY | Correlation spectrocopy |
| DALYs | Disability-adjusted life years |
| DMSO | Dimethyl sulfoxide |
| GC/MS | Gas chromatography/ Mass spectrometry |
| HMBC | Heteronuclear multiple bond correlation |
| HPLC | High performance liquid chromatography |
| HSQC | Heteronuclear single quantum correlation |
| KEMRI | Kenya medical research institute |
| LC | Lethal concentration |
| $LC_{50}$ | Lethal concentration killing 50% of parasite |
| $LC_{90}$ | Lethal concentration killing 90% of parasite |
| NMR | Nuclear magnetic resonance |
| PTLC | Preparative thin layer chromatography |
| $R_f$ | Retention factor |
| SPSS | Statistical package for social science |
| STH | Soil transmitted helminth |
| TB | Tuberculosis |
| TEEAL | The essential electronic agricultural library |
| TLC | Thin layer chromatography |

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background information

Helminths belong to two major groups: platyhelminthes and nematodes (Ijagbone and Olagunju, 2006). They include roundworms, hook worms and whip worms which cause high morbidity and mortality in humans. These parasites are common in sub-Saharan Africa due to poor sanitation, poverty, malnutrition, ignorance, lack of protective clothing, lack of clean water as well as limited access to preventive measures and health care (Zvi *et al*., 2010). These factors also make helminths co-infections in human such as with *Plasmodium falciparum*, HIV/AIDS and tuberculosis prevalent. Helminthic morbidity is also common with obesity, cardiovascular diseases, allergy and diabetes (Perry and Randolph, 1999; Zvi *et al*., 2010).

Gastrointestinal parasites have also been reported to be a major menace facing animals and birds. These parasites are known to reduce the animals' survival, growth rate and reproductive performance thus affecting their overall production. A study conducted in Kenya, showed that helminthiosis accounted for the second most important disease accounting for 21% of the deaths by parasitic conditions, between 1989 and 1998 in chicken (Permin *et al.*, 1999). It has been estimated that more than 750 million chickens, guinea fowls and ducklings in Africa die each year as a result of various infections, helminthosis being a major contributor (Matur *et al.,* 2010). Therefore, the economic and social impact of helminths cannot be underestimated. In humans and livestock their effects range from stunted growth, reduced weight gain, diarrhoea, anaemia, respiratory problems, reduced productivity and death especially in the tropics and developing countries (Jozef *et al.,* 2011; Velkers *et al.,* 2011).

The use of synthetic drugs has been effective in controlling the helminths. However, development of anti-helmintic resistance, the problem of drug residues, deficient livestock extension services and the high cost of conventional anti-helmintics, has led to the evaluation of medicinal plants as an alternative source of anti-helmintics for treatment of human and livestock against helminthes (Jozef *et al.,* 2011).

## 1.2 Statement of the problem

Helminth infections cause adverse effects on human and livestock such as haematological and biochemical disturbances, loss of body weight, death leading to economic losses. The use of synthetic anti-helmintics has for a long time been considered the only effective way of controlling these parasitic infections in humans and livestock. However, these drugs are very expensive, unaffordable and at times unavailable to poor people in rural areas particularly in sub-Saharan Africa. In addition, synthetic drugs have developed some serious problems such as drug resistance, ecological pollution and residues in food. Additionally, there is a general stagnation in the development of new safe, efficacious and reliable conventional drugs, which has led to an increased need for the treatment and control of helminth infections. Thus people have resorted to the continued use of plant based de-wormers. The use of ethno-medicine or ethno-botanical medicine and ethno-veterinary medicine seem as feasible alternative anti-helmintics and is therefore gaining a lot of importance.

## 1.3 Objectives

## 1.4 General objective

The general objective of this study was to isolate and characterize secondary metabolites from *T. nobilis* and *R. melanophloeos* and determine their anti-helmintic activity, against *Schistosoma mansoni in vitro*.

## 1.5 Specific objectives

i. To isolate and determine the chemical composition of essential oil from *T. nobilis.*
ii. To evaluate anti-helmintic activity of *T. nobilis* essential oil against *S. mansoni.*
iii. To determine anti-helmintic activity of non-volatiles from *T. nobilis* and *R. melanophloeos* against *S. mansoni*.
iv. To isolate and elucidate the structure(s) of *T. nobilis* and *R. melanophloeos* anti-helmintic compounds.

## 1.6 Hypotheses

i. The chemical composition of essential oil from *T. nobilis* cannot be determined.
ii. Essential oil of *T. nobilis* has no anti-helmintic activity.

iii. Non-volatile extracts of *T. nobilis* and *R. melanophloeos* have no anti-helmintic activity.

iv. The structure of the active anti-helmintic compounds cannot be elucidated.

## 1.7 Justification

Plant anti-helmintics for a long time have lacked scientific evaluation and medical awareness. Studies on such plants need to go beyond mere anthropological curiosity. This is because they are readily applicable elements of ethno-medicine in human and livestock development. Thus studying these herbal medicines can serve to validate and enhance existing local uses and can give clues to remedies with further potential. Plant metabolites also offer cheaper, more sustainable, available, efficacious and reliable alternatives to synthetic drugs. They also offer treatment methods that are more environmentally friendly, since they are less toxic, produce fewer side effects, are more biodegradable, accumulate no drug residues in meat or faeces and do not trigger anti-helmintic chemo-resistance. *T. nobilis* and *R. melanophloeos* have been ethno-medicinally used as anti-helmintics, as analgesics and for respiratory problems among other uses. In this study, anti-helmintic properties of their secondary metabolites against *S. mansoni* were evaluated.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Helminthosis

Helminthosis is of both economic and public health importance as well as being one of the world's most important neglected tropical diseases (Maduike *et al*., 2012). According to recent reports by WHO, helminthosis burden is equivalent to 50% of that of malaria and 25% of that of HIV/AIDS with approximately 2.9 billion people being infected with nematode (WHO, 2010). It is estimated that, about 90% of the 200 million people with Schistosomiasis live in Africa. Similarly, Africa bears 198 million people with Hookworm, 173 million with *Ascaris lumbricoides* and 162 million with *Trichuris trichiura* (Zvi *et al*., 2010, WHO; 2010). Hookworm morbidity associated with anaemia, likely contributing to maternal mortality, among pregnant women currently stands at 44 million. Also 12% of the total disease burden among children aged 5-14 years is contributed by intestinal worms (Jozef *et al*., 2011).

Soil transmitted helminth (STH) infections have been estimated to cause 135,000 deaths annually, mainly due to anaemia, caused by hookworm and whipworm infections, intestinal, or biliary obstruction and chronic dysentery caused by roundworms and whipworms (Jozef *et al*., 2011). STH infections are as important as malaria and TB in terms of disability-adjusted life years (DALYs) lost, that is the number of healthy years lost to premature death or disability, (Stephenson *et al*., 2000). It is estimated that 22.1 million DALYs are lost due to hook worm, 10.5 million due to *Ascaris lumbricoides*, 6.4 million due *Trichuris trichiura*, and 37 million for the three combined while that of malaria is approximately 35.7 million globally (Jozef *et al*., 2011).

Helminthic infections, HIV, TB and malaria have also been reported to have extensive overlap in sub-Saharan Africa (Jozef *et al*., 2011) with both helminths and HIV-1 estimated to be over 22 million (Judd *et al.*, 2010). De-worming individuals with HIV-1 has been suggested to delay HIV-1 disease progression by up to 25% and delay AIDS development by upto 3.5 years (Judd *et al.*, 2010). According to Midzi *et al.* ( 2011) 45 million (25%) school-aged children are also at risk of Hookworm and malaria infection.

## 2.2 *Schistosoma mansoni*

*S. mansoni* is a blood dwelling digenetic trematode which causes intestinal Schistosomiasis or bilharziasis. It is ranked as the second most important parasitic infection after malaria in terms of morbidity and mortality. In 2011, at least 243 million people were reported to require treatment. In sub-Saharan Africa, an estimated mortality of 280,000 people is reported annually (Boissier *et al*., 2009).

*S. mansoni* has a complex life cycle. Briefly, parasitic eggs excreted in faeces from infected persons on reaching fresh water hatch into larvae called miracidiae which then seek out *Biomphalaria alexandriana* snails. Upon infection of snail, the miracidiae are transformed into mother sporocytes which then develop into daughter sporocytes. These in turn develop into mature cercariae. The cercariae are released into the water, where they infect human by penetration of the skin. The head of the cercariae transform, in the skin, into an endoparasitic larva called schistosomulae which enters the vascular system. The schistosomulae migrates to the lung, develop further and move to the liver. In the liver, they feed on the red blood cells and mature worms pair up. The worm pairs travel to the mesenteric veins, where they produce eggs. The eggs pass through the walls of the blood vessels through the intestinal wall to be passed out in faeces (Mohamed *et al*., 2005; Frelick, 2012).

## 2.3 Anti-helmintics

An anti-helmintic or de-wormer is a substance that expels or destroys gastrointestinal worms. Anti-helmintics eliminates worms by either paralyzing or starving them to death. Worms must eat almost continuously to meet their metabolic needs since they do not have means to store energy. Thus any disruption in the feeding for 24 hours or less process results in energy depletion and eventual death. Worm paralysis and temporally lose of the ability to maintain posture in the gut also leads to death (Shaziya and Goyal, 2012). Chemotherapy is mostly the method of choices in helminthes control. However, the world wide long-term application of the drugs coupled with the recent development of anti-helmintic tolerant strains (Shaziya and Goyal, 2012; Boissier *et al*., 2009), in parasites of high economic significance generates concern over the development of new drugs.

Modern synthetic anti-helmintics are majory grouped into four classes:

I. Benzimidazoles such as albendazole and mebendazole. They are broad spectrum drugs that bind to free $\beta$-tubulin, inhibiting its polymerization and so interfering with microtubule-dependent glucose uptake by the parasite.

II. Imidazothiazoles and tetrahydropyrimidines such as levamisole and pyrantel which stimulate the nicotinic acetylcholine receptors, resulting in over-stimulation, blockage of the neuromuscular junctions, and rigid paralysis of the worms. The parasites are then unable to move in the intestinal tract and are swept out by the peristaltic action in the intestine.

III. Macrocyclic lactones such as Ivermectin which open glutamate-gated chloride channels, increasing chloride ion conductance, leading to defects in neurotransmission and flaccid paralysis.

IV. Heterocyclic ethyleneamines, of which the best known member, piperazine, is only used against *A. lumbricoides* and *E. vermicularis*. This drug acts by reversibly inhibiting neuromuscular transmission by stimulating gamma-aminobutyric acid (GABA) receptors in nematode muscle, causing flaccid paralysis of the worms, which are then removed by normal intestinal peristalsis (Stepek *et al*., 2004; Stepek *et al*., 2006).

In the developing countries, ethno-veterinary medicines have been used for a long time as anti-helmintic (Singh and Shalini, 2000; Lalchhandama, 2011; Nalule *et al.,* 2011; Velkers *et al.,* 2011; Shaziya and Goyal, 2012). Plants containing proteolytic enzymes of the cysteine catalytic class such as papaya, which contain enzymes such as papain, chymopapain and lysozymes in the latex as well as in leaves, have mostly been used (Dakpogan, 2005). Moreover, various plants have been studied and reported to have anti-helmintic activity. Some of these plants include *Einocostemma littorale* whose extracts have been shown to cause paralysis of *Pheretima pothera* worms (Vidyadhar *et al*., 2010). The plants *Annona senegalensis, Anogeissus leiocarpus, Lippia rogosa, Strerospermum kontihianum, Vernonia tonoreana* (Mali and Mehta 2008) and *Corriandum sativum* (Eguale *et al*., 2006) have been shown to have effects against *Heamonchus contortus*. Furthermore, *Ocimum sanctum, Piliostigma thoningii, Moghania vestite, Mimusops elergi, Punica granatum, Alotropic procera, Capparic deciduas, Butea monosperma, Aloe vera, Neolamarckia cadamba, Xylopia aethiopica, Cynadropsis gynandra, Evolvus alsinoides, Carica papaya, Piper longum, Nigella sativa, Ficus inspida, Nicotiana tabacum, Cannabis sativa,*

*Cleome icosandra, Trifolium repers, Strobilanthas discolor* among others have been shown to have anti-helmintic activity (Munglo *et al*., 2006). Several phytochemicals such as nefuridin, linalool, chavicol, kaempferol among others (**1-9**) have been isolated and also reported to have anti-helmintic properties. Some of these compounds are shown in figure 1 below (Chitwood, 2002; Lasisi and Kareem, 2011).

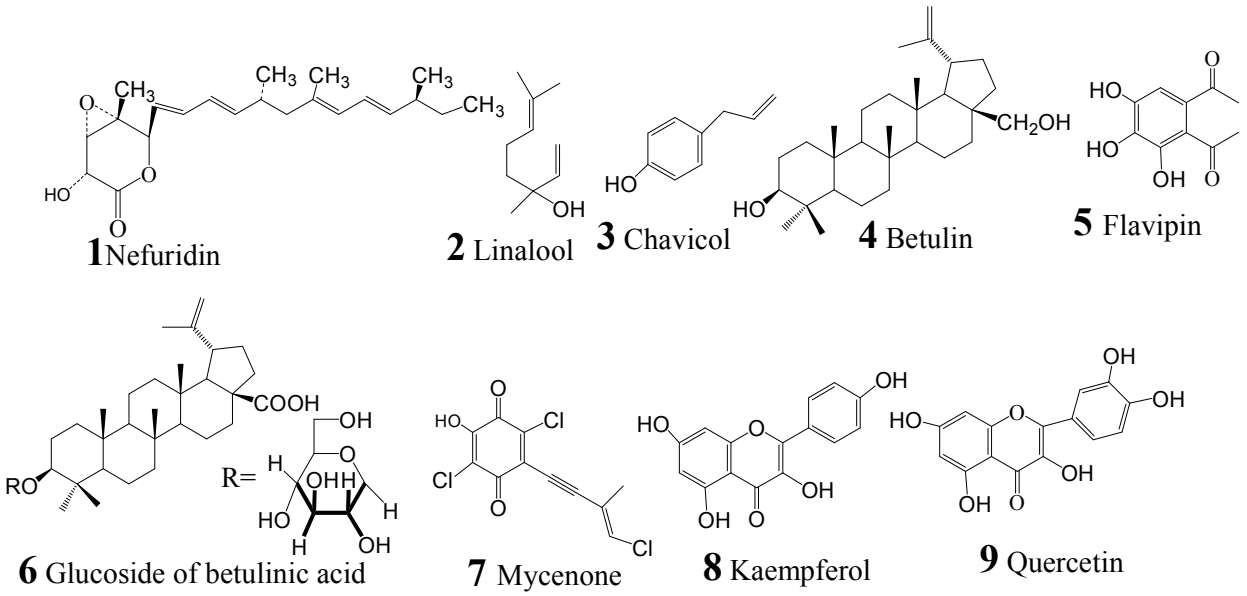



**Figure 1: Phytochemicals with anti-helmintic activity**

## 2.4 *Teclea nobilis*

*T. nobilis* is an ever green shrub or tree of the Rutaceae family with a smooth grey bark native to Ethiopia, Kenya, Tanzania, and Uganda rain forests. It has simple leaves on glabrous branchlets. A photograph of the plant is shown in figure 3 below. It is reported to have been used ethno-medicinally in many African countries and in Saudi Arabia (Adnan *et al*., 2001a).

The leaves and stem bark has been used as a remedy for gonorrhea. It has been reported to have anti-inflammatory, analgesic and antipyretic effect (Adnan *et al*., 2001a). Quinoline and furoquinolie alkaloids, limonoids, triterpenes, lignin and flavonoid have been reported in Rutaceae family in which this plant belongs. In a previous study the presence of two isomeric axane and oppositane sesquiterpene ketones (**15-16**), teclenone A [1α-(1-Oxo-2-methylpropyl)-3aα-methyl-7-methyleneoctahydroinden-4α-ol] and teclenone B [1α-(1-Oxo-2-methylpropyl)-

3aβ-methyl-7-methyleneoctahydroinden-4β-ol] and alkaloids (**10-14**) from aerial parts (Figure 4) of *T. nobilis* were reported (Adnan *et al.*, 2002; Adnan *et al.,* 2003).

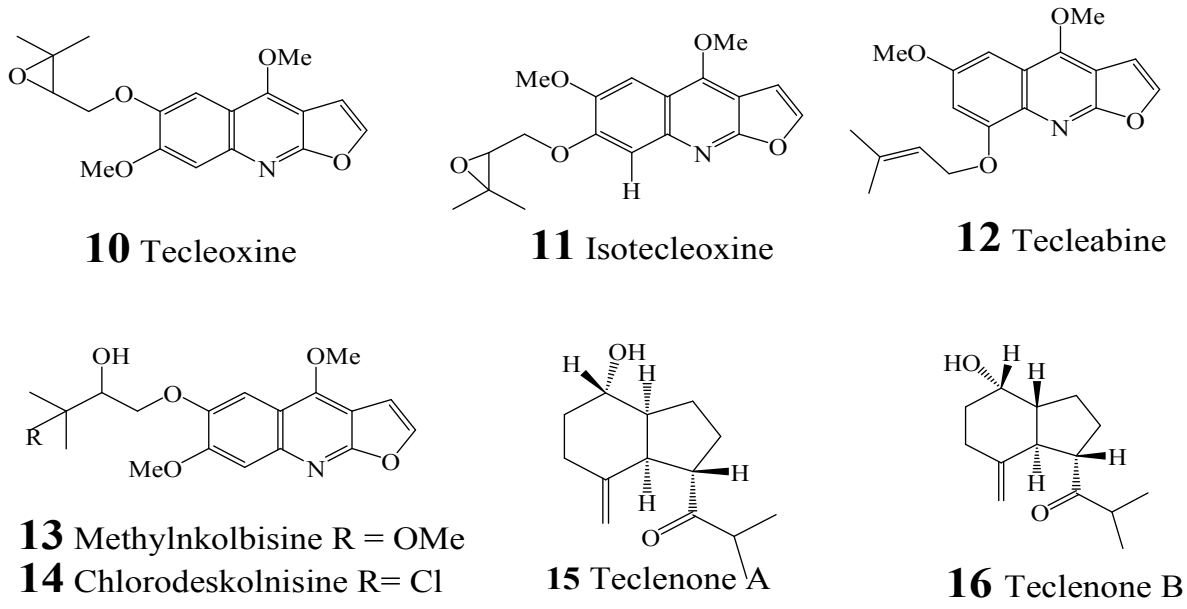



**Figure 2: Alkaloids and sesquiterpene ketones isolated from *T. nobilis*.**

The essential oil from *T. nobilis* are also said to contain Germacrene-d, Ocimene isomer, Gualol, Elemol and Bulnesol as the main components. The oil is also reported to show antipyretic activity and sedative effects (Adnan, 2001b).

**Figure 3: Photographs of *T. nobilis***

## 2.5 *Rapanea melanophloeos*

*R. melanophloeos* is an evergreen tree of the Myrsinaceae family widespread in Kenyan upland forests (Mosa *et al*., 2010). When mature, leaves are simple oblong-lanceolate, dark green, paler below, leathery and dull with reddish leaf stalks and are clustered mostly at the last part of the branches. When young, leaves are pale green and maroon. Small, whitish or creamy yellow clusters of flowers appear on the branchlets in June to December. The fruits are thinly fleshed and round in shape, green when young and purple when mature (Githiori, 2004).

**Figure 4: Photographs of *R. melanophloeos***

The grey bark and roots are used ethno-medicinally for respiratory problems, stomach, muscular and heart complaints. The bark, roots and fruits are reported to have anti-helmintic properties. The fruits are particularly used as anti-helmintic in livestock and humans, primarily against cestodes by either being chewed or eaten in porridge (Midiwo *et al*., 2002). According to Githiori *et al.* (2002) the plant has some *in vivo* anti-helmintic activity against the nematode parasite, *Haemonchus contortus*.

Triterpenoid saponins (**19**) isolated from *Rapanea melanophloeos* have been scientifically proven to have molluscicidal and antifungal activities (Kazuhiro *et al*., 2002). The compound 3β-Hydroxylanosta-9,24-dien-21-oic acid (**17**) isolated from the bark, has been reported to exhibit anti-platelet aggregation activity hence can be used to treat clotting related ailments (Gwala, 2011). This property is attributable to its antioxidant activity. Phytochemical analysis shows that the plant contains saponin, terpenoids, tannins, alkaloids, flavanoids, cardiac glycosides and phlobatanins. The plant also contains benzoquinones particularly Embelin (**18**), 2,5-dihydroxy-3-undecyl-1,4-benzoquinone, which has larvicidal effects against *Aedes egypti,* antibacterial activity, antitumor, anti-inflammatory, anti-helmintic, antioxidant and analgesic activity (Marston *et al*., 2000; Joy and Lakshimi, 2010).

The family Myrsinaceae is reported to contain benzoquinones as the major compounds. The family also accumulates flavonols in their glycoside form as well as alkaloids but none have been reported in *R. melanophloeos*. Calcium oxalate crystals have also been found in the tissues of plants in this family. The family exhibits a range of biological activity including acaricidal, antimicrobial, insecticidal, nematicidal and phototoxic activity (Lukhele, 2010).

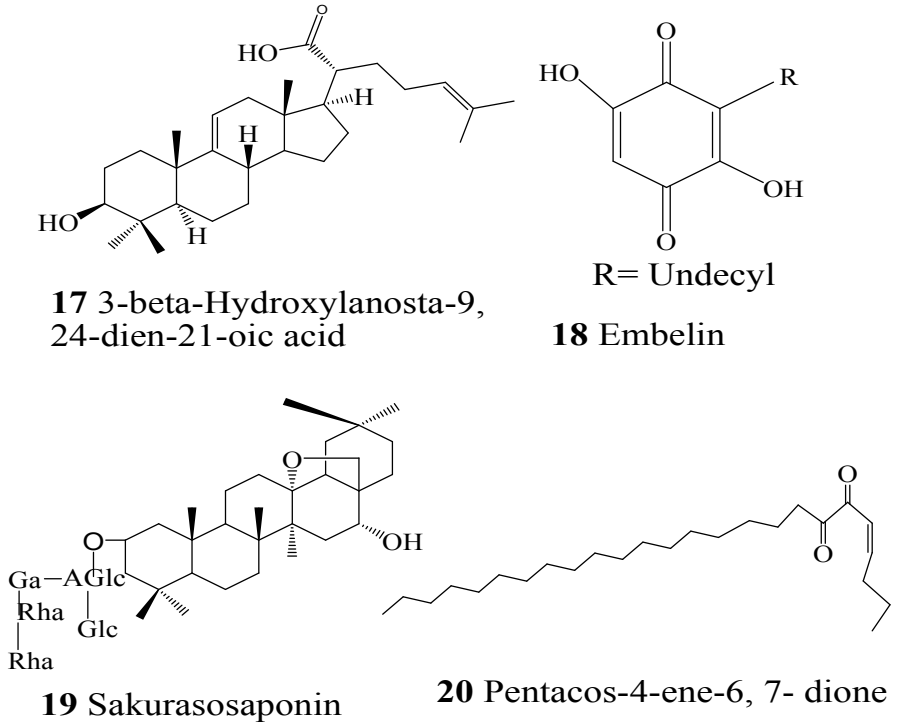



**Figure 5: Some of the compounds isolated from *R. melanophloeos*** (Joy and Lakshimi 2010; Gwala, 2011).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Collection of plant material

Plant materials of *T. nobilis* and *R. melanophloeos* were collected from Kakamega rain forest ($0^0$ 10' – $0^0$ 21' N $34^0$ 44' – $34^0$ 58' E) and Sururu Forest, Mau Narok, Nakuru County (0°66′ 55.2″S 36°1′ 47.3″E) respectively. They were identified by a taxonomist at the Department of Biological Sciences, Egerton University, where a voucher specimen was deposited. The *T. nobilis* materials were divided into two. One batch of the *T. nobilis* and *R. melanophloeos* materials were taken to the centre for Herbal research in Egerton University where they were dried under shade for three weeks. The other batch of *T. nobilis* was taken to Biotechnology laboratory for extraction of essential oil.

## 3.2 Extraction and analysis of essential oil

### 3.2.1 Distillation of essential oil

The fresh leaves of *T. nobilis* were subjected to hydro distillation in a modified Clevenger apparatus to extract essential oils. These leaves were cut into pieces of about 2 X 2 cm and 100g boiled with 500 ml of distilled water in a modified Clevenger apparatus until oil distillation ceased after 4-6 h. The essential oil in the distillate was dried over anhydrous $Na_2SO_4$ and refrigerated at $4^oC$.

### 3.2.2 Essential oil chemical composition determination and spectroscopic analysis

The essential oil was analysed by use of an Agilent GC-MSD apparatus equipped with an Rtx-5SIL MS ('Restek') (30 m x 0.25 mm, 0.25 μm film thickness) fused-silica capillary column. Briefly, the following protocol was applied. The essential oil was diluted in methyl-t-butyl ether (MTBE) (1:100). The carrier gas used was Helium (at 0.8 mL/min). Sample was injected in the split mode at a ratio of 1:10 – 1:100. The injector was kept at $250^oC$ and the transfer line at $280^oC$. The column was maintained at $50^oC$ for 2 min and then programmed to $260^oC$ at $5^oC$/min and held for 10 min at $260^oC$.

The MS was operated in the electron impact ionization (EI) mode at 70 eV, in *m/z* range 42-350. The identification of the compounds was performed by comparing their retention indices and mass spectra with those found in literature and supplemented by Wiley 7N.l, HPCH 1607.L and FLAVORS.L GC-MS libraries. The relative proportions of the essential oil constituents are expressed as percentages obtained by peak area normalization, all relative response factors being taken as one. A summary of the extraction is shown as per the flow diagram in figure 6.

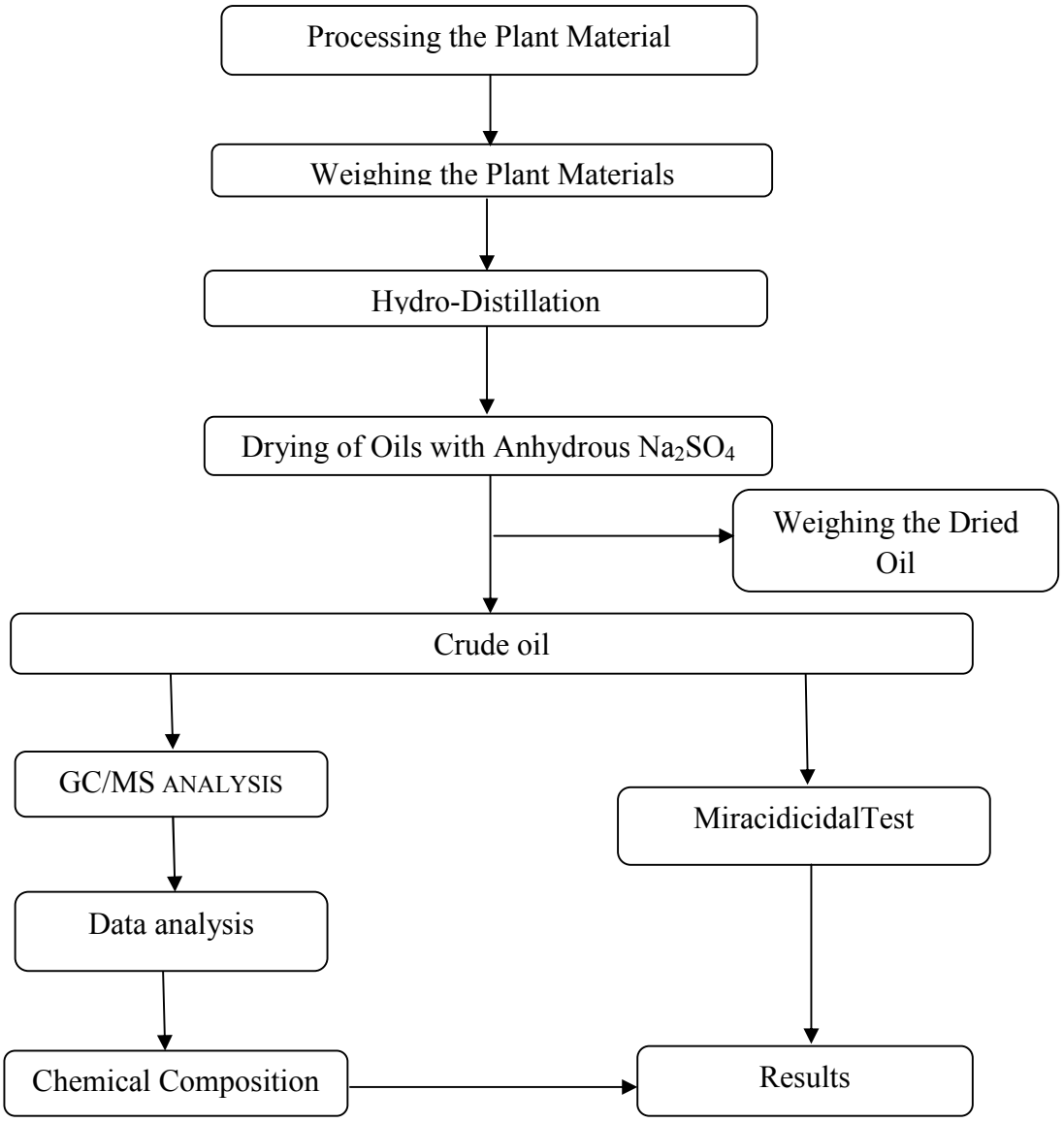



**Figure 6: A flow chart showing the summary of isolation and analysis of essential oil**

### 3.3 Extraction, isolation and structure elucidation of non-volatiles

Extraction was done using solvents of different polarities acquired from Indo laboratories. The solvents were of GPR grade and were distilled before use. These solvents included methanol, ethyl acetate and hexane. The procedure of the extraction, isolation and structure elucidation of the pure compounds is briefly described below and summarized in figure 7.

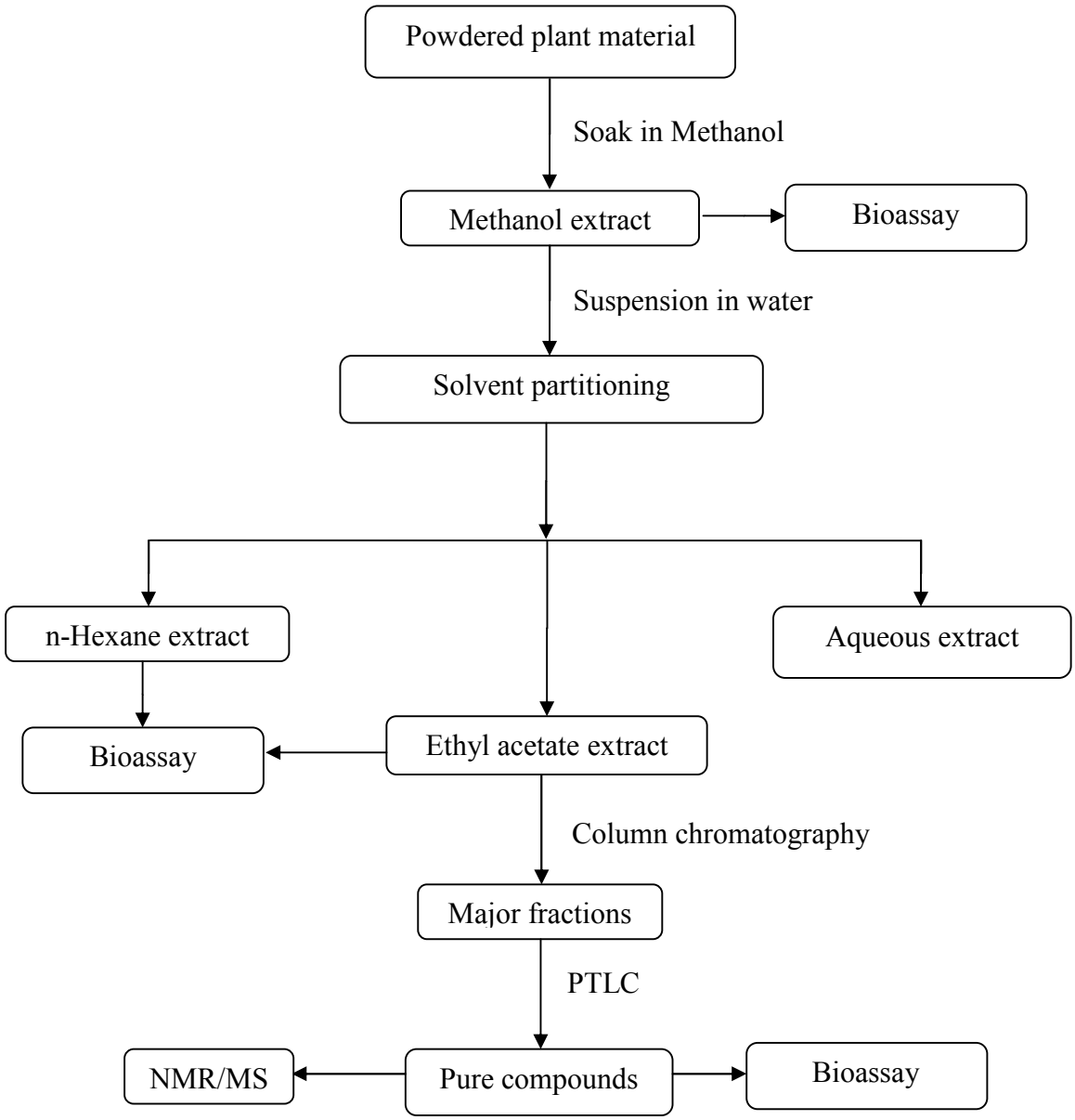



**Figure 7: A summary of isolation and structure elucidation of non-volatiles**

### 3.3.1 Extraction of phytochemicals

Leaves of *T. nobilis* and *R. melanophloeos* were dried under shade and at ambient temperatures to avoid loss of labile compounds and also avoid formation of other artefacts. To avoid the formation of mould during drying, the plant materials were periodically turned over. The materials were separately ground to a fine powder using a Thomas-Wiley mill model 4. Four and five kilograms of the *T. nobilis* and *R. melanophloeos* ground materials were respectively soaked in methanol at room temperature for 24 hours with periodical shaking. The contents were filtered through Whatman no. 1 filter paper and the filtrate evaporated to dryness in vacuum at 40°C using Buchi Rotavapor R-205 rotary evaporator. The methanol crude extracts were dried in a fume hood.

### 3.3.2 Solvent partitioning of crude methanol extract

The crude methanol extracts of *T. nobilis* and *R. melanophloeos* were subjected to liquid-liquid fractionation by suspending them in water and sequentially extracting with hexane and ethyl acetate to afford three extracts namely; hexane, ethyl acetate and aqueous extracts. From the hexane and ethyl acetate extracts, the solvents were removed from the hexane and ethyl acetate extracts in a rotary evaporator at $40^0$C and residues dried completely in a fume hood. Ethyl acetate extracts were then subjected to extensive TLC analysis and column chromatography as outlined below.

### 3.3.3 Thin layer chromatography (TLC)

Analytical TLC was performed on silica gel GF 254nm, (Merck, Germany) 0.25mm thickness. Briefly, the dry extracts were reconstituted in ethyl acetate to make up a final concentration of about 10mg/cm$^3$. To ensure homogeneity all extracts were thoroughly mixed. Preliminary analysis was performed to identify optimum solvent systems for use as mobile phases. Ethyl acetate and hexane solvent mixtures were tried and modified accordingly to give optimum separation for ethyl acetate extracts. The solvent mixtures that were giving optimum separations were, 5:5 ethyl acetate-hexane mixtures for *R. melanophloeos* and 6:4 ethyl acetate-hexane mixtures for *T. nobilis*. Visualization was done by illumination under UV lamp (Uvitec-LF-204.LS) at 254 nm and 365nm.

Samples were spotted on 4×5cm or 2×5cm aluminium backed TLC plates. The spotting was done with care being taken not to over load the plate. For each of the different plants, samples were spotted on separate plates. In each plate samples were about half a centimeter from the base and developed to a distance of 4cm in an aluminium foil covered 100ml glass beaker pre-saturated with the corresponding mobile phase. The developed chromatograms were then visualized under UV light at 254 nm and 365 nm to detect UV visible compounds.

### 3.3.4 Column chromatography

After intensive TLC analysis of *T. nobilis* and *R. melanophloeos* ethyl acetate extracts to identify the optimum solvent systems that were giving good separation of compounds, the extracts were then prepared for column chromatography. The dry extracts were re-dissolved in minimum amount of ethyl acetate and separately loaded on evenly packed silica gel columns, by dripping on the column walls, cautioning the disturbance of the silica gel layer. Silica gel 60 0.06-0.2mm (70-230mesh ASTM) supplied by Scharlau Lab supplies Limited was used for the column chromatography. The columns were eluted gradually with the appropriate mobile phase. The lengths of the columns were 50 cm with a diameter of 20mm and the flow rates were maintained at approximately 20ml/5min.

Fractions of equal volumes were collected and the TLC of each fraction done. Fractions with nearly similar TLC patterns were pooled. The *T. nobilis* ethyl acetate; extract yielded five major fractions while *R. melanophloeos* yielded four major fractions. Fraction three of *R. melanophloeos* was added in activated charcoal, filtered, concentrated in vacuo and eluted on a column chromatography with a pre-determined mobile phase of 4:6 Ethyl acetate-Hexane mixtures. From this, three sub-fractions were collected then subjected to PTLC. This yielded two purified fractions $RMF_3C_2$(**23**) and $RMF_3C_3$(**24**). *T. nobilis* fractions three and four were also subjected to PTLC. This yielded two purified fractions $TNF_3C_1$(**10**, **13** and **21**) and $TNF_4C_1$(**22**).

### 3.3.5 Purification of compounds

The preparative TLC (PTLC) were prepared using silica gel ‘G’ (for TLC, containing 13% calcium sulphate) supplied by Laborama limited on 20 cm × 20 cm glass plates. 90 g of silica gel was added to 20 ml of distilled water to make slurry. The slurry was then evenly spread on the glass plates to ensure uniform thickness. Care was taken to avoid very thick

plates. Fractions that were completely dry were re-dissolved in ethyl acetate and spotted on a preparative TLC plate with care not to overload using pasture pipette. The plates were then developed to a distance of 18 cm in a closed TLC tank pre-saturated with the corresponding pre-determined solvent mixtures. The plates were then visualized under 254 nm UV radiation and the bands containing the target compounds scraped off. The compounds were then extracted with ethyl acetate, filtered and then concentrated in vacuum at $40^0$C. The purity of the compounds was determined by TLC. Where the compounds did not give clear single spots they were re-spotted and developed on PTLC. They were then dried under a current of air in the fume hood. The dry samples were weighed and the w/w percentage yield calculated. The compounds were taken for further analysis using 1 and 2D high field NMR spectroscopy and mass spectrometry.

