PHYTOCHEMICAL AND ANTHELMINTIC STUDY OF THE ROOT BARK OF *TECLEA TRICHOCARPA*, ENGL. (RUTACEAE)

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(U59/71105/2007)

A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Pharmacy in Pharmaceutical Analysis

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Declaration

This research thesis is my original work and has not been presented elsewhere for examination.

29.11.2013

STANLEY MULE MUEMA Date

This research thesis has been submitted for examination with our approval as university supervisors.

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Title of the work PHYTOCHEMICAL AND ANTIHELMINTIC STUDY OF ROOT BARK OF TECLEA TRICHOCARPA, ENGL. (RUTACEAE)

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Dedication

This thesis is dedicated to my wife Lilian Moraa; my son Michael Muema; my parents Peninah Nduku and Richard Kitonga.

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TABLE OF CONTENTS

List of Figures

List of Tables

List of Appendices

Abbreviations continued ...

µl	Microlitres
µm	Micrometers
MHz	Mega hertz
min	Minute(s)
ml	Milliliters
mp	Melting point
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
RB	Root bark
R_f	Retention factor
RR_f	Relative retention factor
s	Singlet
t	Triplet
TLC	Thin layer chromatography
UV	Ultra-violet
v/v	Volume by volume
WHO	World Health Organization

Abstract

Plants are used by various communities all over the world for their medicinal values. Many plants including *Teclea trichocarpa* have been used traditionally for their anthelmintic activity. Scientific research to isolate active ingredients and determine their biological activities is important to support the ethno-medicinal uses of plants. This study was carried out to isolate and characterize pure compounds from the root bark of *Teclea trichocarpa* and to determine their anthelmintic activities.

The plant materials were collected from Ngong Forest in Kenya, dried at room temperature and ground into a fine powder. Phytochemical screening tests were carried out. Cold maceration extractions were done using dichloromethane-methanol (1:1) as the solvent. The dry extract was fractionated by open column chromatography on normal phase silica gel powder (32-63μm) connected to a fraction collector. Ethyl acetate was used as the mobile phase for the system. The eluate was collected in test tubes. Crystals that formed in test tubes were re-crystallized and their purity was monitored using thin layer chromatography and confirmed using high pressure liquid chromatography. Egg hatch assay and larval development assay was done on the crude extract and the isolates to determine their anthelmintic activity. Brine shrimp lethality test (BSLT) was done on the *Teclea trichocarpa* root bark extract.

The root bark of *Teclea trichocarpa* was found to contain alkaloids and tannins. Three pure compounds were isolated and their molecular structures determined as the triterpene lupeol and the alkaloids melicopicine and 6-methoxytecleanthine, based on their ultra violet, fourier transform infra red, carbon-13 and proton nuclear magnetic resonance spectroscopy and mass spectrometry data. Both melicopicine and 6-methoxytecleanthine inhibited hatching of the sheep

nematode (strongyles) eggs while the three isolates did not inhibit the development of the strongyloides larvae. The LD_{50} for brine shrimp lethality test done on the crude extract was 41.64 µg/ml.

This is the first time lupeol is reported in this plant. The anthelmintic activities of melicopicine and 6-methoxytecleanthine support the ethno-medicinal use of the root bark of *Teclea trichocarpa* as an anthelmintic. This study concludes that the root bark of *Teclea trichocarpa* contains lupeol, melicopicine and 6-methoxytecleanthine and recommends further work to be done to isolate more compounds. This plant can therefore be source of lead structures for development of new anthelmintic agents.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

The World Health Organization (WHO) defines traditional medicine (TM) as the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 1989). It has been used for thousands of years with great contributions made by practitioners to human health, particularly as primary health care providers at the community level. It is estimated that 80% of the world's population use herbal medicines for their healthcare needs (WHO, 1989). Traditional medicine is often the only affordable treatment available to the poor people and those in remote communities, due to inaccessibility of conventional medicines (Carlos, 2002). Herbs have been used for thousands of years to prevent or treat various ailments and the knowledge of their use has been transferred from generation to generation. Many of the remedies employed by the herbalists provide effective treatment to various disease conditions.

Ethno-medicinal uses of plants provide a rich source for identification of novel lead structures and subsequent development of new therapeutic agents. The interest in medicinal plants is now directed towards identifying therapeutically active compounds from crude plant extracts, which are quantifiable, with no serious toxicities. The active principles in medicinal plants are usually secondary metabolites such as alkaloids, terpenoids and flavonoids. Herbal plant materials can be administered in different ways. These include taking whole herb, syrup, essential oils, ointments,

and rubs. Capsules and Tablets containing ground or powdered form of a raw herb or its dried extract are also used.

Plants are sources of many conventional drugs that are in clinical use today. *Artemisia annua* is an old antimalarial medicinal herb widely applied in traditional medicine. Its antimalarial activity is associated with a sesquiterpine lactone, artemisinin. Artemisinin and its derivatized analogues are well tolerated antimalarial drugs that are now in use conventionally (Weici and Gerhard, 2011). Quinine, an alkaloid from *Cinchona ledgeriana* bark originally used in South America for treatment of fever, is conventionally useful in treatment of malaria (Wagner and Wolff, 1976). Another alkaloid isolated from the same plant is quinidine a cardiotonic agent. Other cardiotonic agents isolated from plants are digoxin, digitoxin and other digitalis glycosides from foxglove (*Digitalis spp.*). They are used for treatment of congestive cardiac failure (Weici and Gerhard, 2011).

Morphine is the active constituent of the plant opium poppy (*Papaver somniferum*) and it is used conventionally together with its synthetic analogues as an opioid analgesic. *Erythroxylum coca* is the source of the drug cocaine which is precursor of the local anaesthetic agents lidocaine, xylocaine and tetracaine (Weici and Gerhard, 2011).

The Madagascar periwinkle Vinca (*Catharanthus roseus*) was traditionally used for treating diabetes but further investigation yielded the anticancer alkaloids vincristine and vinblastine. These alkaloids are used as antileukemic agents. The anticancer agent podophyllotoxin (currently in clinical use) was isolated from the mayapple plant *Podophyllum peltatum*. Etoposide and tenoposide are semisynthetic derivatives of epipodophyllotoxin from the same plant, which are also used as anticancer agents. Another antitumor agent isolated from plants is colchicines from

the plant *Colchicum autumnale*. Paclitaxel, an anticancer agent, discovered from the pacific yew tree (*Taxus brevifolia*), is used in the treatment of various types of cancers (Weici and Gerhard, 2011).

Calabar bean (*Physostigma venenosum*) is the source of anticholinesterase physiostigmine. *Ephedra sinica* is the source of the sympathomimetic agents ephedrine and pseudoephedrine. Ephedrine is used in combination with theophylline from the plant *Theobroma cacao* in the management of asthma. The anticholinergic agent, atropine, used as an ophthalmic miotic agent and before surgery to reduce secretions, is derived from the plant *Atropa belladonna* (Wagner and Wolff, 1976; Michael *et al.*, 2004).

From the plant *Cephaelis ipecacuanha*, the anti-emetic emetine was isolated. *Salix capensis* has been used in south eastern Africa for centuries as a pain killer and antipyretic and is known to contain esters of salicylic acid, a compound whose acetylated form is a conventional analgesic aspirin (Maurice, 1993).

There is need for more research to be conducted to develop new drugs. The current study aims at isolating compounds from the root bark of *Teclea trichocarpa* and testing for their anthelmintic activity.

1.2 Literature Review

Herbal medicines are the most lucrative form of traditional medicine, generating billions of dollars in revenue globally. However a number of challenges face use of herbs in management of various disease conditions. Counterfeit, poor quality or adulterated herbal products in international markets are serious patient safety threats. Regulation of herbal medicine is also a challenge in many countries owing to the fact that some of the traditional practitioners claim that

their herbal preparations are food supplements and not drugs (WHO, 1989). Some of the herbs used by traditional healers have not been researched to authenticate their claimed activities. The dosage regimen is also not clear in most of these herbs. This results in people taking under-dose or over-dose of their prescribed herbs. There is no standardization, and components of a herbal extract or a product are likely to vary significantly between batches and producers. There is need to standardize the herbal preparations to ensure consistency in formulations and dosage. Aspects of Good Manufacturing Practices (GMP) need to be incorporated in the formulation of herbal preparations to guarantee quality and safety of herbs. The traditional medical practitioners therefore need basic training in GMP. The cleanliness of the equipments used by these healers also needs to be guaranteed.

1.2.1 Helminthiasis

Parasitic worms are cestodes, nematodes and trematodes. The classification with examples of the host target tissues and the drugs used for treatment are summarized in Table 1. There are a billion people globally infected by *Ascaris lumbricoides*, nine hundred million people are infected with *Ancylostoma duodenale* and five hundred million people with *Trichuris trichura.* (WHO, 1989). Helminthic infections are more common in people living in poverty and in resource limited areas. They have been a major cause of human suffering for centuries due to the fact that they live, feed and receive protection from the human host while causing weakness by consuming the host's nutrition. In developing countries, they still pose a large threat to public health and contribute to the prevalence of anaemia, malnutrition, eosinophilia and pneumonia. Diseases from parasitic worms causing severe morbidity include lymphatic filariasis (elephantiasis), fasciolosis, schistosomiasis and onchocercosis (WHO, 1989).

Table 1: Human parasitic worms*

Class of parasites	Examples	Host target tissues	Treatment
Cestodes (Tapeworms)	*Taenia solium*, *Taenia saginata*. *Diphyllobothrium latum*	Intestines	praziquantel and niclosamide
Nematodes	Hookworm (*Ancylostoma duodenale*) Intestinal roundworm (*Ascaris lumbricoides*) Whipworm (*Trichuris trichura*) Filarial worm (*Wuchereria bancrofti*) Pinworm (*Enterobius vermicularis*)	Intestines, blood , lymphaticic sytem	albendazole, mebendazole, levamisole, ivermectin and nitazoxamide
Trematodes	*Schistosoma mansoni*, *Schistosoma hematobium*, *Schistosoma japonicum* and *Fasciola hepaticum*	Intestines, blood, lungs and liver	praziquantel, diethylcarbamazine, metrifornate, oxamniquine and albendazole

*(WHO, 1989; Goering *et al.*, 2008; Stephen *et al.*, 2010; Sean, 2011)

1.2.2 Conventional drugs used in the treatment of parasitic worms

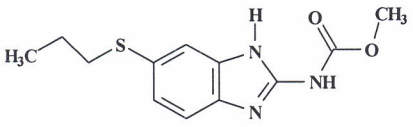
Some of the drugs (for example praziquantel and pyrantel pamoate) used to treat worm infections affect the nervous system of the parasite resulting in muscle paralysis while others like albendazole and mebendazole inhibit the parasitic uptake of glucose thus reducing the parasite's energy stores. Molecular structures of these anthelmintic drugs are shown in Figure 1.

The primary drug used for cestode infections is praziquantel. It causes spastic paralysis and also damages the membranes of the worm, which activates host defense mechanisms. It is readily

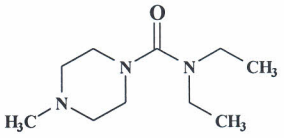
absorbed from the intestinal tract and it is a broad-spectrum anthelmintic affecting both cestodes and trematodes. Niclosamide is used to treat invasive cestodes (Goering, *et al.*, 2008).

Praziquantel is the most effective drug for treatment of intestinal, liver and lung flukes and is the drug of choice in the treatment of schistosomiasis. It is even safe to be used in pregnancy. Metrifornate and oxamniquine are alternative drugs for treatment of *S. haematobium* and *Schistosoma mansoni*, respectively. Triclabendazole is used in the treatment of fasciolosis (Stephen, *et al.*, 2010; Sean, 2011).

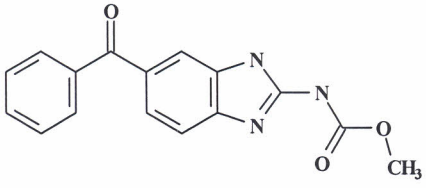
Treatment of blood and tissue round worms require use of drugs that are absorbed from the intestinal tract and penetrate into tissues. The primary drugs used for treatment of nematodes are albendazole, mebendazole, levamisole, nitazoxamide and pyrantel pamoate. Diethylcarbamazine and ivermectin, are readily absorbed from the intestinal tract. They are used in the treatment of filarial worm infections Blood levels are reached quickly, and action against the microfilariae is rapid. A severe allergic or febrile reaction due to the death of the microfilariae can follow the use of these drugs. Pyrantel pamoate is the drug of choice in treatment of pinworm. Most of the drug is not absorbed from the intestinal tract, resulting in high levels in the intestinal lumen. Levamisole is used in the treatment of lungworm infections (Goering, *et al.*, 2008; Stephen, *et al.*, 2010; Sean, 2011).



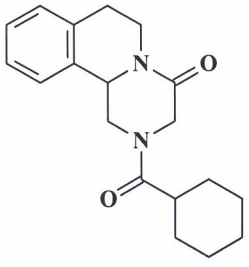
Albendazole



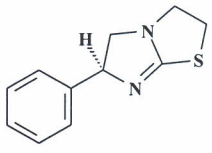
Diethylcarbamazine



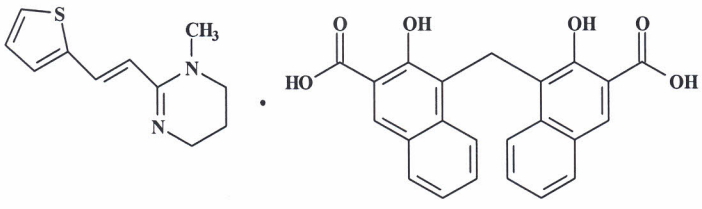
Mebendazole



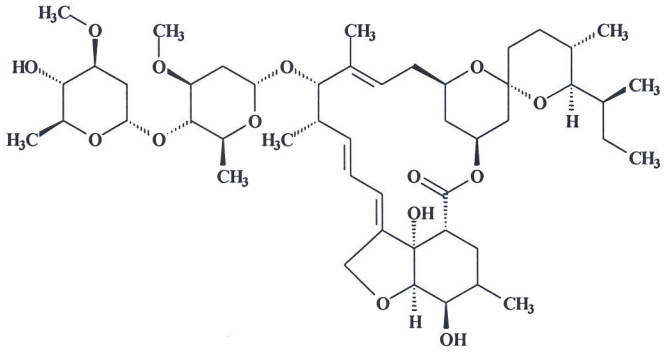
Praziquantel



Levamisole



Pyrantel pamoate



Ivermectin

Figure 1: Chemical structures of major anthelmintic drugs (Goering, *et al.*, 2008; Stephen, *et al.*, 2010; Sean, 2011).

1.2.3 Use of plants in the treatment of parasitic infections

A large number of medicinal plants, claimed to possess anthelmintic properties, are used by different ethnic communities worldwide to treat parasitic worms infections. Some of these plants are recognized by various pharmacopoeias. People use plant based preparations to treat parasitic worms because of limited availability of modern anthelmintic medicines. In Kenya, some plants are reported to have been used for centuries for treatment of intestinal worms in man and domestic animals. Some of them like *Albizia anthelmintica*, *Albizia gummifera*, *Albizia julibrissin*, *Croton megalocarpus*, *Embelia schimperi* and *Myrsine africana* have been investigated for active ingredients and for biological activity but there have been no efforts to develop drugs from them (Kokwaro, 1993; Beentje, 1994; Liu and Weller, 1996).