### 3.3.6 Nuclear magnetic resonance (NMR) spectroscopy

The $^1$H, APT, HSQC, COSY and HMBC NMR spectra were recorded on the Bruker Advance 500 MHz NMR spectrometer at the Technical University of Berlin, Germany. All the readings were done in Deuterated chloroform and chemical shifts assigned by comparison with the residue proton and carbon resonance of the solvent. Tetramethylsilane (TMS) was used as an internal standard and chemical shifts were given as δ (ppm). The structures were simulated using ACD NMR manager program to obtain the chemical shifts of proton.

### 3.3.7 Two dimensional NMR spectroscopy

The off- diagonal elements were used to identify the spin – spin coupling interactions in the $^1$H –$^1$H COSY (Correlation spectroscopy). The proton-carbon connectivity, up to three bonds away, was identified using $^1$H–$^{13}$C HMBC (Heteronuclear Multiple Bond Correlation) spectrum. The $^1$H–$^{13}$C HSQC spectrum (Heteronuclear Single Quantum Coherence) was used to determine the connectivity of hydrogen to their respective carbon atoms. The APT (Attached proton test) spectrum was used to identify the resonances of Quartenary, methines, methylene and methyl carbon atoms.

### 3.3.8 Mass spectrometry

The mass spectra of the compounds were recorded on Finnigan Triple Stage Quadrupol Spectrometer (TSQ-70) with electro spray ionization (ESI) Method. The Thermo Xcalibur Qual computer software was used in analysis of the mass chromatograms.

## 3.4 *In vitro* anti-helmintic activity test

*In vitro* anti-helmintic activity was assessed through miracidia motility assays for *S. mansoni.*

### 3.4.1 Collection and preparation of *S. mansoni* miracidia

Stool samples were collected from volunteers who work as car washers and sand harvesters in Usoma village Kisumu County. Diagnosis was established by the help of field teams of KEMRI Schisto branch by use of the Kato-Katz faecal thick smear.

The cellophane fecal thick smears were prepared according to the method described by Lofty (2009) with some modifications. A small mound of faecal material was placed on a scrap paper and a piece of 80 mesh nylon screen (30-35mm) pressed on top so that some of the faeces sieved through the screen and accumulated on top. A flat-sided spatula was scraped across the upper surface of the screen to collect the sieved faeces. A plastic Kato-Katz template, with a hole of 6mm on a 1.5mm thick template delivering 41.7mg of faeces, was then placed on the centre of the microscope slide and faeces from the spatula added so that the hole was completely filled. The template was then passed over using the side of the spatula to remove excess faeces from the edge of the hole. The template was carefully removed so that the cylinder of faeces was left on the slide. The faecal material was covered with hydrophilic cellophane, 34µm thick, pre-soaked in glycerol and 1ml of 3% aqueous malachite green. The microscope slide was inverted and the faecal sample firmly pressed against the hydrophilic strip on another microscope slide. The faecal material was evenly spread between the microscope slide and the cellophane strip. Carefully the slide was removed by gently sliding it sideways to avoid separating the cellophane strip. The slide was then placed on the bench with the cellophane upward to allow water to evaporate while glycerol cleared the faeces. The slide was kept at room temperature for one hour to clear the faecal material, prior to examination under the microscope. The number of eggs

counted from the two slides was multiplied by 24 to obtain the number of eggs per gram stool. The samples found positive by the Kato-Katz fecal thick smears were prepared for hatching.

*S. mansoni* eggs were recovered from positive stools as described below. Fresh fecal sample was taken and emulsified in about 250ml of de-chlorinated water. The homogenized fecal sample was placed on a bank of arranged nested sieves in descending order of pore size from the top (750, 410, 212 and 45 μm) and passed through with aid of a cap of an inverted 50ml Tube while splashing with water using a wash bottle. The top most sieve was removed after the filtrate passed through. The same procedure was repeated with the other sieves. The schistosome ova were collected at the 45 μm sieve. The last sieve was splashed with water while agitating until clean water was seen to pass through. The filtrate containing eggs was Collected in a 50ml centrifuge tube and stored at $4^0$C. To confirm the presence of schistosome ova, a direct smear was prepared using the supernatant in the tube and observed under a dissecting Microscope.

When hatching schistosome ova, all the filtrate contained in the 50ml tube was poured into a 1litre flat bottomed flask and filled with water to the brim. The flask was exposed to the open light (next to the window) for 1 hour. The flask was covered with a dark cloth for 10 minutes. The top part of the water was poured into a Petri-dish and observed for miracidia under 10X objective under a dissecting microscope. The miracidia were picked using a micropipette and transferred into 24 well multiwell plates.

### 3.4.2 Effect of the extracts on miracidia

24 wells multiwell plates were used as test chambers to observe the viability and death of the miracidia under a dissecting microscope. Twenty miracidia were picked using 100μl pipette and placed in each well. Serial dilutions of the crude extracts and major fractions were added to each well as follows; 4000, 2000, 1000, 500, 450, 400, 350, 300, 250, 200, 150, 100 and 50 ppm. Two negative controls were also set up using de-chlorinated water and 1% DMSO in de-chlorinated water, respectively. Praziquantel was used as a positive control. Three replicates were prepared for each test and mortality observed after 30 minutes. Percentage mortality was calculated and $LC_{50}$ for each sample determined by use of IBM SPSS 15.0.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

## 4.1 Structure elucidation of isolated compounds

Six compounds were isolated and their structures elucidated. Of the six, four were from *T. nobilis* that is, Tecleoxine **10**, Methylnkolbisine **13**, Kokusagine **21** and Nkolbisine **22**. From *R. melanoploeos*, two benzoic acid derivatives; Myrsinoic acid B **23** and Myrsinoic acid C **24** were obtained. The structure elucidation of these compounds is explained in the following sections.

### 4.1.1 Structure elucidation of compounds 10, 13 and 21

These compounds were obtained as an oily dark mixture with a mass of 213.1 mg and an extended purple spot was observed under 254 nm UV irradiation on TLC. They were not resolved individually into purified forms due to their minimal amounts and close $R_f$ values. The analysis of their NMR spectra indicated that they were three closely related alkaloids, that is, **10**, **13** and **21**. The difference between these three compounds was determined to be the substituent at C-6 (see figures 8, 10 and 12). The 1D and 2D NMR spectral data of **10** are summarized in table 1.

Compound **10** had eighteen carbon atoms, nineteen hydrogen atoms and hence the molecular formula $C_{18}H_{19}NO_5$ with ten hydrogen deficiencies. The $^{13}C$ APT NMR spectrum showed the presence of four aromatic CH signals at δ 142.5, 104.7, δ102.5 and 106.4. A total of seven aromatic quaternary carbons at δ102.2, 155.9, 112.7, 146.7, 152.9, 142.3, 163.9 and a non-aromatic one at δ58.6 were observed. Two methoxy carbons were also observed at δ55.9 and 58.9.

The HSQC spectrum showed correlations of the carbon atoms with the protons directly attached to them. From the spectrum there were correlations between protons at δ7.47, 6.96, 7.43, 7.24, 4.12, 3.17, 1.30, 1.28, 4.32, 3.90 and C-2, C-3, C-5, C-8, C-1', C-2', C-4',C-5', 4-OMe, 7-OMe respectively. The 4-Omethyl group at δ 4.32 is characteristic for furoquinoline alkaloids. However, the coupling constants and the proton multiplicities were not determined as the H spectrum had distorted baseline with presence of broad peaks (Appendix 36).

The proton-carbon HMBC spectrum showed proton correlations with carbon atoms which are two bonds or three bonds away. This helps in identification of carbon atoms which are next to each other and those which are two bonds away from each other.

**Table 1: NMR data of compound 10**

| Carbon | $^{13}C(\delta)$ | $^{1}H(\delta)$ | APT | COSY | HMBC | $^{13}C(\delta)$ Literature* |
|---|---|---|---|---|---|---|
| 2 | 142.5 | 7.47 | CH | 3 | 3, 3a, 9a | 142.7 |
| 3 | 104.7 | 6.96 | CH | 2 | 2, 3a, 9a | 104.9 |
| 3a | 102.2 | - | C | - | - | 102.2 |
| 4 | 155.9 | - | C | - | - | 155.9 |
| 4a | 112.7 | - | C | - | - | 113.1 |
| 5 | 102.5 | 7.43 | CH | - | 4, 4a, 6, 7, 8a | 102.7 |
| 6 | 146.7 | - | C | - | - | 147.0 |
| 7 | 152.9 | - | C | - | - | 153.1 |
| 8 | 106.4 | 7.24 | CH | - | 4a, 6, 7, 8a | 107.2 |
| 8a | 142.3 | - | C | - | - | 143.1 |
| 9a | 162.9 | - | C | - | - | 163.5 |
| 1’ | 67.8 | 4.12 | $CH_2$ | 2’ | 6, 2’, 3’ | 68.1 |
| 2’ | 61.2 | 3.17 | CH | 1’ | 1’, 3’, 4’, 5’ | 61.6 |
| 3’ | 58.6 | - | C | - | - | 58.4 |
| 4’ | 24.5 | 1.30 | $CH_3$ | - | 2’, 3’, 5’ | 24.9 |
| 5’ | 19.0 | 1.28 | $CH_3$ | - | 2’, 3’, 4’ | 19.4 |
| 4-OMe | 58.9 | 4.32 | $CH_3$ | - | 4 | 59.2 |
| 7-OMe | 55.9 | 3.90 | $CH_3$ | - | 7 | 56.3 |

*Adnan *et al*., 2003

From the HMBC spectrum the proton H-2 absorbing at δ 7.47 showed correlation with aromatic carbons C-3 and C-3a which are one bond away and a quaternary carbon, C-9a, which is two bonds away. Similarly, H-3 absorbing at δ6.96 correlated with carbons at position C-2, C-3a, and C-9a. The aromatic proton H-5 absorbing at δ7.43 showed correlations with C-4, C-4a,

C-6, C-7 and C-8a while H-8 absorbing at δ7.24 correlated with carbons at position C-4a, C-6, C-7 and C-8a. The protons H-1' (δ1.30) and H-2' (δ1.28) correlates with C-6, C-2', C-3' and C-1', C-3', C-4', C-5', respectively. This helped in confirmation of the substituent at C-6. In the same note, the methoxy protons at δ 4.32 and δ3.90 also helped in confirmation of these methoxy groups to their respective positions as they correlated with C-4 and C-7, respectively.

The proton-proton COSY correlation for the compound **10** was also determined. COSY spectrum gave information on protons which are attached to adjacent carbons. Aromatic protons H-2 (δ 7.47) and H-3 (δ6.96) correlated with each other. Protons H-1' (δ4.12) and H-2' (δ 3.17) also showed correlations. The structure is shown in figure 8.

The high resolution positive electron impact mass spectrometry (HREIMS) of this compound at 9.55 minutes retention time showed a molecular ion peak at *m/z* 330.13 ($[M+H]^+$) (calculated for $[C_{18}H_{19}NO_5 + H]$; *m/z* 330.14). The mass spectrum of the compound is shown in figures 9.

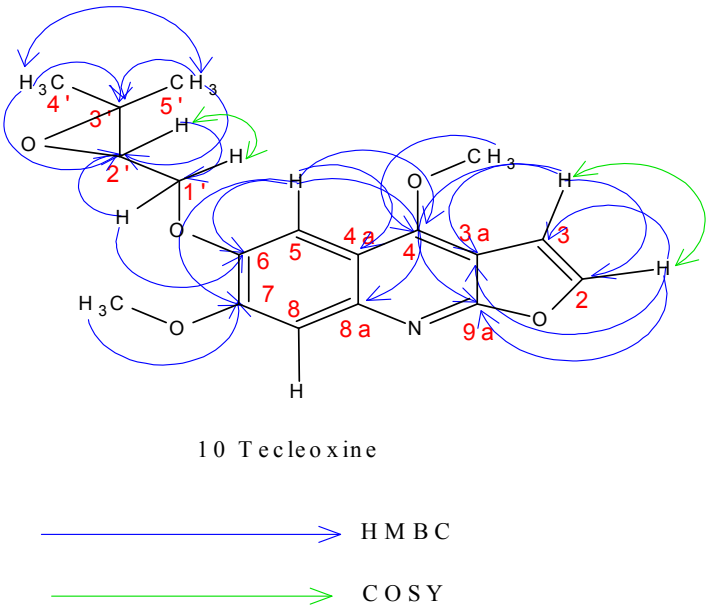



**Figure 8: Structure of compound 10 showing COSY and HMBC**

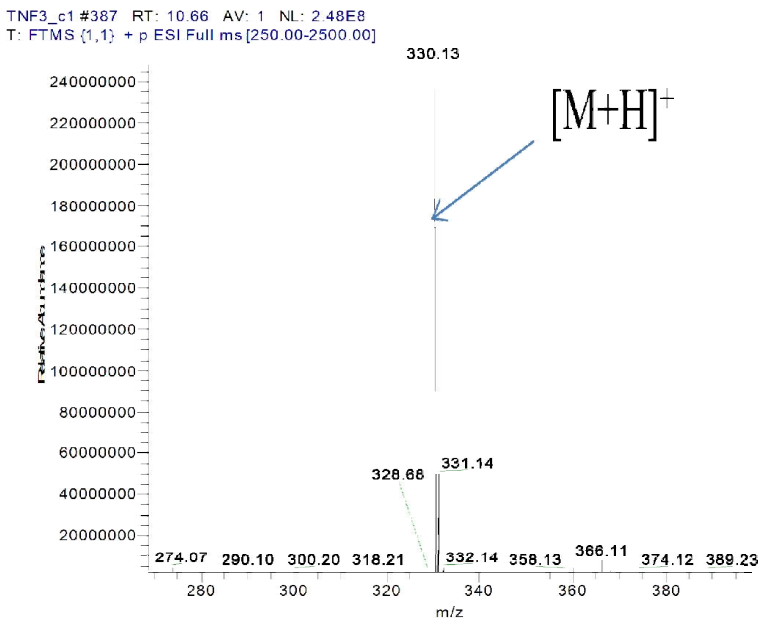



**Figure 9:** Mass spectrum of compound 10

### 4.1.2 Structure elucidation of compound 13

The NMR spectra of this compound were similar to that of compound **10** with the difference only being in the substituent at C-6. From the $^{13}C$ APT NMR spectrum, the substituent had six carbons, one CH (C-1'- δ68.3), one $CH_2$ (C-2'- δ71.0), one Cq (C-3'- δ71.8), two $CH_3$ (C-4'- δ26.4 and C-5'- δ26.8) and one methoxy carbon (3'-OMe- δ52.5). The 1D and 2D NMR spectral data are summarized in table 2.

HSQC spectrum showed that the protons absorbing at δ3.42/3.37, δ3.63, δ1.34 and δ 3.62 correlated with carbon atoms C-1', C-2', C-4'/C-5' and C-3'-OMe respectively. The proton-proton COSY showed correlations between protons H-1' and H-2' absorbing at δ3.42 and δ3.63 respectively. The HMBC spectrum showed proton H-1' absorbing at δ3.42/3.37 correlated with carbons C-6, C-2' and C-3'. Proton H-2', absorbing at δ3.63 correlated with carbons C-1', C-3', C-4', C-5'and protons H-4' and H-5' absorbing at δ1.34 correlated with carbons C-2' and C-3'. The methoxy protons absorbing at δ3.62 also showed correlation with carbon C-3'. This helped in confirmation of the position of the methoxy group in the substituent.

**Table 2: NMR data of compound 13**

| Carbon | $^{13}C(\delta)$ | $^{1}H(\delta)$ | APT | COSY | HMBC | $^{13}C(\delta)$ Literature* |
|---|---|---|---|---|---|---|
| 2 | 142.5 | 7.47 | CH | 3 | 3, 3a, 9a | 142.2 |
| 3 | 104.7 | 6.96 | CH | 2 | 2, 3a, 9a | 104.3 |
| 3a | 102.2 | - | C | - | - | 101.9 |
| 4 | 155.9 | - | C | - | - | 155.4 |
| 4a | 112.7 | - | C | - | - | 112.7 |
| 5 | 102.5 | 7.43 | CH | - | 4, 4a, 6, 7, 8a | 102.3 |
| 6 | 146.7 | - | C | - | - | 146.8 |
| 7 | 152.9 | - | C | - | - | 152.7 |
| 8 | 106.4 | 7.24 | CH | - | 4a, 6, 7, 8a | 106.2 |
| 8a | 142.3 | - | C | - | - | 142.6 |
| 9a | 162.9 | - | C | - | - | 163.0 |
| 1’ | 71.0 | 3.42, 3.37 | $CH_2$ | 2’ | 6, 2’, 3’ | 70.1 |
| 2’ | 68.3 | 3.63 | CH | 1’ | 1’, 3’, 4’, 5’ | 74.6 |
| 3’ | 71.8 | - | C | - | - | 75.9 |
| 4’ | 26.4 | 1.34 | $CH_3$ | - | 2’, 3’, 5’ | 21.1 |
| 5’ | 26.8 | 1.34 | $CH_3$ | - | 2’, 3’, 4’ | 20.4 |
| 3’-OMe | 52.5 | 3.62 | $CH_3$ | - | 3’ | 49.2 |
| 4-OMe | 58.9 | 4.32 | $CH_3$ | - | 4 | 58.6 |
| 7-OMe | 55.0 | 3.90 | $CH_3$ | - | 7 | 55.7 |

*Adnan *et al*., 2003

The compound was identified as with molecular ion peak *m/z* 362.2 ($[M+H]^+$) (calculated for [$C_{19}H_{23}NO_6$+ H] (*m/z* 362.15) using high resolution positive electron impact mass spectrometry (HREIMS) at 10.62 minutes retention time. The mass spectrum of the compound is shown in figure 11.

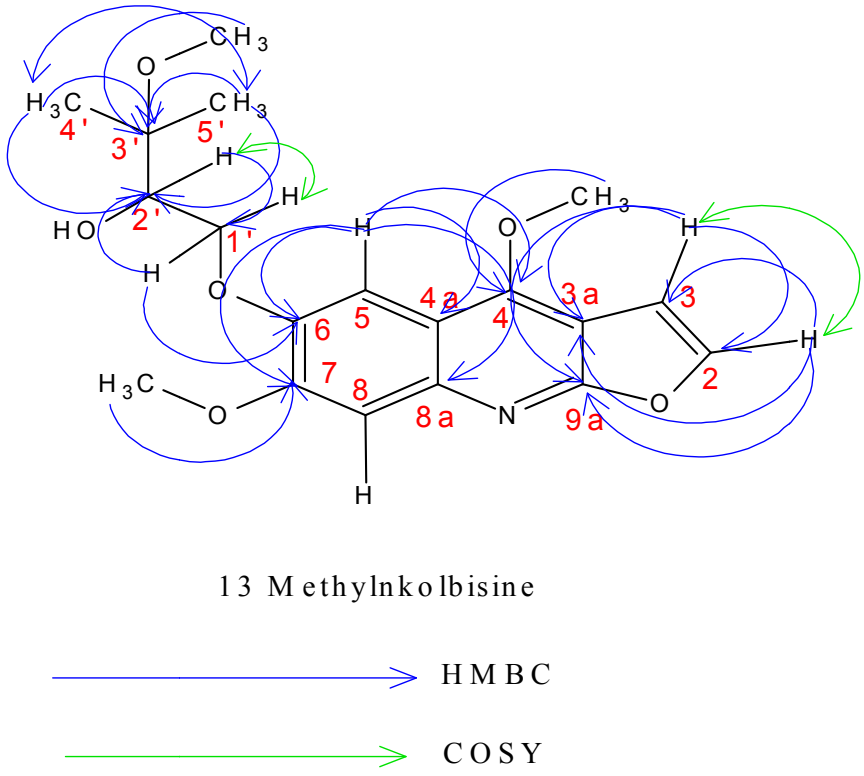



**Figure 10: Structure of compound 13 showing COSY and HMBC**

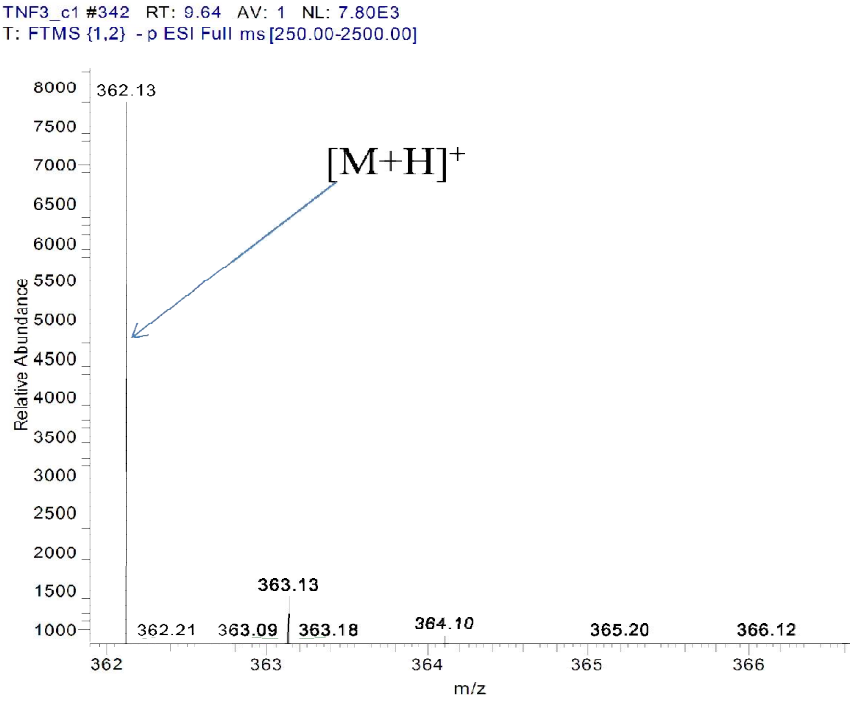



**Figure 11: Mass spectrum of compound 13**

### 4.1.3 Structure elucidation of compound 21

The NMR spectra of this compound were similar to that of compound **10** with the difference only being in the substituent at C-6. From the $^{13}C$ APT NMR spectrum, the substituent had one methoxy carbon (6-OMe) absorbing at δ52.5. The position of the methoxy group was confirmed using HMBC in which the proton 6-OMe absorbing at δ 3.73 showed correlation with carbon C-6. The structure of the compound is shown in figure 12 below. The 1D and 2D spectral data are summarized in table 3.

The compound was identified as with molecular ion peak *m/z* 260.1 ($[M+H]^+$) (calculated for $[C_{14}H_{13}NO_4 + H]$ (*m/z* 260.3) using high resolution positive electron impact mass spectrometry (HREIMS) at 10.62 minutes retention time. The mass spectrum of the compound is shown in figure 13.

**Table 3: NMR data of compound 21**

| Carbon | $^{13}C(\delta)$ | $^{1}H(\delta)$ | APT | COSY | HMBC | $^{13}C(\delta)$ Literature* |
|---|---|---|---|---|---|---|
| 2 | 142.5 | 7.47 | CH | 3 | 3, 3a, 9a | 142.7 |
| 3 | 104.7 | 6.96 | CH | 2 | 2, 3a, 9a | 104.8 |
| 3a | 102.2 | - | C | - | - | 102.4 |
| 4 | 155.9 | - | C | - | - | - |
| 4a | 112.7 | - | C | - | - | 112.9 |
| 5 | 102.5 | 7.43 | CH | - | 4, 4a, 6, 7, 8a | 100.2 |
| 6 | 146.7 | - | C | - | - | - |
| 7 | 152.9 | - | C | - | - | - |
| 8 | 106.4 | 7.24 | CH | - | 4a, 6, 7, 8a | 105.8 |
| 8a | 142.3 | - | C | - | - | 142.7 |
| 9a | 162.9 | - | C | - | - | - |
| 4-OMe | 58.9 | 4.32 | $CH_3$ | - | 4 | 59.1 |
| 6-OMe | 56.3 | 3.73 | $CH_3$ | - | 6 | 56.2 |
| 7-OMe | 55.0 | 3.90 | $CH_3$ | - | 7 | 56.0 |

*Adnan *et al.,* 2003

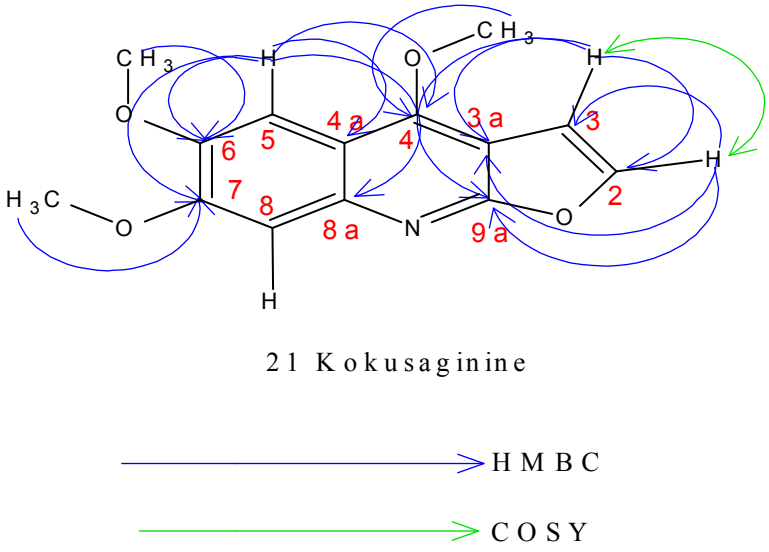



**Figure 12: Structure of compound 21 showing COSY and HMBC**

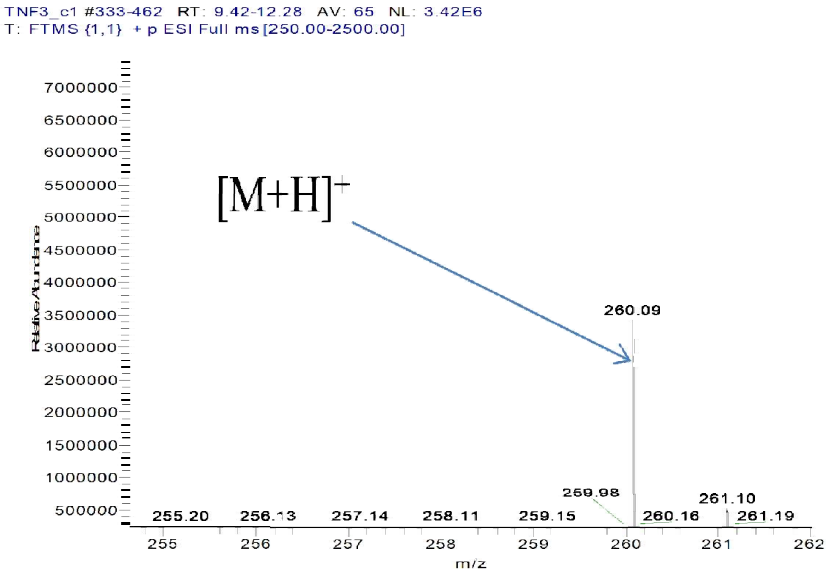



**Figure 13: Mass spectrum of compound 21**

### 4.1.4 Structure elucidation of compound 22

The compound **22** was obtained as an oily dark substance with a mass of 192.6 mg. The $^{13}C$ APT and $^{1}H$ NMR spectra of the compound, like the previously identified compounds, showed great similarity with the compound **10** with the major difference being on the substituent at C-6. The 1D and 2D spectral data are summarized in table 4.

The $^{13}C$ APT NMR spectrum showed the presence of five carbon atoms attributed to the substituent at C-6. These included a CH signal at δ75.2, a $CH_2$ at δ70.9, a Cq at δ72.1 and two $CH_3$ at δ26.4 and δ25.9. From HSQC spectrum, there was clear correlations between, C-1' and the proton absorbing at δ4.31/4.14, C-2' and the proton absorbing at δ3.83, C-4' and the proton absorbing at δ1.29 and C-5' and the proton absorbing at δ1.27. However, the coupling constants and the proton multiplicities were not determined as the H spectrum had distorted baseline with presence of broad peaks (Appendix 37).

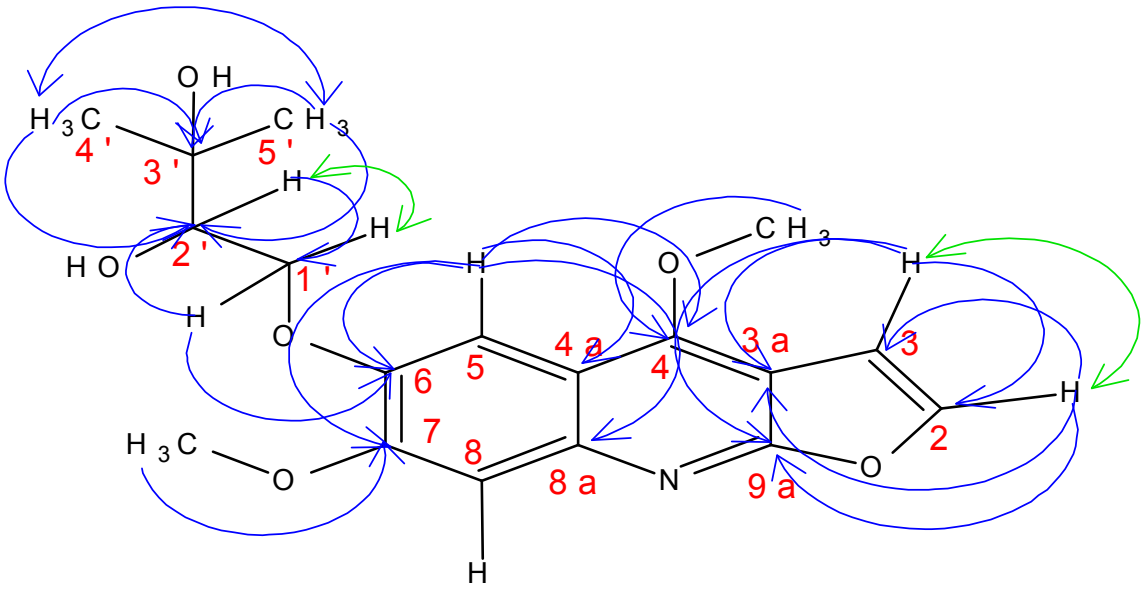
**Table 4: NMR data of compound 22**

| **Carbon** | **$^{13}C$(δ)** | **$^{1}H$(δ)** | **APT** | **COSY** | **HMBC** | **$^{13}C$(δ)# Literature(DMSO)** |
|---|---|---|---|---|---|---|
| 2 | 142.5 | 7.52 | CH | 3 | 3, 3a, 9a | 143.6 |
| 3 | 104.8 | 7.01 | CH | 2 | 2, 3a, 9a | 105.9 |
| 3a | 102.3 | - | C | - | - | 102.5 |
| 4 | 155.9 | - | C | - | - | 155.7 |
| 4a | 112.9 | - | C | - | - | 112.8 |
| 5 | 102.1 | 7.42 | CH | - | 4, 4a, 6, 7, 8a | 101.4 |
| 6 | 146.6 | - | C | - | - | 147.7 |
| 7 | 152.6 | - | C | - | - | 153.1 |
| 8 | 106.4 | 7.28 | CH | - | 4a, 6, 7, 8a | 107.0 |
| 8a | 142.3 | - | C | - | - | 142.3 |
| 9a | 163.0 | - | C | - | - | 163.1 |
| 1' | 70.9 | 4.31, 4.14 | $CH_2$ | 2' | 6, 2', 3' | 71.6 |
| 2' | 75.2 | 3.83 | CH | 1' | 1', 3', 4', 5' | 76.3 |
| 3' | 72.1 | - | C | - | - | 71.0 |
| 4' | 26.4 | 1.29 | $CH_3$ | - | 2', 3', 5' | 24.8 |
| 5' | 25.9 | 1.27 | $CH_3$ | - | 2', 3', 4' | 27.9 |
| 4-OMe | 58.9 | 4.38 | $CH_3$ | - | 4 | 59.8 |
| 7-Ome | 56.0 | 3.93 | $CH_3$ | - | 7 | 56.2 |

# Cao *et al*., 2009

The proton-carbon HMBC spectrum showed that the proton H-1' absorbing at δ4.31/4.14 correlated with carbons C-6, C-2'and C-3'. Similarly, the proton absorbing at δ3.83 correlated with carbons C-1', C-3', C-4' and C-5'. There was also correlations between proton absorbing at δ1.29 and carbons C-2', C-3' and C-5'. The carbons C-2', C-3' and C-4' also had strong correlation with proton absorbing at δ1.27. The proton-proton COSY correlation showed correlations between H-1' and H-2' which absorbed at δ4.31/4.14 and δ3.83 respectively. The structure of the compound is shown in figure 14.

The compound was identified as with molecular ion peak *m/z* 348.1 ($[M+H]^+$) (calculated for $[C_{18}H_{22}NO_6 + H]$ (*m/z* 348.4) using high resolution positive electron impact mass spectrometry (HREIMS) at 9.55 minutes retention time. The mass spectrum of the compound is shown in figure 15.




**2 2** N k o lb is in e

H M B C

C O S Y

**Figure 14: Structure of compound 22 showing COSY and HMBC**

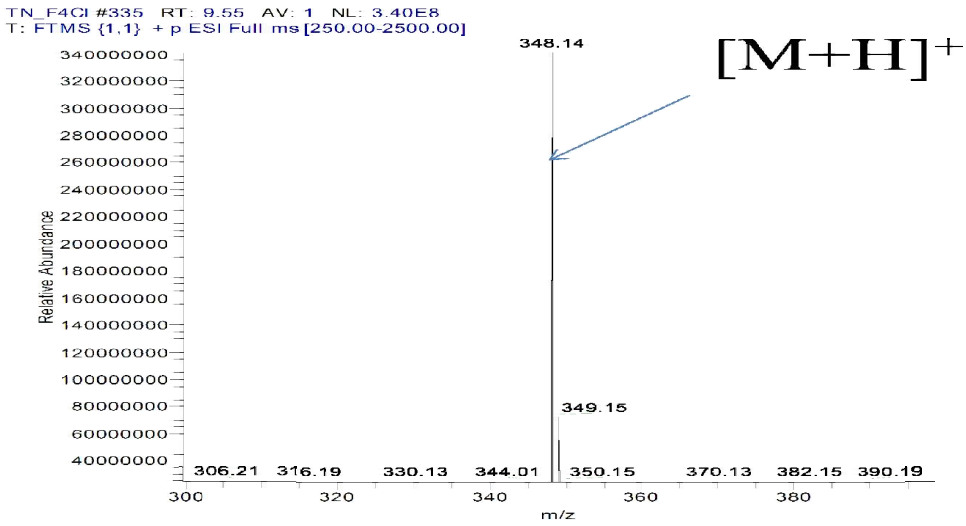



**Figure 15: Mass spectrum of Compound** 22

The compounds **10, 13** and **21** were confirmed to be alkaloids Tecleoxine, Methylnkolbisine and Kokusaginine earlier isolated from *T. nobilis* (Adnan *et al*., 2003). The compounds were confirmed to be the actual compounds by comparison of their spectroscopic data and their mass spectrometric data with that from the literature. Kokusagine has also been isolated from the plants *Melicope bonickii, Haplophylum species* and *Esenbeckia leiocarpa* (Komala *et al*., 2006 Elaine *et al*., 2010; Ulubelen and Oztork, 2008). These compounds have also been isolated from other plants of the family Rutaceae including the genus *Teclea* which is well endowed with furoquinoline alkaloids (Tarus, 2005; Rios *et al*., 2002; Ayafor and Okogun, 1982; Riyanto *et al*., 2001; Terezen *et al.,* 2010; Wansi *et al*., 2008; Kiplimo, 2012).

Compound **22** was identified as Nkolbisine. This was determined by comparison of its NMR and mass spectral data with those reported in literature (Cao *et al*., 2009). This compound has been isolated in other plants (Ulubelen and Oztork, 2008; Kiplimo, 2012) but it is the first time it is being reported in *T. nobilis*.

### 4.1.5 Structure elucidation of compound 23

The compound **23** was obtained as an oily yellow substance with a mass of 450.2mg. The analysis of APT NMR spectra of this compound revealed the presence of 22 carbon atoms with ten of them being olefinic inclusive those of the benzene ring. Of these there were two aromatic

methine signals at δ124.2 and δ131.3. Two other olefinic methine signals at δ121.5 and δ124.2 and a hydroxymethine at δ89.3 were also observed. There were also a total of eight quarternary carbon atoms absorbing at δ73.9, 121.8, 122.9, 127.1, 31.9, 132.9, 162.4 and 171.5. Moreover, a total of four methylene carbon atoms absorbing at δ21.9, 28.3, 29.9 and 37.6 were observed. Five methyl carbon atoms absorbing at δ17.6, 17.8, 22.2, 25.7 and 25.7 were also observed.

The HSQC spectrum showed correlations of the protons to the carbon atoms in which they were directly bonded. From the spectrum the protons absorbing at δ4.77, 3.13/3.25, 7.72, 7.73, 1.55/1.63, 2.09/2.20, 5.12, 1.67, 1.29, 1.72, 3.26/3.31, 5.29, 1.73 and 1.63 and the carbons absorbing at δ89.3, 29.9, 124.9, 131.3, 37.6, 21.9, 124.2, 25.7, 22.2, 17.8, 28.3, 121.5, 25.7 and 17.6, respectively showed correlations.