In preliminary work carried out Prof. Grace Thoithi at the School of Pharmacy, University of Nairobi in May 2010, 29 plants were screened for their anthelmintic activity. The plants were selected based on previous knowledge that they are used for treatment of intestinal worms and other common ailments like malaria, diarrhea, skin infections, coughs, and abdominal ailments. *In-vitro* anthelmintic screening on the ethanolic extracts of the plants was done against nematode *Nemastospiroides dubius*. Five of the plants, *Albizia gummifera*, *Crotalaria axillaris*, *Manilkara discolour*, *Teclea trichocarpa* and *Zanthoxylum usambarense* were found to have high activity against round worm and were recommended for further work (the findings were not published but were contained in a project funded by the Kenya National Commission for Science, Technology and Innovation (NACOSTI) – South Africa (National Research Fund). The present study was designed to investigate anthelmintic activity of *Teclea trichocarpa* root bark as well as isolation of the active principles determining their anthelmintic activities. The toxicity of the crude extract will also be determined.

1.2.4 The Rutaceae family

Rutaceae family, commonly known as the rue or citrus family, is a family of flowering plants, usually placed in the order Sapindales. It is comprised of herbs, shrubs, and trees with glandular punctate, commonly strongly smelling herbage comprising about 150 genera and 1,500 species distributed throughout the world, especially in warm temperate and tropical regions. The largest numbers are found in Africa and Australia, often in semiarid woodlands. The members of this family are characterized by the common occurrence of spines and conspicuous inflorescence flowers containing petioles divided into four or five wings. The fruits of this family vary, for example; capsules (genus *Ruta*), follicles (*Zanthoxylum*), drupes (*Amyris*), berries (*Triphasia*), samaras (hop tree) and schizocarps (*Helietta*).

The most economically important genus in the family is *Citrus*, which includes the sweet orange (*Citrus sinensis*), sour orange (*Citrus aurantium*), lemon (*Citrus limon*), grapefruit (*Citrus paradisi*), and key lime (*Citrus aurantifolia*). All these plants are grown for their fruits. Other important fruits in this family are the kumquat (genus *Fortunella*), bael (genus *Aegle*), elephant apple (*Limonia acidissima*), and Japanese pepper (*Zanthoxylum piperitum*).

Other members of this family are grown as ornamentals, and they have attractive white flowers and red berries. These include; poncirus, a spiny hedge shrub of temperate regions, Japanese skimmia (*Skimmia japonica*) and Chinese skimmia (*Skimmia reevesiana*). Orange jessamine (*Murraya exotica*) is native to Southeast Asia and is widely grown in the tropics as an ornamental. The burning bush (*Dictamnus albus*), is a non-woody poisonous perennial herb that has attractive white flowers. *Boronia* is a large Australian genus, with some members being

plants with highly fragrant flowers which are used in commercial oil production. Other large genera include *Zanthoxylum*, *Melicope* and *Agathosma* (Chase *et al.*, 1999).

Some of the genera of Rutaceae whose species have been used in traditional medicine include *Zanthoxylum*, *Evodia*, *Ckausena*, *Teclea* and *Phellodendron*. *Zanthoxylum* has about 30 species and all have been used in traditional medicine (Evans, 2005). The family elaborates alkaloids of which the acridone alkaloids are mainly found in the family Rutaceae (Jean, 1999).

1.2.5 The genus *Teclea*

Teclea is a genus in the family Rutaceae, consisting of about 80 species, most of them found in mainland Africa, Madagascar (30 endemic) and 1 in India. Several *Teclea* species are medicinally used in the East African region. *Teclea amaniensis* (Engl.) occurs in Kenya, Tanzania and Mozambique and has unifoliolate leaves. *Teclea eugeniifolia* (Engl.) occurs in Ethiopia, Somalia, Kenya and Tanzania. *Teclea uguenensis* (Engl.) occurs in Kenya and Tanzania. In Kenya the Pokot people take a root infusion to treat malaria. *Teclea nobilis*, occurs in wet highland Forests of Kenya, Tanzania and Uganda where the leaves and roots are used to treat cold and chest problems. Other species in the genus *Teclea* include; *Teclea carpopunctifera* (endemic to Côte d'Ivoire), *Teclea crenulata*, *Teclea ouabanguiensis*, *Teclea fischeri*, *Teclea myrei*, *Teclea rogersii*, *Teclea villosa*, *Teclea afzelii*, *Teclea natalensis* and *Teclea trichocarpa* (Bussmann, 2006).

Compounds isolated from Teclea *nobilis* include: furoquinoline alkaloids like tecleabine, tecleoxine, isotecleoxine, methylnkolbisine, flindersiamine, maculosidine, syringaldehyde and chlorodesnkolbisine; limonoids like methyl-uguenenoate and triterpenoid and lupeol (Schmelzer, 2011). From the root of *Teclea uguenensis*, limonoid derivative, methyl-uguenenoate, the azole

uguenenazole, the amide guenenonamide, together with the known furoquinoline alkaloids flindersiamine, maculosidine and syringaldehyde were isolated. From the methanolic extract of *Teclea afzelii*, lupeol was isolated (Cheplogoi *et al.*, 2008).

1.2.6 Taxonomic position of *Teclea trichocarpa*

The common name of *Teclea trichocarpa* is "furry-fruited teclea" and the local name is *Munderendu-wa-ikurari* in Kikuyu language (Kenya). It is synonym to *Vepris trichocarpa* and *Teclea gerrardii* (Schmelzer, 2011). The taxonomic classification of *Teclea trichocarpa* places it in the kingdom, Plantae; phylum, Magnoliophyta; class, Angiospermae; order, Sapindales; family, Rutaceae; and genus, *Teclea* (Kokwaro, 1993; Beentje, 1994).

1.2.7 Morphological description of *Teclea trichocarpa*

Teclea trichocarpa is an evergreen much-branched shrub tree 2-10 m tall; stem 10 cm in diameter; bark grey, smooth, young branchlets short-hairy. Leaves, shown in Figure 2, are alternate, 3-foliolate, dark green and aromatic when crushed. Stipules are absent; petioles are furrowed or flattened above and sometimes narrowly winged. Glandular-dotted with oil glands are present on the leaves and other parts of the plant. The leaves also contain 8-14 pairs of parallel veins. The flowers are sweet scented, bisexual, zygomorphic with four to five connate sepals, four to five connate petals, eight to ten stamens and superior ovary. The fruit is an ellipsoid drupe, base short-hairy, wrinkled, one-seeded. The seeds are ellipsoid with a longitudinal groove, dark green to dark brown (Beentje, 1994; Schmelzer, 2011; Evans, 2005).

Figure 2: Photograph of *Teclea trichocarpa* leaves taken at Ngong Forest in Kenya.

1.2.8 Distribution of *Teclea trichocarpa* in Kenya

Teclea trichocarpa is widely spread in coastal and upland Forests and grasslands, often near rivers. It also occurs in dune bush, Forest edges and lowland rain Forest, riverine Forest, dry semi-deciduous Forest, especially in rocky localities, from sea-level up to 2300 m altitude. It is indigenous to Kenya where it is found in Ngong Forest, Marakwet District and Keiyo District (Muriithi *et al.*, 2002; Schmelzer, 2011).

1.2.9 Medicinal uses of *Teclea trichocarpa* in Kenya

The Kamba people use the leaf extracts to treat malaria, fever and as an anthelmintic (Beentje, 1994; Muriithi *et al.*, 2002; Joseph and Paul, 2009). The vapour of leaves in hot water is inhaled to treat fever. The Giriama people in Kenya put the strong-smelling leaves in the nose of their hunting dogs to improve their scenting powers (Beentje, 1994). The stem bark infusion is taken to treat malaria. The smoke of green twigs on a fire is applied to treat body pain and hepatitis. The leaf decoction is taken to treat pneumonia (Schmelzer, 2011).

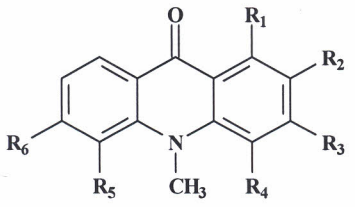
1.2.10 Previous studies on *Teclea trichocarpa*

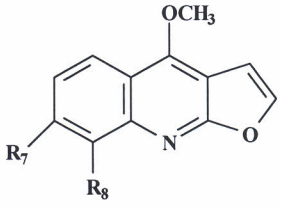
From the stem bark of *Teclea trichocarpa*, acridone alkaloids (the chemical structures of these isolates are shown in Figure 3) namely; melicopicine, tecleanthine, 6-methoxy tecleanthine, tegerrardin A, tegerrardin B and arborinine and the acridone precursor tecleanone were isolated (Mwangi and Erastus, 2007). The furoquinoline evoxine and the rare cyclobutaquinoline alkaloid cyclomegistine were also isolated (Schmelzer, 2011).

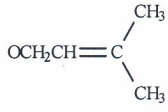
From the leaves the following acridone alkaloids were isolated; normelicopicine, arborinine, melicopicine, tecleanthine, 3β-sitosterol and 6-methoxytecleanthine. Furoquinoline alkaloids

skimmianine and dictamnine, and the triterpenoid α-amyrin (Figure 4) were also isolated (Mwangi, 2007; Schmelzer, 2011; Kiplimo *et al.*, 2011).

The fruit yielded the acridone alkaloids melicopicine and 1,2,3-trimethoxy-*N*-methylacridone, and the furoquinoline alkaloid skimmianine (Mwangi and Erastus, 2007; Schmelzer, 2011).





	R_1	R_2	R_3	R_4	R_5	R_6
Normelicopicine	OH	OCH_3	OCH_3	OCH_3	H	H
Arborinine	OH	OCH_3	OCH_3	H	H	H
Melicopicine	OCH_3	OCH_3	OCH_3	OCH_3	H	H
Tecleanthine	OCH_3	-	OCH_2O	H	OCH_3	H
6-Methoxytecleanthine	OCH_3	-	OCH_2O	H	OCH_3	OCH_3
Tegerrardin A	H	H	H	H	OCH_3	OH
Tegerrardin B	H	H	H	H		OH
1,2,3-Trimethoxy-N-acridone	OCH_3	OCH_3	OCH_3	H	H	H

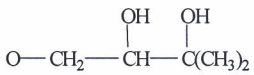
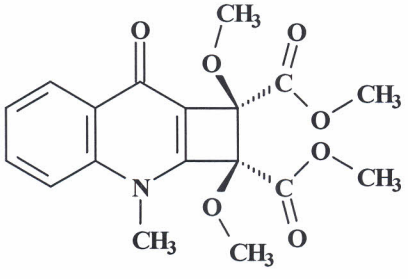
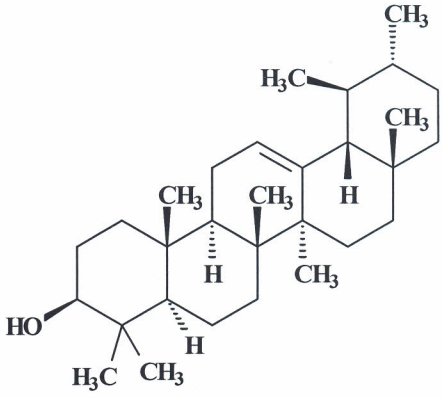
	R_7	R_8
Dictamnine	H	H
Evoxine	OCH3	
Skimmianine	OCH_3	OCH_3

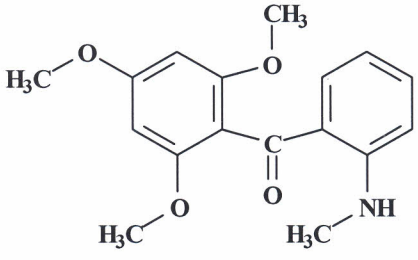
Figure 3: Structures of alkaloids previously isolated from *Teclea trichocarpa* (Muriithi *et al.*, 2002; Mwangi *et al.*, 2010).



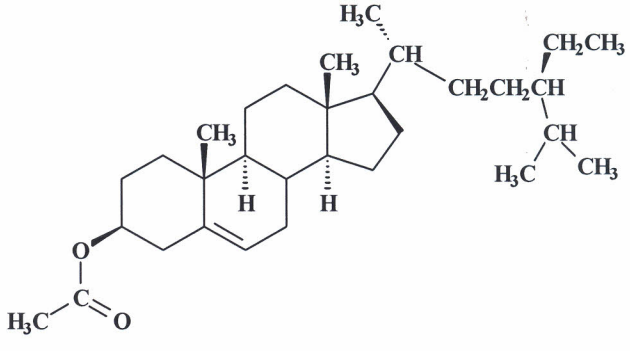
Cyclomegistine



α-Amyrin



Tecleanone



3-β-Acetyl sitosterol

Figure 4: Structures of other compounds previously isolated from *Teclea trichocarpa* (Muriithi *et al.*, 2002, Mwangi *et al.*, 2010, Mwangi *et al.*, 2012).

Some of the compounds isolated from this plant have been tested for biological activities. Melicopicine and tecleanthine exhibited mild antifeedant activity against the African army worm, *Spodoptera exempta*. Normelicopicine and arborinine, displayed limited *in vitro* activities against HB3 and K1 strains *Plasmodium falciparum*, but there appeared to be a cross-resistance with chloroquine. Normelicopicine was found to have some activity against *Plasmodium berghei* in mice (32% suppression of parasitaemia at a dose of 25 mg/kg/day). Melicopicine, tecleanthine and 6-methoxytecleanthine showed antimicrobial activity against the bacterium *Bacillus subtilis*, and the fungus *Cladosporium cucumerinum*. Antibacterial activity tests for the n-hexane extract of *Teclea trichocarpa* (leaves) showed an inhibition zone of 6, 12, 10 and 6 mm against *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*, respectively; while the dichloromethane extract showed an inhibition zone of 6, 12, 9 and 6 mm on the same bacteria, respectively. Both n-hexane and dichloromethane extracts of the leaves being active towards both Gram-negative and Gram-positive bacteria, have potential for identification of lead structures. The hexane extract of the leaves showed the highest insecticidal activity at 600 ppm against maize weevil, *Sitophilus zeamais*. Therefore the plant is a potential source of maize protectant compounds (Mwangi and Erastus, 2007; Schmelzer, 2011, Mwangi *et al.*, 2012).

1.3 Problem statement

Parasitic diseases cause severe morbidity affecting mainly people living in endemic areas with major economic and social consequences. Resistance of parasites to the existing drugs and their high costs warrants the search for newer anthelmintic molecules. An ideal anthelmintic agent should have broad spectrum of action, high percentage of cure with a single therapeutic dose, selective toxicity to the parasite but safe to the host and should be cost-effective.

The research questions arising from the above problem are:

a) Is the traditional use of herbs attributable to isolable compounds?

b) Are these isolates responsible for toxicity of herbs?

1.4 Hypothesis of the study

1.5.1 The null hypothesis of the study is that 'the anthelmintic activity of the root bark of *Teclea trichocarpa* is not due to isolaTable active principles'.