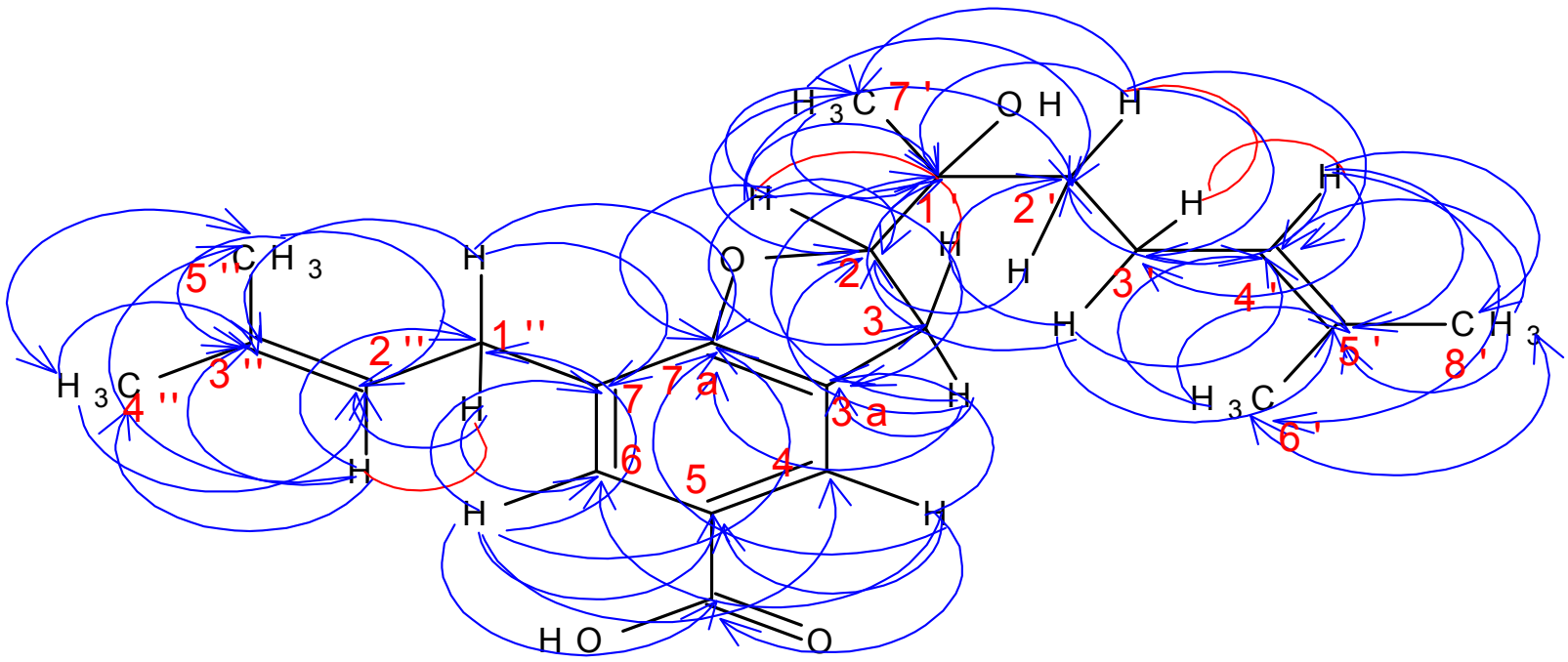
The HMBC spectrum helps in identification of carbon atoms which are next to each other and those two bonds away. These correlations are shown in table 5 and figure 16 below. The COSY spectrum showed correlations of H-2 and H-3, H-3' and H-4' as well as H-1'' and H-2''. The protons that showed correlations indicated that they were bonded to adjacent carbon atoms. A summary of the NMR spectral data is shown in table 5.

The compound was determined to be with eight hydrogen deficiencies thus the molecular formula $C_{22}H_{30}O_4$. It was identified as with molecular ion peaks *m/z* 359.22 ($[M+H]^+$) (calculated for [$C_{22}H_{30}O_4$ + H] (*m/z* 359.47), *m/z* 381.20 ($[M+Na]^+$) (calculated for [$C_{22}H_{30}O_4$ + Na] (*m/z* 381.47) and *m/z* 397.20 ($[M+K]^+$) (calculated for [$C_{22}H_{30}O_4$ + K] (*m/z* 397.47), using high resolution positive electron impact mass spectrometry (HREIMS) at 10-12 minutes retention time. The mass spectra of the compound are shown in figures 17, 18 and 19. The structure of the compound is shown in figure 16.

**Table 5: NMR data of compound 23**

| No. | $^{13}C(\delta)$ | $^{1}H(\delta)$ | APT | COSY | HMBC | $^{13}C(\delta)$# |
|---|---|---|---|---|---|---|
| 2 | 89.3 | 4.74 t, J= 8.99Hz | CH | 3 | 3,3a,,7a,1',7',2' | 89.6 |
| 3 | 29.9 | 3.13 dd, J= 9.76, 9.50 Hz 3.25 dd, J= 8.22, 7.70 Hz | $CH_2$ | 2 | 2,3a,7a,4,1' | 29.9 |
| 3a | 121.8 | - | C | - | - | 123.1 |
| 4 | 124.9 | 7.72 s | CH | - | 3,3a,5.6,7a,**C**OOH | 131.4 |
| 5 | 122.9 | - | C | - | - | 121.8 |
| 6 | 131.3 | 7.73 s | CH | - | 4,5,7,7a,1'', **C**OOH | 125.0 |
| 7 | 127.1 | - | C | - | - | 127.2 |
| 7a | 162.4 | - | C | - | - | 162.4 |
| 1' | 73.9 | - | C | - | - | 73.8 |
| 2' | 37.6 | 1.55, 1.63 m | $CH_2$ | 3' | 2,1',7',3'4' | 37.1 |
| 3' | 21.9 | 2.09,2.20 m | $CH_2$ | 2',4' | 1',2',4'5' | 22.0 |
| 4' | 124.2 | 5.12 t, J= 6.94, 7.19Hz | CH | 3' | 2',3',5'6', 8' | 124.1 |
| 5' | 132.9 | - | C | - | - | 132.2 |
| 6' | 25.7 | 1.67 s | $CH_3$ | - | 4',5',8' | 25.7 |
| 7' | 22.2 | 1.29 s | $CH_3$ | - | 2, 1',2' | 22.6 |
| 8' | 17.8 | 1.72 s | $CH_3$ | - | 4',5',6' | 17.7 |
| 1'' | 28.3 | 3.26,3.31 m | $CH_2$ | 2'' | 6,7,7a,2'', 3'' | 28.3 |
| 2'' | 121.5 | 5.29 t, J=7.45, 7.19 Hz | CH | 1'' | 1'',3'',4'',5'' | 121.4 |
| 3'' | 131.9 | - | C | - | - | 133.2 |
| 4'' | 25.7 | 1.73 s | $CH_3$ | - | 2'',3''5'' | 25.8 |
| 5'' | 17.6 | 1.62 s | $CH_3$ | - | 2'',3'',4'' | 17.9 |
| **C**OOH | 171.5 | - | C | - | - | 172.0 |

# Hirota *et al*., (2002)




**2 3** M Y R S IN O IC A C ID B

H M B C

C O S Y

**Figure 16: Structure of compound 23 showing COSY and HMBC**

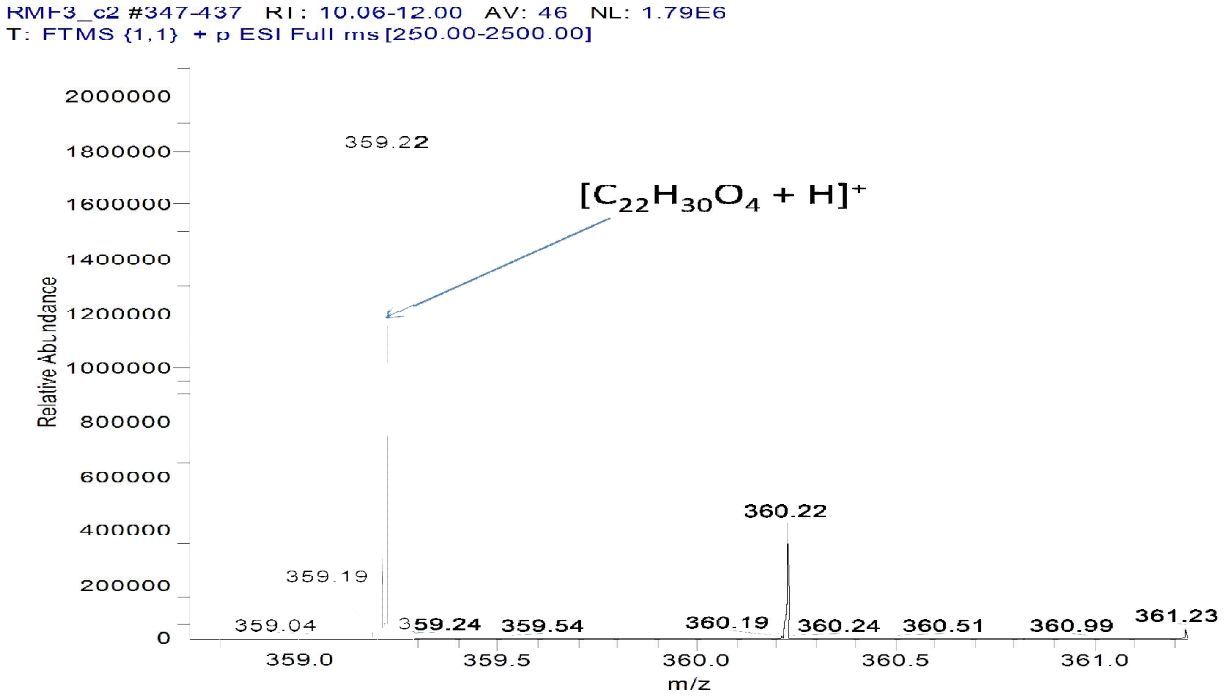



**Figure 17: M+H mass spectrum of compound 23**

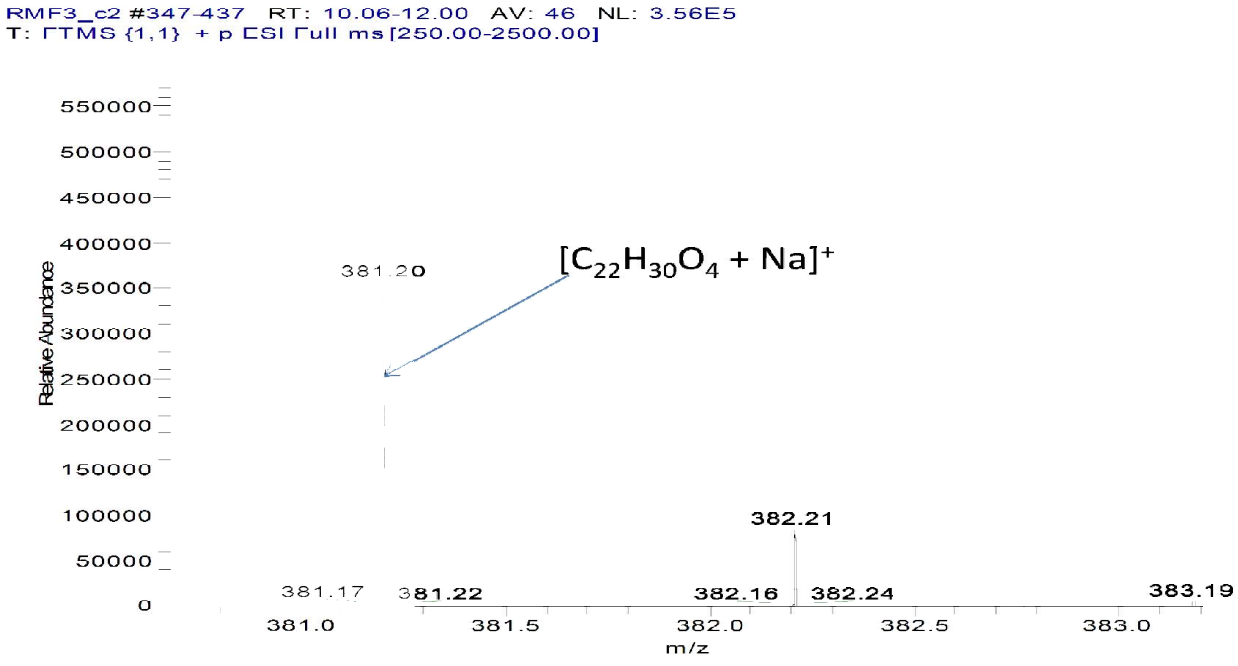



**Figure 18: M+Na mass spectrum of compound 23**

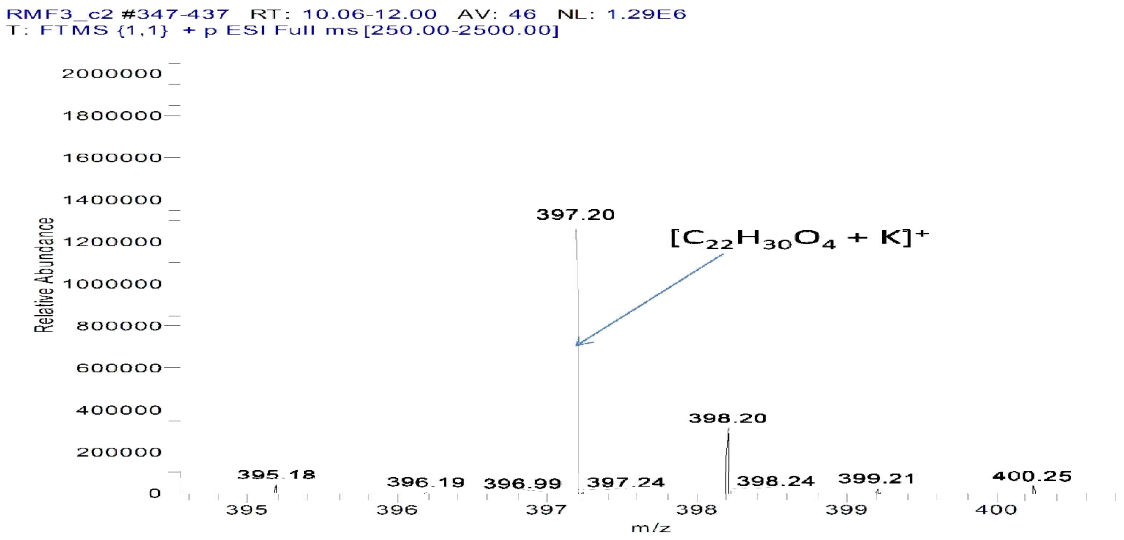



**Figure 19: M+K mass spectrum of compound 23**

### 4.1.6 Structure elucidation of compound 24

The compound **24** was obtained as an oily yellow substance with a mass of 345.3mg. The analysis of NMR spectra of this compound revealed that the compound was closely related to the compound **23**. The 1D and 2D NMR spectral data of **24** are summarized in table 6.

The compound **24** was identified to have twenty two carbon atoms and thirty two protons and hence the molecular formula $C_{22}H_{30}O_4$. The APT indicated two aromatic methine signals at δ131.3 and 124.9. Three other methane signals were observed at δ121.2, 124.1 and 67.8. A total of eight quaternary carbons at δ76.9, 127.1, 123.0, 133.0, 162.3, 132.0, and δ123.2 were observed. Four methylene signals were observed at δ21.6, 37.4, 29.5 and 29.9 and five methyl signals absorbing at δ17.8, 25.8, 22.3, 17.9 and 25.7.

The HSQC spectrum showed correlations between protons resonating at δ3.91, 1.32/3.13, δ7.73, 1.53/1.63, 2.06/2.17, 5.11, 1.71, δ1.73, 1.20/1.30, 3.32, 5.30, 1.61 and 1.68 with carbon atoms absorbing at δ67.8, 29.5, 124.9/131.3, 37.4, 21.7, 124.1, 17.9, 25.7, 22.3, 29.9, 121.1, 17.8 and 25.8, respectively. This implied that these protons were attached to their respective carbons in which they showed correlations. However, the coupling constants and the proton multiplicities were not determined as the H spectrum had distorted baseline with presence of broad peaks (Appendix 27).

The HMBC and COSY correlations observed are shown in the figure 20 and also in table 6. The HMBC correlations indicated the carbons that are adjacent to each other and those two bonds away while COSY confirmed the protons bonded to two adjacent carbon atoms (see figure 20).

**Table 6: NMR data of compound 24**

| Carbon | $^{13}C(\delta)$ | $^{1}H(\delta)$ | APT | COSY | HMBC | $^{13}C(\delta)$ Literature# |
|---|---|---|---|---|---|---|
| 2 | 76.9 | - | C | - | - | 79.8 |
| 3 | 67.8 | 3.91 | CH | 3 | - | 67.9 |
| 4 | 29.5 | 1.32,3.13 | $CH_2$ | 4 | 2,3,4a,8a,5 | 31.1 |
| 4a | 123.2 | - | C | - | - | 120.9 |
| 5 | 124.9 | 7.73 | CH | - | 4a,8a,7,6, COOH | 129.8 |
| 6 | 123.0 | - | C | - | - | 118.6 |
| 7 | 131.3 | 7.73 | CH | - | 5,6,8,8a,1'', COOH | 130.6 |
| 8 | 133.0 | - | C | - | - | 130.2 |
| 8a | 162.3 | - | C | - | - | 155.5 |
| 1' | 37.4 | 1.53,1.62 | $CH_2$ | 2' | 2,2',3',7' | 37.6 |
| 2' | 21.7 | 2.06,2.17 | $CH_2$ | 1',3' | 2,1',3',4' | 21.7 |
| 3' | 124.1 | 5.11 | CH | 2' | 1',2',3',4'5',6' | 123.8 |
| 4' | 132.0 | - | C | - | - | 132.3 |
| 5' | 17.9 | 1.71 | $CH_3$ | - | 3',4',6' | 17.6 |
| 6' | 25.7 | 1.73 | $CH_3$ | - | 3',4',5' | 25.6 |
| 7' | 22.3 | 1.20,1.30 | $CH_3$ | - | 2,3,1'2' | 19.0 |
| 1'' | 29.9 | 3.32 | $CH_2$ | 2'' | 7,8,8a,2'' | 28.5 |
| 2'' | 121.2 | 5.30 | CH | 1'' | 1'',3'',5'',4'' | 121.9 |
| 3'' | 127.1 | - | C | - | - | 132.8 |
| 4'' | 17.8 | 1.61 | $CH_3$ | - | 2'',3''5'' | 17.9 |
| 5'' | 25.8 | 1.68 | $CH_3$ | - | 2'',3'',4'' | 25.8 |
| COOH | 171.4 | - | C | - | - | 171.7 |

# Hirota *et al*., (2002)

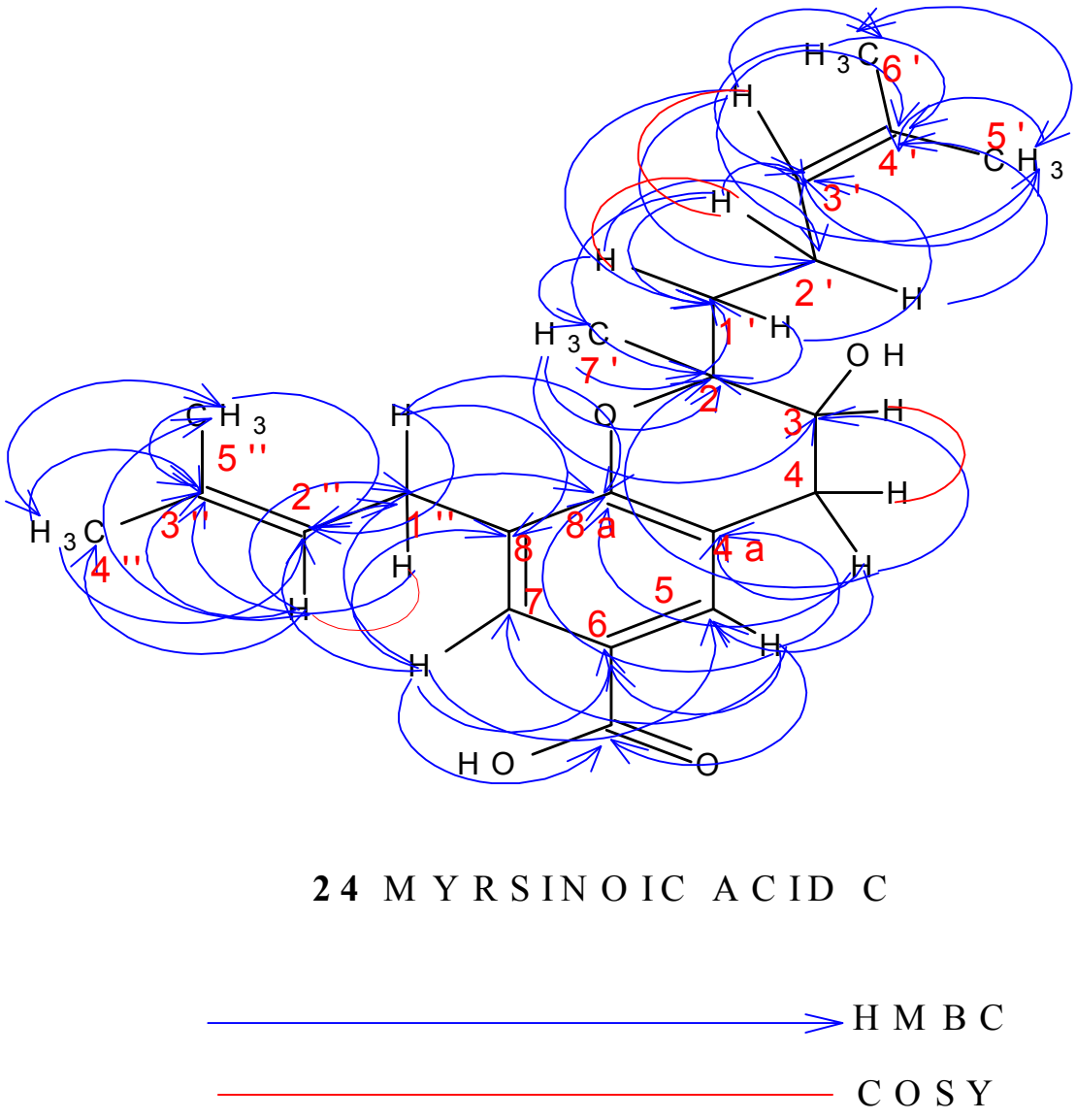



**Figure 20: Structure of compound 24 showing COSY and HMBC**

The compound was identified as with molecular ion peaks *m/z* 359.21 ($[M+H]^+$) (calculated for $[C_{22}H_{30}O_4 + H]$ (*m/z* 359.47) and *m/z* 397.20 ($[M+K]^+$) (calculated for $[C_{22}H_{30}O_4 + K]$ (*m/z* 397.47), using high resolution positive electron impact mass spectrometry (HREIMS) at 10-11 minutes retention time. The mass spectra of the compound are shown in figures 21 and 22. The structure of the compound is shown in figure 20.

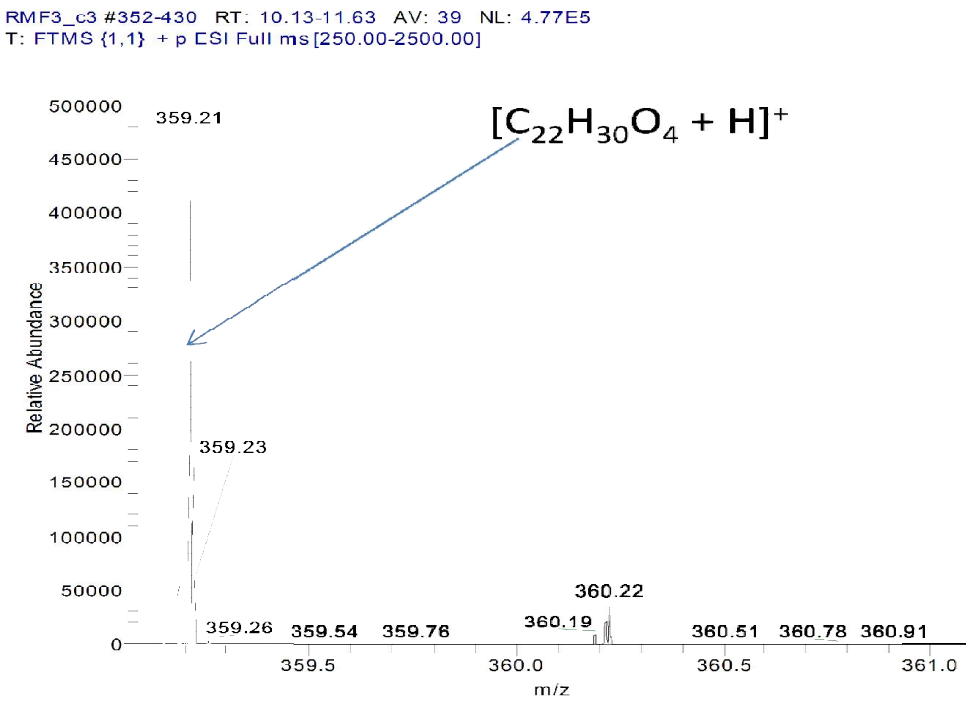



**Figure 21: M+K mass spectrum of compound 24**

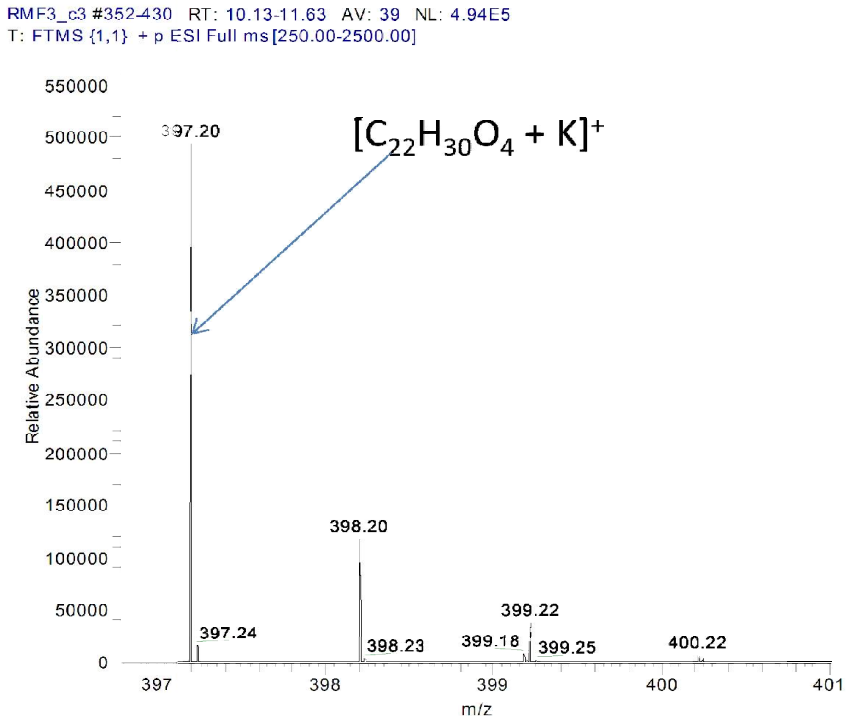



**Figure 22: M+H mass spectrum of compound 23**

These compounds **23** and **24** were confirmed, by comparison of their spectral data and that in literature, to be Myrsinoic acid B and Myrsinoic acid C, respectively. These compounds had previously been isolated from other plants of the family Myrsinaceae including the genus *Rapanea* and proven to have a range of biological activity. These plants include *Rapanea Umbellate, Myrsine seguinii, Rapanea ferruginea, Myrsine coriacea, Myrsine floculosa* and *Gaballeria ferruginea* (Januario *et al*., 1991; Hirota *et al*., 2002; Ito *et al*., 2008; Hess *et al*., 2010; Cruz *et al*., 2013). However, these compounds are reported here for the first time from the plant *R. melanophloeos* and shown to have anti-helmintic activity against *S. mansoni*.

### 4.2 Miracidicidal assay of the methanol crude extract of *T. nobilis*

The *S. mansoni* miracidia were subjected to methanol crude extract in triplicate. The test solutions were prepared as described in section 3.4.2. The percentage mortality of the miracidia after 30 minutes exposure to the various serial dilutions is listed in table 1. IBM SPSS 15.0 software was used to calculate $LC_{90}$ and $LC_{50}$ using Log probit regression analysis at 95% confidence level. The negative controls used were 1% DMSO and dechlorinated water which showed no activity against the miracidia after 30 minutes. The positive control was praziquantel that gave a mortality of 100% at 1000 ppm. The results on table 7 shows that there is a positive correlation between the percentage mortality values and the crude extract concentrations. That is, the percentage mortality depends on the concentration of the extract with 1000 ppm having 100% mortality and 50ppm with the lowest mortality of 1.7%. Through log probit regression analysis at 95% confidence level, the crude extract had $LC_{50}$ and $LC_{90}$ values as 261.69 and 575.74 ppm, respectively. Thus, it's evident that there are miracidicidal compounds in the methanol crude extract.

These results have correlations with other extracts isolated from other plants. The plants *Zanthoxylum naranjillo* (Braguine *et al*., 2009) and *Haplophyllum tuberculatum*, both from Rutaceae family have been reported to have reduced egg production by *S. mansoni* worm and reduced shedding of cercariae by *Biomphalaria alaxandrina* snails, respectively (Rizk *et al.,* 2012). Mohamed *at al.,* (2005) and Abozeid *et al*., (2012) also indicated that sativa seeds and *Punica granatum* extracts, respectively, can be used to control *S. mansoni* miracidia. Similarly other plants from different families have also been reported to have schistosomicidal activity at various stages of *S. mansoni* life cycle (Muchika, 2010; Parrakh, 2010; Ismail *et al*., 2007).

**Table 7: Miracidicidal activity of *T. nobilis* methanol crude extract**

| Conc. (ppm) | Mean mortality (%) | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 500 | 96.7±2.4 | | |
| 450 | 81.7±2.4 | | |
| 400 | 70±0 | | |
| 350 | 61.7±2.4 | | |
| 300 | 55±2.4 | 261.69 (228.79-296.47) | 575.74 (474.71-760.53) |
| 250 | 43.3±4.7 | | |
| 200 | 30±4.1 | | |
| 150 | 20±4.1 | | |
| 100 | 8.3±2.4 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel | 100±0.0 | | |

a- Negative control, b – Positive control

### 4.3 Miracidicidal assay of *T. nobilis* ethyl acetate and hexane extracts

The *T. nobilis* methanol crude extract was partitioned among water, ethyl acetate and hexane. The ethyl acetate and hexane extracts were subjected to miracidicidal assays and the results tabulated in tables 8 and 9. The $LC_{50}$ and $LC_{90}$ were obtained through log probit regression analysis at 95 confidence level and reported in tables 8 and 9.

The ethyl acetate and hexane extracts were active against miracidia but to different extents. From table 8 and 9 it is evident that, the percentage mortality at 500 ppm was 88.3% for ethyl acetate extract and 83.3% for hexane extract. At 50 ppm the ethyl acetate had a mortality of 1.7% while hexane did not show any activity at the same concentration. Similarly, the ethyl acetate had an $LC_{50}$ of 320.48 while that observed for hexane extract was 334.70. This indicates that ethyl acetate extracts was slightly more active than hexane extract. However, it was evident

that the two extracts do contain compounds that were quite active against miracidia and thus can be used for the control of the same.

**Table 8: Miracidicidal activity of *T. nobilis* ethyl acetate crude extract**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 98.3 ±2.4 | | |
| 500 | 88.3±2.4 | | |
| 450 | 78.3±4.7 | | |
| 400 | 68.3±2.3 | | |
| 350 | 55 ± 0 | | |
| 300 | 48.3±6.2 | | |
| 250 | 33.3±2.4 | 289.28(252.00-329.65) | 672.79(547.23-940.74) |
| 200 | 25±0 | | |
| 150 | 20±4.1 | | |
| 100 | 11.7±2.4 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

**Table 9: Miracidicidal activity of *T. nobilis* hexane crude extract**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 500 | 83.3±6.2 | | |
| 450 | 75±4.1 | | |
| 400 | 63.3±2.4 | 307.92(273.05- | 638.73(531.23- |
| 350 | 56.7±2.4 | 347.23) | 854.03) |
| 300 | 41.7±2.4 | | |
| 250 | 33.3±2. | | |
| 200 | 25±4.1 | | |
| 150 | 16.7±2.4 | | |
| 100 | 1.7±2.4 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

### 4.4 Miracidicidal assay of fractions from ethyl acetate crude extract

The *T. nobilis* ethyl acetate crude extract was fractionated into four fractions namely; $F_1$, $F_3$, F4 and MeOH fraction through column chromatography on silica gel. These fractions were then subjected to anti-miracidia assay and the results obtained tabulated in tables 10, 11, 12 and 13 respectively. In these tables, also are listed $LC_{50}$ and $LC_{90}$ obtained through log probit regression analysis.

All the fractions were potent against the miracidia to different extents. The fraction $F_3$ was the most active with an $LC_{50}$ and $LC_{90}$ of 254.01 and 467.67 ppm, respectively. This was followed by $F_4$ which had $LC_{50}$ and $LC_{90}$ of 324.69 and 491.86 ppm, respectively. The fraction $F_1$ was less active with $LC_{50}$ and $LC_{90}$ of 439.36 and 787.20 ppm, respectively. The MeOH fraction was the least active with an observed $LC_{50}$ and $LC_{90}$ of 535.86 and 727.54 ppm, respectively. These four fractions generally do have anti-helmintic compounds active against *S.*

*mansoni* miracidia as it is clearly seen from tables 10, 11, 12 and 13. However, fractions $F_3$ and $F_4$, which recorded the highest mortality of the four fractions, were the ones which were purified further to yield pure compounds **10, 13, 21** and **22,** respectively.

**Table 10: Miracidicidal activity of *T. nobilis* ethyl acetate $F_1$**

| Conc.(ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 800 | 96.7±2.4 | | |
| 750 | 88.3±2.4 | | |
| 700 | 80±4.1 | | |
| 650 | 75±0 | | |
| 600 | 71.7±2.4 | 439.36 (402.89- | 787.20 (703.50- |
| 550 | 68.3±2.4 | 475.28) | 922.10) |
| 500 | 61.7±2.4 | | |
| 450 | 51.7±2.4 | | |
| 400 | 45±4.1 | | |
| 350 | 28.3±2.4 | | |
| 300 | 21.7±2.4 | | |
| 250 | 11.7±2.4 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

**Table 11: Miracidicidal activity of *T. nobilis* ethyl acetate $F_3$**

| Conc.(ppm) | Mean % mortality | $LC_{50}$ | $LC_{90}$ |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 500 | 91.7±2.4 | | |
| 450 | 85±0 | | |
| 400 | 81.7±2.4 | 227.28 (195.64- | 519.78 (434.15- |
| 350 | 71.7±2.4 | 258.76) | 680.45) |
| 300 | 65±4.1 | | |
| 250 | 51.7±2.4 | | |
| 200 | 38.3±2.4 | | |
| 150 | 26.7±2.4 | | |
| 100 | 13.3±2.4 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

**Table 12: Miracidicidal activity of *T. nobilis* ethyl acetate $F_4$**

| Conc.(ppm) | Mean % mortality | $LC_{50}$ | $LC_{90}$ |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 500 | 91.7±2.4 | | |
| 450 | 78.3±2.4 | | |
| 400 | 70±0 | 301.30(270.19- | 564.62(482.97- |
| 350 | 58.3±2.4 | 335.21) | 719.07) |
| 300 | 48.3±2.4 | | |
| 250 | 33.3±2.4 | | |
| 200 | 13.3±2.4 | | |
| 150 | 8.3±2.4 | | |
| 100 | 5±4.1 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

**Table 13: Miracidicidal activity of *T. nobilis* ethyl acetate methanol fraction**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 750 | 96.7±2.4 | | |
| 700 | 86.7±2.4 | | |
| 650 | 73.3±2.4 | 520.05(489.09- | 756.59(693.72- |
| 600 | 61.7±2.4 | 551.99) | 860.285) |
| 550 | 50±0 | | |
| 500 | 43.3±4.7 | | |
| 450 | 33.3±2.4 | | |
| 400 | 21.7±2.4 | | |
| 350 | 8.3±2.4 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

## 4.5 Bio-assay of purified compounds isolated from *T. nobilis*

The fractions $F_3$ and $F_4$ were purified as described in section 3.3.5. Fraction $F_3$ yielded a mixture of compounds **10**, **13** and **21** while fraction $F_4$ yielded compound **22**. These compounds were then subjected to miracidicidal tests in three replicate for each serial dilution. Two negative controls of de-chrolinated water and 1% DMSO were also set up which showed no activity. The results observed are tabulated in tables 14 and 15.

**Table 14: Miracidicidal activity of compounds 10, 13 and 21 mixtures**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 1000 | 98.3±2.4 | | |
| 500 | 86.7±2.4 | | |
| 450 | 78.3±2.4 | | |
| 400 | 70.0±0.0 | 270.18(232.55- | 690.93(552.63- |
| 350 | 61.7±2.4 | 312.12) | 979.49) |
| 300 | 48.3±2.4 | | |
| 250 | 41.7±2.4 | | |
| 200 | 30.0±0.0 | | |
| 150 | 20.0±4.1 | | |
| 100 | 8.3±4.1 | | |
| 50 | 3.3±2.4 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

The purified compounds from *T. nobilis* leaves showed activity against miracidia after 30 minutes of exposure. Compound **22**, which had $LC_{50}$ and $LC_{90}$ of 287.97 and 631.73 ppm, respectively, had lower activity than the compounds **10**, **13** and **21** mixtures which had $LC_{50}$ and $LC_{90}$ of 270.18 and 690.93 ppm, respectively. It was also clear that fraction $F_3$ had a higher activity than the purified compounds **10**, **13** and **21** mixtures. This may be due to synergistic effects with other compounds within the crude extract.

**Table 15: Miracidicidal activity of compound 22**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 1000 | 100±0 | | |
| 500 | 88.3±2.4 | | |
| 450 | 76.7±2.4 | | |
| 400 | 66.7±4.7 | | |
| 350 | 56.7±2.4 | 287.97 (253.01- | 631.73 (521.44- |
| 300 | 46.7±4.7 | 326.61) | 853.51) |
| 250 | 41.7±2.4 | | |
| 200 | 28.3±2.4 | | |
| 150 | 16.7±6.2 | | |
| 100 | 3.3±2.4 | | |
| 50 | 1.7±2.4 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

To the best of my knowledge there is no reported study on the evaluation of miracidicidal activity of isolated alkaloids. However, the four alkaloids isolated, that is, compounds **10**, **13**, **21** and **22** had schistosomicidal activity and comparisons of their LC values showed excellent toxicities against *S. mansoni* miracidia. The miracidia mortality of the compounds had dose dependent effect, that is, the activity was proportional to the concentration. There are few reported studies of miracidicidal activity of isolated pure compounds on *S. mansoni* miracida. However, alkaloids both synthetic and from natural sources have been shown to be excellent anti-helmintics. For instance, Glycoalkaloids from *Solanum* spp have been reported to have *in vitro* anti-schistosomal effects. Similarly, epiisopiloturine and Imidazole alkaloids from *Pilocarpus microphyllus* (Rutaceae) leaves have been reported to have in vitro effect on the survival time of *S.mansoni* Schistosomulae and adult worm stages (Rai and Kon , 2013). Moreover, the drugs Praziquantel and Triclabendazole (figure 23) are alkaloids which are known to cause tegumental damage and paralysis of the trematodes (How, 2007). According to Satou *et al. (*2002a), the isoquinoline alkaloids; Allocyptopine, Dehydrocaryodaline and Papaverine have

been shown to cause larval mobility inhibition against the helminth *Toxocara canis.* The alkaloids Protopine, d-Corydoline and l-Stylopine have also been reported to have anthelmintic activity against *Strongyloides ratti* and *Strongyloides venezuelensis* (Satou *et al*., 2002b). Satou *et al*. (2005) also reported that β-Carboline alkaloids isolated from the plants *Picrasma quassoides* and *Ailanthus altissima* exhibit larval toxicity against *Toxocara canis*.