1.5.2 The alternative hypotheis of the study is that 'the anthelmintic activity of the root bark of *Teclea trichocarpa* is due to isolaTable active principles'.

1.5 Study Justification

The origin of many effective drugs is found in the traditional medicine practices and in view of this, there is need to evaluate more folklore plants for their claimed anthelmintic efficacy. Isolation of compounds from plants is an important step in the discovery of new drugs (Liu and Weller1996; Temjenmongla and Yadav, 2005; Dharani and Yenesew, 2010). Determination of the concentration of the active constituents helps in standardization of herbal preparations which is fundamental in assay of crude herbs. Phytochemical work done may yield compounds that can serve as templates from which more effective, efficacious and safer anthelmintics can be synthesized. The root bark of *Teclea trichocarpa* has been previously used traditionally for its anthelmintic activity. No study has been done to date to isolate compounds from the root bark of this plant, which is the part used for treatment of intestinal worms. In view of this, there is need to isolate ingredients from the root bark of *Teclea trichocarpa* and further determine the molecular structure of the major constituents and their anthelmintic activity.

1.6 Objectives of the study

The general objective of the study is to determine the anthelmintic activity of *Teclea trichocarpa* root bark and to isolate and characterize the active principles.

The specific objectives are:

a) To isolate and characterize the active ingredients from the root bark of *Tecleatrichocarpa.*

b) To determine the anthelmintic activity of *Teclea trichocarpa* root bark extract and the isolated compounds.

c) To establish the toxicity of the root bark of *Teclea trichocarpa* root bark crude extract using brine shrimp lethality testing (BSLT).

CHAPTER 2

EXPERIMENTAL

Plant material for phytochemical investigation was first tested for presence of glycosides, saponins, alkaloids and tannins to give preliminary information on the nature of compounds present. Three consecutive cold maceration extractions were done using dichloromethane-methanol (1:1) solvent mixture. The filtrate was dried *in vacuo* and the percentage yield determined. The crude extract was kept at 4 °C for use on column chromatography, brine shrimp lethality testing and anthelmintic study.

2.1 Solvents, reagents and materials

2.1.1 Solvents and reagents

General purpose reagent (GPR) grade solvents, namely; dichloromethane, chloroform, acetonitrile, methanol and ethyl acetate were from Sigma-Aldrich GmbH (Seelze, Germany). These solvents were distilled before use. Normal phase silica gel powder 32-63 μm was also obtained from Sigma-Aldrich GmbH. Analytical grade acetone and ethanol used for crystallization were from Scharlab S.L (Gato Perez, Spain). Dimethyl sulfoxide and concentrated sulphuric acid were from Fischer Scientific (Leicester, UK). Iodine resublimed general reagent was from Merck (Damstadt, Germany) while vanillin was from BDH Chemicals Ltd., (Poole, England). Mercuric chloride powder for phytochemical test was from BDH Chemicals Ltd. while lead acetate was from Unilab Ltd., (Nairobi, Kenya). Anhydrous ferric chloride was from Loba Chemie PVT. Ltd. (Mumbai, China) while ammonium acetate was from Hopkin and Williams (Essex, U.K). Bismuth subnitrate was from Aldrich Chemical Company Inc. (Milwaekee, USA).

2.1.2 Material

2.1.2.1 Plant material collection and preservation

The root bark of *Teclea trichocarpa* was collected from Ngong Forest, Kajiado County, in October 2012. A voucher specimen of the plant was also collected and pressed on site and assigned a reference number TRB/2012. Taxonomical identification was done at the Department of Botany Herbarium, University of Nairobi, where the voucher specimen is deposited.

2.1.2.2 Chromatographic and filtration materials

The liquid chromatography glass columns were laboratory fabricated at Catholic University (Leuven, Belgium). HPLC was done using hypersil ODS2 5µm: 4.6 mm × 250 mm column from Shandon Scientific Ltd. (Chenshire, UK). The Whatman filter paper No. 1 was from Whatman International Ltd. (Maidstone, UK) while sintered glass filter funnel No. 4 was from Schott Duran GmbH, Co. (Wertheim, Germany). TLC GF_{254} pre-coated aluminium plates were from Sigma-Aldrich GmbH.

2.1.2.3 Biological materials

The sheep nematode strongyloides eggs were obtained from fresh stools from a sheep at Kabete Animal Farm, University of Nairobi, while Artemia brine shrimp eggs, from Great Salt Lake (Heinsberg, U.S.A) were purchased from pet stores within the city of Nairobi, Kenya.

2.2 Equipment

The Heizbad WB rotary vacuum evaporator from Heidolph Electro GmbH, Co. KG (Kelheim, Germany) was connected to a Polyscience cooler from Polyscience (Niles, USA), a WB2000 water bath from Heidolph Electro GmbH, Co. KG and a N820.3 Laboport diaphragm vacuum pump from KNF Neuberger GmbH (Freiburg, Germany). A Samsung refrigerator was from

Samsung Electronics (Seoul, South Korea) while AUW220D analytical balance was from Shimadzu Corporation. PB3002 DeltaRange top loading balance was from Mettler Toloedo AG (Greifensee, Switzerland). Fractions were collected using a SuperFrac™ automatic fraction collector from Pharmacia LKB Biotechnology (Uppsala Sweden) while Min UVIS ultraviolet light lamp was from Desaga GmbH (Heidelberg, Germany).

Melting points of the re-crystallized isolates were determined using melting point apparatus from Barloworld Scientific Ltd. (Staffordshire, UK). The ultraviolet spectrum was obtained using a T90 UV/Visible scanning spectrophotometer from PG Instruments Ltd. (Cambridge, UK). Nuclear Magnetic Resonance (NMR) spectroscopic data was obtained using Varian-Mercury™ 200 MHz spectrometer from Varian Inc. Palo (Alto, USA) with data acquisition by an online computer from Sun Micro-Systems (California, USA) and analyzed using Varian™ software. The components of the HPLC machine consisted of SPD - 20A Prominence UV/Vis detector, LC - 20 AT, Prominence pump and DGU - $20A_3$ Prominence degasser and LTO - 10A S_VP oven all from Shimadzu Corporation (Kyoto, Japan). Infrared spectroscopic data was obtained by IR Prestige-21 fourier transform infrared (FTIR) spectrophotometer from Shimadzu Corporation. Mass spectrometric data was obtained by Electron Impact Mass Spectrometry (EIMS) using 70 and 20 or 30 eV ionizing energies on a VG 12-250™ quadrupole mass spectrometer from VG Analytical (Manchester, UK) operated at 8×10^{-6} Pa vacuum pressure, 180 °C ion source temperature and 400 °C heated solid probe temperature. The mass spectra were acquired using an online computer and processed using MassLynx software from Microsoft Corporation (Redmond, USA).

2.3 Procedures

2.3.1 Preparation of reagents

Dragendorff's Reagent was prepared as follows using about 1.7 g of bismuth nitrate, 20 g tartaric acid and 20 ml glacial acetic acid which were dissolved in 80 ml of distilled water to form solution A. About 16 g of potassium iodide was dissolved in 40 ml distilled water to form solution B. Stock solution was obtained by mixing solution A and solution B in the ratio 1:1 (v/v). Dragendorff's Reagent was them made by adding 5 ml of the stock solution to 10 g tartaric acid in 50 ml of water.

Iodine was prepared by taking about 2 g of iodine crystals in a glass tank, covering and allowing the iodine to sublime. The tank was allowed to saturate with the iodine vapour for one hour.

A 1 % w/v vanillin in concentrated sulphuric acid was prepared by putting one gram of vanillin into a conical flask. About 99 ml of 36 N sulphuric acid was then added into the flask and the contents swirled until all the vanillin had dissolved to form 1% w/v vanillin which is a light yellowish solution.

Meyer's reagent was prepared by dissolving a mixture 1.36 g of mercuric chloride and 5.00 g potassium iodide in 100 ml water.

2.3.2 Phytochemical tests

2.3.2.1 Test for alkaloids

One gram of dry powdered root bark of *Teclea trichocarpa* was extracted by warming on water bath with 10 ml of 10 % sulphuric acid for 5 min. It was then filtered and the filtrate portion tested by adding one to two drops of Meyer's Reagent. Formation of a white buffy precipitate indicated presence of alkaloids (Evans, 2005).

The rest of the extract was alkalinized to pH of 12 with dilute ammonia solution. It was extracted with 2 ml of chloroform and the chloroform layer separated and washed with water then filtered through a small cotton plug. The filtrate was evaporated to dryness. The residue was dissolved in 0.2 ml of 1 % sulphuric acid and to a 0.1 ml of the filtrate solution, a drop of Dragendorff's Reagent was added. Formation of a precipitate was indicative of presence of alkaloids (Evans, 2005).

2.3.2.2 Test for tannins

One gram of the dry and powdered root bark of *Teclea trichocarpa* was extracted by boiling in 5 ml of water. Few drops of a solution of ferric chloride were added. Formation of a blue precipitate was an indication of presence of tannins (Evans, 2005).

2.3.2.3 Test for glycosides

To 1 gram of the dry powdered root bark of *Teclea trichocarpa*, 10 ml of 70 % ethanol was added and heated on water bath for 2 min, then cooled. Ten millilitres of water were added and five drops of 60 % lead sub-acetate solution were added and then 10 % sulphuric acid added drop wise until no further precipitation formed. Successive 5 ml portions of chloroform were added to the extract. The two chloroform extracts were combined and washed with 1 ml of distilled water. The chloroform portion was separated and divided into two equal parts for the following two tests:

Test for unsaturated lactone ring of the aglycone: one of the two chloroform portions was evaporated to dryness and one drop of 90 % ethanol added. Two drops of 2 %

3,5-dinitrobenzoic acid in 90 % ethanol added. The solution was made alkaline with 20 % sodium hydroxide solution. A colour change to purple indicated the presence of unsaturated lactone ring of aglycone (Evans, 2005).

Test for 2-deoxy sugar (Keller Killian Test): The other chloroform portion was evaporated to dryness and then 0.4 ml of glacial acetic acid containing trace of ferric chloride was added. Carefully down the test tube, 0.5 ml of concentrated sulphuric acid was added. A colour change to green blue in the upper acetic acid layer was an indication of the presence of glycosides (Evans, 2005).

2.3.2.4 Test for saponins

About one gram of the powdered root bark of *Teclea trichocarpa* was added to a test tube and shaken slowly. Persistent frothing is an indication of presence of saponins (Evans, 2005).

In an alternative test, 0.2 g portion of the powdered plant material was extracted with 10 ml of warm water and filtered retaining the filtrate. Then 2 ml of 1.8 % sodium chloride solution was added to two test tubes. To one of these test tubes, 2 ml of distilled water was added and to the other, 2 ml of the extract was added. One drop of fresh blood from a mouse was added to each test tube and the tubes were inverted gently to mix contents. Haemolysis of red blood cells indicated presence of saponins. When there is no heamolysis, only red deposits are seen at the bottom of the test tube but when there is haemolysis all the contents of the test tube are coloured red (Evans, 2005).

2.3.3 Extraction of the root bark of *Teclea trichocarpa*

The plant material was air dried in the shade under room temperature, cut into small pieces, ground and kept in labeled plastic containers until use. The powdered plant material (924 g) was

transferred to a 3 L conical flask and subjected to four consecutive cold maceration extractions. The first extraction was done by adding 2.8 L dichloromethane-methanol (1:1) to the conical flask containing the powdered plant material and allowed to stand for 12 h with regular stirring after which the extract was filtered. Each of the other two consecutive extractions was done by adding a 1:1 mixture of methanol and dichloromethane in the conical flask and allowing it to stand for 24 h with regular stirring and each extract filtered. A fourth cold maceration extraction was done by adding 1.2 L of methanol to the conical flask and allowing it to stand for 24 h with regular stirring and the extract filtered. The four extracts were combined and dried *in vacuo* using a rotary evaporator producing 77 g of dry extract which represented 8.3 % yield. The dry extract was stored in a refrigerator at 4 °C until use.

2.3.4 Isolation of compounds

The crude extract was first adsorbed on silica gel and dry loaded on to the column coupled to a fraction collector. The fractions that formed crystalline compounds were cleaned in suitable solvents and repeatedly re-crystallized in suitable crystallization solvents to obtain pure compounds. The purity of the compounds was monitored using thin layer chromatography (TLC) and confirmed by high performance liquid chromatography (HPLC).

2.3.4.1 Choice of mobile phase

Thin layer chromatography (TLC) was performed on silica gel GF_{254} to determine the solvent systems for isolation. The solvents tested were chloroform, chloroform-methanol (97:3), chloroform-methanol (95:5), dichloromethane, dichloromethane-methanol (97:3), dichloromethane-methanol (95:5), ethyl acetate, ethyl acetate-methanol (97:3) and ethyl acetate-methanol (95:5). The spots in the developed chromatograms were observed under both short UV (254 nm) and long UV (366 nm), exposure to iodine, spraying with 1% w/v vanillin and spraying

with Dragendorff's reagent in that order. Dragendorff's reagent was sprayed because the phytochemical tests done showed presence of alkaloids.

The solvent system that showed best separation was 100 % ethyl acetate though the point of sample spotting in the TLC tested positive for alkaloids when sprayed with Dragendorff's Reagent after developing with ethyl acetate. This indicated traces of alkaloids were present at the point of sample spotting. A three dimensional TLC was therefore done on this point of origin using ethyl acetate-methanol (97:3) and ethyl acetate-methanol (95:5) both of which showed three spots. Based on the above information, the mobile phase for elution was ethyl acetate, ethyl acetate-methanol (97:3) and ethyl acetate-methanol (95:5) in that order.

2.3.4.2 Sample preparation

The dried extract was not completely soluble in the proposed mobile phase and therefore needed to be first adsorbed in silica gel in the ratio of 1:1. To achieve adsorption, 20 g of dry extract was re-dissolved in dichloromethane-methanol (1:1) then dried *in vacuo* using rotary evaporator and ground to fine powder using mortar and pestle. This adsorbed sample was introduced to open column chromatographic column.

2.3.4.3 Packing of column and sample loading

A 1 cm layer of acid washed sand was added to the column followed by slurry of 189 g silica gel in 1.5 L ethyl acetate. The column was compacted using a hand held pump to produce a uniform silica packing. About 32 g of adsorbed extract was added and covered with upper layer of acid washed sand. The mobile phase was allowed to flow at the rate of 5 ml per minute. The fractions were collected (by a fraction collector) in test tubes labeled F_1 to F_{589}.

The TLC profile was established by spotting the eluate in every 10^{th} test tube. The test tubes that produced crystals were targeted for isolation. The TLC profile of the solutions in these test tubes was determined by visualizing under both short UV (254 nm) and long UV (366 nm), exposure to iodine and spraying with 1 % w/v vanillin in that order.

The fractions targeted for isolation were F_{54} to F_{63}, F_{81} to F_{110} and F_{194} to F_{220} which produced crystals within 1 week, 1 week and 2 weeks respectively.

2.3.4.4 Crystallization of compounds

The crystals in test tubes F_{54} to F_{63} were cleaned with acetone and re-dissolved in ethyl acetate for re-crystallization. Subsequent re-crystallizations produced a pure white crystalline compound which produced a single TLC spot that was not visible in both short and long UV but was visualized in the iodine chamber. This compound was coded compound **1** and stored at 4 °C.

The crystals in test tubes F_{81} to F_{110} were cleaned in n-hexane and re-dissolved in warm ethyl acetate to re-crystallize. Several re-crystallizations in ethyl acetate afforded a pure yellow crystalline compound. The compound produced a single spot on TLC when visualized in long UV radiation (366 nm). This compound was coded compound **2** and stored at 4 °C.

The crystals in test tubes F_{194} to F_{220} were cleaned with cold ethanol and re-dissolved in warm ethyl acetate for re-crystallization. Subsequent re-crystallizations produced a pure compound that showed a single TLC spot when visualized under both short and long UV radiation. The compound was coded compound 3 and stored at 4 °C.

The summary of the physical properties of isolated compounds is shown in Table 2. The process of isolation of the three compounds is summarized in Figure 5.

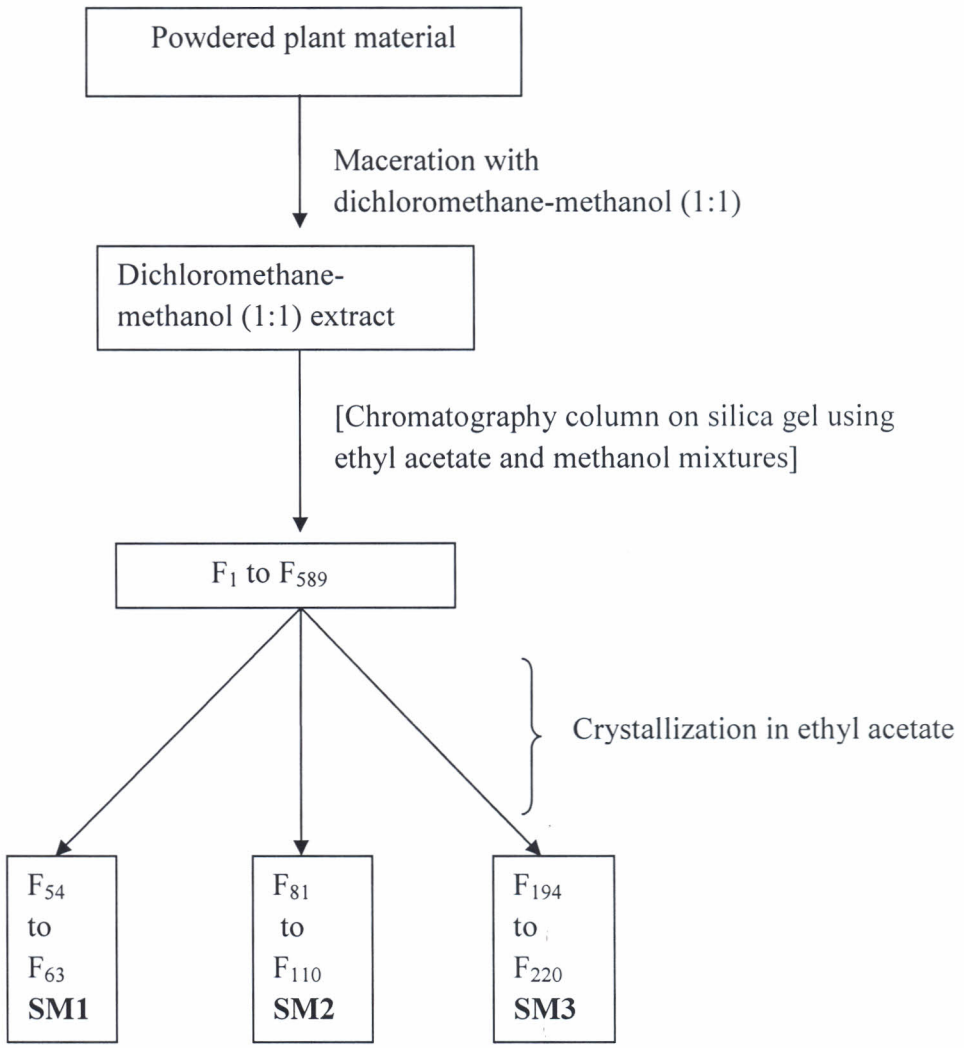


Figure 5: Scheme for isolation of compounds from the root bark of *Teclea trichocapa*.

Table 2: Physical properties of isolated compounds

Compound	Compound 1	Compound 2	Compound 3
Description	White crystals	Yellow crystals	Green crystals
R_f value*	0.70	0.5	0.3
Colour in Iodine	Brown	Brown	None
Colour in Drangendorff's reagent	None	Orange	Orange
Colour under UV light	None	Green	Blue
Melting point (in °C)	211-213	128-130	163-165

*solvent system is ethylacetate and the chromatogram is shown in Appendix 1

2.3.5 Egg hatch assay

Each compound was dissolved in dimethylsufoxide (DMSO)-water (3:97) solution. About 55 eggs of nematodes (*Strongyle* species) were transferred to microtitre plates containing 20 µL of serial dilutions of each pure isolate solution and to 20 µL of DMSO (control). The final volume of the wells was made to 80 µL using distilled water. The plates were incubated for 48 hours. The number of hatched larvae and the number of eggs remaining in each well was counted and the percentage inhibition of hatching at each concentration determined.

2.3.6 Larval development assay

The nematodes eggs at concentration of about 55 eggs in 80 µL were incubated for 48 h at 27 °C without the pure isolates for them to hatch. To each well, 20 µL (5000µg/ml) of the pure isolate was added and the mixture incubated for a further five days to allow the larvae to develop. The number of larvae that did not develop were counted and the percentage inhibition of development at each concentration determined.

2.3.7 Brine shrimp lethality testing

A plastic chamber was divided into two and filled with artificial sea water (made up of 33 g of marine salt and 6 mg of baker's yeast in 1 L of distilled water). The chambers were separated by a plastic wall having several holes of 2 mm diameter. One chamber was darkened while the other was illuminated by an electric bulb lit above the covered tank. About 50 mg of brine shrimp eggs were sprinkled in the darkened side. After 48 h, the shrimps hatched to *Artemia nauplius* larvae that then migrated to the illuminated side. The larvae were harvested using a pipette. The presence of the swimming shrimps was an indication of the viability of the procured lot of eggs/cysts. The test sample was prepared by weighing 50 mg of the dichloromethane-methanol (1:1) extract and then dissolving in 5 ml dimethylsulfoxide (DMSO). From this stock solution, 500 µl, 50 µl and 5 µl were transferred in vials corresponding to 1000 µg/ml, 100 µg/ml and 10 µg/ml, respectively. For each concentration, triplicate vials were prepared giving a total of nine vials for the three concentrations. Ten shrimps were counted per vial and the volume adjusted with brine to 5 ml/vial. The vials were placed uncovered with a lamp lit on top to provide warmth. Care was taken to ensure that the vials were not overheated by the lamp. After 24 h, the surviving shrimps in each vial were counted and recorded. The data obtained was entered in MS Excel and exported to Graphpad prism 4 (April 3, 2003) computer program for analysis to determine the IC_{50} values at 95 % confidence (Ndwigah *et al.*, 2005).

2.3.8 Melting point determination

Melting point of the pure isolates was determined as follows by adding small quantities of finely powdered crystals of the isolated compounds in an oven at 40 °C for 24 h. For each isolate, a small portion was transferred to a capillary tube and the powder packed by tapping on a hard

surface to form a tightly packed column of 4 to 6 mm in height. The metal block of the melting point apparatus was heated at a rapid rate to 190 °C and then the heating rate readjusted to 1 °C/min. The temperature at which the last solid particle of the compact column of each isolate, in the capillary tube, passed into the liquid phase was noted (*British Pharmacopoea*, 2008). The melting point of compound **1**, which was later elucidated as lupeol, is in agreement with the literature melting point as reported by Ndwigah *et al.* (2005). The literature melting points of the acridone alkaloids (compound **2** and compound **3**) was not found.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Phytochemical tests

The results of the phytochemical test done are shown in Table 3. The root bark of *Teclea trichocarpa* was found to contain alkaloids and tannins. *Teclea trichocarpa* is known to contain acridone alkaloids.In this study, two acridone alkaloids were isolated and identified as melicopicine and 6-methoxytecleanthine. Acridone alkaloids have been previously isolated from other parts of this plant. This suggests that they are distributed in all parts of *Teclea trichocarpa.* Acridone alkaloids are restricted to the Rutaceae family (Mwangi, 2007; Schmelzer, 2011).

No glycosides have been isolated from this plant before. A negative test for glycosides was therefore consistent with the previous findings on other parts of the plant.

Table 3: Screening results of the root bark of *Teclea trichocarpa*

Test	Result	Inference
Tannins	Positive blue precipitate with ferric chloride	Tannins present
Dragendorff's Test	Precipitation observed	Alkaloids present
Meyer's Reagent	A white buffy precipitate observed	Alkaloids present
Saponins	No frothing and no haemolysis of red blood cells	Saponins absent
Unsaturated lactone ring of aglycone	No colour change to purple	Glycosides absent
Keller-Kiliani Test	No green blue colour formed in the upper glacial acetic acid layer	Glycosides absent

3.2 Structure elucidation

Crystals of the pure isolates were subjected to UV, FTIR, ^{1}H-NMR, ^{13}C-NMR and MS analyses for structure elucidation. The molecular structures of compounds **1**, **2** and **3** were determined as lupeol, melicopicine and 6-methoxytecleanthine respectively, based on their spectroscopic data.

3.2.1 Compound 1 (Lupeol)

Compound **1** (lupeol) was isolated as white feathery crystals. It was not visible under both short and long UV light at 254 nm and 366 nm, respectively. It produced a brown spot on exposure to iodine powder.

Melting point: 211-213 °C.

IR (KBr) cm^{-1}: 3393 (O-H stretch), 2943 (=C-H stretch), 2868 (aliphatic C-H stretch), 1638 (C=C stretch) and 881 (out of plane C-H bend) (Appendix 2).

^{1}H-NMR (200 MHz, $CDCl_3$) δ ppm: 0.67 (d), 0.77 (d), 0.83 (s), 0.95 (d), 1.03 (s, H-23), 1.25 (s, C-24), 1.28 (s, H-25), 1.30 (d), 1.36 (s, H-26), 1.37 (s, H-27), 1.41 (d, H-28), 1.53 (m), 1.58 (s, 30), 1.63 (s), 1.68 (s, OH), 1.86 (m, 1H), 2.32 (m, 1H), 3.20 (m, 1H, H-3) and 4.57 (d, 2H, H-29) (Appendix 3).

^{13}C-NMR (50 MHz, $CDCl_3$) δ ppm: 38.9 (C-1), 27.7 (C-2), 79.2 (C-3), 39.1 (C-4), 55.5 (C-5), 18.5 (C-6), 34.5 (C-7), 41.1 (C-8), 50.7 (C-9), 37.3 (C-10), 21.1 (C-11), 25.4 (C-12), 38.2 (C-13), 43.1 (C-14), 27.7 (C-15), 35.8 (C-16), 43.2 (C-17), 48.5 (C-18), 48.2 (C-19), 151.2 (C-20), 29.9 (C-21), 40.2 (C-22), 28.2 (C-23), 15.6 (C-24), 16.3 (C-25), 16.2 (C-26), 14.8 (C-27), 18.2 (C-28), 109.6 (C-29) and 19.5 (C-30) (Appendix 4).

EI-MS (*m/z*): 426 (100%) [M^+], 411 (53.4%), 383 (15.8%), 365 (20.9%) and 344 (9.4%) (Appendix 6).

In the infra red spectrum, the band at 3393 cm^{-1} is due to O-H stretch. The presence of IR bands at 2943 cm^{-1} and 1638 cm^{-1}, are due to C-H stretch and C=C stretch respectively, which indicates presence of unsaturated system. Infra red bands at 2868 cm^{-1} and 881 cm^{-1} are due to C-H stretching and out of plane C-H bending, respectively indicating presence of methyl, methylene and/or methine groups (Kalsi, 2004; Robert *et al.*, 2005).

In the ^{1}H-NMR spectrum, the chemical shift at δ 1.68 is due to hydroxyl proton while the signal at δ 4.57 is due to double bond protons. The ^{1}H-NMR δ value at 3.20 is due to oximethine proton. The three proton singlets at δ_H 0.83, 1.03, 1.25, 1.28, 1.36, 1.37 and 1.58 are due to 7 methyl groups.

The ^{13}C-NMR spectrum showed a total of 30 peaks corresponding to a total of 30 carbons. The ^{13}C-NMR chemical shifts at δ 109.6 and 151.2 are due to two sp^2 hybridized carbons. The DEPT spectrum (Appendix 5a and 5b) shows presence of 7 methyl, 10 methylene and 7 methine carbon atoms, leaving the remaining six carbons to be quaternary.

The mass spectrum shows a molecular ion at *m/z* 426 as the base peak. The molecular weight of 426 corresponds to the molecular formular of $C_{30}H_{50}O$.

Based on the above information, and comparison with literature data, compound **1** was identified as lupeol whose structure is shown in Figure 6. The comparison of ^{13}C-NMR spectrum data of compound **1** and the literature data for lupeol is shown in Table 4. This is the first time lupeol is reported in *Teclea trichocarpa*.

The probable fragmentation pattern of lupeol is illustrated in Figure 7. A molecular ion at *m/z* 426 was formed by removal of one electron from the compound. Fragmentation of the molecular ion by removal of a methyl and a C_6H_{10} group produces fragment ions of *m/z* 411 and 344, respectively. The fragment ion at *m/z* 411 further fragments by losing CH_2=CH_2 group to yield a fragment ion at *m/z* 383 which further fragments by losing water molecule to produce a fragment ionat *m/z* 365. This fragmentation is similar to that reported by Ndwigah *et al.* (2005).

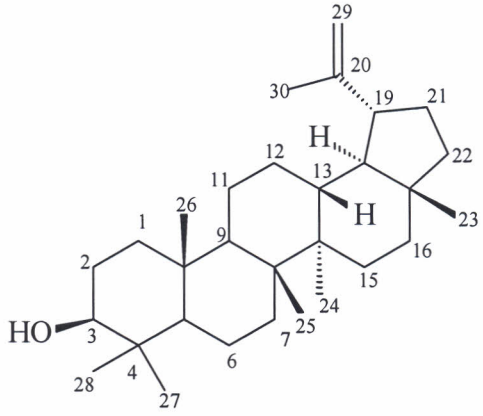


Figure 6: Molecular structure of lupeol.

Table 4: Comparison of ^{13}C-NMR δ values for lupeol and literature values*

Carbon	Compound **1**	Lupeol*
1	38.9	38.7
2	27.7	27.4
3	79.2	79.0
4	39.1	38.8
5	55.5	55.3
6	18.5	18.3
7	34.5	34.2
8	41.1	40.8
9	50.7	50.4
10	37.3	37.1
11	21.1	20.9
12	25.4	25.1
13	38.2	38.0
14	43.1	42.8
15	27.7	27.4
16	35.8	35.6
17	43.2	43.0
18	48.5	48.2
19	48.2	48.0
20	151.2	150.9
21	29.9	29.8
22	40.2	40.0
23	28.2	28.0
24	15.6	15.4
25	16.3	16.1
26	16.2	15.9
27	14.8	14.6
28	18.2	18.0
29	109.6	109.3
30	19.5	19.4

*(Enamul *et al.*, 2006). The data is also in agreement with the ^{13}C-NMR δ values for lupeol reported by Ndwigah *et al.*, 2005; and Soumia, 2012.