## 4.6 Miracidicidal assay of *R. melanoploeos* methanol, ethyl acetate and hexane crude extracts

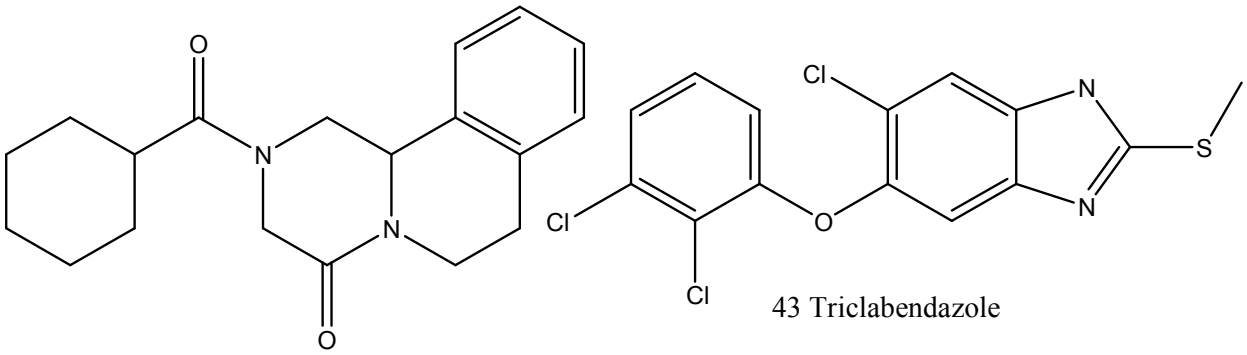
The methanol crude extract was subjected to solvent-solvent partitioning after suspension in water to yield ethyl acetate, hexane and methanol/water fractions. Serial dilutions of methanol, ethyl acetate and hexane extracts were then subjected to *S. mansoni* bio-assay in three replicates for each concentration as described in section 3.4. The percentage mortality was recorded after 30 minutes of exposure as tabulated in tables 16, 17 and 18. The $LC_{50}$ and $LC_{90}$ were then calculated through log probit regression at 95% confidence level using IBM SPSS 11.5 software. Dechlorinated water and 1% DMSO which were used as negative controls showed 0% mortality. Praziquantel, the positive control, showed 100% mortality at 1000ppm.

From tables 16, 17 and 18, it is clearly evident that the methanol, ethyl acetate and hexane extracts were all active against *S. mansoni* miracidia to different extents. At concentrations of 300 ppm, 500 ppm and 400 ppm the methanol, ethyl acetate and hexane extracts had 100% mortality respectively. This is a clear indication that the methanol extract was the most active followed by hexane and lastly ethyl acetate extract. This was further confirmed by the $LC_{50}$ and $LC_{90}$. The methanol, ethyl acetate and hexane extracts had $LC_{50}$ of 96.57, 242.05 and 150.73 ppm, respectively. Similarly, the $LC_{90}$ were calculated to be 257.98, 558.43 and 327.73 for methanol, ethyl acetate and hexane respectively.

**Table 16: Miracidicidal activity of *R. melanophloeos* methanol crude extract**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 1000 | 100 ±0 | | |
| 500 | 100 ±0 | | |
| 400 | 100 ±0 | | |
| 350 | 100 ±0 | | |
| 300 | 100 ±0 | | |
| 250 | 98.3±2.9 | | |
| 200 | 81.7±2.9 | 96.57(79.73- | 257.98(197.49- |
| 150 | 66.7±7.6 | 116.83) | 394.76) |
| 100 | 48.3±7.6 | | |
| 75 | 33.3±7.6 | | |
| 50 | 15±5 | | |
| 25 | 8.3±2.9 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control




42 Praziquantel

43 Triclabendazole

**Figure 23: Structures of praziquantel and triclabendazole**

**Table 17: Miracidicidal activity of *R. melanophloeos* ethyl acetate crude extract**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 500 | 100 ±0 | | |
| 450 | 88.3±2.9 | | |
| 400 | 75±5 | | |
| 350 | 65±5 | 242.05(209.68- | 558.43(462.89- |
| 300 | 51.7±5.9 | 275.89) | 740.30) |
| 250 | 43.3±5.8 | | |
| 200 | 35±5 | | |
| 150 | 20±5 | | |
| 100 | 15±5 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

**Table 18: Miracidicidal activity of *R.melanophloeos* hexane crude extract**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 500 | 100 ±0 | | |
| 450 | 100 ±0 | | |
| 400 | 100 ±0 | | |
| 350 | 95±5 | 150.73 (126.77- | 327.73 (279.03- |
| 300 | 88.3±7.6 | 173.61) | 408.96) |
| 250 | 70±5 | | |
| 200 | 53.3±2.9 | | |
| 150 | 43.3±7.6 | | |
| 100 | 23.3±2.9 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

It is therefore evident that the extracts do contain anti-helmintic compounds with anti-schistosomal activity against *S. mansoni* miracidia. However, the hexane extract was not fractionated to isolate the compounds because of unfavourable TLC patterns. From the ethyl acetate extract two compounds with activity against miracidia were isolated. The anti-helmintic activity shown by this plant is in agreement with the literature according to Githiori (2002) who reported that the plant *R. melanophloeos* shows some in vivo activity against the helminth *Haemonchus contortus* in sheep. Other plants from the family Myrsinaceae such as *Embelia schimperi* have been reported to have in vitro anti-helmintic activity thus confirming the said activity (Bogh *et al*., 1996).

## 4.7 Bio-assay of fractions from ethyl acetate extract

The *R. melanophloeos* ethyl acetate crude extract was subjected to column chromatography to yield four fractions which were then tested for schistosomicidal activity against *S. mansoni* miracidia. The percentage mortality, $LC_{50}$ and $LC_{90}$ (at 95% confidence level) observed are tabulated in tables 19-22.

**Table 19: Miracidicidal activity of *R. melanophloeos* ethyl acetate $F_1$**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 500 | 100 ±0 | | |
| 450 | 83.3±7.6 | | |
| 400 | 66.7±5.8 | | |
| 350 | 55±5 | | |
| 300 | 48.3±2.9 | 283.17 (219.34- | 618.42 (456.57- |
| 250 | 28.3±7.6 | 362.26) | 1219.19) |
| 200 | 21.7±2.9 | | |
| 150 | 13.3±2.9 | | |
| 100 | 6.7±7.6 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

All the four fractions did show activity against miracidia as indicated in tables 19-22. The fractions $F_1$, $F_4$ and methanol showed 100% mortality at a concentration of 500 ppm while $F_3$ had 100% mortality at 400 ppm. The $LC_{50}$ was calculated to be 283.17, 182.28, 238.93 and 215.93 for fractions $F_1$, $F_3$, $F_4$ and methanol, respectively. Similarly, $F_1$, $F_3$, $F_4$ and methanol fractions had $LC_{90}$ of 618.42, 344.42, 472.12 and 451.28 ppm, respectively. This indicates that $F_3$ was the most active followed by methanol, then $F_4$ and lastly $F_1$.

**Table 20: Miracidicidal activity of *R. melanophloeos* ethyl acetate $F_3$**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 500 | 100 ±0 | | |
| 450 | 100 ±0 | | |
| 400 | 100 ±0 | | |
| 350 | 91.7±7.6 | | |
| 300 | 78.3±2.9 | 182.28 (158.19- | 344.42 (300.12- |
| 250 | 61.7±2.9 | 205.00) | 417.85) |
| 200 | 50±5 | | |
| 150 | 36.7±7.6 | | |
| 100 | 16.7±7.6 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

**Table 21: Miracidicidal activity of *R. melanophloeos* ethyl acetate $F_4$**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 500 | 100 ±0 | | |
| 450 | 96.7 ±5.8 | | |
| 400 | 86.7±7.6 | | |
| 350 | 65±5 | 238.93 (210.48- | 472.12 (405.09- |
| 300 | 55±5 | 267.53) | 592.19) |
| 250 | 46.7±2.9 | | |
| 200 | 33.3±7.6 | | |
| 150 | 21.7±7.6 | | |
| 100 | 8.3±2.9 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

**Table 22: Miracidicidal activity of *R. melanophloeos* ethyl acetate methanol fraction**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 500 | 100 ±0 | | |
| 450 | 93.3±7.6 | | |
| 400 | 86.7±7.6 | | |
| 350 | 73.3±2.9 | 215.95 (187.28- | 451.28 (384.80- |
| 300 | 61.7±5.8 | 243.88) | 570.65) |
| 250 | 55±5 | | |
| 200 | 43.3±5.8 | | |
| 150 | 28.3±7.6 | | |
| 100 | 15±5 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

### 4.8 Bio-assay of purified compounds isolated from *R. melanophloeos*

Fraction $F_3$ was subjected to PTLC where it yielded two pure compounds. On subjecting these compounds to *S. mansoni* miracidia acitivty, all the compounds were found to be active. The percentage mortalities, $LC_{50}$ and $LC_{90}$ calculated are tabulated in Tables 23 and 24.

The compounds were found to cause 100% mortality for **23** and **24** at the concentrations of 350 ppm and 300ppm respectively. The $LC_{50}$ at 95% confidence level, were calculated to be 139.89 and 98.06 for **23** and **24,** respectively. The $LC_{90}$ at 95% confidence level was similarly found to be 314.23 and 236.52 for compounds **23** and **24,** respectively. From this, it is clear that compound **24** was the most active, followed by compound **23**. Compound **24** was contaminated with compound **23** and synergism of the two compounds may have been the cause of the observed high activity in compound **24**. However, the activity of all of them compared favourably well with those of commercial Praziquantel which was used as the reference standard.

**Table 23: Miracidicidal activity of compound 23**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 1000 | 100±0 | | |
| 500 | 100±0 | | |
| 450 | 100±0 | | |
| 400 | 100±0 | | |
| 350 | 100±0 | | |
| 300 | 95±5 | | |
| 250 | 71.7±7.6 | 139.89 (111.35- | 314.23 (250.00- |
| 200 | 53.3±7.6 | 170.07) | 445.02) |
| 150 | 41.7±7.6 | | |
| 100 | 28.3±7.6 | | |
| 75 | 16.7±2.9 | | |
| 50 | 10±5 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

**Table 24: Miracidicidal activity of compound 24**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 1000 | 100±0 | | |
| 500 | 100±0 | | |
| 450 | 100±0 | | |
| 400 | 100±0 | | |
| 350 | 100±0 | | |
| 300 | 100±0 | | |
| 250 | 95±5 | 98.06 (81.92-114.55) | 236.51 (197.25-301.31) |
| 200 | 71.7±7.6 | | |
| 150 | 58.3±2.8 | | |
| 100 | 48.3±2.8 | | |
| 75 | 36.7±7.6 | | |
| 50 | 16.7±7.6 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control, b – Positive control

The comparisons of the LC values of the two compounds **23** and **24** showed excellent toxicities against *S. mansoni* miracidia. The miracidia mortality of the compounds had dose dependent effect, that is, the activity was proportional to the concentration. There are few reported studies of miracidicidal activity of isolated pure compounds on *S. mansoni* miracida. Tannins extracted from *Punica granatum* showed high activity against this miracidia with 100% mortality being registered with concentrations as low as 0.39 and 50 ppm within 50-150 minutes and 5-15 minutes respectively (Abozeid *et al*., 2012). The methanolic extract of *Pleclanthus tenuiflorus* has also been shown to have an $LC_{50}$ of 24.37 mg/100ml on *S. mansoni* miracidia (Abdel-Aziz *et al*., 2011). Aqueous extracts from *Phytolacca dodecandra* at a concentration of 4 ppm was determined to prevent *S. mansoni* miracidia from infecting snails (Birrie *et al*., 1998) and also shown to have an $LC_{50}$ of 8.2 ppm (Madhina and Shiff, 1996). The plant *Iris pseudacorus* leaves extract at a concentration as low as 0.9mg/l have been shown to have miracidicidal effects (Ahmed and El Hamshary, 2005). *Nigella sativa* crushed seeds at a

concentration of 4ppm were reported to have miracidicidal activity within one minutes against *S. mansoni* miracidia (Azza *et al*., 2005). A concentration of 400ppm extracts from *Tetrapleura tetraptera* have been shown to have lethal effects against *S. haematobium*, *S. bovis* and *S. mansoni* miracidia (Aladesanmi, 2007). Saponins isolated from *Furcraea selloea* were shown to kill 100% of miracidia at 50 ppm with the saponin 3-O-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1-3)-β-D-xylopyranoside gloriogenin causing a 100% mortality at a concentration of 6ppm (El-Nahas *et al*., 2005). Isoflavonoids isolated from *Milletis thoniningii* have also been shown to have miracidicidal activity (Lyddiard *et al*., 2002). Eissa *et al*. (2011), also reported that miltefosine, the oral drug licensed for the treatment of Leishmaniasis, has lethal effects against both *S. mansoni* and *S. haematobium* miracidia.

### 4.9 Anti-miracidia assay of *T. nobilis* essential oil

The percentage yield of the essential oil obtained from *T. nobilis* leaves was calculated to be 0.33% v/w with the density determined to be 0.99 g/ml. The oil anti-helmintic activity against *S. mansoni* miracidia was evaluated and the results observed tabulated in table 25.

At a concentration of 400 ppm, the oil was found to cause 100% miracidia mortality within 30 minutes. Decreasing the concentration to 350 ppm induced 93.3% mortality. At 50 ppm, the lowest concentration tested, an observed mortality of 5% was recorded. Through log probit regression analysis, at 95% confidence level, the $LC_{50}$ and $LC_{90}$ were determined to be 196.29 and 367.24 respectively after 30 minutes of exposure. Therefore, it is evident that the constituents of the oil have anti-helmintic compounds active against *S. mansoni* miracidia.

The activity of the essential oil was in strong agreement with other plants essential oils that have been shown to have schistosomicidal activity. Rai and Kon (2013), reported that essential oils from *Baccharis dracucolifolia* had *in vitro* activity against *S. mansoni* at a concentration of 10µg/ml while those of *Bidens pilosa* and *Tagetes erecta* at a concentration of 100µg/ml. *Piper cubeba* and *Ageratum conyzoides* have also been reported to be active against adult *S. mansoni* worms. Furthermore, essential oils from *Apium graveolens*, *Piper cubeba*, *Piper marginatum, Eucalyptus* spp and *Zingiber officinale* have been reported to be active against *S. mansoni* cercariae (Rai and Kon, 2013).

**Table 25: Miracidicidal activity of *T. nobilis* essential oil**

| Conc. (ppm) | Mean % mortality | $LC_{50}$ (ppm) | $LC_{90}$ (ppm) |
|---|---|---|---|
| 4000 | 100 ±0 | | |
| 2000 | 100 ±0 | | |
| 1000 | 100 ±0 | | |
| 500 | 100 ±0 | | |
| 450 | 100 ±0 | | |
| 400 | 100 ±0 | | |
| 350 | 93.3±2.9 | 196.29 (153.12- | 367.24 (296.12- |
| 300 | 75±5 | 237.58) | 541.19) |
| 250 | 56.7±7.6 | | |
| 200 | 46.7±5 | | |
| 150 | 21.7±7.6 | | |
| 100 | 11.7±2.9 | | |
| 1% DMSO[a] | 0.0±0.0 | | |
| Praziquantel (1,000 ppm)[b] | 100±0.0 | | |

a- Negative control b- Positive control

### 4.10 Determination of the chemical composition of the oil extract

The chemical composition of the essential oil extracted from *T. nobilis* was determined by the GC-MS. The identification of the compounds in the oil was determined by comparing the electron impact mass spectrum of the compounds in the oil and those in the Wiley7N.l, FLAVORS.L and HPCH1607.L computer library databases. From the data obtained, it was revealed that monoterpenes (42.21%) and sesquiterpenes (33.09%) are the main constituents of the oil. The major monoterpenes were found to be β- Ocimene, γ- Terpinene, α- Pinene, Limonene and Methyl Eugenol and with concentrations of 10.15, 6.11, 3.95, 3.34 and 2.68%, respectively. Sesquiterpenes β- Cadinene, 1,6-Germacradien-5-ol, α-Amorphine, tau-Cadinol and Germacrene D having concentrations of 4.98, 4.38, 3.96, 3.56 and 3.06%, respectively were determined to be the major ones.

**Table 26: Major constituents of *T. nobilis* essential oil**

| Compound Number | R.T (min) | Compound Name | %Concentration | Detection method |
|---|---|---|---|---|
| **25** | 6.81 | α – Pinene | 3.95 | GC/MS |
| **26** | 8.01 | γ – Terpinene | 6.11 | GC/MS |
| **27** | 8.45 | β – Myrcene | 2.17 | GC/MS |
| **28** | 9.50 | Limonene | 3.34 | GC/MS |
| **29** | 10.29 | β – Ocimene | 10.15 | GC/MS |
| **30** | 11.65 | Linalool | 1.13 | GC/MS |
| **31** | 12.55 | Neo-allo-Ocimene | 3.68 | GC/MS |
| **32** | 20.21 | Methyl Eugenol | 2.68 | GC/MS |
| **33** | 23.89 | Elemicin | 2.89 | GC/MS |
| **34** | 19.59 | β – Gurjunene | 1.26 | GC/MS |
| **35** | 20.44 | Germacrene D | 3.06 | GC/MS |
| **36** | 22.39 | α – Amorphine | 3.96 | GC/MS |
| **37** | 23.08 | β – Cadinene | 4.98 | GC/MS |
| **38** | 23.16 | δ – Cadinene | 1.28 | GC/MS |
| **39** | 24.84 | Guaiol | 1.01 | GC/MS |
| **40** | 25.88 | Tau- Cadinol | 3.56 | GC/MS |
| **41** | 26.25 | α – Cadinol | 2.57 | GC/MS |

The compound (**25**) was observed to occur at 6.81 minutes retention time and was identified as α–Pinene. The compound has a molecular weight of 136amu and constituted 3.95% of the total oil. Analysis of the MS spectrum showed the presence of a small peak at *m/z* 136 corresponding to $[C_{10}H_{16}]^+$. Another peak at *m/z* 121 was observed which corresponded with $[M\text{-}CH_3]^+$. The peaks at *m/z* 105, 93 and 77 were corresponding to $[M\text{-}C_2H_5]^+$, $[M\text{-}H\text{-}C_3\ H_7]^+$ and $[M+H\text{-}C_4\ H_{10}]^{+,}$ respectively. The structure of the compound and its MS spectra are shown in figure 24.

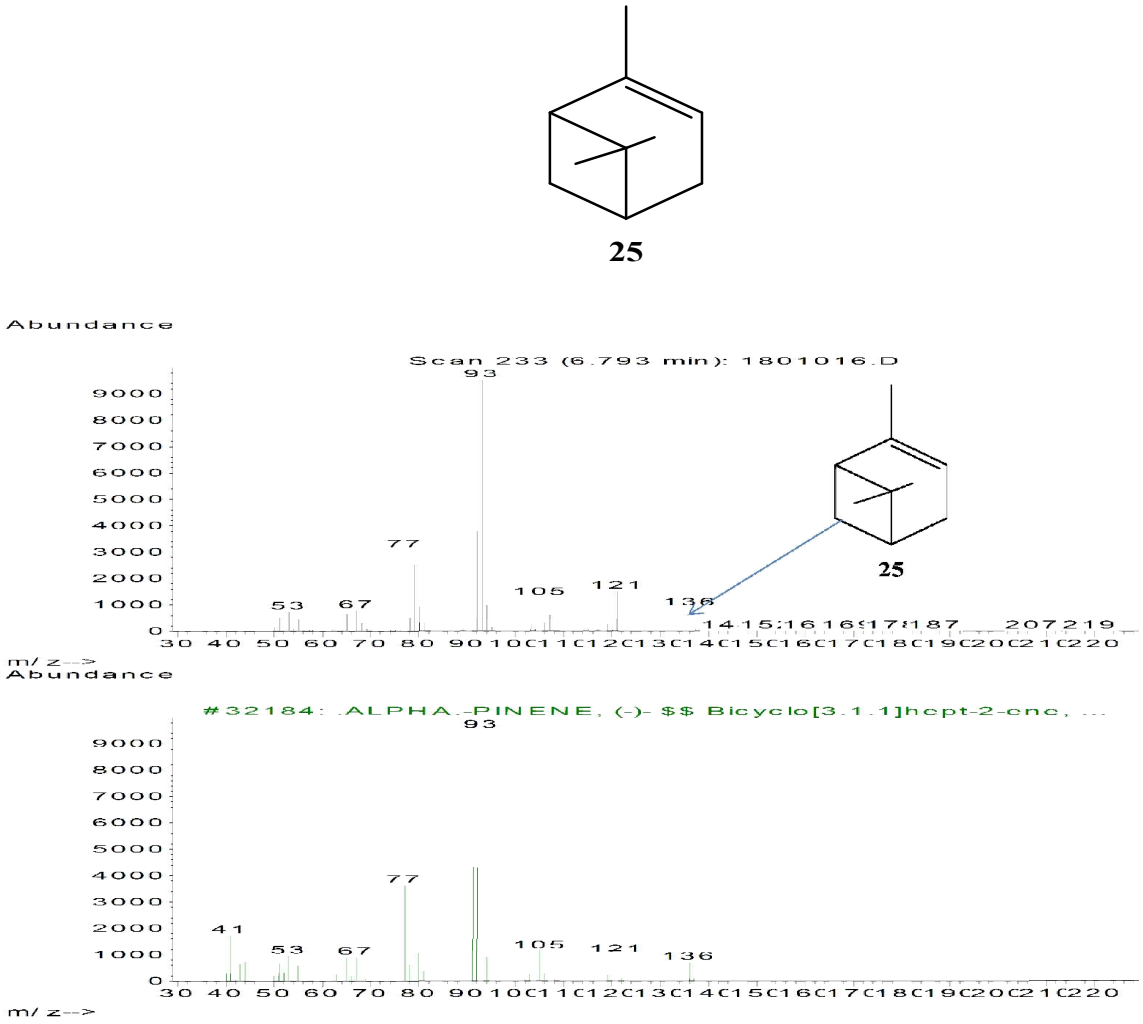
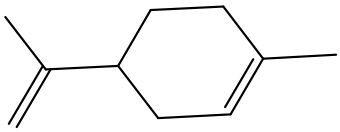



**Figure 24: Mass spectra of compound 25**

This compound has been reported to be the major constituent in the essential oil of *Plectranthus neochilus*. This oil was reported to have anthelmintic properties against *S. mansoni* adult worms causing 100% mortality at a concentration of 0.1mg/ml. It also reduces hatching of *S.mansoni* eggs (Caixeta *et al*., 2011). Its isomer β-pinene is a major constituent of *Piper cubeba* essential oil which was reported to cause mortality of *S. mansoni* adult worms at a concentration of 0.13mg/ml within 120 h (De Oliviera *et al*., 2012). Therefore, even though the compounds' individual activity has not been reported, its effect against *S. mansoni* miracidia may not be ruled out.

The compound (**28**) occurred at the retention time 9.50 and was identified as limonene with molecular weight of 136 and was 3.34% of the total oil. The GC-MS spectrum of the oil showed a peak at *m/z* 121 corresponding to the fragment $[C_9H_{13}]^+$ after the loss of a methyl group. The peaks at *m/z* 68 $[C_5H_8]^+$ and at *m/z* 67 $[C_5H_7]^+$ are due to a retro Diels Alder reaction the compound undergoes. The peaks at *m/z* 107, 93 and 79 corresponds to $[C_8H_{13}]^+$, $[C_7H_9]^+$, and $[C_6H_7]^+$ respectively. Limonene is a monoterpene as it has ten carbon atoms. The compound has not been reported to show antischistosomal activity or any anthelmintic activity. However, (+) Limonene epoxide isomers has been shown to have similar effects to those of praziquantel at 25µg/ml on *S. mansoni* adult worms with reduction in motility and death after 120 h (Moraes *et al*., 2013). The compound has been reported to be a major constituent of essential oils from the plants *Eucalyptus citridora, Eucalyptus staigeriana* and *Eucalyptus globules*. These oils have been shown to have antihelmintic activity against *Haemonchus contortus* by reduction of egg hatching and impairing larval development (Macedo *et al*., 2009; Macedo *et al*., 2010; Macedo *et al.*, 2011). The structure and the mass spectra for Limonene are shown in figure 25 below.



**28**

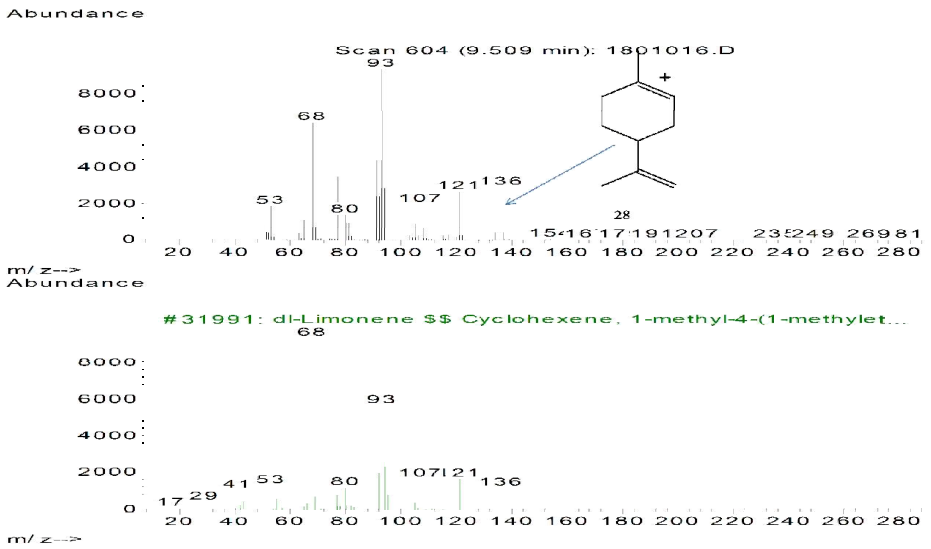
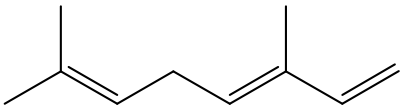



**Figure 25: Mass spectra of compound 28**

Ocimene isomers were found to be the major constituents of *T. nobilis* oil, with β – Ocimene constituting 10.15% of the total oil. β–Ocimene (29) was observed at retention time 10.29 minutes and identified to have a molecular weight of 136. Analysis of the MS spectrum showed the presence of a small peak at *m/z* 136 corresponding to $[C_{10}H_{16}]^+$. Another peak at *m/z* 121 was observed which corresponded with $[M\text{-}CH_3]^+$. The peaks at *m/z* 105, 93 and 79 were corresponding to $[M\text{-}C_2H_5]^+$, $[M\text{-}H\text{-}C_3H_7]^+$ and $[M+H\text{-}C_4H_8]^+$, respectively. The structure of the compound and its MS spectra are shown below. Compound (**29**) has not been reported to have anthelmintic activities. However, its activity may not be ruled out.



**29**

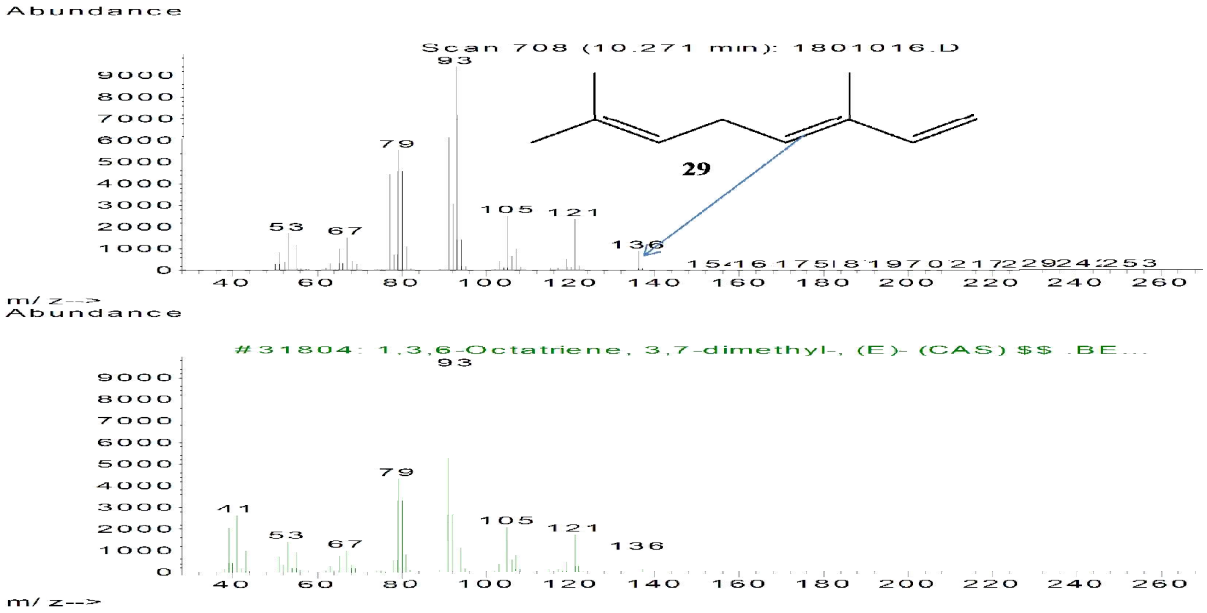
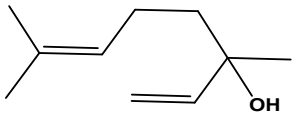



**Figure 26: Mass spectra of compound 29**

Compound (**30**) occurring at retention time 11.65 was identified as Linalool constituting 1.13% of the total oil. The compound was identified with molecular weight of 154. The molecular ion peak *m/z* 154 was observed in both spectra though the peaks were very small. The

peaks at *m/z* 136, 121, 107, 93, 71 and 41 were observed. These peaks are shown in figure 27 below and correspond to fragments shown as per the scheme in figure 28 below.




**30**

Dearth of literature is available on the activity of Linalool against helminthes. However, it has been reported to reduce the infection of *S. mansoni* miracidia (Ahmed, 2006). The activity of the oil observed may not be solely attributed to Linalool.

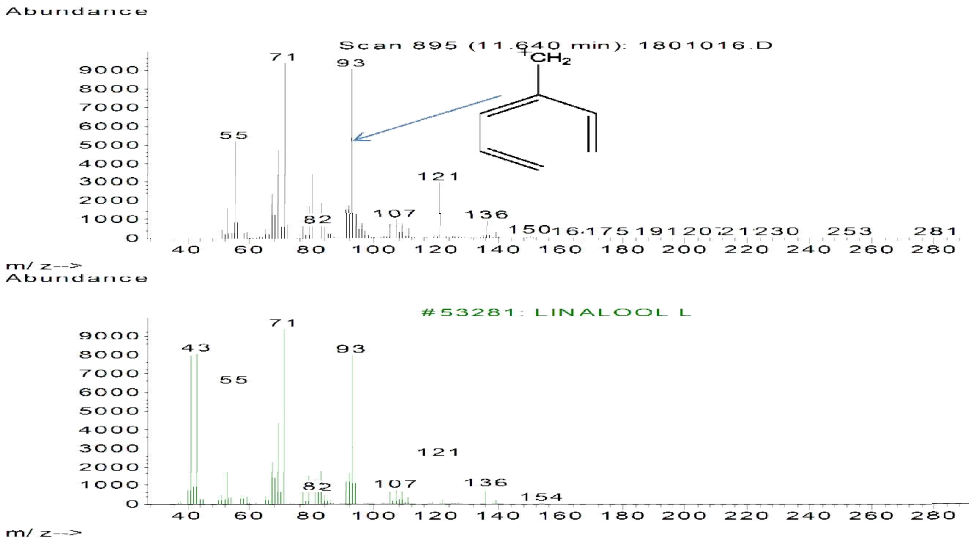



**Figure 27: Mass spectra of compound 30**

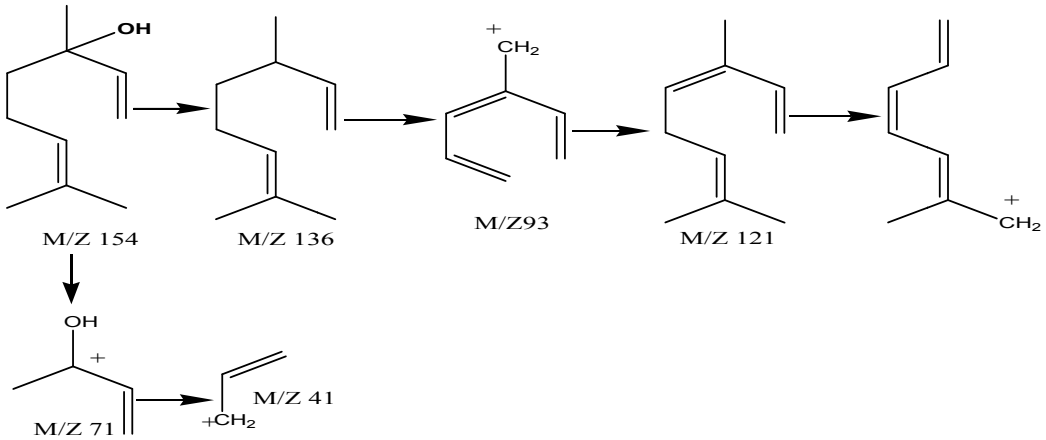
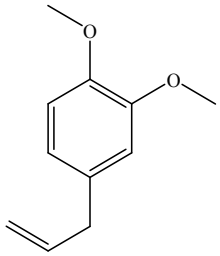



**Figure 28: Scheme showing fragmentation pattern of compound 30**

The compound (**32**) was identified as methyl eugenol. It had a concentration of 2.68% and appeared at 20.21 retention time. It has a molecular weight of 78 amu. The analysis of the ms spectra showed the presence of a strong peak at m/z 178 corresponding to $[C_{11}H_{14}O_2]^+$. There was another peak at m/z 163 corresponding to the fragment $[C_{10}H_{11}O_2]$+ after loss of a methyl group. The fragment $[C_{10}H_{11}O]$+ which occurred after loss of a methoxy group showed a peak at m/z 147. The spectra (figure 29) and structure of the molecule are shown below.




**32**

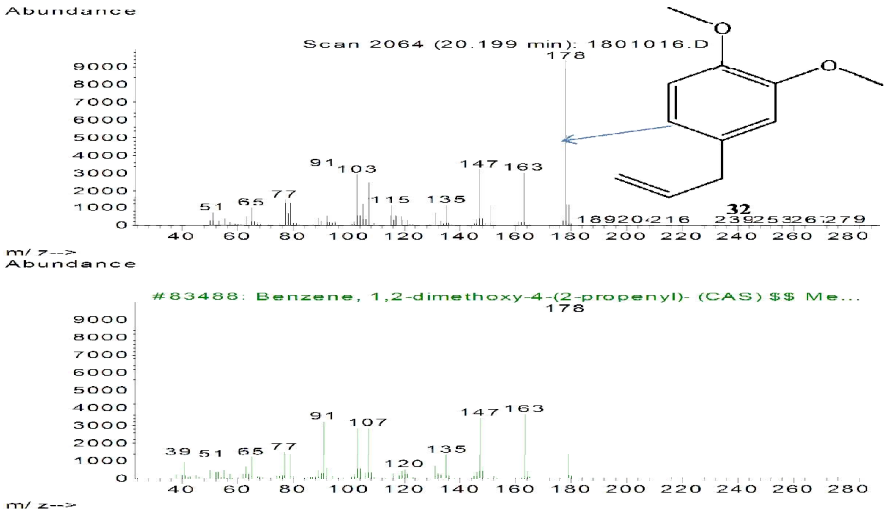



**Figure 29: Mass spectra of compound 32**

The compound Eugenol which belongs to the same class of compounds as compound **32** has been reported to have lethal effects against cercariae and miracidia larval stages of *S. mansoni* (Mansour *et al*., 2003). It has also been shown to reduce the infection of snails by *S. mansoni* miracidia (Ahmed, 2006). Eugenol has also been reported to be the major constituent of

*Piper longum* essential oil which has been shown to have antihelmintic activity against *Fasciola gigantica* by causing paralysis of the worms (Singh *et al*., 2009). Therefore, compound **32** may have contributed to the activity shown by the oil.

The compounds **26, 27, 31, 33, 34, 35, 36, 37, 38, 39, 40** and **41** shown in figure 30 were also found in appreciable amount each constituting more than one percent of the total oil. None of these compounds however have been reported to show anti-helmintic activity. Therefore, their individual anthelmintic activity needs evaluation.

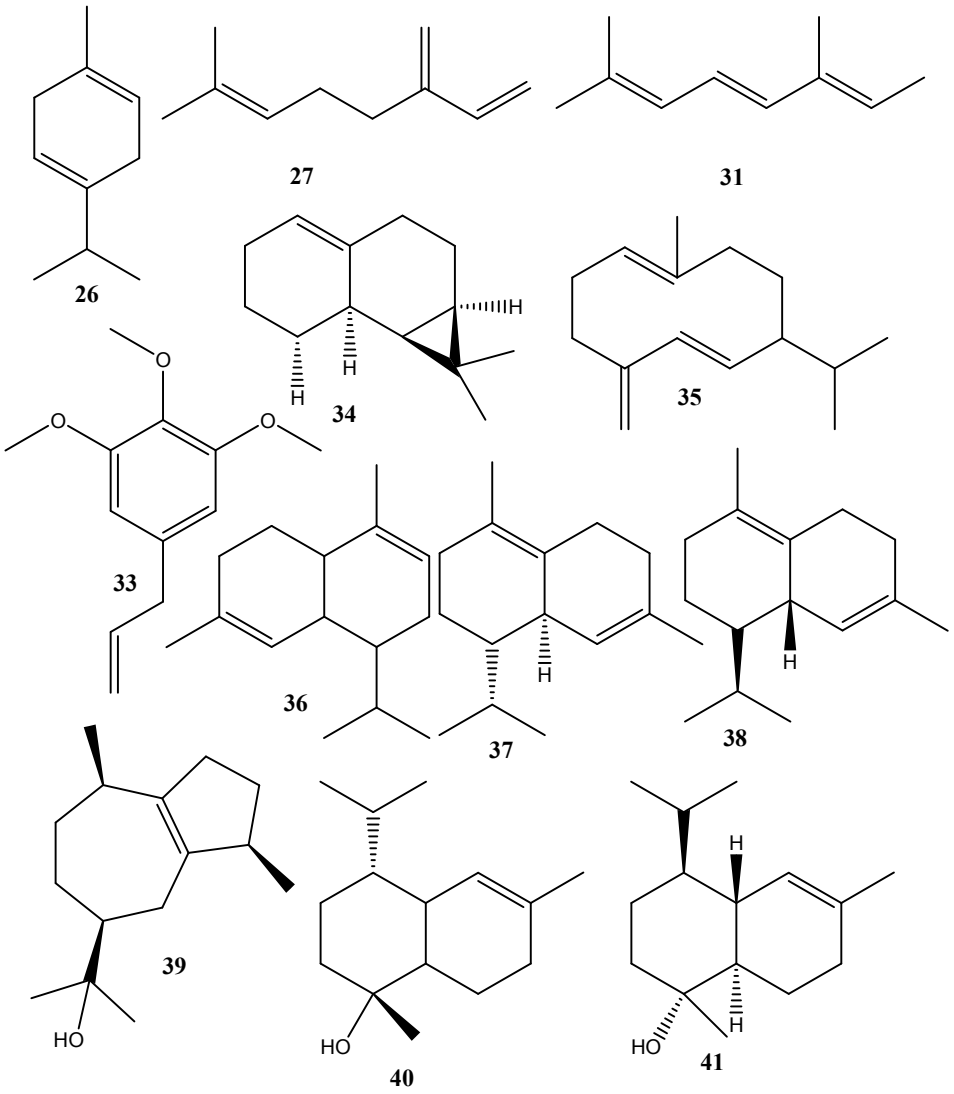



**Figure 30: Some major compounds of *T. nobilis* essential oil**

# CHAPTER FIVE

# CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Conclusion

The essential oil from *T. nobilis* was determined to be majorly constituted of monoterpenes (42.21%) and sesquiterpenes (33.09%). The major compounds were β-Ocimene, γ-Terpinene, β-Cadinene and 1,6-Germacradien-5-ol which had concentrations of 10.15%, 6.11%, 4.98% and 4.38% respectively. The essential oil showed lethal anti-helmintic effects against *S. mansoni* miracidia with recorded $LC_{50}$ and $LC_{90}$ values of 196.29 ppm and 367.24 ppm respectively.