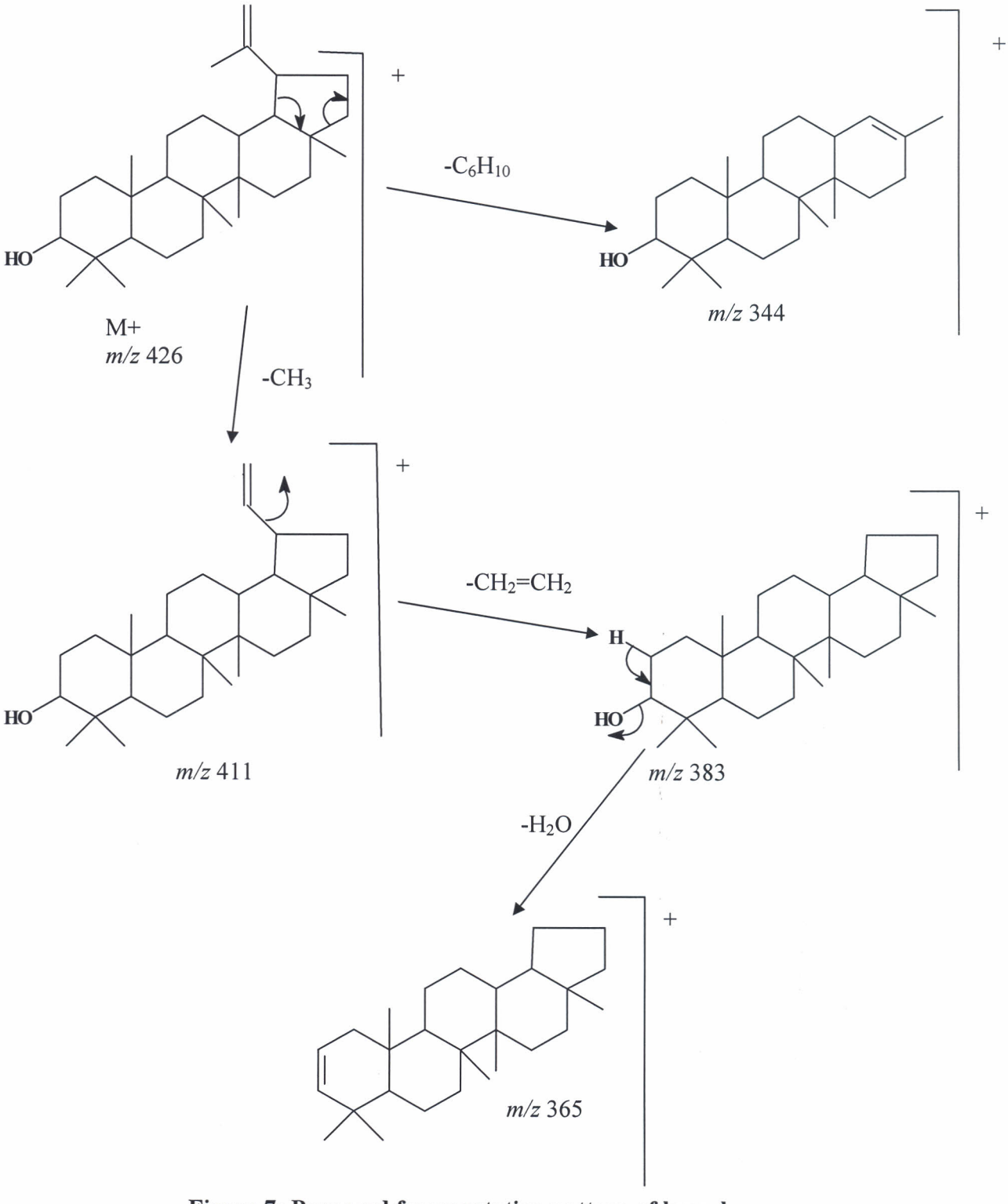


Figure 7: Proposed fragmentation pattern of lupeol.

3.2.2 Compound 2 (Melicopicine)

Compound **2** was isolated as yellow crystals. It showed a green fluorescent spot under UV light (366 nm). It produced a brown spot on exposure to iodine vapour and showed a single HPLC peak (Appendix 7).

Melting point: 128-130 °C.

UV λ_{max} (Acetonitrile) nm: 267 and 401 (Appendix 8).

Infra red (IR) ν_{max} (KBr) cm^{-1}: 3420, 3263 (O-H stretch of the enol form), 2995, 2940, 2868 and 2843 (C-H stretch), 1634 (C=O stretch of the cyclic α,β-unsaturated ketone), 1591 (N-H bend), 1122 (C-O stretch) and 980 (out of plane C-H bend) (Appendix 9).

^{1}H-NMR (200 MHz, $CDCl_3$) δ ppm: 8.31 (d, H-8), 7.36 (d, H-7), 7.65 (m, H-6), 7.15 (m, H-5), 4.09 (s, 3H, H-11), 3.98 (s, 3H, H-12), 3.96 (s), 3.93 (s, 3H, H-13), 3.09 (s), 3.88 (s, 3H, H-14) and 3.75 (s, 3H,H-15) (Appendix 10).

^{13}C-NMR (50 MHz, $CDCl_3$) δ ppm: 152.7 (C-1), 145.1 (C-2), 142.1 (C-3), 150.4 (C-4), 116.1 (C-4a), 115.8 (C-5), 121.5 (C-6), 127.2 (C-7), 133.3 (C-8), 139.1 (C-8a), 177.5 (C-9), 137.5 (C-9a), 124.4(C-10a), 62.2 (C-11), 62.0 (C-12), 61.9 (C-13), 61.7 (C-14) and 42.0 (C-15) (Appendix 11).

EI-MS (*m/z*): 329 (24.9%) molecular ion (M^+), 314 (100%), 299 (7%), 286 (6.9%), 284 (38.4%), 271 (37.4%) and 256 (75.1%) (Appendix 13).

UV absorption at λ_{max} 267 and 401 corresponds to an acridone chromophore (Kiplimo *et al.*, 2011).

The IR bands at 2995 cm^{-1} and 2940 cm^{-1} are due to aromatic C-H stretch (Kalsi, 2004). The IR band at 1634 cm^{-1} is due to C=O stretch of α,β-unsaturated ketone. The infra red bands at 2868 cm^{-1} and 2843 cm^{-1} are due to aliphatic C-H stretch indicating presence of an aliphatic system. This is supported by IR band at 980 due to out of plane C-H bend.

The ^{1}H-NMR spectrum showed five deshielded three proton singlets at δ_H 4.08, 3.98, 3.96, 3.93 and 3.88 indicating the presence of five methoxy and/or N-methyl groups. The corresponding ^{13}C-NMR signals appear at δ_C 62.2, 62.0, 61.9 and 61.7 for four of the methoxyl carbon atoms while the fifth ^{13}C-NMR signal at δ_C 42.0 corresponds to N-methyl group (Kalsi, 2004). Presence of additional ^{13}C-NMR signals at δ_C 152.7, 150.4, 145.1 and 142.1 are due to four aromatic carbon atoms, each attached to an electronegative atom, all of which correspond to the A-ring carbon atoms on the acridone skeleton. The DEPT spectrum shows four methine carbon atoms which are all aromatic due to their ^{13}C-NMR signals at δ_C 133.3, 127.2, 121.5 and 115.8 (Appendix 12). The corresponding ^{1}H-NMR signals appear at δ_H 7.15, 7.35, 7.57 and 8.30 as mutually coupled four aromatic protons which corresponds to unsubstituted C-ring protons of acridone alkaloid (Figure 8). The presence of the low intensity ^{13}C-NMR signals at δ_C 139.1, 137.5, 124.4 and 116.1 are due to four quaternary aromatic carbons. The ^{13}C-NMR signals at δ_C 177.5 correspond to a carbonyl carbon (C-9) (Kalsi, 2004; Robert *et al.*, 2005).

The mass spectrum shows a molecular ion at *m/z* 329 corresponding to the molecular structure of $C_{18}H_{19}O_5N$. Based on the above information, compound **2** is likely to be melicopicine. This is an acridone alkaloid whose molecular structure is shown in Figure 8. The identity was confirmed through comparison with literature data (Table 5).

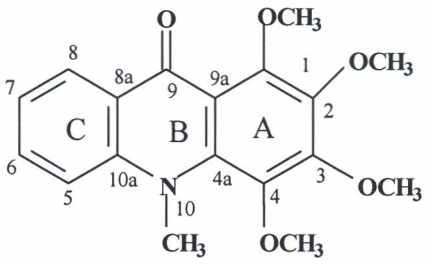


Figure 8: Molecular structure of melicopicine.

The probable MS fragmentation pattern of melicopicine is shown in Figure 9. The molecular ion at *m/z* 329 is formed by loss of one electron. It fragments by losing CH_3 group to form the base peak at *m/z* 314. This peak is most abundant (100%) because any of the four methoxy methyl groups and the N-methyl group can be lost. This fragment ion further fragments either by loss of a methyl group to form a fragment ion at *m/z* 299 or by loss of CO group to form a fragment ion at *m/z* 286. The fragment ion at *m/z* 299 fragments further by either losing a methyl group to form a fragment ion at *m/z* 284 or losing $N{=}CH_2$ group to form a fragment ion at *m/z* 271. The fragment ion at *m/z* 284 further breaks by losing CO group to produce a peak at *m/z* 256.

Table 5: Comparison of ^{13}C-NMR δ values for melicopicine (compound 2) and literature*

Carbon	Melicopicine measured δ values (ppm)	Melicopicine literature δ values (ppm)
1	152.7	152.5
2	145.2	144.9
3	142.1	141.8
4	150.4	150.1
4a	116.1	115.9
5	115.8	115.6
6	121.5	121.3
7	127.2	127.0
8	133.3	133.0
8a	139.1	138.8
9	177.5	177.5
9a	137.5	137.3
10a	124.4	124.9
11	62.2	62.0
15	42.0	41.7
12	62.0	61.6
13	61.9	61.7
14	61.7	61.4

*(Rasoanaivo *et al.*, 1999)

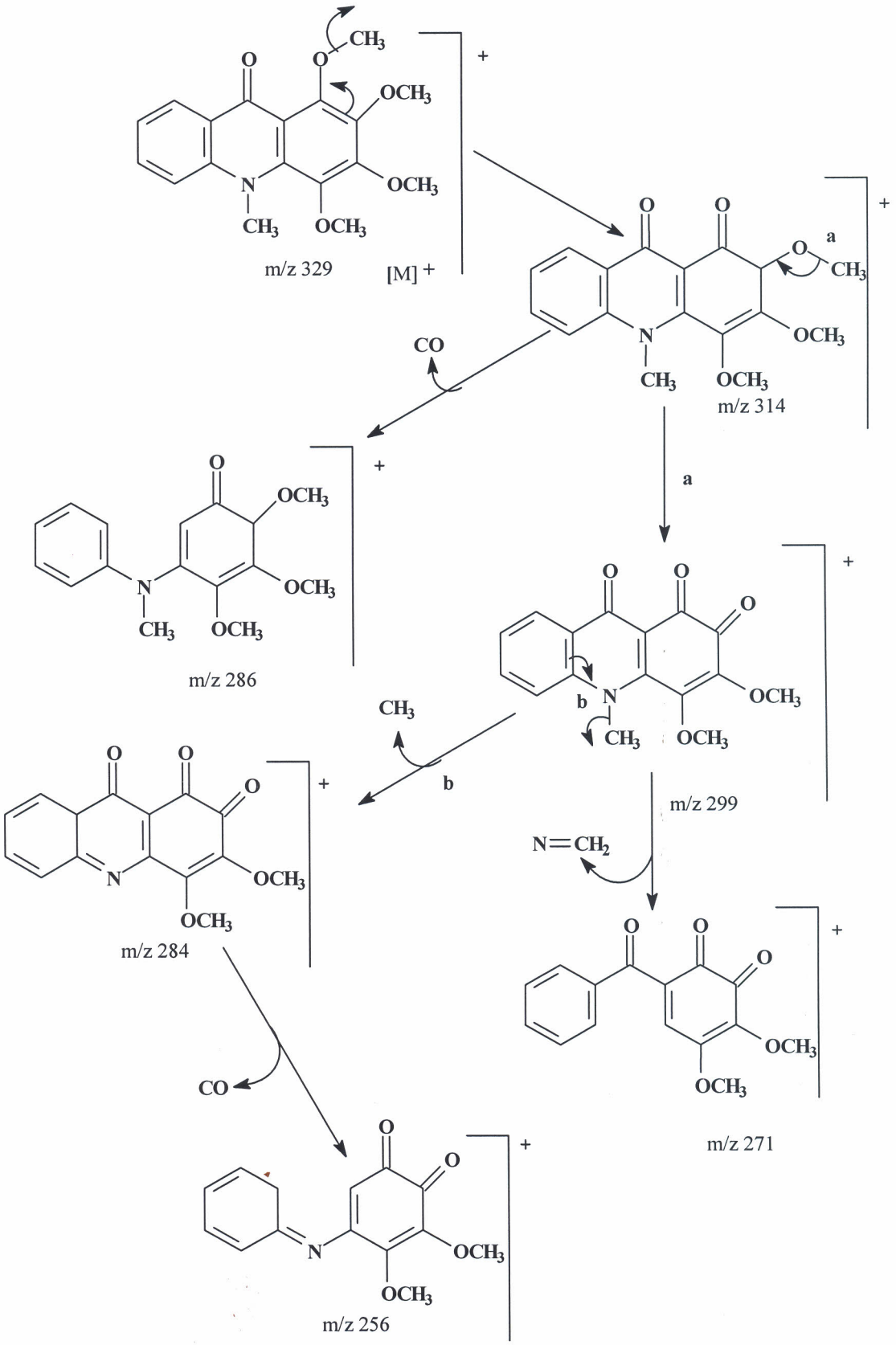


Figure 9: Proposed fragmentation pattern of melicopicine (compound 2).

3.2.3 Compound 3 (6-methoxytecleanthine)

Compound **3** (6-methoxytecleanthine) was isolated as green feathery crystals. It showed a blue fluorescent spot under both short and long UV (254 nm and 366 nm) light. It could not be visualized on exposure to iodine vapour, but showed a single HPLC solute peak and a small noise peak (Appendix 14).

Melting point: 163-165 °C

UV λ_{max} (acetonitrile) nm: 272 and 391 nm (Appendix 15).

Infra red (IR) (KBr) cm^{-1}: 3451 (O-H stretch of the enol form), 2943 (aromatic C-H stretch), 2851, 2628 (aliphatic C-H stretch), 1636 (C=O stretch of the cyclic α,β-unsaturated ketone), 1601 (C=C stretch), 1231 (C-O stretch) and 1060 (out of plane C-H bend) (Appendix 16).

^{1}H-NMR (200 MHz, $CDCl_3$) δ ppm: 8.18 (d, 1 H, H-8), 6.90 (d, 1H, H-7), 6.64 (d, 1H, H-4), 6.01(s, 2H, H-11), 4.13 (s, 3H, H-12), 3.98 (s, 3H, H-13), 3.89 (s, 3H, H-14) and 3.78 (s, 3H, H-15) (Appendix 17).

^{13}C-NMR (50 MHz, $CDCl_3$) δ ppm: 156.7 (C-1), 137.0 (C-2), 138.3 (C-3), 91.1 (C-4), 121.5 (C-4a), 145.4 (C-5), 153.6 (C-6), 107.6 (C-7), 112.3 (C-8), 143.0 (C-8a), 177.0 (C-9), 123.6 (C-9a), 132.9 (C-10a), 101.9 (C-11), 61.3 (C-12), 41.9 (C-13), 56.5 (C-14) and 61.2 (C-15) (Appendix 18).

MS (*m/z*): 343 100% (molecular ion), 328 (41.7%), 315 (77.2 %), 300 (84%), 298 (77.6%), 283 (68.3%), 269 (57.3%) and 255 (47%) (Appendix 19).

The UV λ_{max} at 272 and 391 nm suggested an acridone chromophore (Kiplimo *et al.*, 2011). The infra red band at 2943 cm^{-1} is due to =C-H stretch of unsaturated system. Infra red band at 1636

cm^{-1} is attributed to C=O stretch of a α,β-unsaturated ketone. The infra red bands at 2851 cm^{-1} and 2628 are due to C-H stretch of an aliphatic system. This is supported by infra red band at 1060 due to out of plane C-H bend.

The signals in the ^{1}H-NMR at δ_H 4.13, 3.98, 3.89 and 3.78 (all singlets, deshielded and with integration of 3 protons) are due to methoxyl and/or N-methyl groups. The ^{1}H-NMR signals at δ_H 8.18 (d, 1 H), 6.90 (d, 1 H), and 6.64 are due to aromatic protons.

The ^{13}C-NMR which showed signals at δ_C 61.3, 61.2 and 56.5 supported the presence of methoxy groups. The ^{13}C-NMR signal at δ_C 41.8 is due to N-methyl group (Kalsi, 2004). The presence of ^{13}C-NMR signals at δ_C 156.7, 153.6, 145.4 and 143.0 are due to four deshielded aromatic carbon atoms each attached to an electronegative atom. The ^{13}C-NMR signals at δ_C 123.6, 107.6 and 91.1 are due to three aromatic methine carbon atoms (C-4, C-7 and C-8). The low intensity ^{13}C-NMR signals at δ_C 138.3, 137.0, 132.9 and 121.5 are due to four quaternary aromatic carbons (C-4a, C-8a, C-9a and C-10a). The ^{13}C-NMR signal at δ_C 177.0 is due to a carbonyl carbon (C-9) (Kalsi, 2004). The ^{1}H-NMR singlet at δ_H 6.64 was assigned to H-4 of ring A which is substituted with methylene dioxy (δ_H 6.01; δ_C 101.8) at C-2/C-3 and a methoxyl at C-1. The two *ortho* coupled doublets (1 H) at δ_H 8.17and 6.90 were assigned to H-8 and H-7 respectively with C-5 and C-6 being substituted with methoxyl groups (Robert *et al.*, 2005).

The mass spectrum shows a molecular ion at *m/z* 343. This corresponds to the atomic structure of $C_{18}H_{17}O_6N$. Based on the above information, compound **3** is likely to be 6-methoxytecleanthine. This is an acridone alkaloid whose structure is shown in Figure 10. The identity of the compound was confirmed through comparison of ^{13}C-NMR data with literature (Table 6).

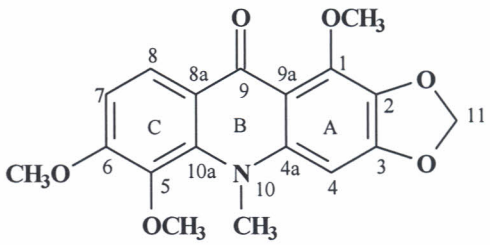


Figure 10: Molecular structure of 6-methoxytecleanthine.

Table 6: Comparison of ^{13}C-NMR δ values for 6-methoxytecleanthine and literature*

Carbon	6-methoxytecleanthine measured δ values	6-methoxytecleanthine literature δ values
1	156.7	156.4
2	137.0	136.9
3	138.3	138.1
4	91.1	90.8
4a	121.5	121.3
5	145.4	145.1
6	153.6	153.3
7	107.6	107.4
8	112.2	112.1
8a	143.0	142.8
9	177.0	176.7
9a	123.6	123.3
10a	132.9	132.7
11	101.9	101.6
12	61.3	61.0
13	41.8	41.5
14	56.5	56.2
15	61.2	60.9

(Rasoanaivo *et al.*, 1999)

The proposed fragmentation pattern of 6-methoxytecleanthine is illustrated in Figure 11. Compound 3 loses one electron to form the molecular ion/base peak at *m/z* 343. This fragments by loss of CO group to form a fragment ion of *m/z* 315. This fragment ion further breaks by either losing a methyl group to form a fragment ion of *m/z* 300 or by losing CH_2O_2 group to form a fragment ion of *m/z* 269. The molecular ion also fragments by consecutively losing methyl groups to form fragment ions of *m/z* 328, 313, 298, 283 and 255.

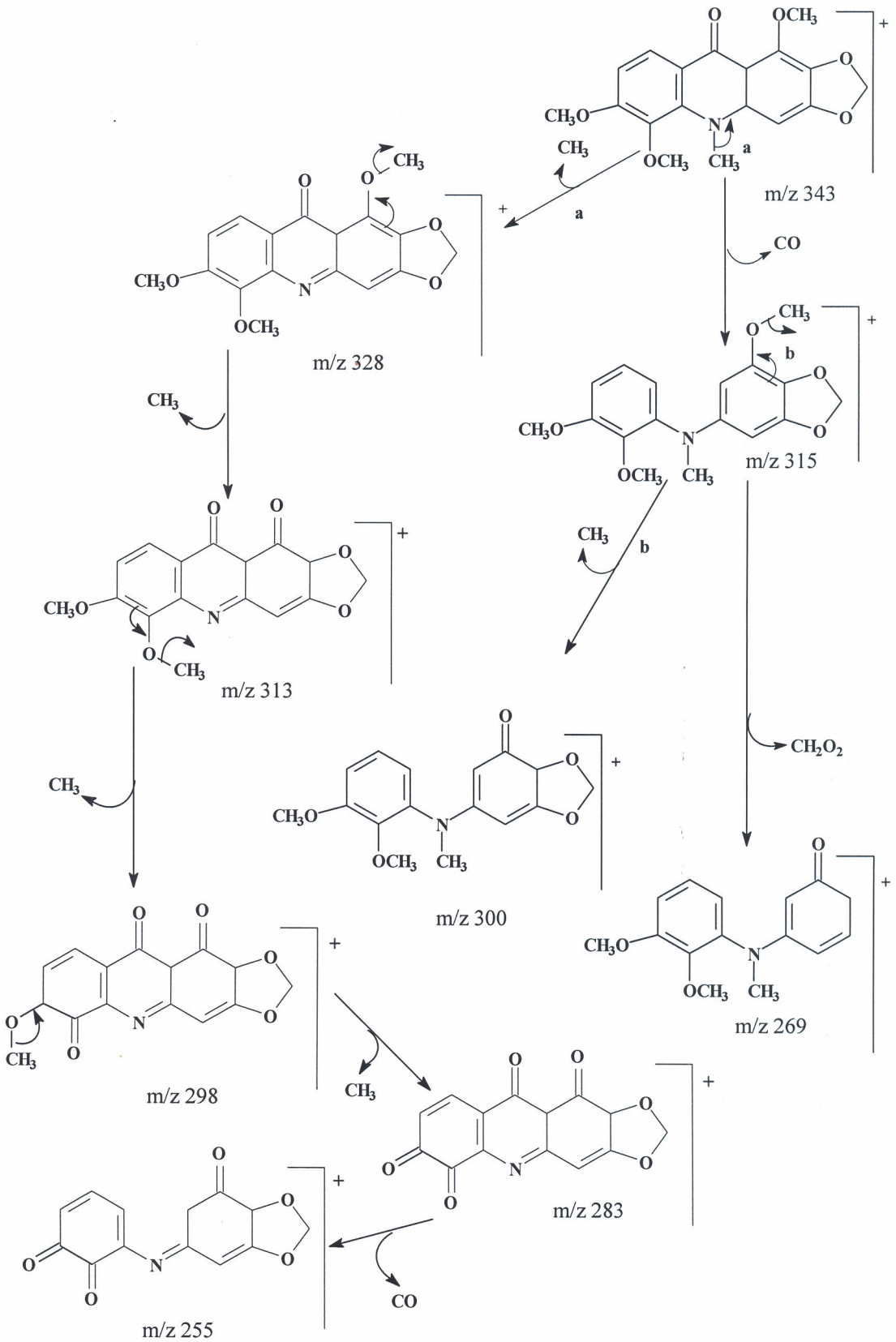


Figure 11: Proposed fragmentation pattern of 6-methoxytecleanthine (compound 3).

Lupeol has been isolated from this plant for the first time. It's a compound that occurs in many plants and has been isolated before from other *Teclea* species (Kiplimo *et al.*, 2011). Acridone alkaloids isolated in this current study are coloured which concurs with literature information that acridone alkaloids are all coloured. The colour is due to high conjugation of these compounds making them absorb light in the visible region of the electromagnetic spectrum (Mwangi, 2007).

3.2.4 Anthelmintic and toxicity study

Two tests, egg hatch assay (EHA) and larval development assay (LDA), were carried out to determine the anthelmintic activities of the crude extract and pure isolates. The sheep nematode (strongyles) was the test organism. Dimethylsulfoxide was the negative control. The toxicity of the crude extract was investigated using brine shrimp lethality test (BSLT).

3.2.4.1 Egg hatch assay

The number of hatched larvae and the number of eggs remaining in each well was counted and the percentage inhibition of hatching at each concentration determined (Appendix 20). Three determinations were done for each isolate, crude extract and DMSO.

*The percentage inhibition was corrected for normal mortality using the Schneider-Orelli's (1947) formula shown below.

$$A\% = \frac{E}{E + L} \times 100$$

Where A is percentage inhibition; E is number of eggs; L is the number of larvae.

The concentration at 50% inhibition (IC_{50}) for melicopicine, 6-methoxytecleanthine and crude extract was found to be 509.28 µg/ml, 352.29 µg/ml and 185.25 µg/ml respectively. This

indicates that the anthelmintic activity of *Teclea trichocarpa* root bark of inhibition of strongyloides egg hatching could be due to melicopicine and 6-methoxytecleanthine. Compound **1** and the negative control (DMSO) did not have any activity at the test concentration range.

The crude extract, melicopicine and 6-methoxytecleanthine show a dose dependent toxicity towards the sheep nematode eggs. From the IC_{50} above, the crude extract is more active in inhibiting the strongyles eggs compared to individual isolates. This could be due to synergistic action of compounds present in the crude extract and further tests could be done to ascertain this. Melicopicine and 6-methoxytecleanthine have been tested previously for antimalarial and antibacterial activities (Mwangi, 2007) but no test has been done to determine their activity in inhibiting the hatching of strongyles eggs. Lupeol exhibited no activity against hatching of the strongyless eggs. Similar observation was seen with DMSO which was also inactive as expected.

3.2.4.2 Larval development assay

The number of larvae that did not develop was counted and the percent inhibition of development at each concentration determined (Appendix 20). Three determinations were done for each isolate, crude extract and DMSO. The IC_{50} values for the three isolates were above 1000 µg/ml which therefore means that the three compounds and the crude extract were inactive against inhibition of the development of the strongyloides larvae. There is no evidence in literature of larval development assay done on the pure isolates and the crude extract of *Teclea trichocarpa* root bark.

3.2.4.3 Brine shrimp lethality assay

From brine shrimp lethality test done on *Teclea trichocarpa* root bark crude extract, the lethal dose at 50% (LD_{50}) was found to be 41.64 µg/ml (Appendix 20). The brine shrimp lethality

assay represents a rapid yet inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates with its cytotoxic and anti tumor properties. According to the criteria of the American National Cancer Institute, the LD_{50} limit to consider a crude extract promising for further purification is lower than 30 μg/ml (McLauglin *et al*, 1993). There is no evidence of any previous study done to determine the toxicity of *Teclea trichocarpa* root bark crude extract.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

4.1 Conclusion

In the study on phytochemistry of the root bark *Teclea trichocarpa*, three compounds were isolated from the dichloromethane: methanol (1:1) extract. These were characterized as lupeol, melicopicine and 6-methoxytecleanthine. Lupeol, a triterpene, was isolated in this plant for the first time while melicopicine and 6-methoxytecleanthine have been previously isolated. Lupeol occurs regularly in many plants and has been isolated before in *Teclea nobilis* (Kiplimo *et al.*, 2011). Two of the compounds are the acridone alkaloids melicopicine and 6-methoxytecleanthine which have been previously isolated from other parts of this plant.

Lupeol did not posses anthelmintic activity. Melicopicine and 6-methoxytecleanthine produced a weak anthelmintic activity by inhibiting the hatching of the strongyloides eggs. All the isolates and the crude extract of the root bark of *Teclea trichocarpa* exhibited no activity against the development of the larvae of sheep nematode (strongyloides). This supports the ethno-medical use of the root bark of *Teclea trichocarpa* as an anthelmintic. The IC_{50} values for the brine shrimp lethality testing done on the crude extract was found to be 41.64 µg/ml.

4.2 Recommendations

Further work should be done to identify more active compounds from the plant. Further work can be done by investigating other biological activities on the crude extract and the pure isolates. This includes study of antibacterial, antimalarial, antipyretic and central nervous system activities. Lupeol is reported to possess antitumor activity. Further work should be done

investigate this. I recommend that anthelmintic study be done to test effects on cestodes and trematodes.