The non-volatiles of both *T. nobilis* and *R. melanophloeos* were active against *S. mansoni* miracidia. The *R. melanophloeos* methanol extract was the most active with $LC_{50}$ and $LC_{90}$ values of 96.57 and 257.98 ppm, respectively. The *T. nobilis* methanol extract recorded $LC_{50}$ and $LC_{90}$ values of 261.69 and 575.74 ppm, respectively. This indicated that the two plants do contain secondary metabolites that contributed to this activity.

Fractionation of *T. nobilis* and *R. melanophloeos* leaf extracts over silica gel column chromatography yielded six compounds which were successfully identified through analysis of their NMR and MS data as well as comparison with literature data. Of the six compounds, *T. nobilis* had four furoquinoline alkaloids; Tecleoxine **10**, Methylnkolbisine **13,** Kokusagine **21** and Nkolbisine **22** while the *R. melanophloeos* had two benzoic acid derivatives Myrsinoic acid B **23** and Myrsinoic acid C **24**. All these compounds were active against *S. mansoni* miracidia. Compound **23** which recorded mortality $LC_{50}$ and $LC_{90}$ values of 98.06 and 236.51 ppm, respectively was the most potent followed by compound **24** which registered $LC_{50}$ and $LC_{90}$ values of 139.89 and 314.23 ppm, respectively. The compounds **10**, **13** and **21** mixture registered $LC_{50}$ and $LC_{90}$ values of 270.18 and 690.93 ppm, respectively and compound **22** which recorded $LC_{50}$ and $LC_{90}$ values of 287.97 and 631.733 ppm, respectively was the least potent.

Results from this study shows that the four furoquinoline alkaloids and the essential oil from *T. nobilis* leaves as well as the two Myrsinoic acid compounds from *R. melanophleoes* have potential applicability in the control of *S. mansoni* miracidia. This study underscores the fact that bioactive plant constituents can be useful agents in the control of Schistosomiasis. These findings

also show that these compounds can be used as lead compounds in the development of new, biodegradable, environmentally benign and more potent anti-helmintics.

## 5.2 Recommendations

In relation to this study, the following recommendations were made;

i. More advanced methods of compound purification such as use of HPLC, be used to purify compounds **10**, **13** and **21** and separate the three alkaloids and their individual miracidicidal activity tested.
ii. Toxicity tests to be carried out for the alkaloids, myrsinoic acids and the essential oil.
iii. The mode of action of each of the pure compounds and that of the essential oil to be investigated.
iv. The components of the essential oil to be isolated and each tested for its miracidicidal activity.

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# APPENDICES

**Appendix 1**: Generated LC values for *Teclea nobilis* methanol crude extract

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 62.544 | 37.114 | 86.206 | 1.796 | 1.570 | 1.936 |
| | .020 | 73.965 | 46.311 | 98.815 | 1.869 | 1.666 | 1.995 |
| | .030 | 82.269 | 53.277 | 107.787 | 1.915 | 1.727 | 2.033 |
| | .040 | 89.126 | 59.189 | 115.093 | 1.950 | 1.772 | 2.061 |
| | .050 | 95.122 | 64.469 | 121.417 | 1.978 | 1.809 | 2.084 |
| | .060 | 100.543 | 69.324 | 127.088 | 2.002 | 1.841 | 2.104 |
| | .070 | 105.549 | 73.873 | 132.292 | 2.023 | 1.868 | 2.122 |
| | .080 | 110.243 | 78.191 | 137.146 | 2.042 | 1.893 | 2.137 |
| | .090 | 114.693 | 82.330 | 141.726 | 2.060 | 1.916 | 2.151 |
| | .100 | 118.948 | 86.326 | 146.090 | 2.075 | 1.936 | 2.165 |
| | .150 | 138.310 | 104.937 | 165.800 | 2.141 | 2.021 | 2.220 |
| | .200 | 155.922 | 122.359 | 183.633 | 2.193 | 2.088 | 2.264 |
| | .250 | 172.809 | 139.370 | 200.772 | 2.238 | 2.144 | 2.303 |
| | .300 | 189.527 | 156.383 | 217.899 | 2.278 | 2.194 | 2.338 |
| | .350 | 206.458 | 173.657 | 235.531 | 2.315 | 2.240 | 2.372 |
| | .400 | 223.922 | 191.380 | 254.148 | 2.350 | 2.282 | 2.405 |
| | .450 | 242.222 | 209.708 | 274.263 | 2.384 | 2.322 | 2.438 |
| | **.500** | **261.693** | **228.791** | **296.475** | **2.418** | **2.359** | **2.472** |
| | .550 | 282.728 | 248.813 | 321.513 | 2.451 | 2.396 | 2.507 |
| | .600 | 305.835 | 270.037 | 350.306 | 2.485 | 2.431 | 2.544 |
| | .650 | 331.704 | 292.873 | 384.091 | 2.521 | 2.467 | 2.584 |
| | .700 | 361.337 | 317.969 | 424.642 | 2.558 | 2.502 | 2.628 |
| | .750 | 396.294 | 346.373 | 474.719 | 2.598 | 2.540 | 2.676 |
| | .800 | 439.213 | 379.861 | 539.070 | 2.643 | 2.580 | 2.732 |
| | .850 | 495.142 | 421.781 | 626.979 | 2.695 | 2.625 | 2.797 |
| | **.900** | **575.739** | **479.707** | **760.528** | **2.760** | **2.681** | **2.881** |
| | .910 | 597.097 | 494.670 | 797.132 | 2.776 | 2.694 | 2.902 |
| | .920 | 621.199 | 511.388 | 839.005 | 2.793 | 2.709 | 2.924 |
| | .930 | 648.824 | 530.349 | 887.714 | 2.812 | 2.725 | 2.948 |
| | .940 | 681.132 | 552.273 | 945.610 | 2.833 | 2.742 | 2.976 |
| | .950 | 719.948 | 578.287 | 1016.439 | 2.857 | 2.762 | 3.007 |
| | .960 | 768.386 | 610.294 | 1106.686 | 2.886 | 2.786 | 3.044 |
| | .970 | 832.424 | 651.914 | 1229.002 | 2.920 | 2.814 | 3.090 |
| | .980 | 925.889 | 711.412 | 1413.288 | 2.967 | 2.852 | 3.150 |
| | .990 | 1094.960 | 815.879 | 1762.568 | 3.039 | 2.912 | 3.246 |

a Logarithm base = 10.

**Appendix 2**: Generated LC values for *Teclea nobilis* hexane extract

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 81.887 | 50.321 | 109.783 | 1.913 | 1.702 | 2.041 |
| | .020 | 95.635 | 61.935 | 124.456 | 1.981 | 1.792 | 2.095 |
| | .030 | 105.532 | 70.635 | 134.809 | 2.023 | 1.849 | 2.130 |
| | .040 | 113.646 | 77.959 | 143.192 | 2.056 | 1.892 | 2.156 |
| | .050 | 120.704 | 84.460 | 150.419 | 2.082 | 1.927 | 2.177 |
| | .060 | 127.056 | 90.406 | 156.877 | 2.104 | 1.956 | 2.196 |
| | .070 | 132.900 | 95.952 | 162.786 | 2.124 | 1.982 | 2.212 |
| | .080 | 138.360 | 101.195 | 168.284 | 2.141 | 2.005 | 2.226 |
| | .090 | 143.521 | 106.202 | 173.461 | 2.157 | 2.026 | 2.239 |
| | .100 | 148.441 | 111.019 | 178.384 | 2.172 | 2.045 | 2.251 |
| | .150 | 170.672 | 133.248 | 200.529 | 2.232 | 2.125 | 2.302 |
| | .200 | 190.692 | 153.768 | 220.474 | 2.280 | 2.187 | 2.343 |
| | .250 | 209.729 | 173.545 | 239.611 | 2.322 | 2.239 | 2.380 |
| | .300 | 228.438 | 193.064 | 258.744 | 2.359 | 2.286 | 2.413 |
| | .350 | 247.263 | 212.605 | 278.488 | 2.393 | 2.328 | 2.445 |
| | .400 | 266.557 | 232.354 | 299.411 | 2.426 | 2.366 | 2.476 |
| | .450 | 286.657 | 252.454 | 322.105 | 2.457 | 2.402 | 2.508 |
| | **.500** | **307.917** | **273.054** | **347.233** | **2.488** | **2.436** | **2.541** |
| | .550 | 330.754 | 294.355 | 375.566 | 2.520 | 2.469 | 2.575 |
| | .600 | 355.695 | 316.666 | 408.054 | 2.551 | 2.501 | 2.611 |
| | .650 | 383.451 | 340.456 | 445.959 | 2.584 | 2.532 | 2.649 |
| | .700 | 415.049 | 366.435 | 491.099 | 2.618 | 2.564 | 2.691 |
| | .750 | 452.075 | 395.696 | 546.332 | 2.655 | 2.597 | 2.737 |
| | .800 | 497.206 | 430.041 | 616.601 | 2.697 | 2.634 | 2.790 |
| | .850 | 555.528 | 472.813 | 711.558 | 2.745 | 2.675 | 2.852 |
| | **.900** | **638.726** | **531.509** | **854.029** | **2.805** | **2.726** | **2.931** |
| | .910 | 660.623 | 546.594 | 892.767 | 2.820 | 2.738 | 2.951 |
| | .920 | 685.262 | 563.411 | 936.937 | 2.836 | 2.751 | 2.972 |
| | .930 | 713.416 | 582.438 | 988.135 | 2.853 | 2.765 | 2.995 |
| | .940 | 746.229 | 604.377 | 1048.754 | 2.873 | 2.781 | 3.021 |
| | .950 | 785.499 | 630.326 | 1122.590 | 2.895 | 2.800 | 3.050 |
| | .960 | 834.285 | 662.136 | 1216.199 | 2.921 | 2.821 | 3.085 |
| | .970 | 898.432 | 703.310 | 1342.310 | 2.953 | 2.847 | 3.128 |
| | .980 | 991.404 | 761.820 | 1530.854 | 2.996 | 2.882 | 3.185 |
| | .990 | 1157.855 | 863.639 | 1884.175 | 3.064 | 2.936 | 3.275 |

a Logarithm base = 10.

**Appendix 3**: Generated LC values for *Teclea nobilis* ethyl acetate extract

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| LOGIT | .010 | 49.512 | 24.674 | 74.215 | 1.695 | 1.392 | 1.870 |
| | .020 | 64.869 | 35.681 | 92.017 | 1.812 | 1.552 | 1.964 |
| | .030 | 76.102 | 44.353 | 104.537 | 1.881 | 1.647 | 2.019 |
| | .040 | 85.333 | 51.824 | 114.583 | 1.931 | 1.715 | 2.059 |
| | .050 | 93.345 | 58.536 | 123.155 | 1.970 | 1.767 | 2.090 |
| | .060 | 100.525 | 64.717 | 130.739 | 2.002 | 1.811 | 2.116 |
| | .070 | 107.096 | 70.503 | 137.610 | 2.030 | 1.848 | 2.139 |
| | .080 | 113.202 | 75.982 | 143.942 | 2.054 | 1.881 | 2.158 |
| | .090 | 118.940 | 81.216 | 149.852 | 2.075 | 1.910 | 2.176 |
| | .100 | 124.379 | 86.249 | 155.423 | 2.095 | 1.936 | 2.192 |
| | .150 | 148.569 | 109.382 | 179.936 | 2.172 | 2.039 | 2.255 |
| | .200 | 169.839 | 130.541 | 201.324 | 2.230 | 2.116 | 2.304 |
| | .250 | 189.684 | 150.762 | 221.346 | 2.278 | 2.178 | 2.345 |
| | .300 | 208.909 | 170.602 | 240.984 | 2.320 | 2.232 | 2.382 |
| | .350 | 228.054 | 190.412 | 260.960 | 2.358 | 2.280 | 2.417 |
| | .400 | 247.553 | 210.444 | 281.924 | 2.394 | 2.323 | 2.450 |
| | .450 | 267.815 | 230.906 | 304.559 | 2.428 | 2.363 | 2.484 |
| | **.500** | **289.276** | **252.000** | **329.651** | **2.461** | **2.401** | **2.518** |
| | .550 | 312.457 | 273.983 | 358.163 | 2.495 | 2.438 | 2.554 |
| | .600 | 338.031 | 297.224 | 391.342 | 2.529 | 2.473 | 2.593 |
| | .650 | 366.934 | 322.301 | 430.904 | 2.565 | 2.508 | 2.634 |
| | .700 | 400.560 | 350.129 | 479.413 | 2.603 | 2.544 | 2.681 |
| | .750 | 441.159 | 382.198 | 541.075 | 2.645 | 2.582 | 2.733 |
| | .800 | 492.707 | 421.111 | 623.547 | 2.693 | 2.624 | 2.795 |
| | .850 | 563.245 | 471.994 | 742.861 | 2.751 | 2.674 | 2.871 |
| | **.900** | **672.786** | **547.229** | **940.737** | **2.828** | **2.738** | **2.973** |
| | .910 | 703.554 | 567.724 | 998.780 | 2.847 | 2.754 | 2.999 |
| | .920 | 739.215 | 591.186 | 1067.306 | 2.869 | 2.772 | 3.028 |
| | .930 | 781.360 | 618.544 | 1149.962 | 2.893 | 2.791 | 3.061 |
| | .940 | 832.440 | 651.212 | 1252.461 | 2.920 | 2.814 | 3.098 |
| | .950 | 896.468 | 691.483 | 1384.377 | 2.953 | 2.840 | 3.141 |
| | .960 | 980.639 | 743.400 | 1563.293 | 2.992 | 2.871 | 3.194 |
| | .970 | 1099.593 | 815.047 | 1826.150 | 3.041 | 2.911 | 3.262 |
| | .980 | 1289.992 | 926.191 | 2269.353 | 3.111 | 2.967 | 3.356 |
| | .990 | 1690.125 | 1148.707 | 3280.647 | 3.228 | 3.060 | 3.516 |

a Logarithm base = 10.

**Appendix 4**: Generated LC values for *Teclea nobilis* fraction $F_1$

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 152.436 | 110.961 | 188.359 | 2.183 | 2.045 | 2.275 |
| | .020 | 172.567 | 129.645 | 208.990 | 2.237 | 2.113 | 2.320 |
| | .030 | 186.697 | 143.073 | 223.275 | 2.271 | 2.156 | 2.349 |
| | .040 | 198.084 | 154.065 | 234.689 | 2.297 | 2.188 | 2.370 |
| | .050 | 207.857 | 163.613 | 244.422 | 2.318 | 2.214 | 2.388 |
| | .060 | 216.554 | 172.193 | 253.042 | 2.336 | 2.236 | 2.403 |
| | .070 | 224.479 | 180.074 | 260.866 | 2.351 | 2.255 | 2.416 |
| | .080 | 231.820 | 187.426 | 268.091 | 2.365 | 2.273 | 2.428 |
| | .090 | 238.705 | 194.363 | 274.848 | 2.378 | 2.289 | 2.439 |
| | .100 | 245.223 | 200.966 | 281.231 | 2.390 | 2.303 | 2.449 |
| | .150 | 274.159 | 230.649 | 309.451 | 2.438 | 2.363 | 2.491 |
| | .200 | 299.572 | 257.115 | 334.173 | 2.477 | 2.410 | 2.524 |
| | .250 | 323.243 | 281.986 | 357.258 | 2.510 | 2.450 | 2.553 |
| | .300 | 346.091 | 306.080 | 379.697 | 2.539 | 2.486 | 2.579 |
| | .350 | 368.702 | 329.899 | 402.162 | 2.567 | 2.518 | 2.604 |
| | .400 | 391.522 | 353.791 | 425.213 | 2.593 | 2.549 | 2.629 |
| | .450 | 414.945 | 378.033 | 449.389 | 2.618 | 2.578 | 2.653 |
| | **.500** | **439.363** | **402.871** | **475.278** | **2.643** | **2.605** | **2.677** |
| | .550 | 465.218 | 428.569 | 503.563 | 2.668 | 2.632 | 2.702 |
| | .600 | 493.049 | 455.457 | 535.089 | 2.693 | 2.658 | 2.728 |
| | .650 | 523.566 | 484.004 | 570.948 | 2.719 | 2.685 | 2.757 |
| | .700 | 557.772 | 514.919 | 612.656 | 2.746 | 2.712 | 2.787 |
| | .750 | 597.196 | 549.334 | 662.494 | 2.776 | 2.740 | 2.821 |
| | .800 | 644.386 | 589.149 | 724.274 | 2.809 | 2.770 | 2.860 |
| | .850 | 704.117 | 637.906 | 805.243 | 2.848 | 2.805 | 2.906 |
| | **.900** | **787.201** | **703.499** | **922.100** | **2.896** | **2.847** | **2.965** |
| | .910 | 808.697 | 720.137 | 953.031 | 2.908 | 2.857 | 2.979 |
| | .920 | 832.714 | 738.589 | 987.903 | 2.920 | 2.868 | 2.995 |
| | .930 | 859.947 | 759.349 | 1027.824 | 2.934 | 2.880 | 3.012 |
| | .940 | 891.416 | 783.138 | 1074.438 | 2.950 | 2.894 | 3.031 |
| | .950 | 928.714 | 811.081 | 1130.326 | 2.968 | 2.909 | 3.053 |
| | .960 | 974.534 | 845.064 | 1199.889 | 2.989 | 2.927 | 3.079 |
| | .970 | 1033.973 | 888.636 | 1291.536 | 3.015 | 2.949 | 3.111 |
| | .980 | 1118.636 | 949.813 | 1424.656 | 3.049 | 2.978 | 3.154 |
| | .990 | 1266.369 | 1054.419 | 1663.626 | 3.103 | 3.023 | 3.221 |

a Logarithm base = 10.

**Appendix 5**: Generated LC values for *Teclea nobilis* fraction $F_3$

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 50.632 | 28.765 | 71.740 | 1.704 | 1.459 | 1.856 |
| | .020 | 60.372 | 36.270 | 82.789 | 1.781 | 1.560 | 1.918 |
| | .030 | 67.503 | 42.005 | 90.691 | 1.829 | 1.623 | 1.958 |
| | .040 | 73.417 | 46.901 | 97.146 | 1.866 | 1.671 | 1.987 |
| | .050 | 78.607 | 51.294 | 102.748 | 1.895 | 1.710 | 2.012 |
| | .060 | 83.313 | 55.350 | 107.782 | 1.921 | 1.743 | 2.033 |
| | .070 | 87.671 | 59.164 | 112.409 | 1.943 | 1.772 | 2.051 |
| | .080 | 91.766 | 62.796 | 116.731 | 1.963 | 1.798 | 2.067 |
| | .090 | 95.656 | 66.287 | 120.814 | 1.981 | 1.821 | 2.082 |
| | .100 | 99.382 | 69.667 | 124.708 | 1.997 | 1.843 | 2.096 |
| | .150 | 116.418 | 85.520 | 142.337 | 2.066 | 1.932 | 2.153 |
| | .200 | 132.017 | 100.519 | 158.323 | 2.121 | 2.002 | 2.200 |
| | .250 | 147.056 | 115.310 | 173.696 | 2.167 | 2.062 | 2.240 |
| | .300 | 162.016 | 130.248 | 189.047 | 2.210 | 2.115 | 2.277 |
| | .350 | 177.233 | 145.572 | 204.816 | 2.249 | 2.163 | 2.311 |
| | .400 | 192.993 | 161.470 | 221.415 | 2.286 | 2.208 | 2.345 |
| | .450 | 209.573 | 178.105 | 239.287 | 2.321 | 2.251 | 2.379 |
| | **.500** | **227.280** | **195.637** | **258.959** | **2.357** | **2.291** | **2.413** |
| | .550 | 246.483 | 214.245 | 281.096 | 2.392 | 2.331 | 2.449 |
| | .600 | 267.659 | 234.170 | 306.571 | 2.428 | 2.370 | 2.487 |
| | .650 | 291.459 | 255.769 | 336.567 | 2.465 | 2.408 | 2.527 |
| | .700 | 318.834 | 279.617 | 372.786 | 2.504 | 2.447 | 2.571 |
| | .750 | 351.269 | 306.676 | 417.855 | 2.546 | 2.487 | 2.621 |
| | .800 | 391.283 | 338.622 | 476.271 | 2.592 | 2.530 | 2.678 |
| | .850 | 443.713 | 378.671 | 556.818 | 2.647 | 2.578 | 2.746 |
| | **.900** | **519.775** | **434.154** | **680.449** | **2.716** | **2.638** | **2.833** |
| | .910 | 540.023 | 448.518 | 714.554 | 2.732 | 2.652 | 2.854 |
| | .920 | 562.913 | 464.584 | 753.670 | 2.750 | 2.667 | 2.877 |
| | .930 | 589.205 | 482.829 | 799.298 | 2.770 | 2.684 | 2.903 |
| | .940 | 620.022 | 503.956 | 853.697 | 2.792 | 2.702 | 2.931 |
| | .950 | 657.142 | 529.066 | 920.474 | 2.818 | 2.724 | 2.964 |
| | .960 | 703.602 | 560.026 | 1005.890 | 2.847 | 2.748 | 3.003 |
| | .970 | 765.244 | 600.388 | 1122.197 | 2.884 | 2.778 | 3.050 |
| | .980 | 855.626 | 658.288 | 1298.464 | 2.932 | 2.818 | 3.113 |
| | .990 | 1020.237 | 760.487 | 1635.456 | 3.009 | 2.881 | 3.214 |

a Logarithm base = 10.

**Appendix 6**: Generated LC values for *Teclea nobilis* fraction $F_4$

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 96.357 | 63.593 | 124.159 | 1.984 | 1.803 | 2.094 |
| | .020 | 110.129 | 75.932 | 138.395 | 2.042 | 1.880 | 2.141 |
| | .030 | 119.870 | 84.951 | 148.306 | 2.079 | 1.929 | 2.171 |
| | .040 | 127.762 | 92.417 | 156.255 | 2.106 | 1.966 | 2.194 |
| | .050 | 134.563 | 98.957 | 163.058 | 2.129 | 1.995 | 2.212 |
| | .060 | 140.637 | 104.875 | 169.101 | 2.148 | 2.021 | 2.228 |
| | .070 | 146.187 | 110.343 | 174.601 | 2.165 | 2.043 | 2.242 |
| | .080 | 151.342 | 115.470 | 179.694 | 2.180 | 2.062 | 2.255 |
| | .090 | 156.188 | 120.329 | 184.470 | 2.194 | 2.080 | 2.266 |
| | .100 | 160.786 | 124.973 | 188.994 | 2.206 | 2.097 | 2.276 |
| | .150 | 181.308 | 146.041 | 209.138 | 2.258 | 2.164 | 2.320 |
| | .200 | 199.470 | 165.039 | 227.015 | 2.300 | 2.218 | 2.356 |
| | .250 | 216.496 | 183.012 | 243.941 | 2.335 | 2.262 | 2.387 |
| | .300 | 233.020 | 200.480 | 260.649 | 2.367 | 2.302 | 2.416 |
| | .350 | 249.455 | 217.747 | 277.665 | 2.397 | 2.338 | 2.444 |
| | .400 | 266.122 | 235.017 | 295.450 | 2.425 | 2.371 | 2.470 |
| | .450 | 283.306 | 252.448 | 314.463 | 2.452 | 2.402 | 2.498 |
| | **.500** | **301.301** | **270.188** | **335.205** | **2.479** | **2.432** | **2.525** |
| | .550 | 320.439 | 288.420 | 358.251 | 2.506 | 2.460 | 2.554 |
| | .600 | 341.130 | 307.395 | 384.303 | 2.533 | 2.488 | 2.585 |
| | .650 | 363.922 | 327.481 | 414.280 | 2.561 | 2.515 | 2.617 |
| | .700 | 389.590 | 349.227 | 449.489 | 2.591 | 2.543 | 2.653 |
| | .750 | 419.326 | 373.478 | 491.953 | 2.623 | 2.572 | 2.692 |
| | .800 | 455.116 | 401.623 | 545.123 | 2.658 | 2.604 | 2.736 |
| | .850 | 500.708 | 436.221 | 615.656 | 2.700 | 2.640 | 2.789 |
| | **.900** | **564.615** | **482.970** | **719.066** | **2.752** | **2.684** | **2.857** |
| | .910 | 581.236 | 494.861 | 746.744 | 2.764 | 2.694 | 2.873 |
| | .920 | 599.848 | 508.062 | 778.095 | 2.778 | 2.706 | 2.891 |
| | .930 | 621.001 | 522.931 | 814.172 | 2.793 | 2.718 | 2.911 |
| | .940 | 645.509 | 539.990 | 856.542 | 2.810 | 2.732 | 2.933 |
| | .950 | 674.643 | 560.055 | 907.681 | 2.829 | 2.748 | 2.958 |
| | .960 | 710.556 | 584.494 | 971.825 | 2.852 | 2.767 | 2.988 |
| | .970 | 757.337 | 615.885 | 1057.131 | 2.879 | 2.789 | 3.024 |
| | .980 | 824.326 | 660.058 | 1182.558 | 2.916 | 2.820 | 3.073 |
| | .990 | 942.142 | 735.838 | 1411.836 | 2.974 | 2.867 | 3.150 |

a Logarithm base = 10.

**Appendix 7**: Generated LC values for *Teclea nobilis* methanol fraction

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 263.318 | 210.733 | 303.551 | 2.420 | 2.324 | 2.482 |
| | .020 | 285.177 | 233.564 | 324.219 | 2.455 | 2.368 | 2.511 |
| | .030 | 299.977 | 249.275 | 338.111 | 2.477 | 2.397 | 2.529 |
| | .040 | 311.615 | 261.759 | 348.988 | 2.494 | 2.418 | 2.543 |
| | .050 | 321.413 | 272.352 | 358.122 | 2.507 | 2.435 | 2.554 |
| | .060 | 329.996 | 281.687 | 366.109 | 2.519 | 2.450 | 2.564 |
| | .070 | 337.710 | 290.118 | 373.279 | 2.529 | 2.463 | 2.572 |
| | .080 | 344.769 | 297.866 | 379.837 | 2.538 | 2.474 | 2.580 |
| | .090 | 351.317 | 305.078 | 385.919 | 2.546 | 2.484 | 2.586 |
| | .100 | 357.455 | 311.858 | 391.621 | 2.553 | 2.494 | 2.593 |
| | .150 | 384.029 | 341.385 | 416.361 | 2.584 | 2.533 | 2.619 |
| | .200 | 406.551 | 366.530 | 437.495 | 2.609 | 2.564 | 2.641 |
| | .250 | 426.922 | 389.251 | 456.857 | 2.630 | 2.590 | 2.660 |
| | .300 | 446.085 | 410.491 | 475.394 | 2.649 | 2.613 | 2.677 |
| | .350 | 464.609 | 430.788 | 493.720 | 2.667 | 2.634 | 2.693 |
| | .400 | 482.897 | 450.490 | 512.313 | 2.684 | 2.654 | 2.710 |
| | .450 | 501.277 | 469.851 | 531.599 | 2.700 | 2.672 | 2.726 |
| | **.500** | **520.047** | **489.088** | **551.998** | **2.716** | **2.689** | **2.742** |
| | .550 | 539.521 | 508.422 | 573.958 | 2.732 | 2.706 | 2.759 |
| | .600 | 560.055 | 528.121 | 597.992 | 2.748 | 2.723 | 2.777 |
| | .650 | 582.100 | 548.538 | 624.739 | 2.765 | 2.739 | 2.796 |
| | .700 | 606.272 | 570.165 | 655.075 | 2.783 | 2.756 | 2.816 |
| | .750 | 633.485 | 593.728 | 690.320 | 2.802 | 2.774 | 2.839 |
| | .800 | 665.228 | 620.383 | 732.665 | 2.823 | 2.793 | 2.865 |
| | .850 | 704.241 | 652.209 | 786.226 | 2.848 | 2.814 | 2.896 |
| | **.900** | **756.596** | **693.719** | **860.285** | **2.879** | **2.841** | **2.935** |
| | .910 | 769.814 | 704.026 | 879.330 | 2.886 | 2.848 | 2.944 |
| | .920 | 784.435 | 715.358 | 900.548 | 2.895 | 2.855 | 2.955 |
| | .930 | 800.833 | 727.984 | 924.525 | 2.904 | 2.862 | 2.966 |
| | .940 | 819.552 | 742.301 | 952.122 | 2.914 | 2.871 | 2.979 |
| | .950 | 841.437 | 758.916 | 984.677 | 2.925 | 2.880 | 2.993 |
| | .960 | 867.895 | 778.843 | 1024.442 | 2.938 | 2.891 | 3.010 |
| | .970 | 901.566 | 803.967 | 1075.656 | 2.955 | 2.905 | 3.032 |
| | .980 | 948.356 | 838.492 | 1147.906 | 2.977 | 2.923 | 3.060 |
| | .990 | 1027.082 | 895.681 | 1272.128 | 3.012 | 2.952 | 3.105 |

a Logarithm base = 10.

**Appendix 8**: Generated LC values for *Teclea nobilis* compounds **10**, **13** and **21**

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 49.139 | 26.395 | 71.667 | 1.691 | 1.422 | 1.855 |
| | .020 | 60.002 | 34.425 | 84.250 | 1.778 | 1.537 | 1.926 |
| | .030 | 68.108 | 40.730 | 93.390 | 1.833 | 1.610 | 1.970 |
| | .040 | 74.920 | 46.212 | 100.936 | 1.875 | 1.665 | 2.004 |
| | .050 | 80.961 | 51.202 | 107.542 | 1.908 | 1.709 | 2.032 |
| | .060 | 86.484 | 55.863 | 113.520 | 1.937 | 1.747 | 2.055 |
| | .070 | 91.637 | 60.290 | 119.051 | 1.962 | 1.780 | 2.076 |
| | .080 | 96.510 | 64.543 | 124.245 | 1.985 | 1.810 | 2.094 |
| | .090 | 101.166 | 68.664 | 129.179 | 2.005 | 1.837 | 2.111 |
| | .100 | 105.651 | 72.682 | 133.908 | 2.024 | 1.861 | 2.127 |
| | .150 | 126.436 | 91.861 | 155.597 | 2.102 | 1.963 | 2.192 |
| | .200 | 145.833 | 110.433 | 175.664 | 2.164 | 2.043 | 2.245 |
| | .250 | 164.830 | 129.062 | 195.335 | 2.217 | 2.111 | 2.291 |
| | .300 | 183.990 | 148.113 | 215.367 | 2.265 | 2.171 | 2.333 |
| | .350 | 203.727 | 167.824 | 236.384 | 2.309 | 2.225 | 2.374 |
| | .400 | 224.410 | 188.368 | 259.015 | 2.351 | 2.275 | 2.413 |
| | .450 | 246.417 | 209.892 | 283.973 | 2.392 | 2.322 | 2.453 |
| | **.500** | **270.181** | **232.551** | **312.116** | **2.432** | **2.367** | **2.494** |
| | .550 | 296.237 | 256.561 | 344.512 | 2.472 | 2.409 | 2.537 |
| | .600 | 325.288 | 282.273 | 382.531 | 2.512 | 2.451 | 2.583 |
| | .650 | 358.312 | 310.255 | 428.033 | 2.554 | 2.492 | 2.631 |
| | .700 | 396.748 | 341.418 | 483.735 | 2.599 | 2.533 | 2.685 |
| | .750 | 442.866 | 377.234 | 553.960 | 2.646 | 2.577 | 2.743 |
| | .800 | 500.556 | 420.199 | 646.297 | 2.699 | 2.623 | 2.810 |
| | .850 | 577.351 | 475.052 | 775.877 | 2.761 | 2.677 | 2.890 |
| | **.900** | **690.932** | **552.631** | **979.485** | **2.839** | **2.742** | **2.991** |
| | .910 | 721.561 | 572.980 | 1036.585 | 2.858 | 2.758 | 3.016 |
| | .920 | 756.377 | 595.858 | 1102.540 | 2.879 | 2.775 | 3.042 |
| | .930 | 796.600 | 621.981 | 1180.083 | 2.901 | 2.794 | 3.072 |
| | .940 | 844.058 | 652.411 | 1273.347 | 2.926 | 2.815 | 3.105 |
| | .950 | 901.644 | 688.818 | 1388.988 | 2.955 | 2.838 | 3.143 |
| | .960 | 974.339 | 734.043 | 1538.658 | 2.989 | 2.866 | 3.187 |
| | .970 | 1071.789 | 793.524 | 1745.389 | 3.030 | 2.900 | 3.242 |
| | .980 | 1216.585 | 879.802 | 2064.580 | 3.085 | 2.944 | 3.315 |
| | .990 | 1485.530 | 1034.548 | 2692.012 | 3.172 | 3.015 | 3.430 |

a Logarithm base = 10.

**Appendix 9**: Generated LC values for *Teclea nobilis* compound **22**

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 69.184 | 40.909 | 95.055 | 1.840 | 1.612 | 1.978 |
| | .020 | 81.767 | 51.125 | 108.817 | 1.913 | 1.709 | 2.037 |
| | .030 | 90.913 | 58.874 | 118.603 | 1.959 | 1.770 | 2.074 |
| | .040 | 98.461 | 65.454 | 126.567 | 1.993 | 1.816 | 2.102 |
| | .050 | 105.061 | 71.334 | 133.459 | 2.021 | 1.853 | 2.125 |
| | .060 | 111.025 | 76.742 | 139.639 | 2.045 | 1.885 | 2.145 |
| | .070 | 116.533 | 81.811 | 145.310 | 2.066 | 1.913 | 2.162 |
| | .080 | 121.696 | 86.624 | 150.598 | 2.085 | 1.938 | 2.178 |
| | .090 | 126.590 | 91.238 | 155.589 | 2.102 | 1.960 | 2.192 |
| | .100 | 131.269 | 95.694 | 160.344 | 2.118 | 1.981 | 2.205 |
| | .150 | 152.552 | 116.445 | 181.836 | 2.183 | 2.066 | 2.260 |
| | .200 | 171.903 | 135.858 | 201.312 | 2.235 | 2.133 | 2.304 |
| | .250 | 190.449 | 154.786 | 220.083 | 2.280 | 2.190 | 2.343 |
| | .300 | 208.803 | 173.667 | 238.913 | 2.320 | 2.240 | 2.378 |
| | .350 | 227.386 | 192.765 | 258.396 | 2.357 | 2.285 | 2.412 |
| | .400 | 246.547 | 212.261 | 279.091 | 2.392 | 2.327 | 2.446 |
| | .450 | 266.620 | 232.296 | 301.599 | 2.426 | 2.366 | 2.479 |
| | **.500** | **287.970** | **253.014** | **326.608** | **2.459** | **2.403** | **2.514** |
| | .550 | 311.030 | 274.609 | 354.943 | 2.493 | 2.439 | 2.550 |
| | .600 | 336.354 | 297.378 | 387.633 | 2.527 | 2.473 | 2.588 |
| | .650 | 364.696 | 321.791 | 426.046 | 2.562 | 2.508 | 2.629 |
| | .700 | 397.153 | 348.581 | 472.155 | 2.599 | 2.542 | 2.674 |
| | .750 | 435.428 | 378.898 | 529.060 | 2.639 | 2.579 | 2.724 |
| | .800 | 482.405 | 414.666 | 602.132 | 2.683 | 2.618 | 2.780 |
| | .850 | 543.596 | 459.477 | 701.913 | 2.735 | 2.662 | 2.846 |
| | **.900** | **631.733** | **521.437** | **853.505** | **2.801** | **2.717** | **2.931** |
| | .910 | 655.082 | 537.443 | 895.068 | 2.816 | 2.730 | 2.952 |
| | .920 | 681.426 | 555.327 | 942.622 | 2.833 | 2.745 | 2.974 |
| | .930 | 711.617 | 575.610 | 997.953 | 2.852 | 2.760 | 2.999 |
| | .940 | 746.919 | 599.060 | 1063.737 | 2.873 | 2.777 | 3.027 |
| | .950 | 789.324 | 626.879 | 1144.246 | 2.897 | 2.797 | 3.059 |
| | .960 | 842.231 | 661.101 | 1246.872 | 2.925 | 2.820 | 3.096 |
| | .970 | 912.157 | 705.585 | 1386.047 | 2.960 | 2.849 | 3.142 |
| | .980 | 1014.180 | 769.145 | 1595.897 | 3.006 | 2.886 | 3.203 |
| | .990 | 1198.640 | 880.647 | 1994.123 | 3.079 | 2.945 | 3.300 |

a Logarithm base = 10.