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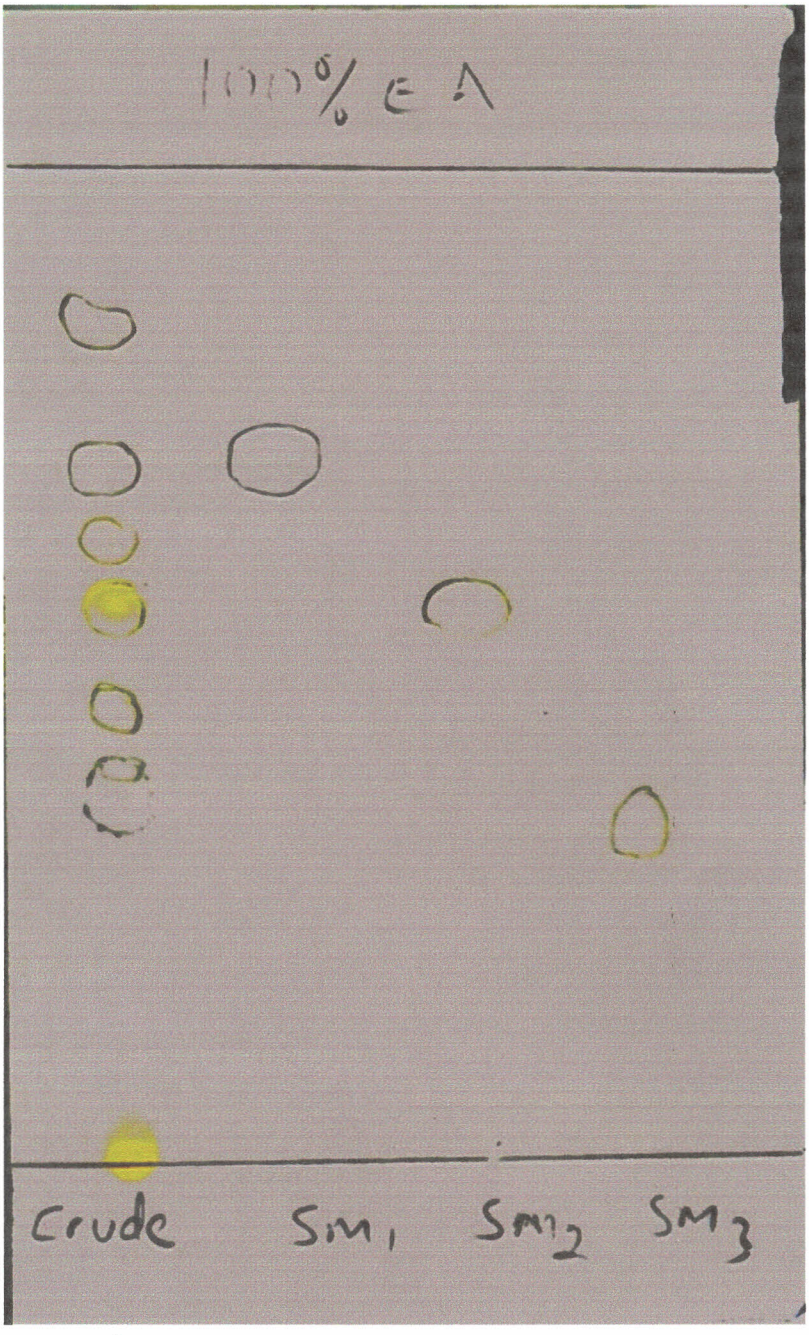
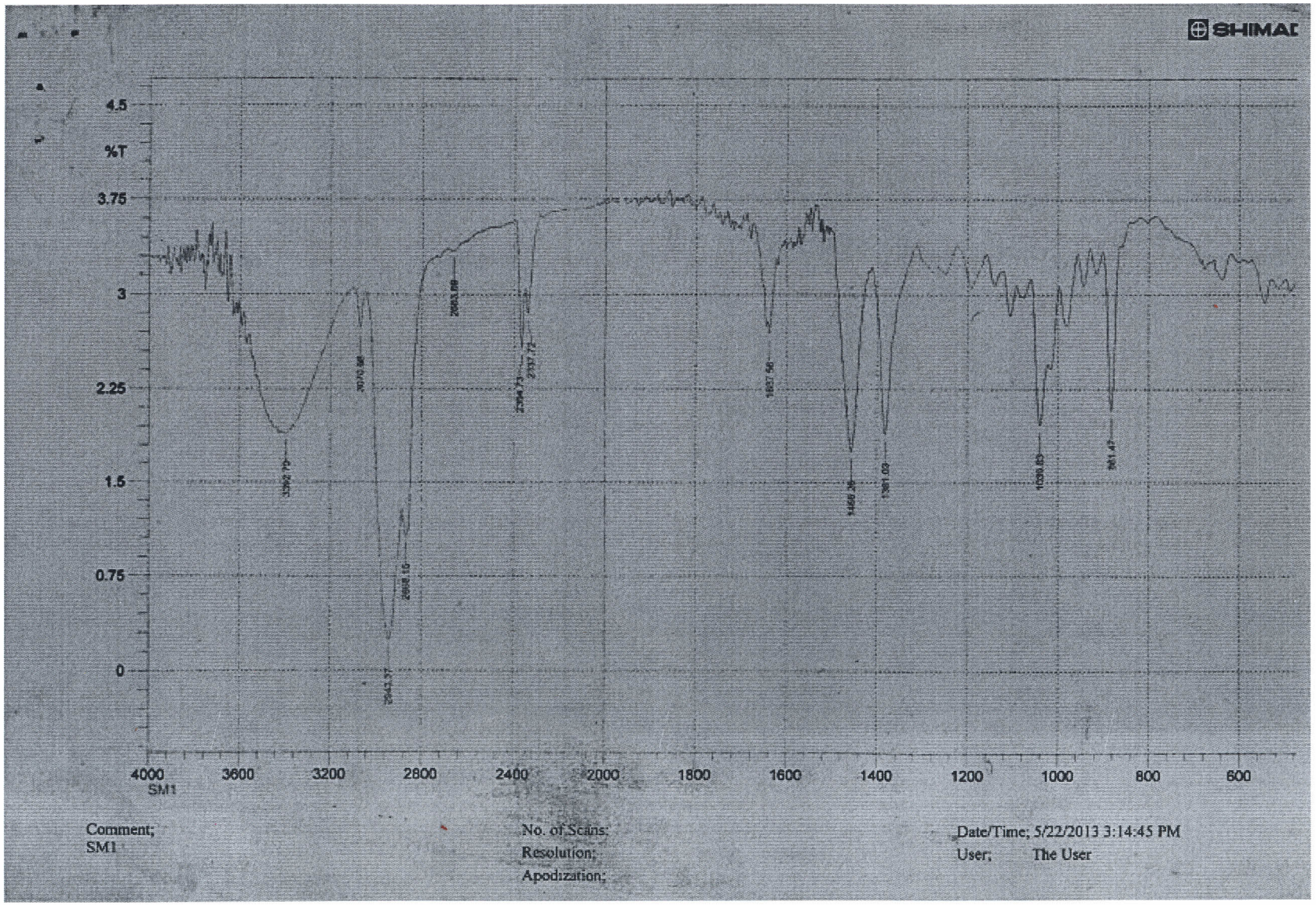
APPENDICES

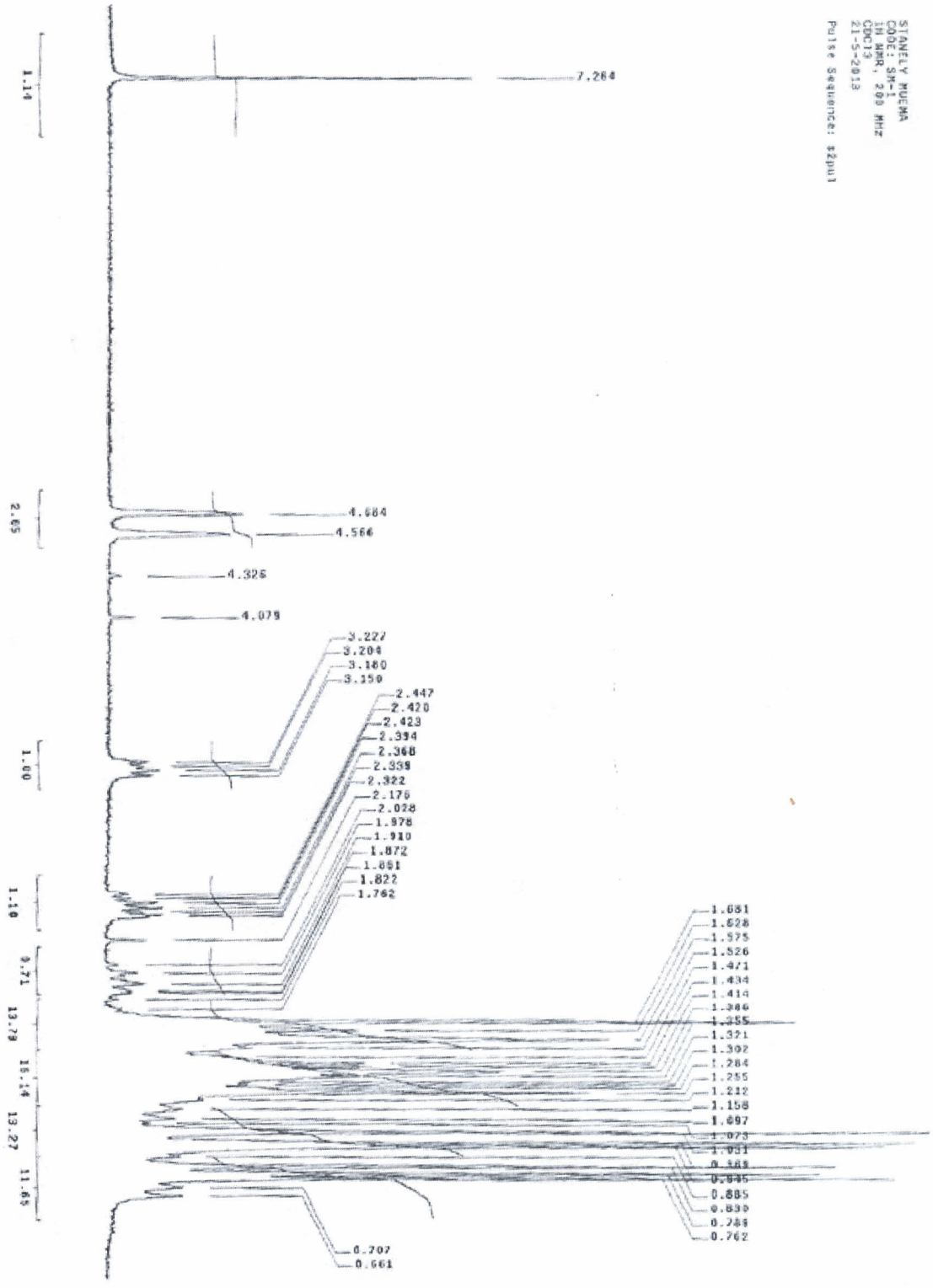
Appendix 1: TLC profiles of *Teclea trichocarpa* root bark extract, compounds 1, 2 and 3*



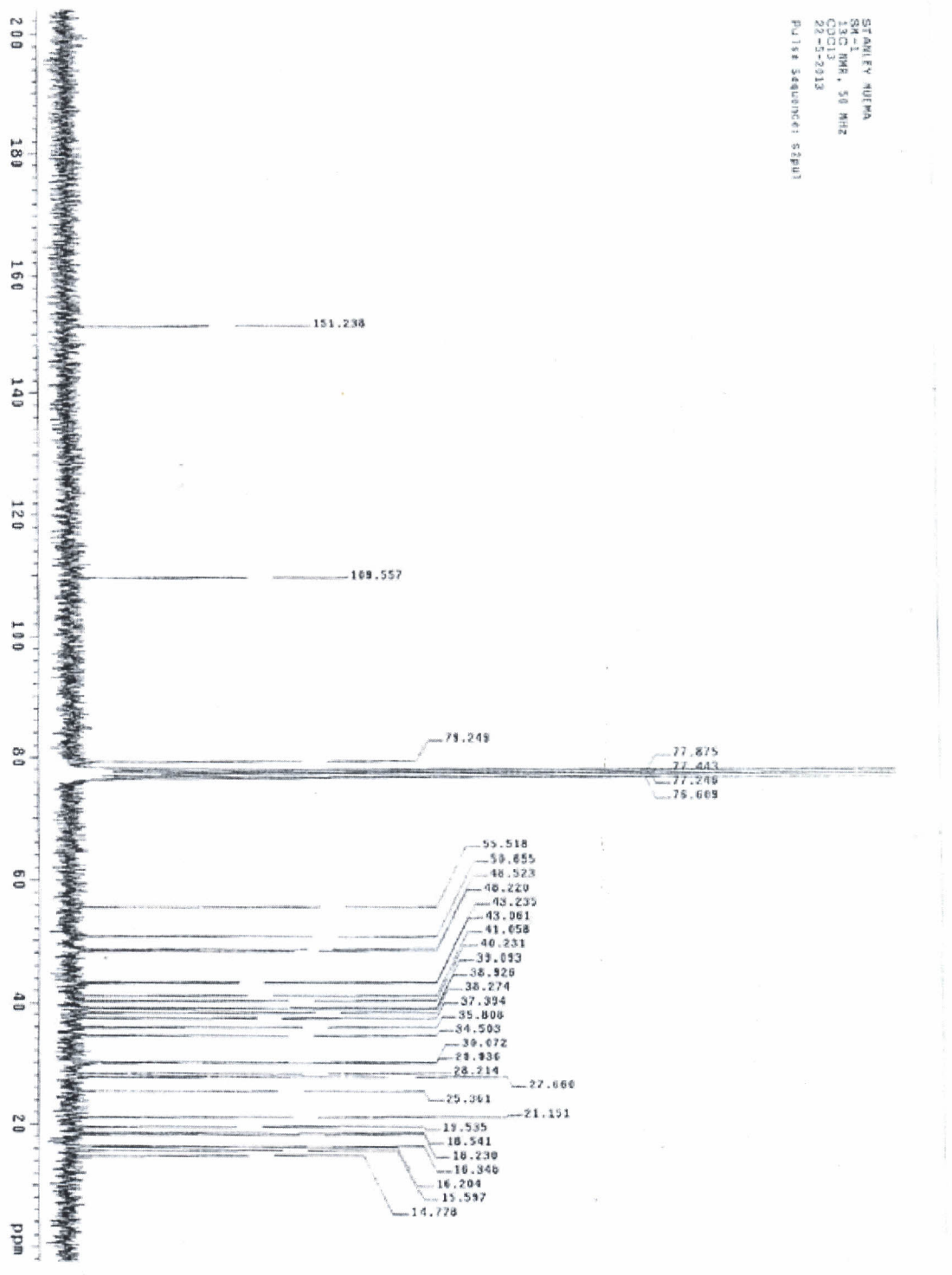
*solvent system is ethylacetate


SHIMADZU
%T
4.5
3.75
3
2.25
1.5
0.75
0
4000
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
SM1
Comment;
SM1
No. of Scans;
Resolution;
Apodization;
Date/Time; 5/22/2013 3:14:45 PM
User; The User