**Appendix 10**: Generated LC values for *Teclea nobilis* Essential oil

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT(b) | .010 | 62.956 | 26.247 | 93.926 | 1.799 | 1.419 | 1.973 |
| | .020 | 71.929 | 32.578 | 103.734 | 1.857 | 1.513 | 2.016 |
| | .030 | 78.275 | 37.349 | 110.529 | 1.894 | 1.572 | 2.043 |
| | .040 | 83.414 | 41.382 | 115.963 | 1.921 | 1.617 | 2.064 |
| | .050 | 87.843 | 44.973 | 120.605 | 1.944 | 1.653 | 2.081 |
| | .060 | 91.797 | 48.265 | 124.722 | 1.963 | 1.684 | 2.096 |
| | .070 | 95.411 | 51.343 | 128.465 | 1.980 | 1.710 | 2.109 |
| | .080 | 98.767 | 54.258 | 131.928 | 1.995 | 1.734 | 2.120 |
| | .090 | 101.921 | 57.047 | 135.173 | 2.008 | 1.756 | 2.131 |
| | .100 | 104.914 | 59.734 | 138.245 | 2.021 | 1.776 | 2.141 |
| | .150 | 118.268 | 72.185 | 151.912 | 2.073 | 1.858 | 2.182 |
| | .200 | 130.084 | 83.755 | 164.030 | 2.114 | 1.923 | 2.215 |
| | .250 | 141.157 | 94.980 | 175.503 | 2.150 | 1.978 | 2.244 |
| | .300 | 151.903 | 106.143 | 186.829 | 2.182 | 2.026 | 2.271 |
| | .350 | 162.589 | 117.422 | 198.368 | 2.211 | 2.070 | 2.297 |
| | .400 | 173.423 | 128.946 | 210.441 | 2.239 | 2.110 | 2.323 |
| | .450 | 184.592 | 140.817 | 223.380 | 2.266 | 2.149 | 2.349 |
| | **.500** | **196.286** | **153.124** | **237.575** | **2.293** | **2.185** | **2.376** |
| | .550 | 208.720 | 165.955 | 253.511 | 2.320 | 2.220 | 2.404 |
| | .600 | 222.162 | 179.416 | 271.824 | 2.347 | 2.254 | 2.434 |
| | .650 | 236.966 | 193.655 | 293.384 | 2.375 | 2.287 | 2.467 |
| | .700 | 253.637 | 208.910 | 319.443 | 2.404 | 2.320 | 2.504 |
| | .750 | 272.944 | 225.589 | 351.927 | 2.436 | 2.353 | 2.546 |
| | .800 | 296.179 | 244.430 | 394.094 | 2.472 | 2.388 | 2.596 |
| | .850 | 325.769 | 266.859 | 452.234 | 2.513 | 2.426 | 2.655 |
| | **.900** | **367.236** | **296.119** | **541.191** | **2.565** | **2.471** | **2.733** |
| | .910 | 378.019 | 303.407 | 565.641 | 2.578 | 2.482 | 2.753 |
| | .920 | 390.092 | 311.437 | 593.629 | 2.591 | 2.493 | 2.774 |
| | .930 | 403.812 | 320.410 | 626.204 | 2.606 | 2.506 | 2.797 |
| | .940 | 419.707 | 330.622 | 664.933 | 2.623 | 2.519 | 2.823 |
| | .950 | 438.601 | 342.531 | 712.319 | 2.642 | 2.535 | 2.853 |
| | .960 | 461.887 | 356.905 | 772.685 | 2.665 | 2.553 | 2.888 |
| | .970 | 492.217 | 375.184 | 854.454 | 2.692 | 2.574 | 2.932 |
| | .980 | 535.639 | 400.610 | 977.509 | 2.729 | 2.603 | 2.990 |
| | .990 | 611.986 | 443.575 | 1210.195 | 2.787 | 2.647 | 3.083 |

a Logarithm base = 10.
b A heterogeneity factor is used.

**Appendix 11**: Generated LC values for *Rapanea melanophloeos* methanol crude extract

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 16.222 | 7.989 | 24.646 | 1.210 | .902 | 1.392 |
| | .020 | 19.994 | 10.610 | 29.161 | 1.301 | 1.026 | 1.465 |
| | .030 | 22.829 | 12.695 | 32.463 | 1.358 | 1.104 | 1.511 |
| | .040 | 25.224 | 14.525 | 35.203 | 1.402 | 1.162 | 1.547 |
| | .050 | 27.357 | 16.202 | 37.612 | 1.437 | 1.210 | 1.575 |
| | .060 | 29.313 | 17.777 | 39.801 | 1.467 | 1.250 | 1.600 |
| | .070 | 31.143 | 19.280 | 41.832 | 1.493 | 1.285 | 1.622 |
| | .080 | 32.879 | 20.730 | 43.746 | 1.517 | 1.317 | 1.641 |
| | .090 | 34.541 | 22.139 | 45.570 | 1.538 | 1.345 | 1.659 |
| | .100 | 36.145 | 23.517 | 47.324 | 1.558 | 1.371 | 1.675 |
| | .150 | 43.619 | 30.144 | 55.430 | 1.640 | 1.479 | 1.744 |
| | .200 | 50.646 | 36.616 | 63.029 | 1.705 | 1.564 | 1.800 |
| | .250 | 57.571 | 43.143 | 70.572 | 1.760 | 1.635 | 1.849 |
| | .300 | 64.593 | 49.841 | 78.346 | 1.810 | 1.698 | 1.894 |
| | .350 | 71.863 | 56.787 | 86.594 | 1.857 | 1.754 | 1.937 |
| | .400 | 79.516 | 64.043 | 95.562 | 1.900 | 1.806 | 1.980 |
| | .450 | 87.695 | 71.667 | 105.530 | 1.943 | 1.855 | 2.023 |
| | **.500** | **96.565** | **79.726** | **116.833** | **1.985** | **1.902** | **2.068** |
| | .550 | 106.333 | 88.317 | 129.896 | 2.027 | 1.946 | 2.114 |
| | .600 | 117.270 | 97.584 | 145.276 | 2.069 | 1.989 | 2.162 |
| | .650 | 129.759 | 107.745 | 163.755 | 2.113 | 2.032 | 2.214 |
| | .700 | 144.362 | 119.141 | 186.494 | 2.159 | 2.076 | 2.271 |
| | .750 | 161.970 | 132.316 | 215.365 | 2.209 | 2.122 | 2.333 |
| | .800 | 184.116 | 148.199 | 253.673 | 2.265 | 2.171 | 2.404 |
| | .850 | 213.779 | 168.563 | 308.050 | 2.330 | 2.227 | 2.489 |
| | **.900** | **257.984** | **197.486** | **394.756** | **2.412** | **2.296** | **2.596** |
| | .910 | 269.966 | 205.092 | 419.315 | 2.431 | 2.312 | 2.623 |
| | .920 | 283.613 | 213.653 | 447.804 | 2.453 | 2.330 | 2.651 |
| | .930 | 299.418 | 223.439 | 481.453 | 2.476 | 2.349 | 2.683 |
| | .940 | 318.112 | 234.854 | 522.133 | 2.503 | 2.371 | 2.718 |
| | .950 | 340.861 | 248.531 | 572.869 | 2.533 | 2.395 | 2.758 |
| | .960 | 369.676 | 265.550 | 638.981 | 2.568 | 2.424 | 2.805 |
| | .970 | 408.459 | 287.980 | 731.050 | 2.611 | 2.459 | 2.864 |
| | .980 | 466.385 | 320.603 | 874.716 | 2.669 | 2.506 | 2.942 |
| | .990 | 574.809 | 379.353 | 1161.603 | 2.760 | 2.579 | 3.065 |

a Logarithm base = 10.

**Appendix 12**: Generated LC values for *Rapanea melanophloeos* Ethyl acetate extract

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 53.072 | 30.733 | 74.517 | 1.725 | 1.488 | 1.872 |
| | .020 | 63.400 | 38.803 | 86.166 | 1.802 | 1.589 | 1.935 |
| | .030 | 70.972 | 44.975 | 94.513 | 1.851 | 1.653 | 1.975 |
| | .040 | 77.258 | 50.248 | 101.342 | 1.888 | 1.701 | 2.006 |
| | .050 | 82.780 | 54.980 | 107.275 | 1.918 | 1.740 | 2.030 |
| | .060 | 87.790 | 59.351 | 112.611 | 1.943 | 1.773 | 2.052 |
| | .070 | 92.432 | 63.461 | 117.521 | 1.966 | 1.803 | 2.070 |
| | .080 | 96.796 | 67.376 | 122.110 | 1.986 | 1.829 | 2.087 |
| | .090 | 100.943 | 71.140 | 126.450 | 2.004 | 1.852 | 2.102 |
| | .100 | 104.918 | 74.784 | 130.591 | 2.021 | 1.874 | 2.116 |
| | .150 | 123.109 | 91.874 | 149.383 | 2.090 | 1.963 | 2.174 |
| | .200 | 139.791 | 108.033 | 166.485 | 2.145 | 2.034 | 2.221 |
| | .250 | 155.893 | 123.950 | 182.996 | 2.193 | 2.093 | 2.262 |
| | .300 | 171.928 | 139.997 | 199.551 | 2.235 | 2.146 | 2.300 |
| | .350 | 188.256 | 156.417 | 216.636 | 2.275 | 2.194 | 2.336 |
| | .400 | 205.180 | 173.399 | 234.708 | 2.312 | 2.239 | 2.371 |
| | .450 | 223.002 | 191.102 | 254.265 | 2.348 | 2.281 | 2.405 |
| | **.500** | **242.052** | **209.685** | **275.891** | **2.384** | **2.322** | **2.441** |
| | .550 | 262.729 | 229.341 | 300.316 | 2.420 | 2.360 | 2.478 |
| | .600 | 285.549 | 250.336 | 328.482 | 2.456 | 2.399 | 2.517 |
| | .650 | 311.220 | 273.081 | 361.663 | 2.493 | 2.436 | 2.558 |
| | .700 | 340.776 | 298.230 | 401.688 | 2.532 | 2.475 | 2.604 |
| | .750 | 375.827 | 326.848 | 451.415 | 2.575 | 2.514 | 2.655 |
| | .800 | 419.118 | 360.769 | 515.760 | 2.622 | 2.557 | 2.712 |
| | .850 | 475.911 | 403.475 | 604.370 | 2.678 | 2.606 | 2.781 |
| | **.900** | **558.427** | **462.893** | **740.301** | **2.747** | **2.665** | **2.869** |
| | .910 | 580.415 | 478.313 | 777.799 | 2.764 | 2.680 | 2.891 |
| | .920 | 605.284 | 495.575 | 820.811 | 2.782 | 2.695 | 2.914 |
| | .930 | 633.860 | 515.196 | 870.991 | 2.802 | 2.712 | 2.940 |
| | .940 | 667.374 | 537.936 | 930.828 | 2.824 | 2.731 | 2.969 |
| | .950 | 707.764 | 564.991 | 1004.302 | 2.850 | 2.752 | 3.002 |
| | .960 | 758.351 | 598.383 | 1098.317 | 2.880 | 2.777 | 3.041 |
| | .970 | 825.523 | 641.967 | 1226.392 | 2.917 | 2.808 | 3.089 |
| | .980 | 924.116 | 704.576 | 1420.627 | 2.966 | 2.848 | 3.152 |
| | .990 | 1103.959 | 815.299 | 1792.369 | 3.043 | 2.911 | 3.253 |

a Logarithm base = 10.

**Appendix 13**: Generated LC values for *Rapanea melanophloeos* hexane extract

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 36.806 | 21.354 | 51.957 | 1.566 | 1.329 | 1.716 |
| | .020 | 43.418 | 26.453 | 59.523 | 1.638 | 1.422 | 1.775 |
| | .030 | 48.216 | 30.294 | 64.902 | 1.683 | 1.481 | 1.812 |
| | .040 | 52.172 | 33.542 | 69.278 | 1.717 | 1.526 | 1.841 |
| | .050 | 55.627 | 36.435 | 73.063 | 1.745 | 1.562 | 1.864 |
| | .060 | 58.749 | 39.088 | 76.454 | 1.769 | 1.592 | 1.883 |
| | .070 | 61.629 | 41.570 | 79.564 | 1.790 | 1.619 | 1.901 |
| | .080 | 64.328 | 43.923 | 82.461 | 1.808 | 1.643 | 1.916 |
| | .090 | 66.885 | 46.175 | 85.193 | 1.825 | 1.664 | 1.930 |
| | .100 | 69.328 | 48.347 | 87.794 | 1.841 | 1.684 | 1.943 |
| | .150 | 80.431 | 58.439 | 99.502 | 1.905 | 1.767 | 1.998 |
| | .200 | 90.510 | 67.872 | 110.027 | 1.957 | 1.832 | 2.041 |
| | .250 | 100.158 | 77.092 | 120.059 | 2.001 | 1.887 | 2.079 |
| | .300 | 109.696 | 86.346 | 129.978 | 2.040 | 1.936 | 2.114 |
| | .350 | 119.342 | 95.805 | 140.053 | 2.077 | 1.981 | 2.146 |
| | .400 | 129.279 | 105.607 | 150.519 | 2.112 | 2.024 | 2.178 |
| | .450 | 139.681 | 115.883 | 161.613 | 2.145 | 2.064 | 2.208 |
| | **.500** | **150.734** | **126.766** | **173.611** | **2.178** | **2.103** | **2.240** |
| | .550 | 162.661 | 138.409 | 186.854 | 2.211 | 2.141 | 2.272 |
| | .600 | 175.748 | 150.996 | 201.796 | 2.245 | 2.179 | 2.305 |
| | .650 | 190.382 | 164.778 | 219.071 | 2.280 | 2.217 | 2.341 |
| | .700 | 207.124 | 180.117 | 239.605 | 2.316 | 2.256 | 2.379 |
| | .750 | 226.847 | 197.589 | 264.848 | 2.356 | 2.296 | 2.423 |
| | .800 | 251.028 | 218.191 | 297.259 | 2.400 | 2.339 | 2.473 |
| | .850 | 282.486 | 243.855 | 341.579 | 2.451 | 2.387 | 2.533 |
| | **.900** | **327.726** | **279.031** | **408.958** | **2.516** | **2.446** | **2.612** |
| | .910 | 339.698 | 288.069 | 427.419 | 2.531 | 2.459 | 2.631 |
| | .920 | 353.200 | 298.147 | 448.528 | 2.548 | 2.474 | 2.652 |
| | .930 | 368.667 | 309.556 | 473.068 | 2.567 | 2.491 | 2.675 |
| | .940 | 386.743 | 322.721 | 502.211 | 2.587 | 2.509 | 2.701 |
| | .950 | 408.443 | 338.309 | 537.823 | 2.611 | 2.529 | 2.731 |
| | .960 | 435.498 | 357.449 | 583.127 | 2.639 | 2.553 | 2.766 |
| | .970 | 471.227 | 382.284 | 644.399 | 2.673 | 2.582 | 2.809 |
| | .980 | 523.301 | 417.707 | 736.429 | 2.719 | 2.621 | 2.867 |
| | .990 | 617.304 | 479.741 | 909.978 | 2.790 | 2.681 | 2.959 |

a Logarithm base = 10.

**Appendix 14**: Generated LC values for *Rapanea melanophloeos* fraction $F_1$

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT(b) | .010 | 68.590 | 20.918 | 110.904 | 1.836 | 1.321 | 2.045 |
| | .020 | 80.988 | 28.075 | 125.019 | 1.908 | 1.448 | 2.097 |
| | .030 | 89.992 | 33.817 | 134.978 | 1.954 | 1.529 | 2.130 |
| | .040 | 97.419 | 38.881 | 143.049 | 1.989 | 1.590 | 2.155 |
| | .050 | 103.910 | 43.541 | 150.017 | 2.017 | 1.639 | 2.176 |
| | .060 | 109.774 | 47.932 | 156.256 | 2.041 | 1.681 | 2.194 |
| | .070 | 115.188 | 52.133 | 161.977 | 2.061 | 1.717 | 2.209 |
| | .080 | 120.262 | 56.195 | 167.312 | 2.080 | 1.750 | 2.224 |
| | .090 | 125.070 | 60.151 | 172.349 | 2.097 | 1.779 | 2.236 |
| | .100 | 129.665 | 64.027 | 177.151 | 2.113 | 1.806 | 2.248 |
| | .150 | 150.559 | 82.730 | 198.953 | 2.178 | 1.918 | 2.299 |
| | .200 | 169.540 | 101.056 | 218.961 | 2.229 | 2.005 | 2.340 |
| | .250 | 187.721 | 119.537 | 238.608 | 2.274 | 2.078 | 2.378 |
| | .300 | 205.704 | 138.425 | 258.813 | 2.313 | 2.141 | 2.413 |
| | .350 | 223.901 | 157.840 | 280.371 | 2.350 | 2.198 | 2.448 |
| | .400 | 242.656 | 177.815 | 304.119 | 2.385 | 2.250 | 2.483 |
| | .450 | 262.294 | 198.328 | 331.026 | 2.419 | 2.297 | 2.520 |
| | **.500** | **283.173** | **219.340** | **362.264** | **2.452** | **2.341** | **2.559** |
| | .550 | 305.714 | 240.861 | 399.278 | 2.485 | 2.382 | 2.601 |
| | .600 | 330.456 | 263.012 | 443.916 | 2.519 | 2.420 | 2.647 |
| | .650 | 358.135 | 286.097 | 498.685 | 2.554 | 2.457 | 2.698 |
| | .700 | 389.818 | 310.668 | 567.274 | 2.591 | 2.492 | 2.754 |
| | .750 | 427.160 | 337.635 | 655.624 | 2.631 | 2.528 | 2.817 |
| | .800 | 472.967 | 368.517 | 774.285 | 2.675 | 2.566 | 2.889 |
| | .850 | 532.596 | 406.102 | 944.584 | 2.726 | 2.609 | 2.975 |
| | **.900** | **618.416** | **456.571** | **1219.189** | **2.791** | **2.660** | **3.086** |
| | .910 | 641.139 | 469.384 | 1297.498 | 2.807 | 2.672 | 3.113 |
| | .920 | 666.771 | 483.608 | 1388.580 | 2.824 | 2.684 | 3.143 |
| | .930 | 696.140 | 499.630 | 1496.475 | 2.843 | 2.699 | 3.175 |
| | .940 | 730.472 | 518.020 | 1627.333 | 2.864 | 2.714 | 3.211 |
| | .950 | 771.699 | 539.664 | 1791.118 | 2.887 | 2.732 | 3.253 |
| | .960 | 823.117 | 566.059 | 2005.412 | 2.915 | 2.753 | 3.302 |
| | .970 | 891.049 | 600.025 | 2305.301 | 2.950 | 2.778 | 3.363 |
| | .980 | 990.109 | 647.960 | 2776.176 | 2.996 | 2.812 | 3.443 |
| | .990 | 1169.070 | 730.606 | 3725.156 | 3.068 | 2.864 | 3.571 |

a Logarithm base = 10.
b A heterogeneity factor is used.

**Appendix 15**: Generated LC values for *Rapanea melanophloeos* fraction $F_3$

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 57.424 | 36.504 | 76.257 | 1.759 | 1.562 | 1.882 |
| | .020 | 65.746 | 43.561 | 85.207 | 1.818 | 1.639 | 1.930 |
| | .030 | 71.642 | 48.718 | 91.444 | 1.855 | 1.688 | 1.961 |
| | .040 | 76.423 | 52.989 | 96.449 | 1.883 | 1.724 | 1.984 |
| | .050 | 80.546 | 56.731 | 100.732 | 1.906 | 1.754 | 2.003 |
| | .060 | 84.231 | 60.119 | 104.537 | 1.925 | 1.779 | 2.019 |
| | .070 | 87.599 | 63.250 | 107.999 | 1.943 | 1.801 | 2.033 |
| | .080 | 90.730 | 66.188 | 111.203 | 1.958 | 1.821 | 2.046 |
| | .090 | 93.674 | 68.974 | 114.206 | 1.972 | 1.839 | 2.058 |
| | .100 | 96.469 | 71.639 | 117.048 | 1.984 | 1.855 | 2.068 |
| | .150 | 108.954 | 83.756 | 129.673 | 2.037 | 1.923 | 2.113 |
| | .200 | 120.019 | 94.741 | 140.805 | 2.079 | 1.977 | 2.149 |
| | .250 | 130.404 | 105.212 | 151.251 | 2.115 | 2.022 | 2.180 |
| | .300 | 140.493 | 115.489 | 161.441 | 2.148 | 2.063 | 2.208 |
| | .350 | 150.538 | 125.780 | 171.668 | 2.178 | 2.100 | 2.235 |
| | .400 | 160.733 | 136.240 | 182.175 | 2.206 | 2.134 | 2.260 |
| | .450 | 171.253 | 147.000 | 193.198 | 2.234 | 2.167 | 2.286 |
| | **.500** | **182.279** | **158.186** | **204.997** | **2.261** | **2.199** | **2.312** |
| | .550 | 194.014 | 169.933 | 217.888 | 2.288 | 2.230 | 2.338 |
| | .600 | 206.713 | 182.403 | 232.277 | 2.315 | 2.261 | 2.366 |
| | .650 | 220.712 | 195.808 | 248.713 | 2.344 | 2.292 | 2.396 |
| | .700 | 236.492 | 210.459 | 267.985 | 2.374 | 2.323 | 2.428 |
| | .750 | 254.789 | 226.848 | 291.297 | 2.406 | 2.356 | 2.464 |
| | .800 | 276.835 | 245.826 | 320.663 | 2.442 | 2.391 | 2.506 |
| | .850 | 304.950 | 269.019 | 359.904 | 2.484 | 2.430 | 2.556 |
| | **.900** | **344.418** | **300.120** | **417.855** | **2.537** | **2.477** | **2.621** |
| | .910 | 354.693 | 307.996 | 433.422 | 2.550 | 2.489 | 2.637 |
| | .920 | 366.202 | 316.727 | 451.077 | 2.564 | 2.501 | 2.654 |
| | .930 | 379.290 | 326.547 | 471.416 | 2.579 | 2.514 | 2.673 |
| | .940 | 394.459 | 337.800 | 495.328 | 2.596 | 2.529 | 2.695 |
| | .950 | 412.503 | 351.018 | 524.217 | 2.615 | 2.545 | 2.720 |
| | .960 | 434.759 | 367.100 | 560.487 | 2.638 | 2.565 | 2.749 |
| | .970 | 463.772 | 387.737 | 608.763 | 2.666 | 2.589 | 2.784 |
| | .980 | 505.359 | 416.756 | 679.794 | 2.704 | 2.620 | 2.832 |
| | .990 | 578.605 | 466.528 | 809.708 | 2.762 | 2.669 | 2.908 |

a Logarithm base = 10.

**Appendix 16**: Generated LC values for *Rapanea melanophloeos* fraction $F_4$

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 69.395 | 44.016 | 92.132 | 1.841 | 1.644 | 1.964 |
| | .020 | 80.213 | 53.216 | 103.722 | 1.904 | 1.726 | 2.016 |
| | .030 | 87.936 | 60.010 | 111.849 | 1.944 | 1.778 | 2.049 |
| | .040 | 94.231 | 65.675 | 118.401 | 1.974 | 1.817 | 2.073 |
| | .050 | 99.683 | 70.667 | 124.027 | 1.999 | 1.849 | 2.094 |
| | .060 | 104.571 | 75.206 | 129.040 | 2.019 | 1.876 | 2.111 |
| | .070 | 109.054 | 79.418 | 133.613 | 2.038 | 1.900 | 2.126 |
| | .080 | 113.231 | 83.382 | 137.856 | 2.054 | 1.921 | 2.139 |
| | .090 | 117.168 | 87.153 | 141.842 | 2.069 | 1.940 | 2.152 |
| | .100 | 120.913 | 90.769 | 145.622 | 2.082 | 1.958 | 2.163 |
| | .150 | 137.736 | 107.318 | 162.510 | 2.139 | 2.031 | 2.211 |
| | .200 | 152.761 | 122.446 | 177.537 | 2.184 | 2.088 | 2.249 |
| | .250 | 166.950 | 136.945 | 191.766 | 2.223 | 2.137 | 2.283 |
| | .300 | 180.813 | 151.227 | 205.781 | 2.257 | 2.180 | 2.313 |
| | .350 | 194.684 | 165.549 | 219.997 | 2.289 | 2.219 | 2.342 |
| | .400 | 208.828 | 180.098 | 234.775 | 2.320 | 2.256 | 2.371 |
| | .450 | 223.491 | 195.028 | 250.482 | 2.349 | 2.290 | 2.399 |
| | **.500** | **238.926** | **210.482** | **267.531** | **2.378** | **2.323** | **2.427** |
| | .550 | 255.427 | 226.619 | 286.424 | 2.407 | 2.355 | 2.457 |
| | .600 | 273.362 | 243.646 | 307.794 | 2.437 | 2.387 | 2.488 |
| | .650 | 293.223 | 261.857 | 332.485 | 2.467 | 2.418 | 2.522 |
| | .700 | 315.717 | 281.713 | 361.691 | 2.499 | 2.450 | 2.558 |
| | .750 | 341.932 | 303.953 | 397.240 | 2.534 | 2.483 | 2.599 |
| | .800 | 373.693 | 329.842 | 442.220 | 2.573 | 2.518 | 2.646 |
| | .850 | 414.456 | 361.765 | 502.571 | 2.617 | 2.558 | 2.701 |
| | **.900** | **472.121** | **405.091** | **592.188** | **2.674** | **2.608** | **2.772** |
| | .910 | 487.212 | 416.150 | 616.367 | 2.688 | 2.619 | 2.790 |
| | .920 | 504.153 | 428.449 | 643.842 | 2.703 | 2.632 | 2.809 |
| | .930 | 523.462 | 442.326 | 675.568 | 2.719 | 2.646 | 2.830 |
| | .940 | 545.902 | 458.282 | 712.968 | 2.737 | 2.661 | 2.853 |
| | .950 | 572.671 | 477.096 | 758.296 | 2.758 | 2.679 | 2.880 |
| | .960 | 605.804 | 500.082 | 815.423 | 2.782 | 2.699 | 2.911 |
| | .970 | 649.175 | 529.718 | 891.827 | 2.812 | 2.724 | 2.950 |
| | .980 | 711.674 | 571.632 | 1004.972 | 2.852 | 2.757 | 3.002 |
| | .990 | 822.618 | 644.088 | 1213.975 | 2.915 | 2.809 | 3.084 |

a Logarithm base = 10.

**Appendix 17**: Generated LC values for *Rapanea melanophloeos* methanol fraction

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 56.665 | 33.964 | 77.759 | 1.753 | 1.531 | 1.891 |
| | .020 | 66.283 | 41.742 | 88.361 | 1.821 | 1.621 | 1.946 |
| | .030 | 73.215 | 47.563 | 95.849 | 1.865 | 1.677 | 1.982 |
| | .040 | 78.903 | 52.463 | 101.915 | 1.897 | 1.720 | 2.008 |
| | .050 | 83.855 | 56.812 | 107.144 | 1.924 | 1.754 | 2.030 |
| | .060 | 88.314 | 60.791 | 111.816 | 1.946 | 1.784 | 2.049 |
| | .070 | 92.418 | 64.502 | 116.090 | 1.966 | 1.810 | 2.065 |
| | .080 | 96.254 | 68.012 | 120.064 | 1.983 | 1.833 | 2.079 |
| | .090 | 99.881 | 71.365 | 123.805 | 1.999 | 1.853 | 2.093 |
| | .100 | 103.340 | 74.593 | 127.360 | 2.014 | 1.873 | 2.105 |
| | .150 | 118.985 | 89.515 | 143.306 | 2.075 | 1.952 | 2.156 |
| | .200 | 133.092 | 103.356 | 157.576 | 2.124 | 2.014 | 2.197 |
| | .250 | 146.519 | 116.789 | 171.142 | 2.166 | 2.067 | 2.233 |
| | .300 | 159.728 | 130.174 | 184.544 | 2.203 | 2.115 | 2.266 |
| | .350 | 173.029 | 143.747 | 198.168 | 2.238 | 2.158 | 2.297 |
| | .400 | 186.673 | 157.688 | 212.354 | 2.271 | 2.198 | 2.327 |
| | .450 | 200.897 | 172.149 | 227.456 | 2.303 | 2.236 | 2.357 |
| | **.500** | **215.954** | **187.278** | **243.883** | **2.334** | **2.272** | **2.387** |
| | .550 | 232.138 | 203.235 | 262.141 | 2.366 | 2.308 | 2.419 |
| | .600 | 249.826 | 220.223 | 282.889 | 2.398 | 2.343 | 2.452 |
| | .650 | 269.526 | 238.529 | 307.018 | 2.431 | 2.378 | 2.487 |
| | .700 | 291.970 | 258.601 | 335.800 | 2.465 | 2.413 | 2.526 |
| | .750 | 318.292 | 281.182 | 371.184 | 2.503 | 2.449 | 2.570 |
| | .800 | 350.405 | 307.565 | 416.461 | 2.545 | 2.488 | 2.620 |
| | .850 | 391.947 | 340.229 | 477.973 | 2.593 | 2.532 | 2.679 |
| | **.900** | **451.285** | **384.803** | **570.647** | **2.654** | **2.585** | **2.756** |
| | .910 | 466.915 | 396.228 | 595.888 | 2.669 | 2.598 | 2.775 |
| | .920 | 484.509 | 408.956 | 624.680 | 2.685 | 2.612 | 2.796 |
| | .930 | 504.621 | 423.347 | 658.065 | 2.703 | 2.627 | 2.818 |
| | .940 | 528.071 | 439.931 | 697.603 | 2.723 | 2.643 | 2.844 |
| | .950 | 556.149 | 459.538 | 745.770 | 2.745 | 2.662 | 2.873 |
| | .960 | 591.051 | 483.567 | 806.837 | 2.772 | 2.684 | 2.907 |
| | .970 | 636.974 | 514.667 | 889.096 | 2.804 | 2.712 | 2.949 |
| | .980 | 703.591 | 558.875 | 1012.031 | 2.847 | 2.747 | 3.005 |
| | .990 | 823.013 | 635.872 | 1242.205 | 2.915 | 2.803 | 3.094 |

a Logarithm base = 10.

**Appendix 18**: Generated LC values for *Rapanea melanophloeos* compound **23**

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT(b) | .010 | 32.197 | 15.675 | 48.552 | 1.508 | 1.195 | 1.686 |
| | .020 | 38.245 | 19.915 | 55.694 | 1.583 | 1.299 | 1.746 |
| | .030 | 42.658 | 23.171 | 60.789 | 1.630 | 1.365 | 1.784 |
| | .040 | 46.311 | 25.959 | 64.946 | 1.666 | 1.414 | 1.813 |
| | .050 | 49.512 | 28.466 | 68.551 | 1.695 | 1.454 | 1.836 |
| | .060 | 52.410 | 30.784 | 71.790 | 1.719 | 1.488 | 1.856 |
| | .070 | 55.090 | 32.967 | 74.766 | 1.741 | 1.518 | 1.874 |
| | .080 | 57.606 | 35.048 | 77.546 | 1.760 | 1.545 | 1.890 |
| | .090 | 59.994 | 37.050 | 80.174 | 1.778 | 1.569 | 1.904 |
| | .100 | 62.279 | 38.989 | 82.680 | 1.794 | 1.591 | 1.917 |
| | .150 | 72.705 | 48.096 | 94.040 | 1.862 | 1.682 | 1.973 |
| | .200 | 82.222 | 56.720 | 104.372 | 1.915 | 1.754 | 2.019 |
| | .250 | 91.374 | 65.222 | 114.343 | 1.961 | 1.814 | 2.058 |
| | .300 | 100.458 | 73.803 | 124.334 | 2.002 | 1.868 | 2.095 |
| | .350 | 109.679 | 82.600 | 134.631 | 2.040 | 1.917 | 2.129 |
| | .400 | 119.211 | 91.722 | 145.493 | 2.076 | 1.962 | 2.163 |
| | .450 | 129.221 | 101.271 | 157.197 | 2.111 | 2.005 | 2.196 |
| | **.500** | **139.893** | **111.354** | **170.070** | **2.146** | **2.047** | **2.231** |
| | .550 | 151.446 | 122.094 | 184.519 | 2.180 | 2.087 | 2.266 |
| | .600 | 164.163 | 133.650 | 201.088 | 2.215 | 2.126 | 2.303 |
| | .650 | 178.430 | 146.242 | 220.533 | 2.251 | 2.165 | 2.343 |
| | .700 | 194.809 | 160.204 | 243.965 | 2.290 | 2.205 | 2.387 |
| | .750 | 214.175 | 176.070 | 273.133 | 2.331 | 2.246 | 2.436 |
| | .800 | 238.015 | 194.764 | 311.056 | 2.377 | 2.290 | 2.493 |
| | .850 | 269.172 | 218.057 | 363.642 | 2.430 | 2.339 | 2.561 |
| | **.900** | **314.232** | **250.005** | **445.021** | **2.497** | **2.398** | **2.648** |
| | .910 | 326.202 | 258.215 | 467.597 | 2.513 | 2.412 | 2.670 |
| | .920 | 339.723 | 267.372 | 493.550 | 2.531 | 2.427 | 2.693 |
| | .930 | 355.238 | 277.736 | 523.901 | 2.551 | 2.444 | 2.719 |
| | .940 | 373.405 | 289.696 | 560.187 | 2.572 | 2.462 | 2.748 |
| | .950 | 395.261 | 303.856 | 604.868 | 2.597 | 2.483 | 2.782 |
| | .960 | 422.578 | 321.240 | 662.221 | 2.626 | 2.507 | 2.821 |
| | .970 | 458.763 | 343.791 | 740.643 | 2.662 | 2.536 | 2.870 |
| | .980 | 511.705 | 375.945 | 860.125 | 2.709 | 2.575 | 2.935 |
| | .990 | 607.824 | 432.224 | 1090.309 | 2.784 | 2.636 | 3.038 |

a Logarithm base = 10.
b A heterogeneity factor is used.

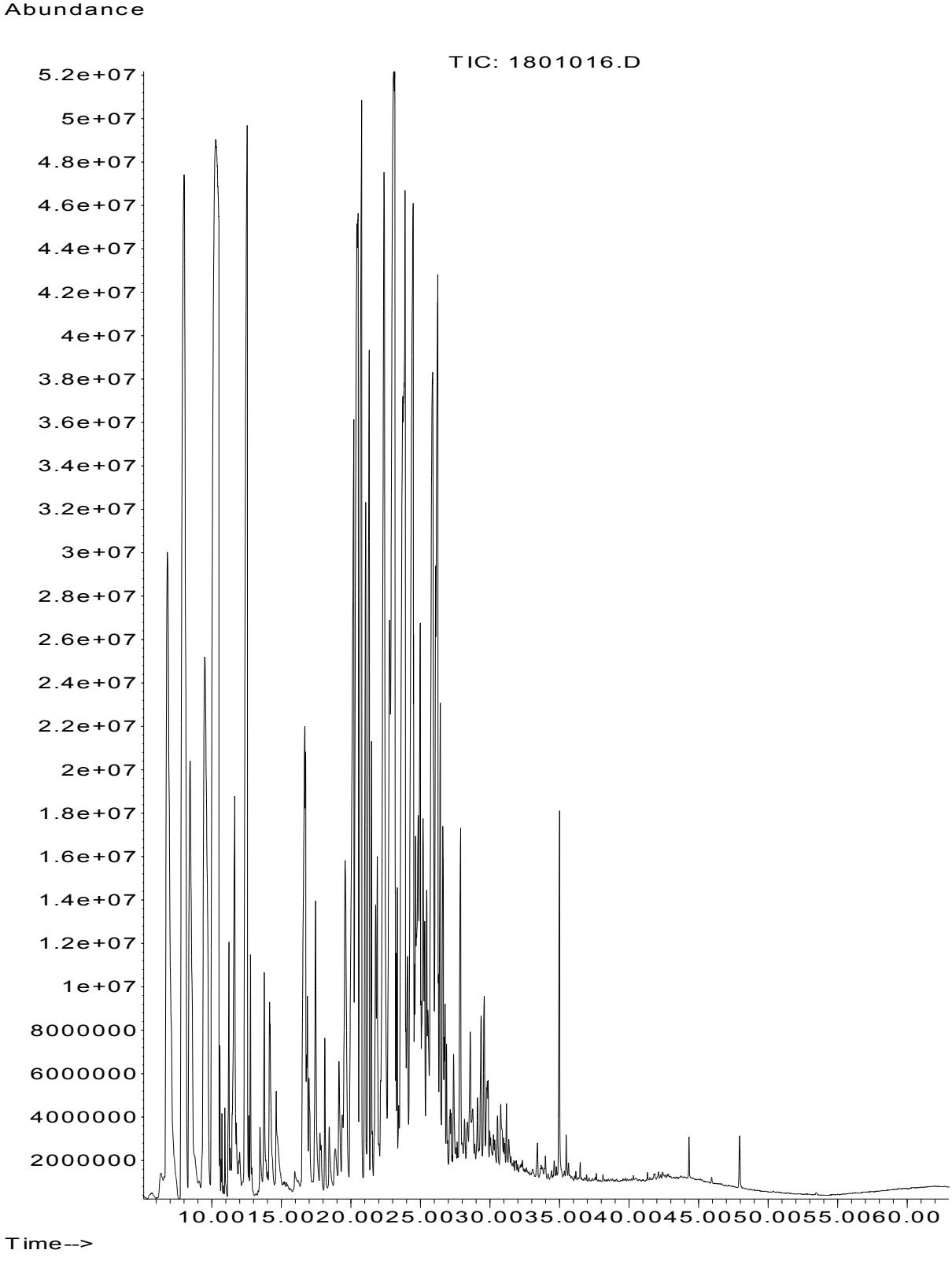
**Appendix 19**: Generated LC values for *Rapanea melanophloeos* compound **24**

**Confidence Limits**

| | | 95% Confidence Limits for conc | | | 95% Confidence Limits for log(conc)(a) | | |
|---|---|---|---|---|---|---|---|
| | Probability | Estimate | Lower Bound | Upper Bound | Estimate | Lower Bound | Upper Bound |
| PROBIT | .010 | 19.832 | 11.622 | 28.307 | 1.297 | 1.065 | 1.452 |
| | .020 | 23.917 | 14.707 | 33.126 | 1.379 | 1.168 | 1.520 |
| | .030 | 26.935 | 17.071 | 36.612 | 1.430 | 1.232 | 1.564 |
| | .040 | 29.453 | 19.093 | 39.483 | 1.469 | 1.281 | 1.596 |
| | .050 | 31.674 | 20.909 | 41.990 | 1.501 | 1.320 | 1.623 |
| | .060 | 33.697 | 22.588 | 44.254 | 1.528 | 1.354 | 1.646 |
| | .070 | 35.576 | 24.168 | 46.344 | 1.551 | 1.383 | 1.666 |
| | .080 | 37.347 | 25.674 | 48.304 | 1.572 | 1.409 | 1.684 |
| | .090 | 39.034 | 27.122 | 50.162 | 1.591 | 1.433 | 1.700 |
| | .100 | 40.654 | 28.526 | 51.940 | 1.609 | 1.455 | 1.716 |
| | .150 | 48.111 | 35.121 | 60.049 | 1.682 | 1.546 | 1.779 |
| | .200 | 55.000 | 41.380 | 67.475 | 1.740 | 1.617 | 1.829 |
| | .250 | 61.692 | 47.573 | 74.664 | 1.790 | 1.677 | 1.873 |
| | .300 | 68.393 | 53.854 | 81.872 | 1.835 | 1.731 | 1.913 |
| | .350 | 75.251 | 60.335 | 89.287 | 1.877 | 1.781 | 1.951 |
| | .400 | 82.392 | 67.109 | 97.080 | 1.916 | 1.827 | 1.987 |
| | .450 | 89.946 | 74.269 | 105.432 | 1.954 | 1.871 | 2.023 |
| | **.500** | **98.057** | **81.918** | **114.553** | **1.991** | **1.913** | **2.059** |
| | .550 | 106.898 | 90.175 | 124.711 | 2.029 | 1.955 | 2.096 |
| | .600 | 116.699 | 99.194 | 136.263 | 2.067 | 1.996 | 2.134 |
| | .650 | 127.774 | 109.187 | 149.707 | 2.106 | 2.038 | 2.175 |
| | .700 | 140.585 | 120.468 | 165.784 | 2.148 | 2.081 | 2.220 |
| | .750 | 155.855 | 133.532 | 185.659 | 2.193 | 2.126 | 2.269 |
| | .800 | 174.818 | 149.230 | 211.344 | 2.243 | 2.174 | 2.325 |
| | .850 | 199.854 | 169.204 | 246.772 | 2.301 | 2.228 | 2.392 |
| | **.900** | **236.509** | **197.252** | **301.310** | **2.374** | **2.295** | **2.479** |
| | .910 | 246.327 | 204.570 | 316.394 | 2.392 | 2.311 | 2.500 |
| | .920 | 257.455 | 212.779 | 333.713 | 2.411 | 2.328 | 2.523 |
| | .930 | 270.272 | 222.131 | 353.940 | 2.432 | 2.347 | 2.549 |
| | .940 | 285.343 | 232.997 | 378.089 | 2.455 | 2.367 | 2.578 |
| | .950 | 303.559 | 245.963 | 407.777 | 2.482 | 2.391 | 2.610 |
| | .960 | 326.453 | 262.021 | 445.818 | 2.514 | 2.418 | 2.649 |
| | .970 | 356.977 | 283.070 | 497.722 | 2.553 | 2.452 | 2.697 |
| | .980 | 402.017 | 313.477 | 576.585 | 2.604 | 2.496 | 2.761 |
| | .990 | 484.817 | 367.716 | 727.907 | 2.686 | 2.566 | 2.862 |

a Logarithm base = 10.

**Appendix 20**: GC/MS spectrum of *Teclea nobilis* essential oil

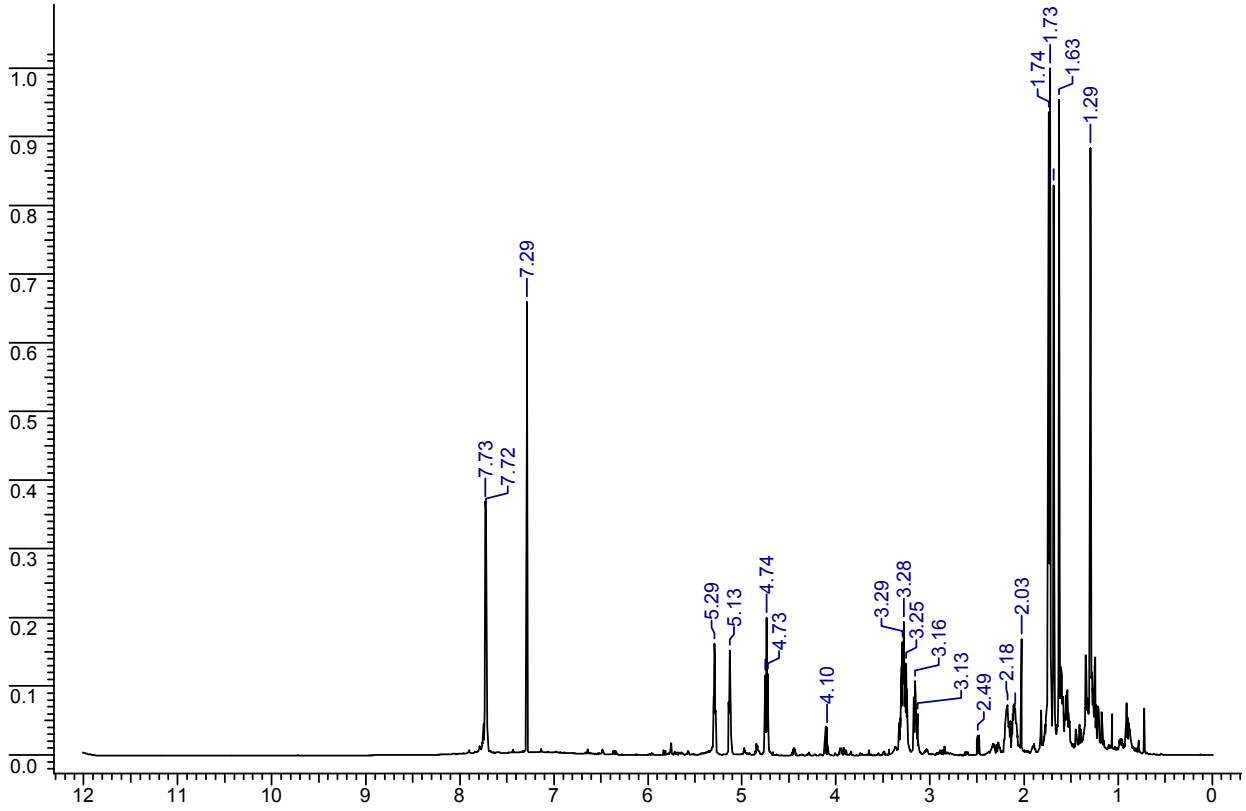


**Appendix 21**: Constituents of *Teclea nobilis* Essential Oil

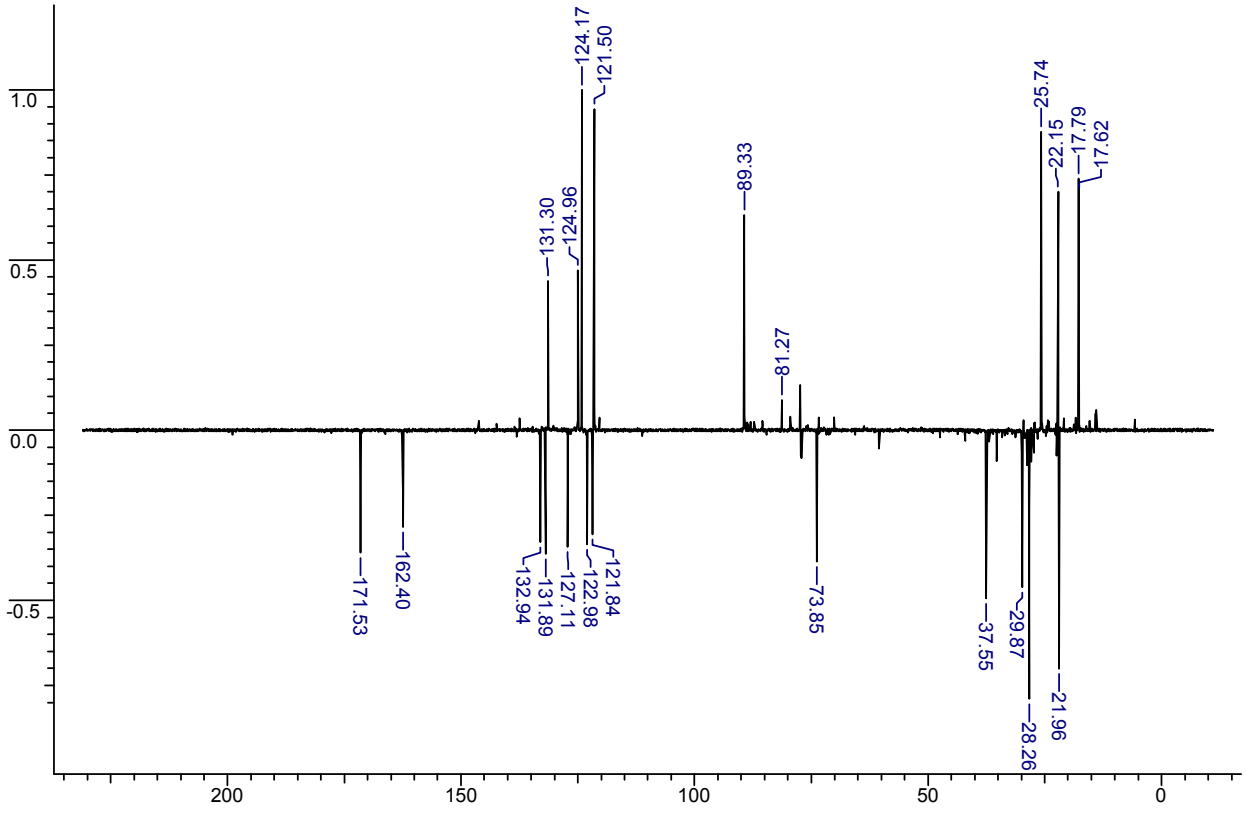
| Compound | S/NO. | Retention Time (Mins) | Conc. % | Detection Method |
|---|---|---|---|---|
| **Monoterpenes** | | | | |
| α – Pinene | 1 | 6.81 | 3.95 | GC/MS |
| γ – Terpinene | 2 | 8.01 | 6.11 | GC/MS |
| β – Myrcene | 3 | 8.45 | 2.17 | GC/MS |
| Limonene | 4 | 9.50 | 3.34 | GC/MS |
| β – Ocimene | 5 | 10.29 | 10.15 | GC/MS |
| α – Terpinolene | 6 | 11.23 | 0.33 | GC/MS |
| Linalool | 7 | 11.65 | 1.13 | GC/MS |
| Neo-allo-Ocimene | 8 | 12.55 | 3.68 | GC/MS |
| Cis-epoxy-Ocimene | 9 | 12.78 | 0.27 | GC/MS |
| 4-methyl-1-(1methyethyl)-3-cyclohexen-1-ol | 10 | 13.77 | 0.37 | GC/MS |
| β – Fenchyl alcohol | 11 | 14.15 | 0.24 | GC/MS |
| α – Terpineol | 12 | 14.19 | 0.37 | GC/MS |
| 3-Ethenyl-Cyclohexane | 13 | 16.81 | 0.13 | GC/MS |
| Anise Camphor | 14 | 16.88 | 0.25 | GC/MS |
| Methyl decadienoate | 15 | 17.46 | 0.71 | GC/MS |
| 1,5,5- Trimethyl-6-methylene-cyclohexene | 16 | 18.13 | 0.22 | GC/MS |
| Methyl Eugenol | 17 | 20.21 | 2.68 | GC/MS |
| 1,2,3,4,4a,5,6,8a-Octahydro-7-methyl-4-methylene-1-(1-naphthalene | 18 | 21.78 | 0.60 | GC/MS |
| 4-ethenyl-α,α,4-trimethyl-3-(-methylethenyl)-[1R-(1α,3α,4β)]-Cyclohexanemethanol | 19 | 23.73 | 2.62 | GC/MS |
| Elemicin | 20 | 23.89 | 2.89 | GC/MS |
| **Total** | **20** | | **42.21** | |
| **Sesquiterpenes** | | | | |
| α – Cubebene | 1 | 18.44 | 0.9 | GC/MS |
| α – Copaene | 2 | 19.15 | 0.32 | GC/MS |
| β – Gurjunene | 3 | 19.59 | 1.26 | GC/MS |
| Germacrene D | 4 | 20.44 | 3.06 | GC/MS |
| α – Amorphine | 5 | 22.39 | 3.96 | GC/MS |
| β – Cadinene | 6 | 23.08 | 4.98 | GC/MS |
| δ – Cadinene | 7 | 23.16 | 1.28 | GC/MS |
| α – Muurolene | 8 | 23.33 | 0.29 | GC/MS |
| Nerolidol | 9 | 24.08 | 0.55 | GC/MS |
| 1,6-Germacradien-5-ol | 10 | 24.48 | 4.38 | GC/MS |

| | | | | |
|---|---|---|---|---|
| Guaiol | 11 | 24.84 | 1.01 | GC/MS |
| β- Elemenone | 12 | 24.99 | 0.96 | GC/MS |
| 1-Methylidene-2b-hydroxymethyl-3,3-dimethyl-4a(3-methylbut-2-enyl)- cyclohexane | 13 | 25.30 | 0.38 | GC/MS |
| 1,2,3,4,6,8a-Hexahydro-1-isopropyl-4,7-dimethyl-Naphthalene | 14 | 25.45 | 0.52 | GC/MS |
| Tau- Cadinol | 15 | 25.88 | 3.56 | GC/MS |
| Trans-Isoelemicin | 16 | 26.09 | 0.83 | GC/MS |
| α – Cadinol | 17 | 26.25 | 2.57 | GC/MS |
| Bulnesol | 18 | 26.43 | 0.83 | GC/MS |
| Isoaromadendrene epoxide | 19 | 26.95 | 0.07 | GC/MS |
| 1-Formyl-2,2,6-trimethyl-3-(3-methyl-but-2-enyl)-6-cyclohexene | 20 | 27.98 | 0.09 | GC/MS |
| Farnesol 2 | 21 | 27.39 | 0.28 | GC/MS |
| (4S,5R)-5-Hydrocaryophyll-8(13)-ene-4,12-epoxide | 22 | 28.17 | 0.17 | GC/MS |
| Caryophylla-3,8-(13)-dien-5-alpha –ol | 23 | 28.91 | 0.08 | GC/MS |
| 6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-ol | 24 | 29.59 | 0.44 | GC/MS |
| E and Z isomers of 1-(2,6,6-trimethyl-1-cyclohexen-1-yl-3-methyl-2-heptene | 25 | 29.87 | 0.13 | GC/MS |
| Dihydrocuminyl aldehyde | 26 | 29.99 | 0.07 | GC/MS |
| 4-methyl-1-(2,3,3-trimethylbicyclo[3.1.0]he-2-yl)-2,4-pentadien-1-one | 27 | 31.36 | 0.12 | GC/MS |
| Ledene | 28 | 41.51 | 0.003 | GC/MS |
| **Total** | **28** | | **33.09** | |
| **Diterpenes** | | | | |
| (1R,3S)-Cembra-4,7,11,15-tetraen-3-ol | 1 | 33.64 | 0.03 | GC/MS |
| Phytol | 2 | 35.00 | 0.60 | GC/MS |
| Docosane | 3 | 41.51 | 0.01 | GC/MS |
| Tricosane | 4 | 44.31 | 0.04 | GC/MS |
| **Total** | **4** | | **0.68** | |
| **Others** | | | | |
| Octasane | 1 | 45.94 | 0.01 | GC/MS |
| Nonacosane | 2 | 47.96 | 0.09 | GC/MS |
| **Total** | **2** | | **0.1** | |

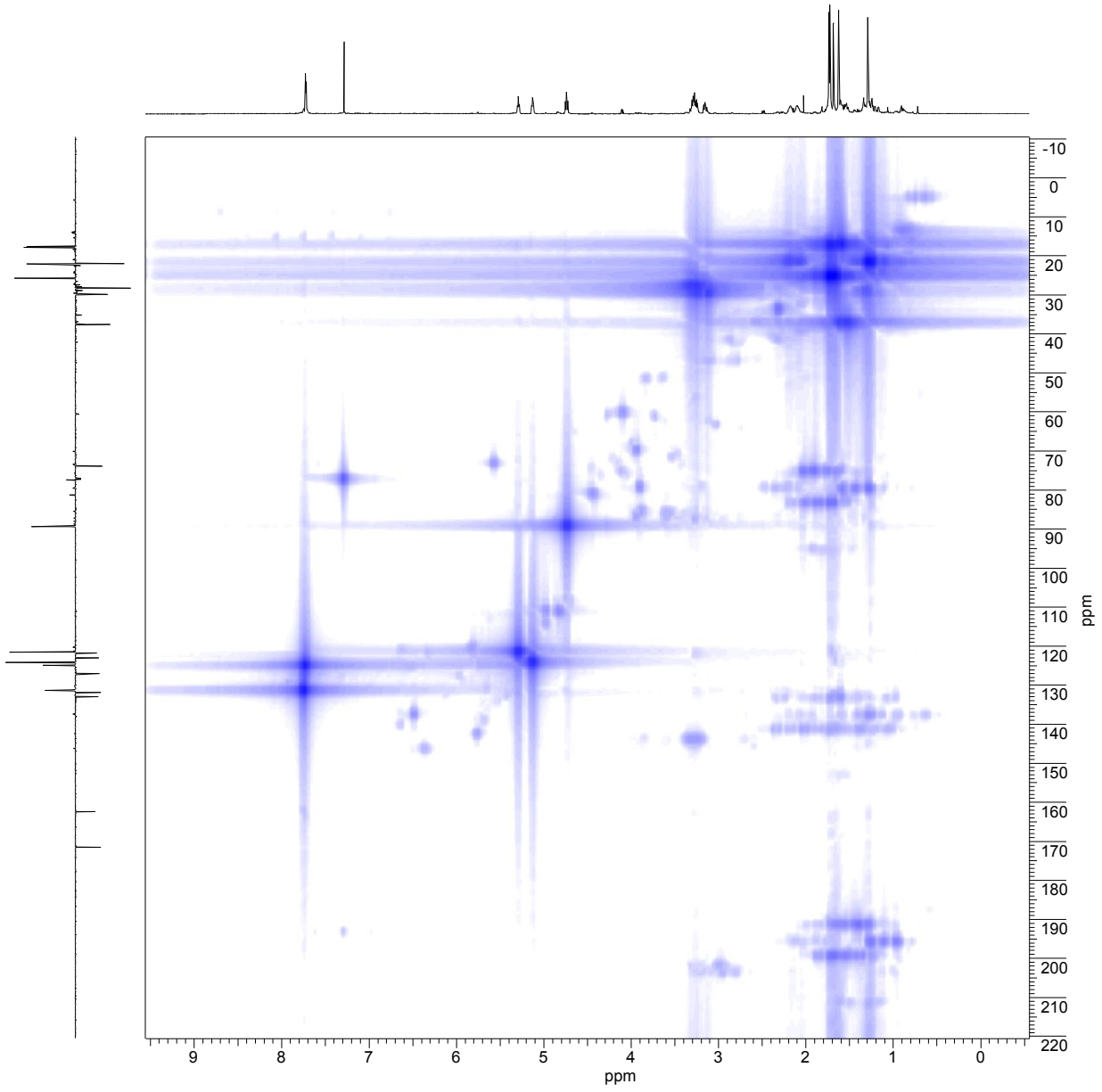
**Appendix 22**: $^{1}$H NMR spectrum of compound **23**



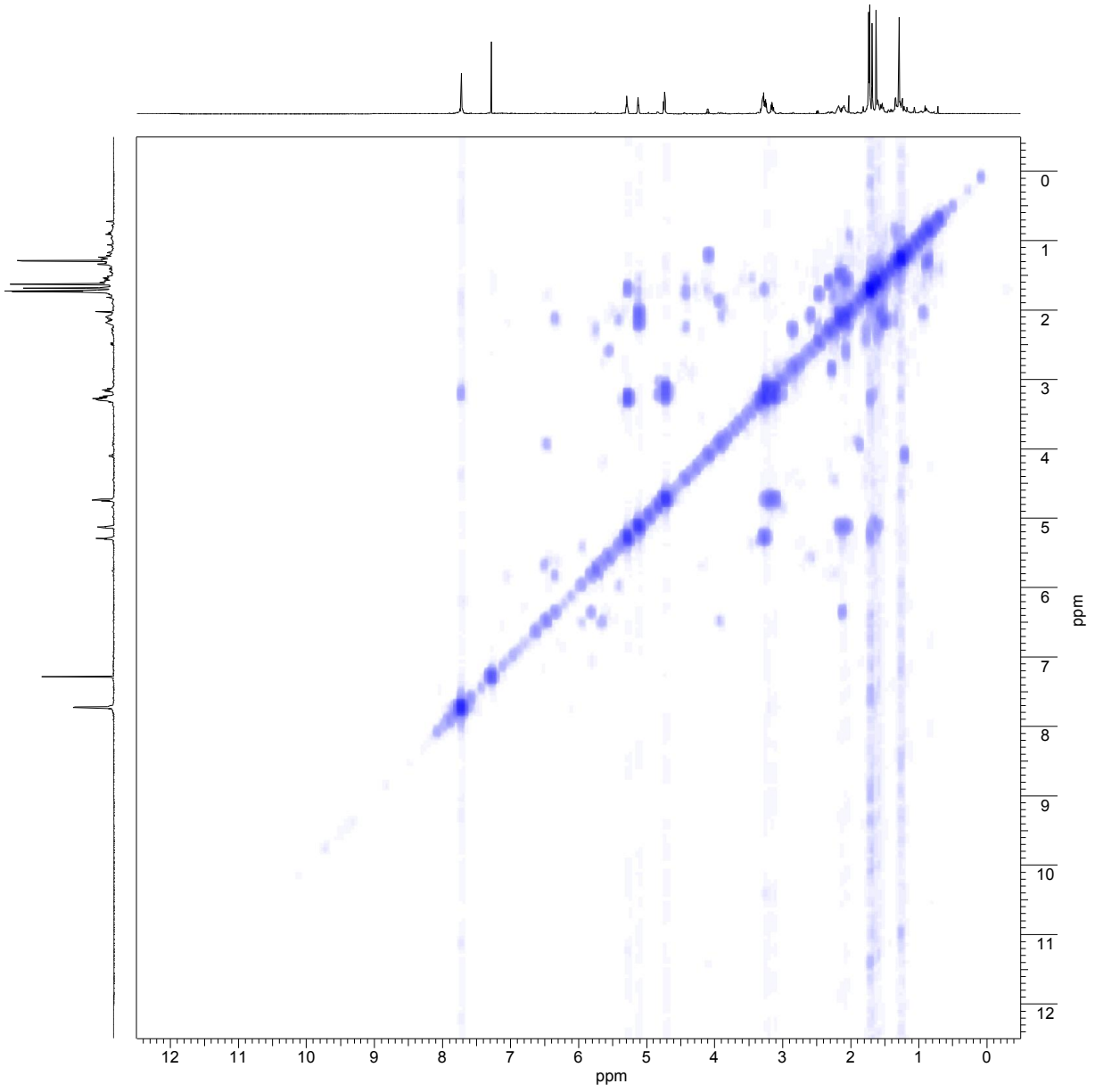
**Appendix 23**: APT spectrum of compound **23**



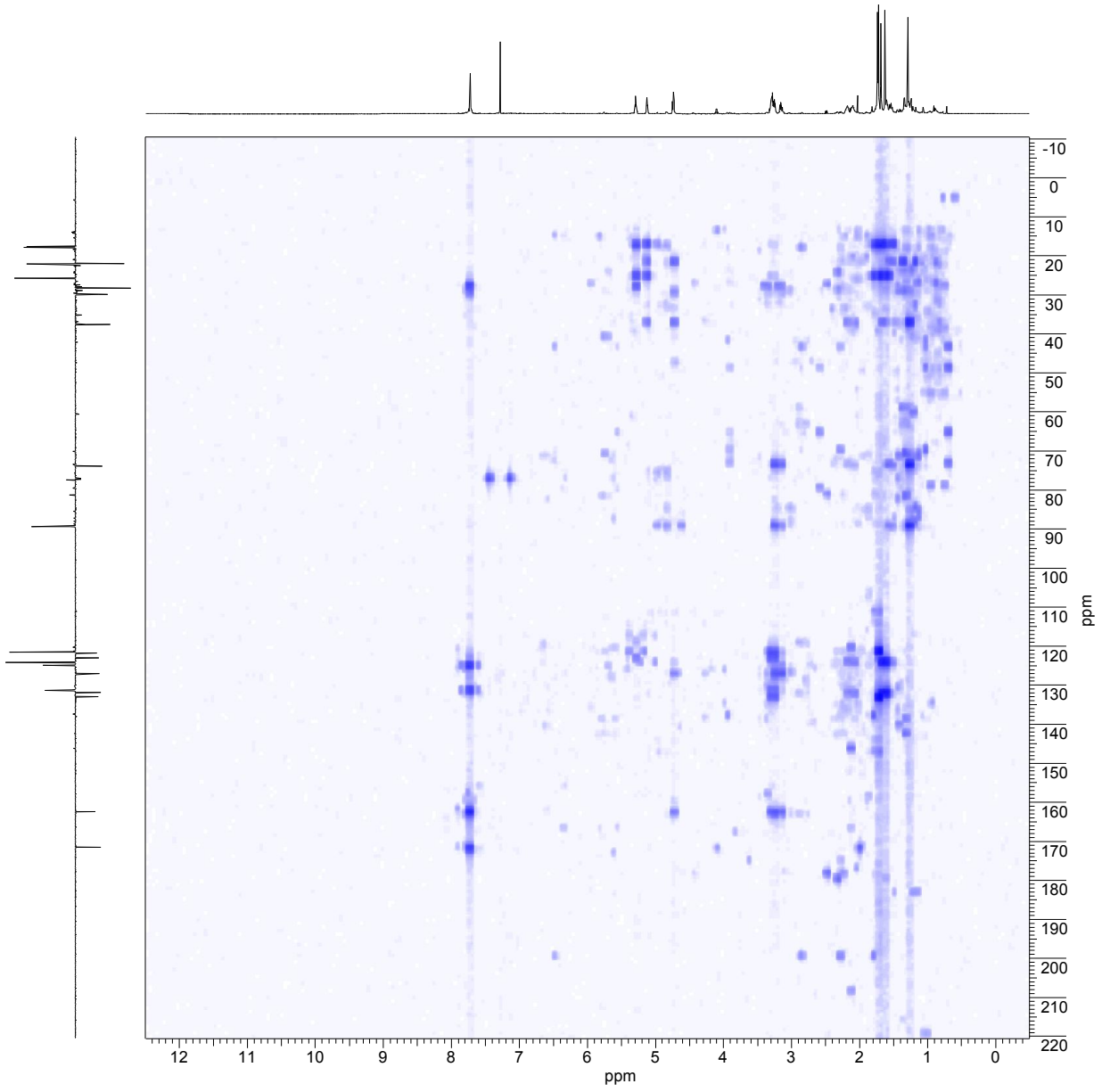
**Appendix 24**: HSQC spectrum for the compound **23**



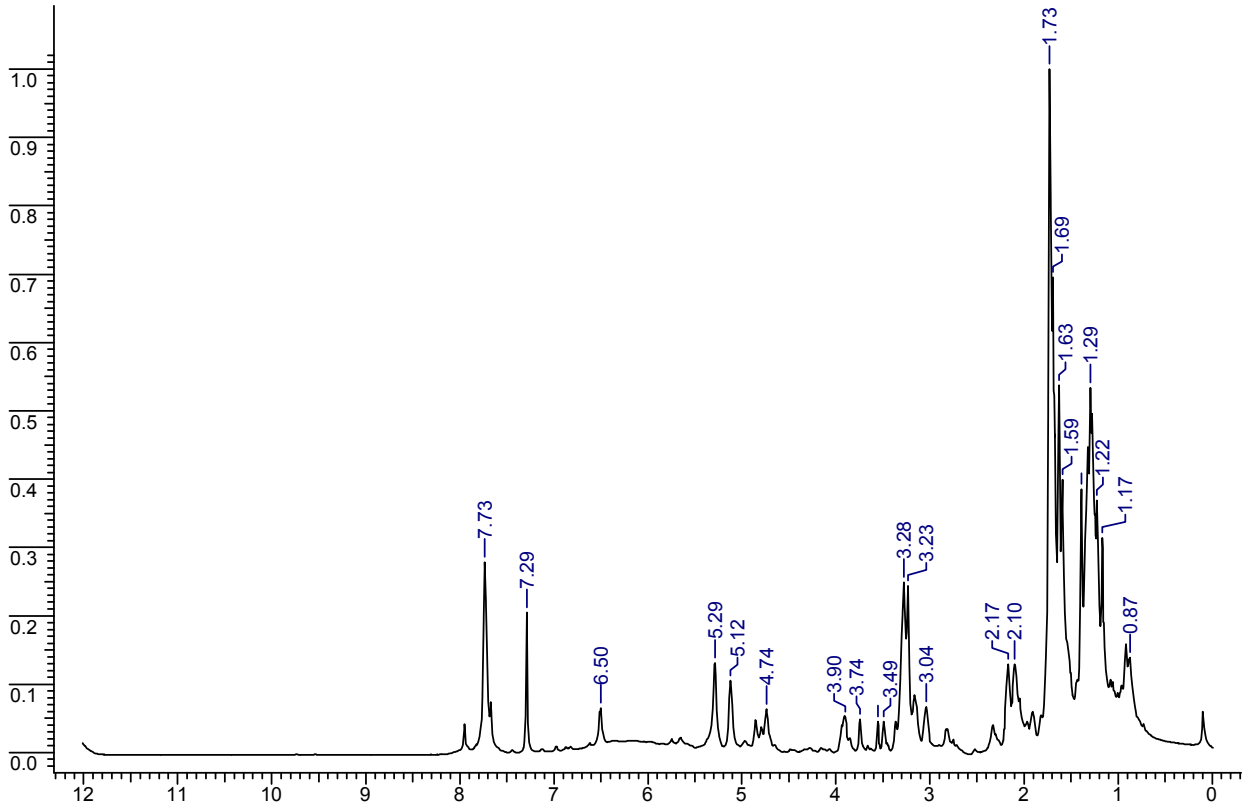
**Appendix 25**: $^{1}$H-$^{1}$H COSY spectrum for the compound **23**



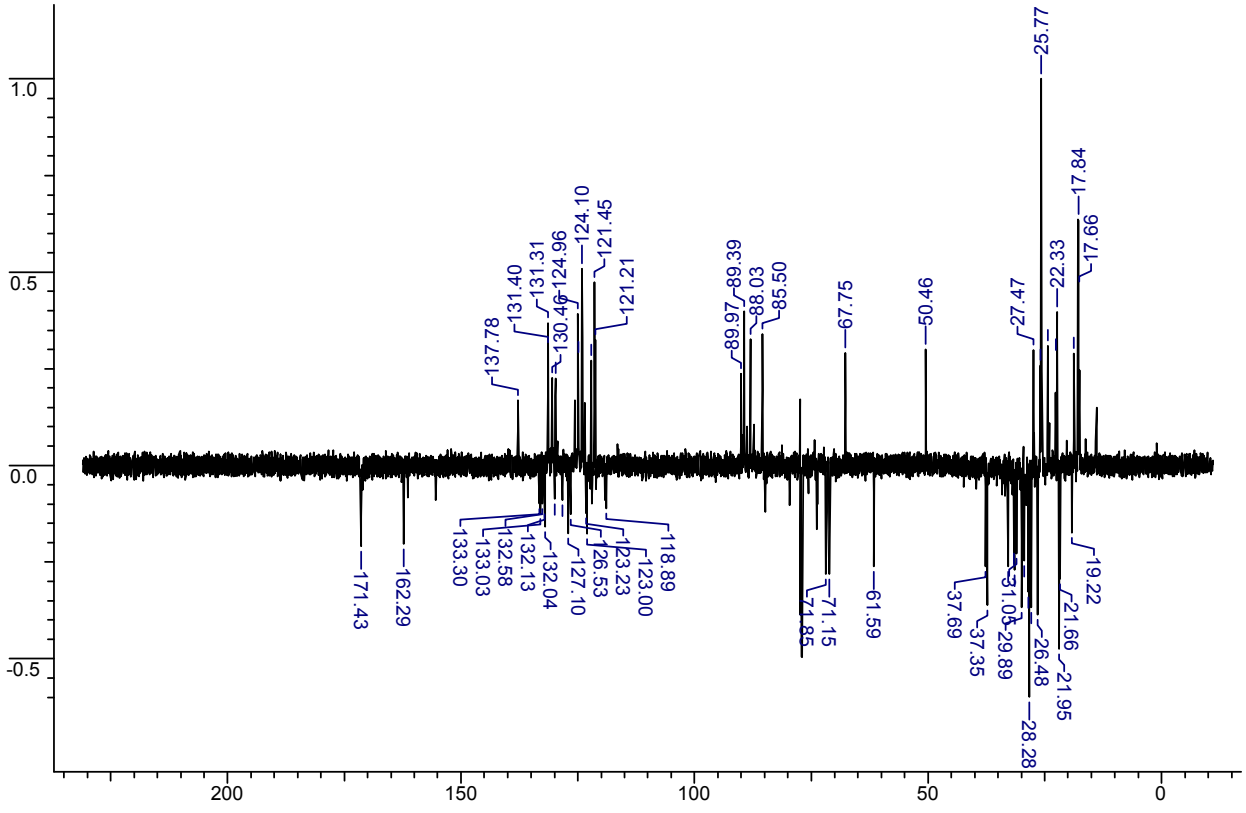
**Appendix 26**: HMBC spectrum for the compound **23**



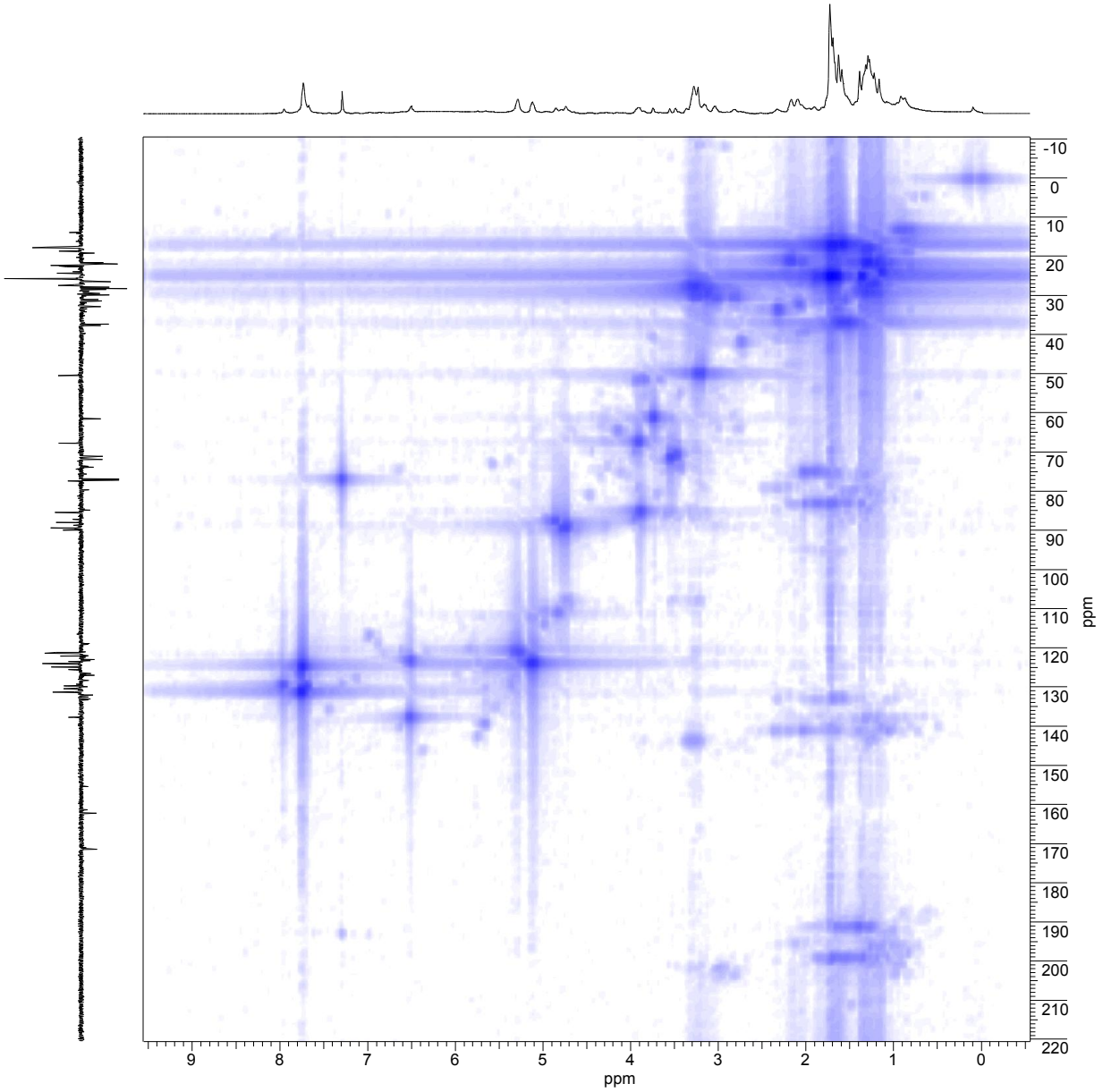
**Appendix 27**: $^{1}$H NMR spectrum of compound **24**



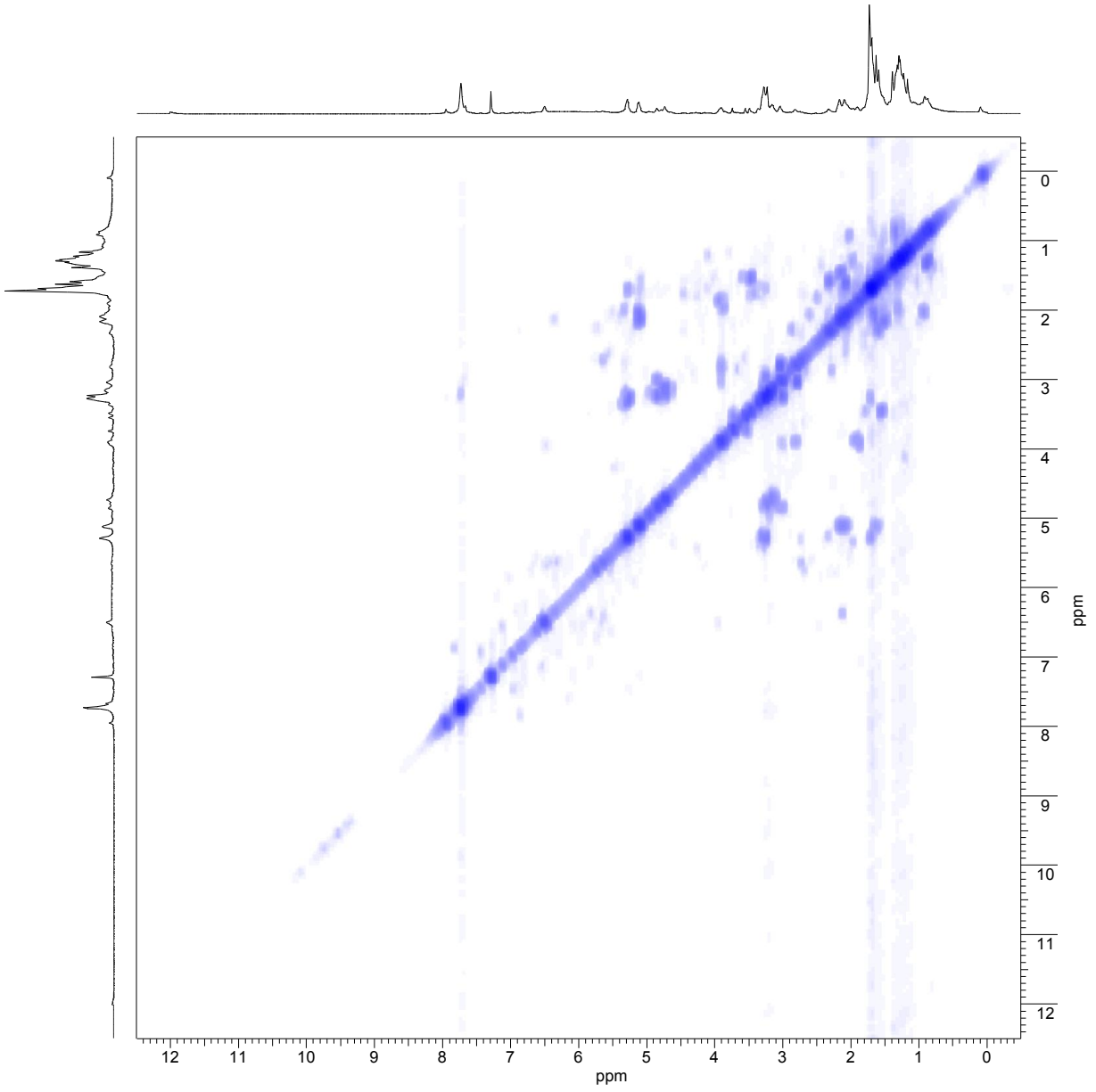
**Appendix 28**: APT NMR spectrum of compound **24**



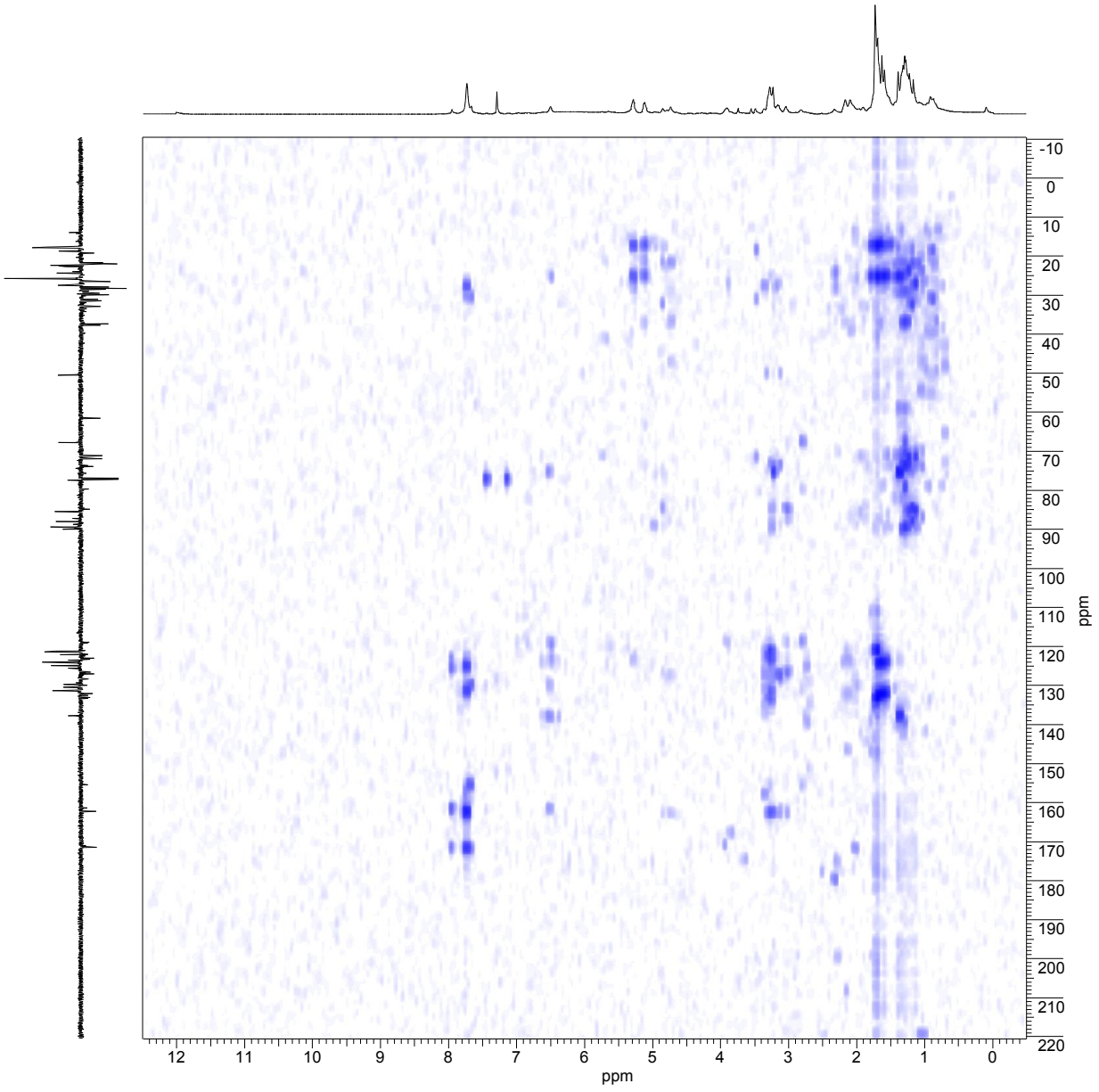
**Appendix 29**: HSQC spectrum of compound **24**



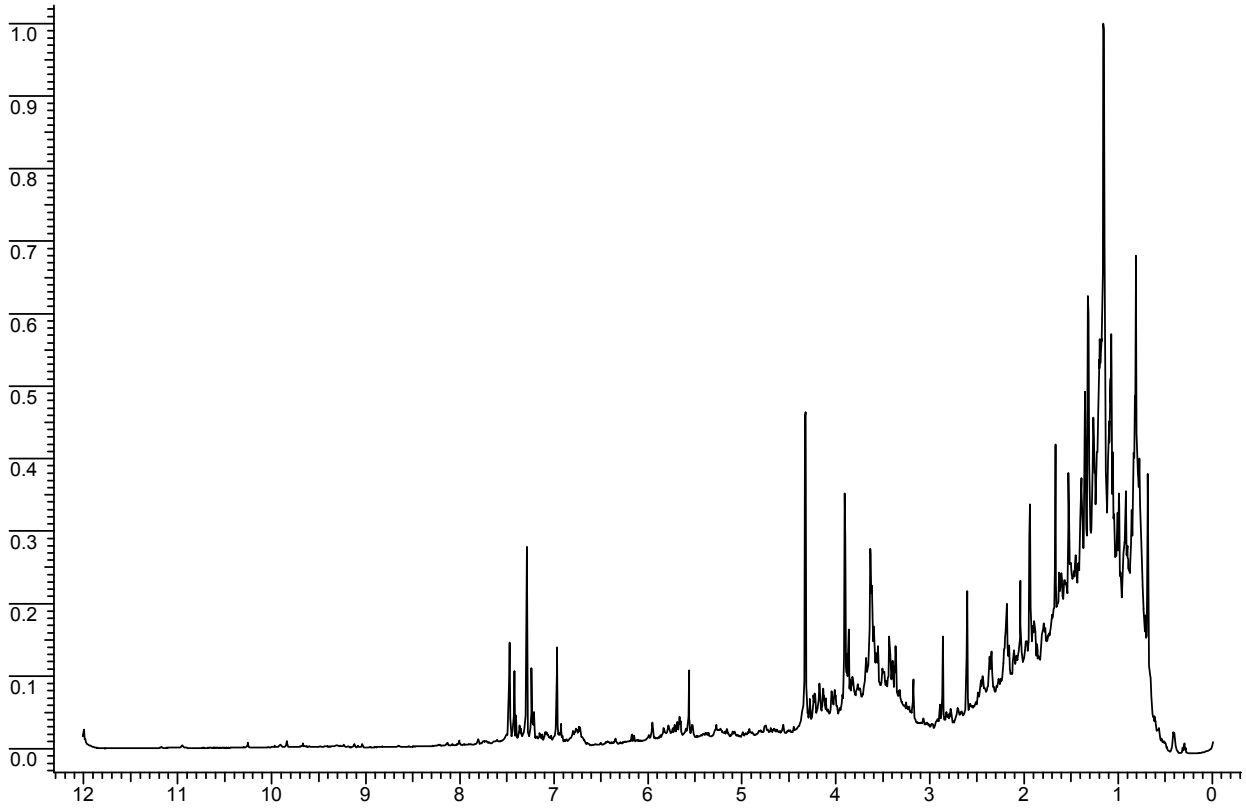
**Appendix 30**: $^{1}H$-$^{1}H$ COSY NMR spectrum of compound **24**



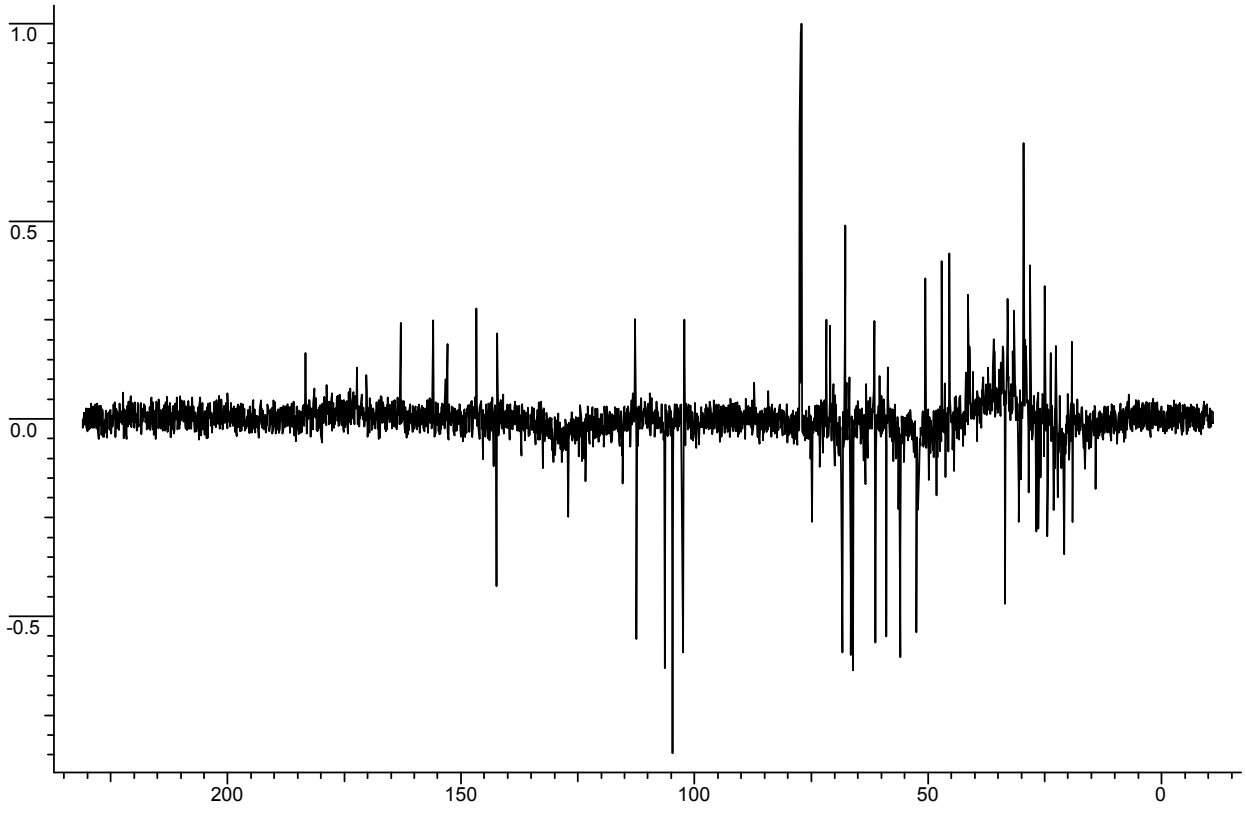
**Appendix 31**: HMBC NMR spectrum of compound **24**



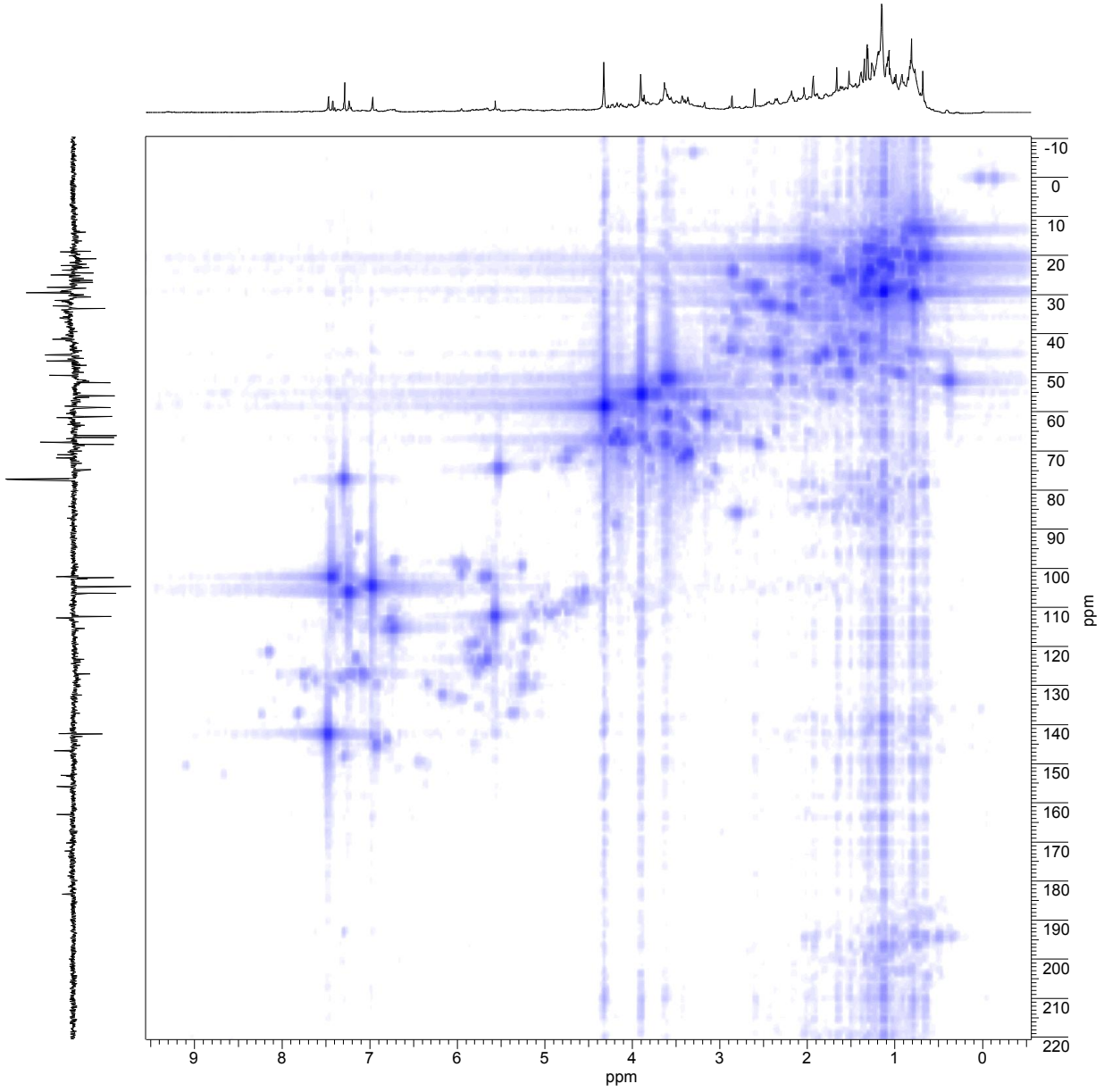
**Appendix 32**: $^{1}$H NMR spectrum of compounds **10**, **13** and **21**



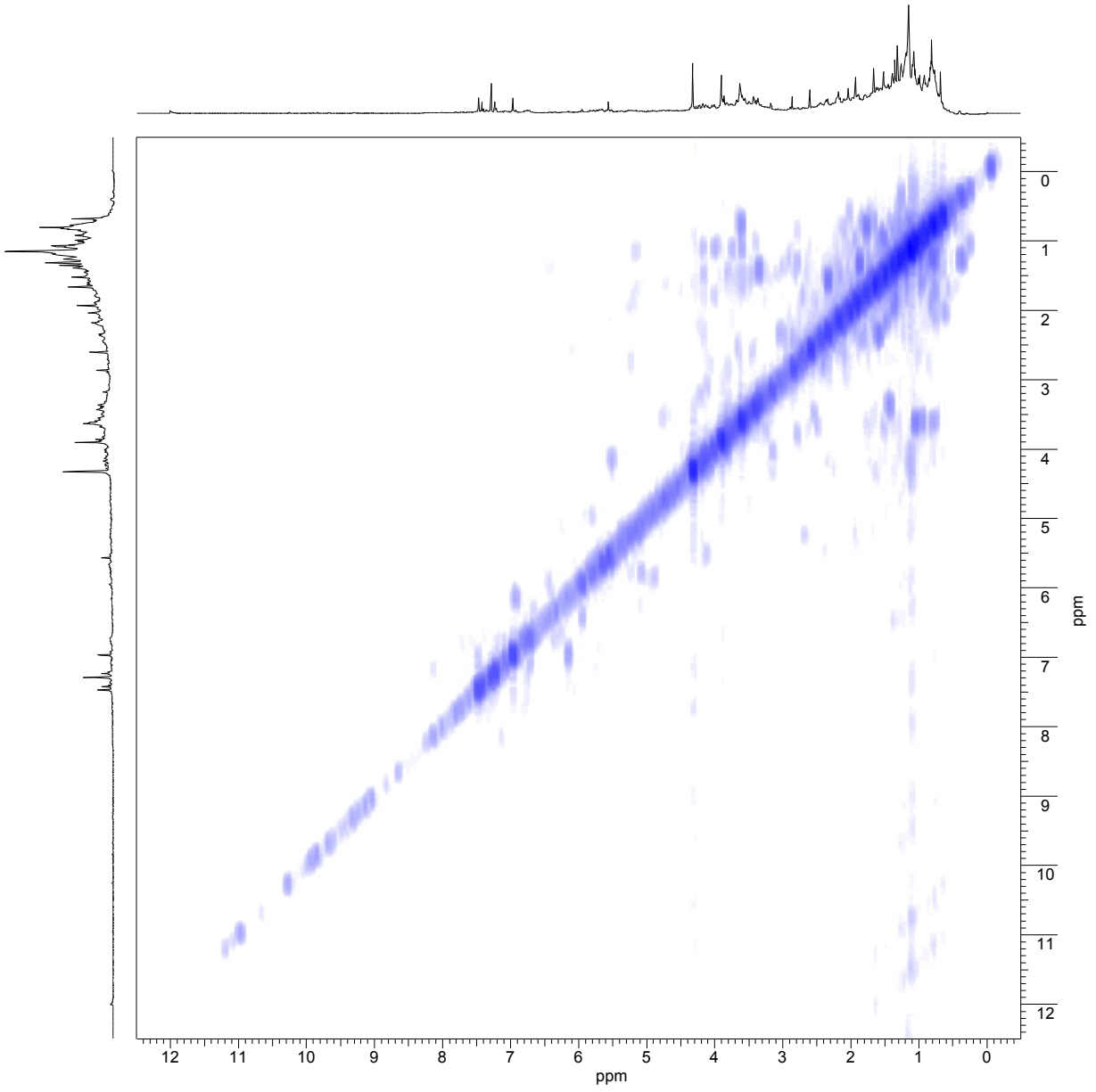
**Appendix 33**: APT NMR spectrum of compounds **10**, **13** and **21**



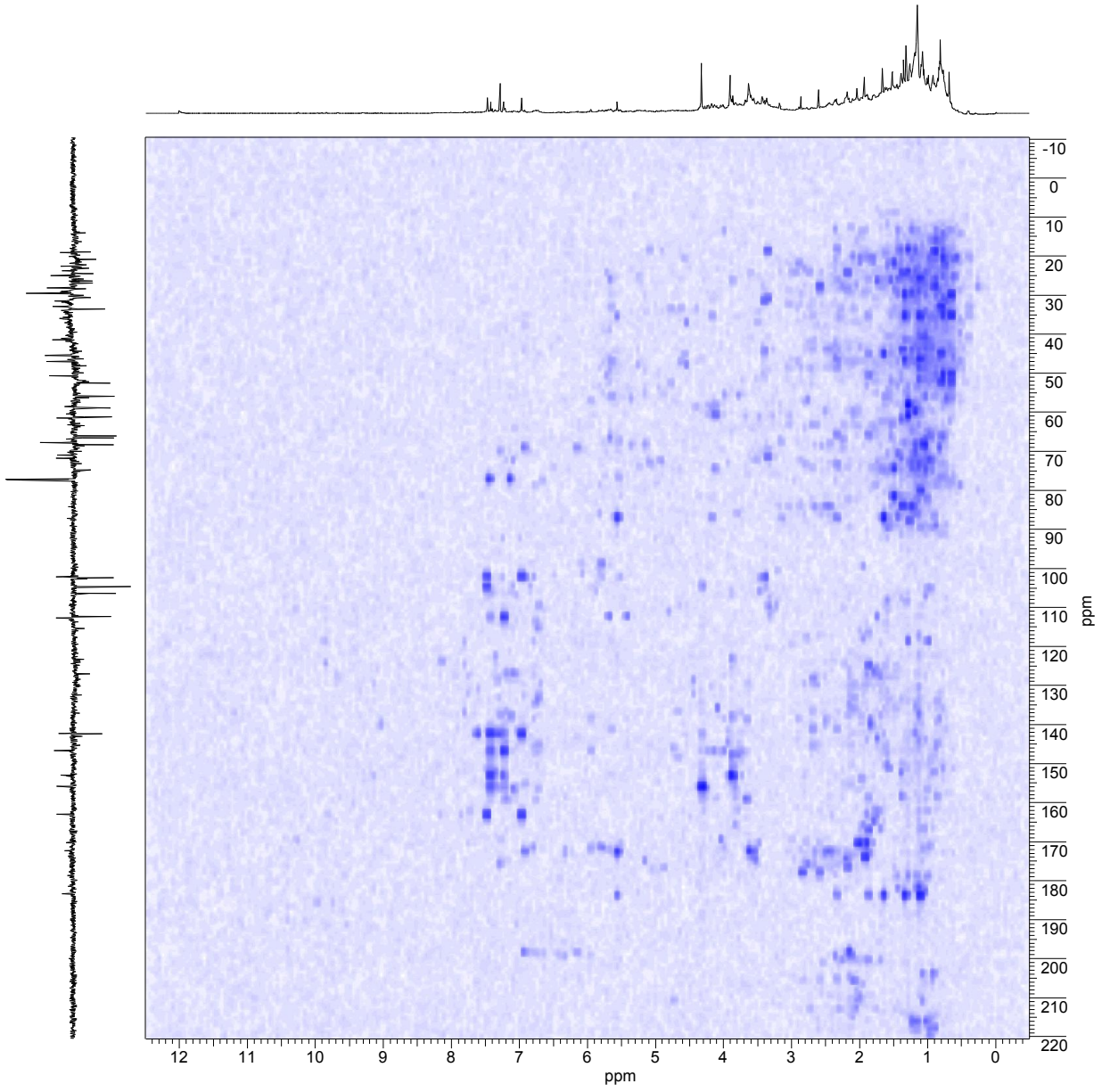
**Appendix 34**: HSQC NMR spectrum of compounds **10**, **13** and **21**



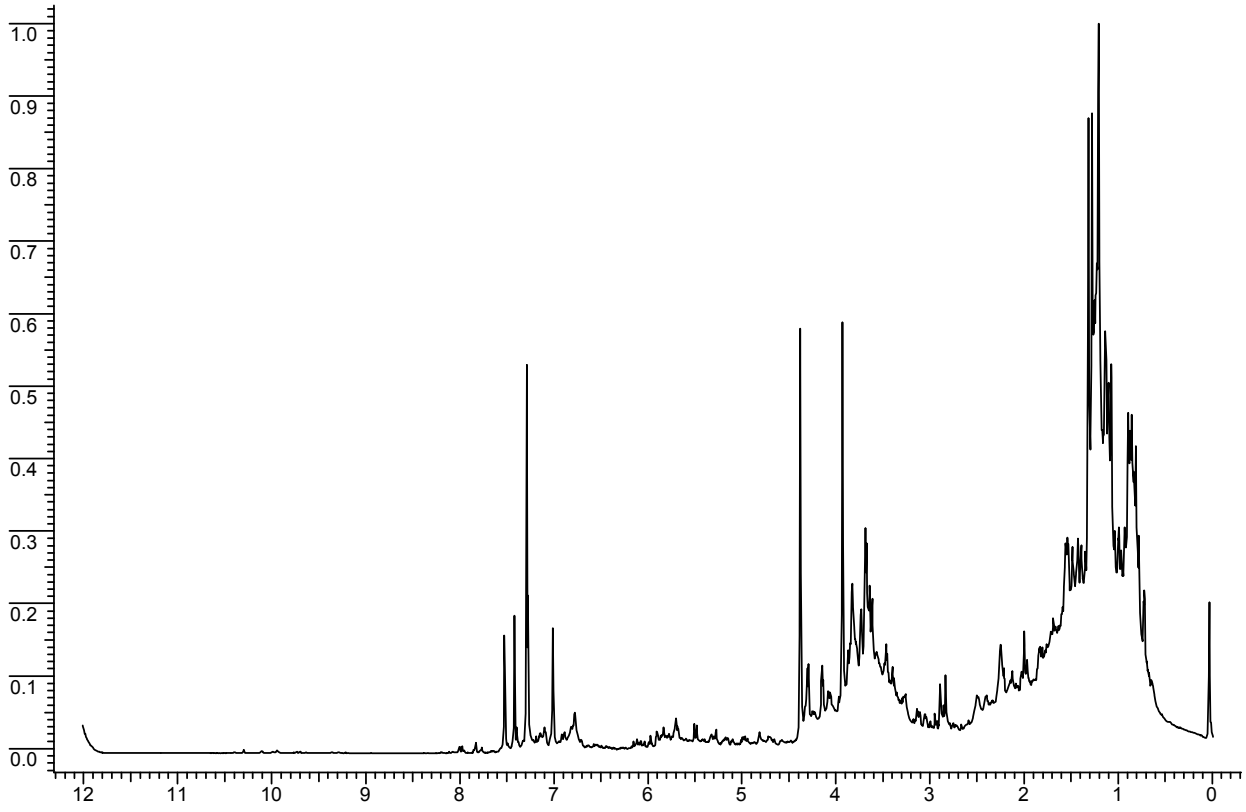
**Appendix 35**: $^{1}$H- $^{1}$H COSY NMR spectrum of compounds **10**, **13** and **21**



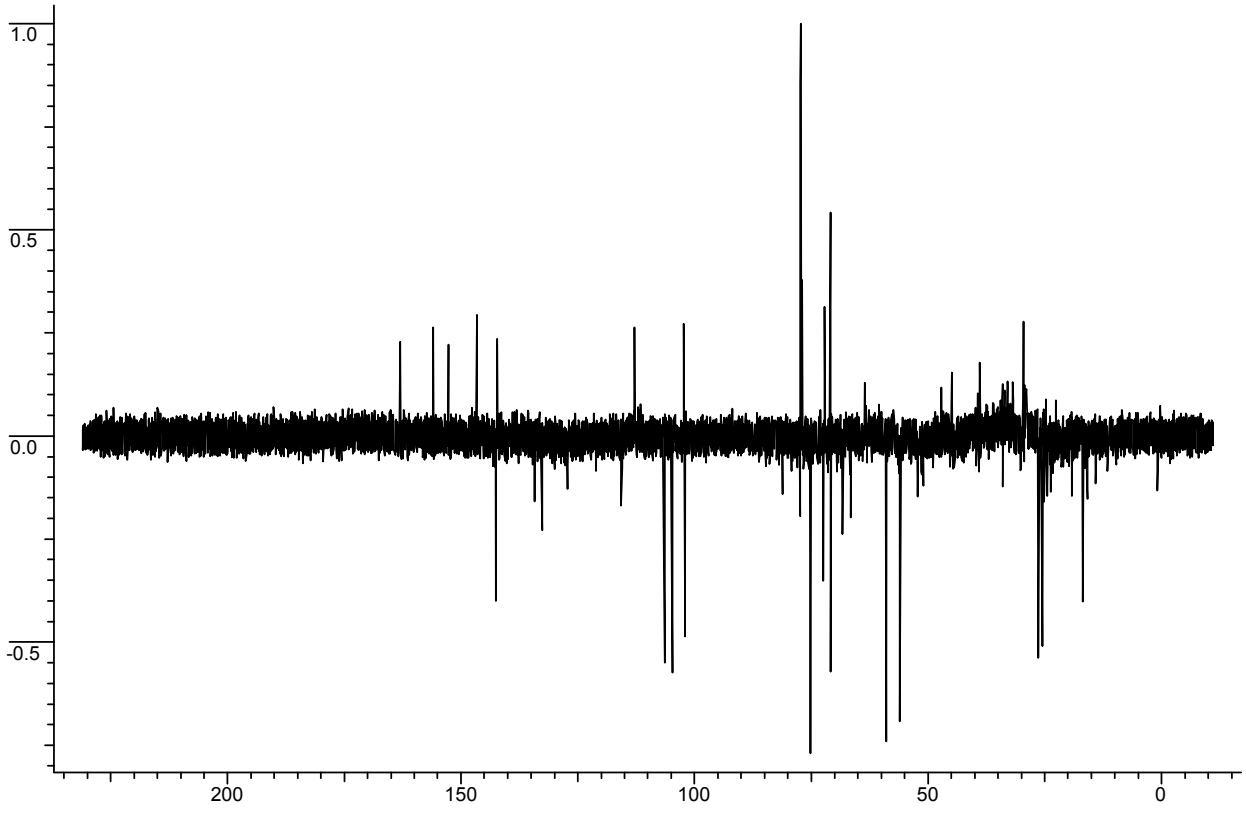
**Appendix 36**: HMBC NMR spectrum of compounds **10**, **13** and **21**



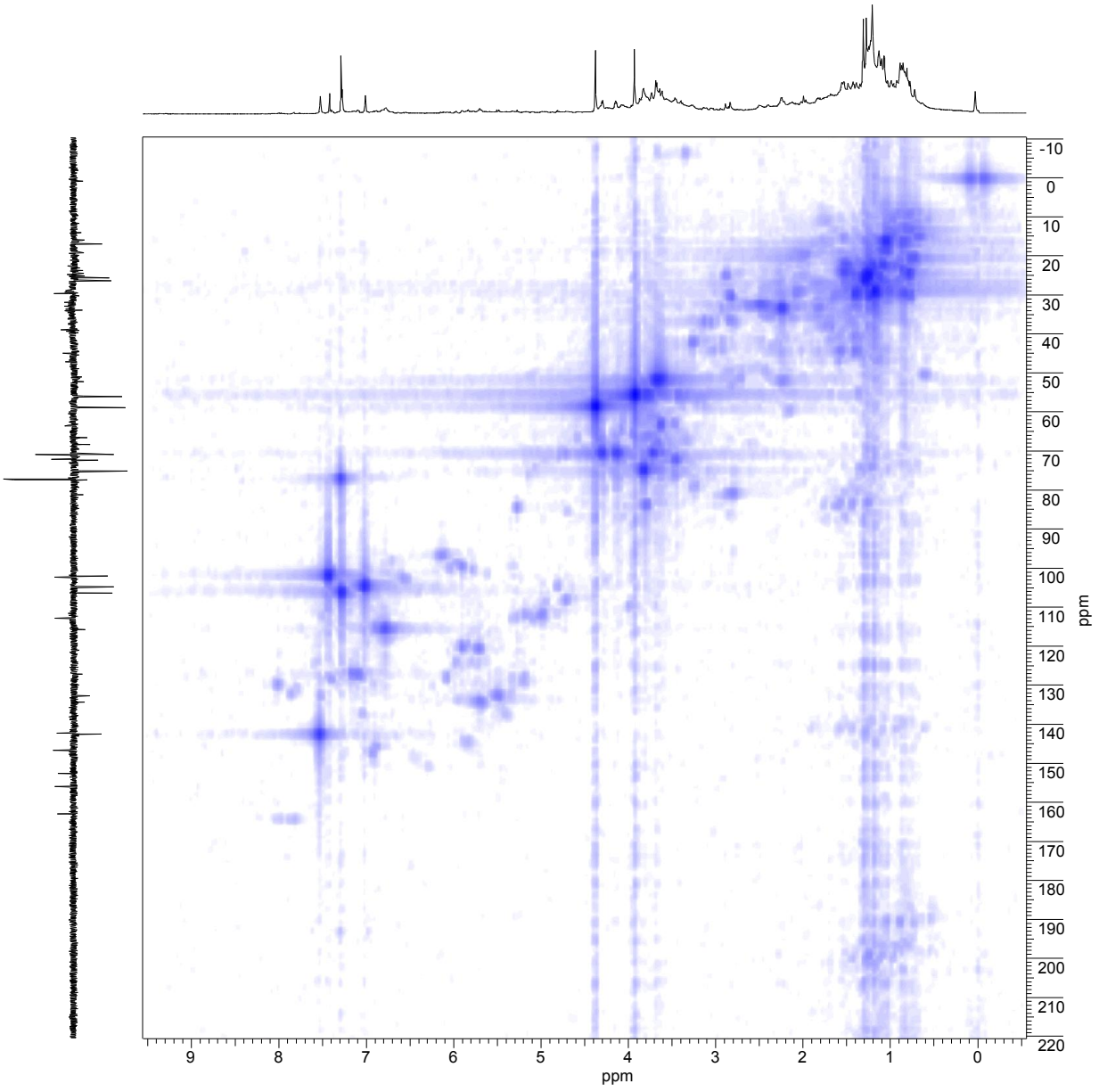
**Appendix 37**: $^{1}$H NMR spectrum of compound **22**



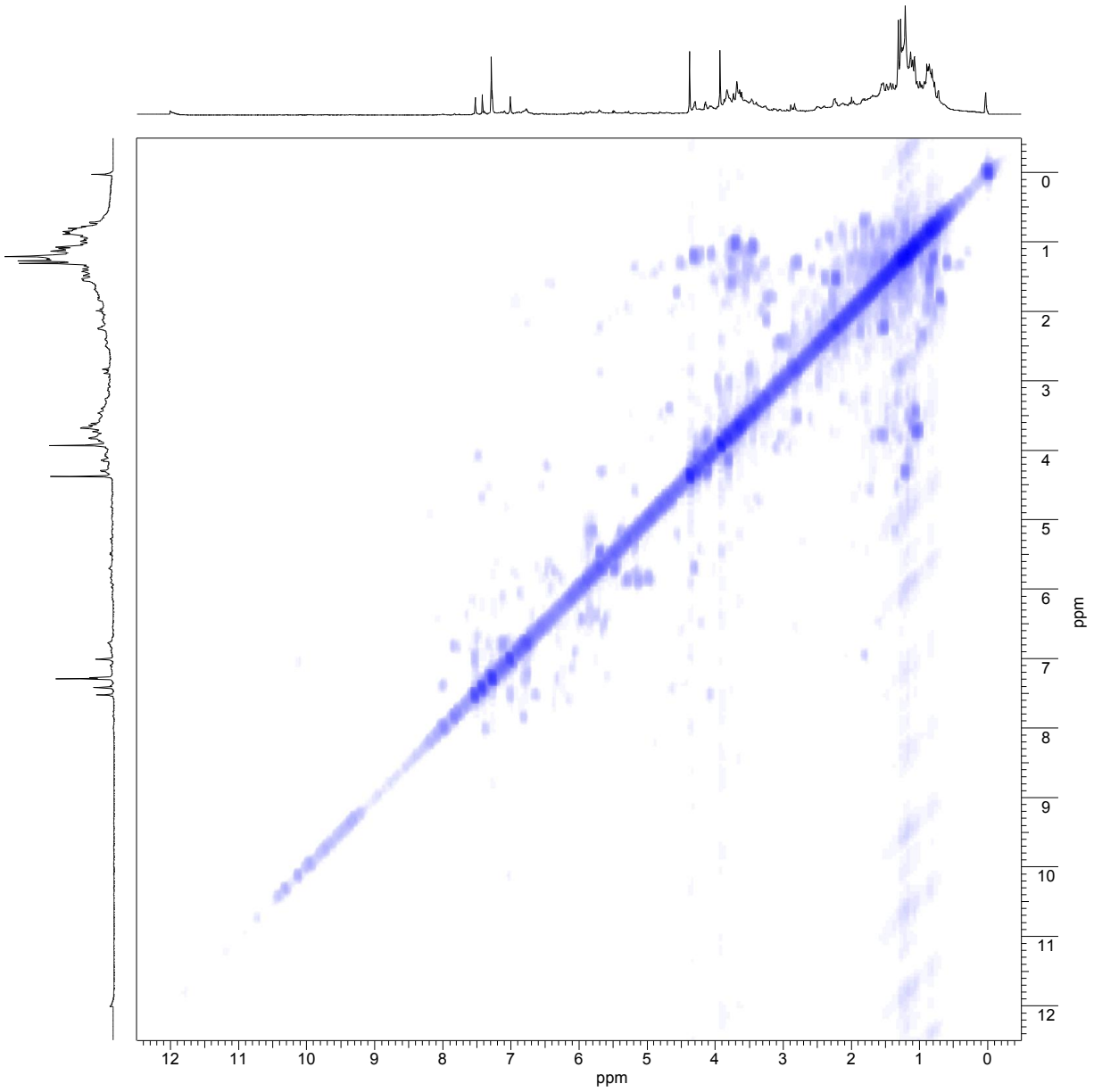
**Appendix 38**: APT NMR spectrum of compound **22**



**Appendix 39**: HSQC NMR spectrum of compound **22**



**Appendix 40**: $^{1}H$-$^{1}H$ COSY NMR spectrum of compound **22**



**Appendix 41**: HMBC NMR spectrum of compound **22**