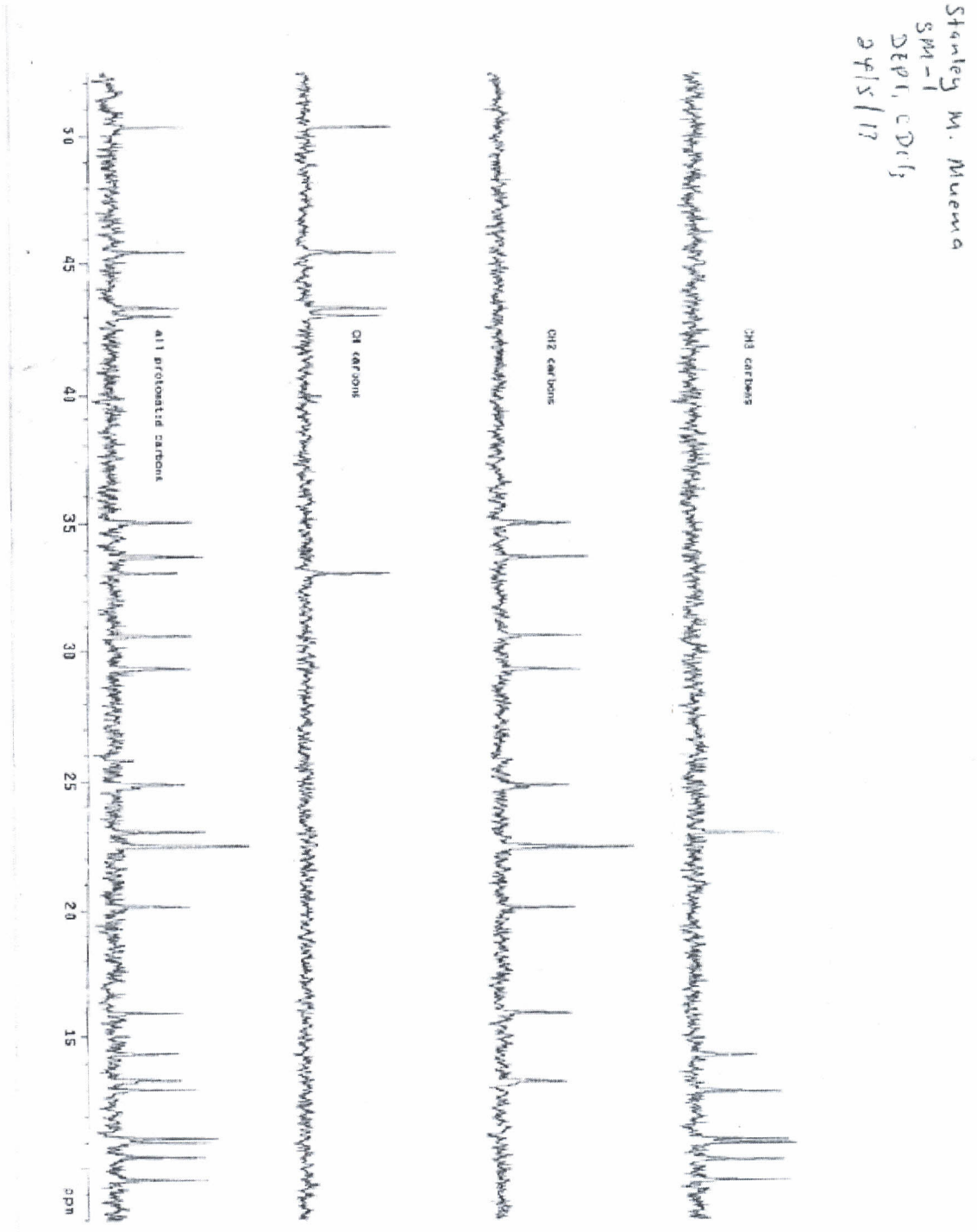
Appendix 3: ^{1}H-NMR of lupeol



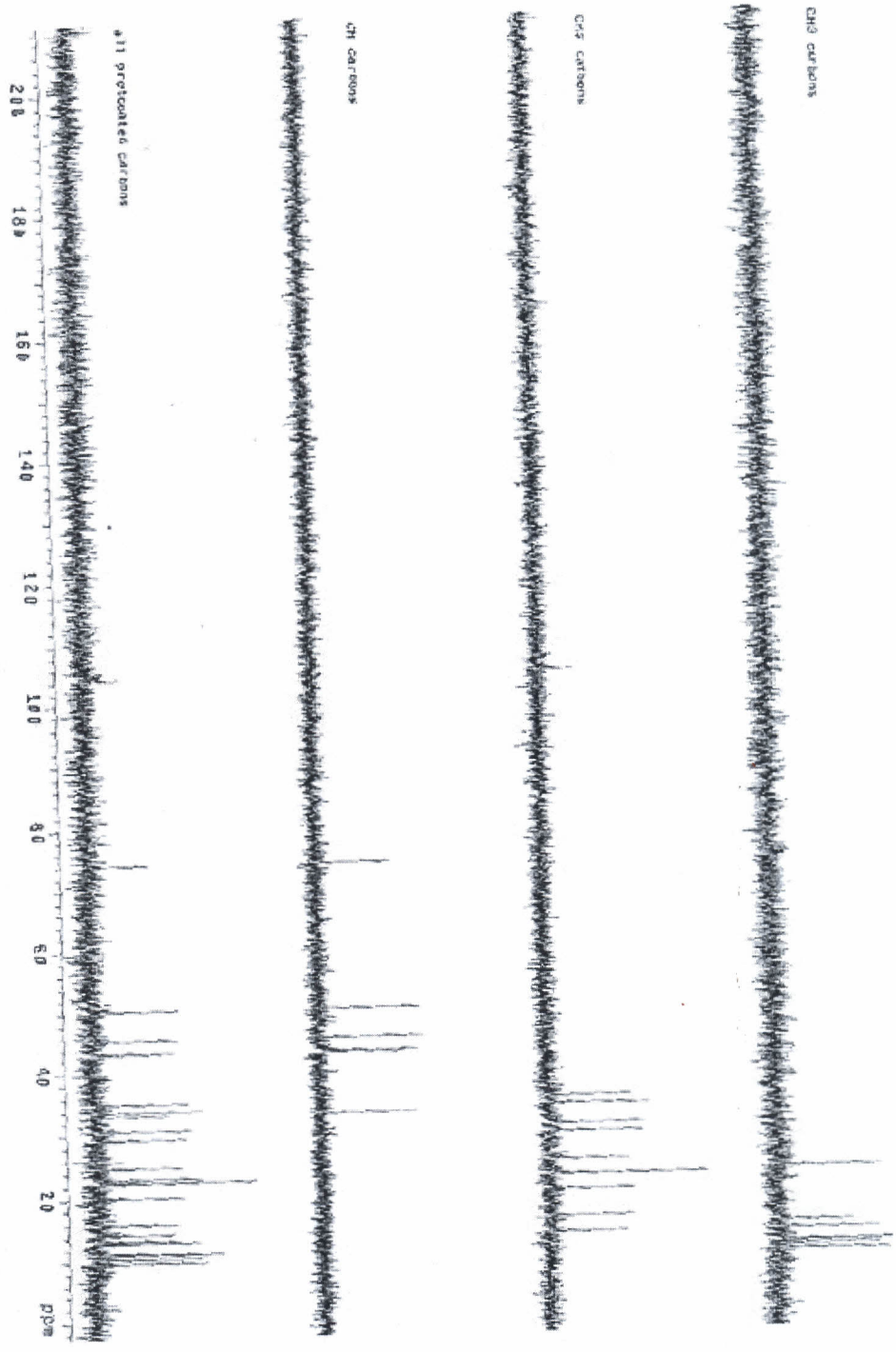
Appendix 4: ^{13}C-NMR spectrum of lupeol



Appendix 5 (a): DEPT spectrum of lupeol



Appendix 5 (b): DEPT spectrum of lupeol

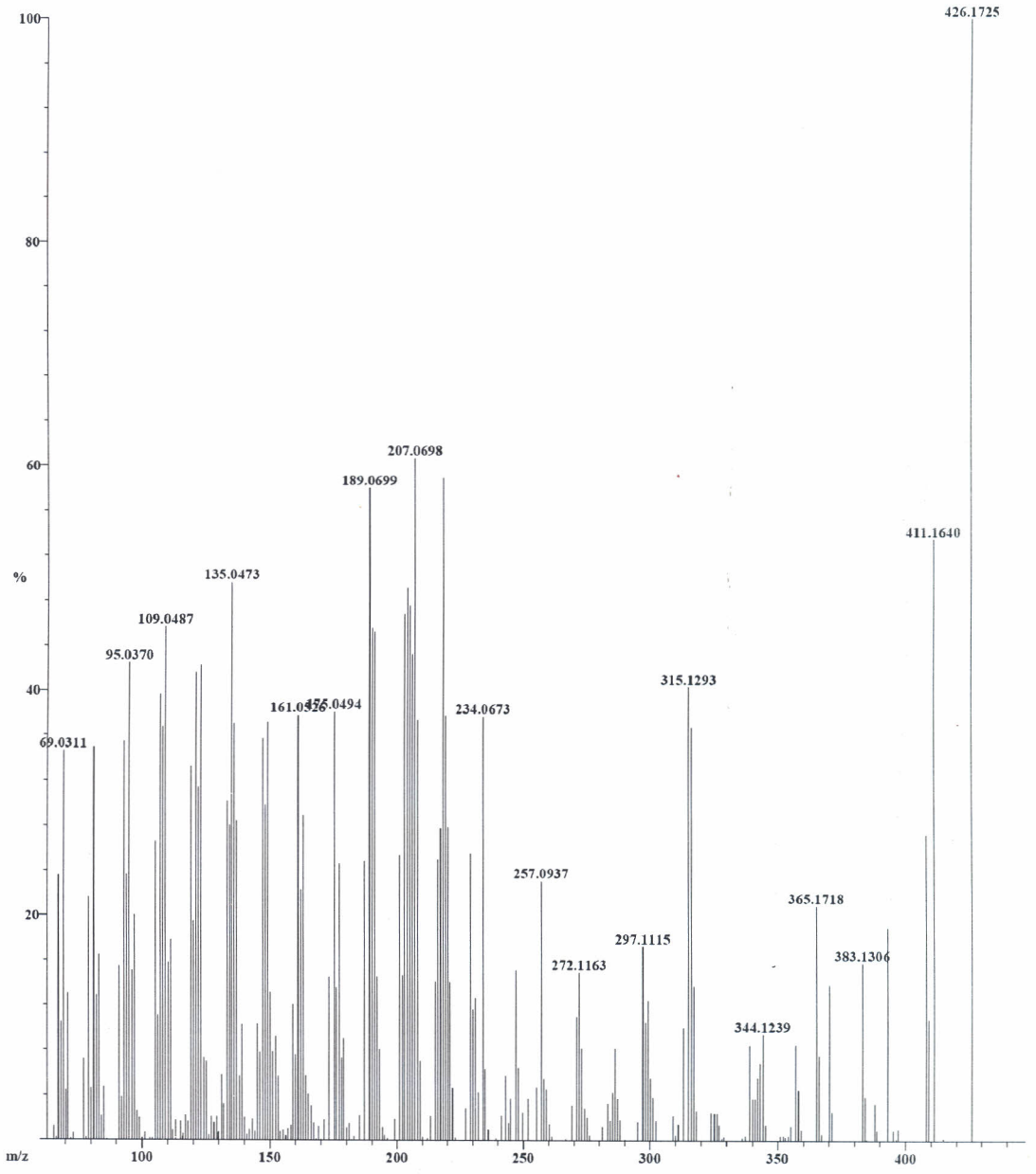


Appendix 6: MS spectrum of lupeol

Univ CapeTown
6/21/2013 Page 1

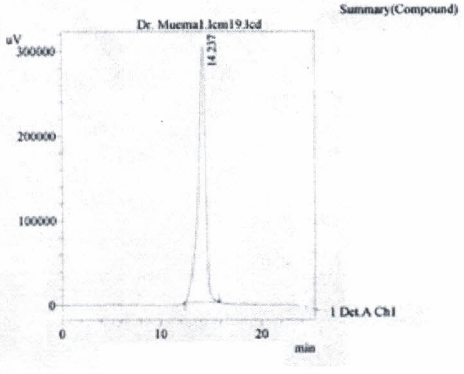
File: 13F21-SM1 **Date Run: 06-21-2013 (Time Run: 12:19:22)**
Sample:
Instrument: JEOL GCmateII
Inlet: Direct Probe **Ionization mode: EI+**

Scan: 52 **R.T.: 1.03**
Base: m/z 426; 30.1%FS TIC: 9004522 **#Ions: 247**

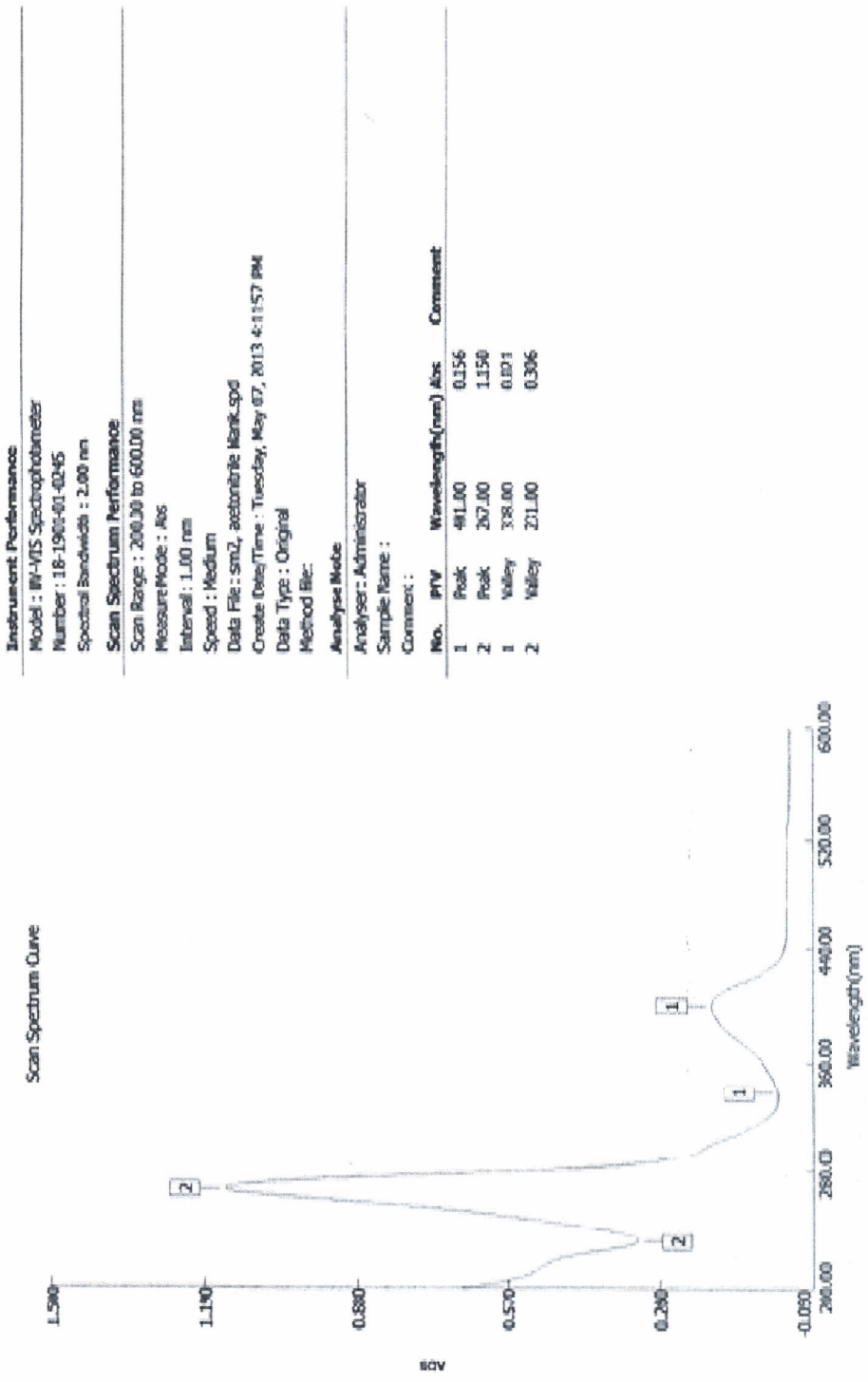


Appendix 7: HPLC chromatogram of melicopicine

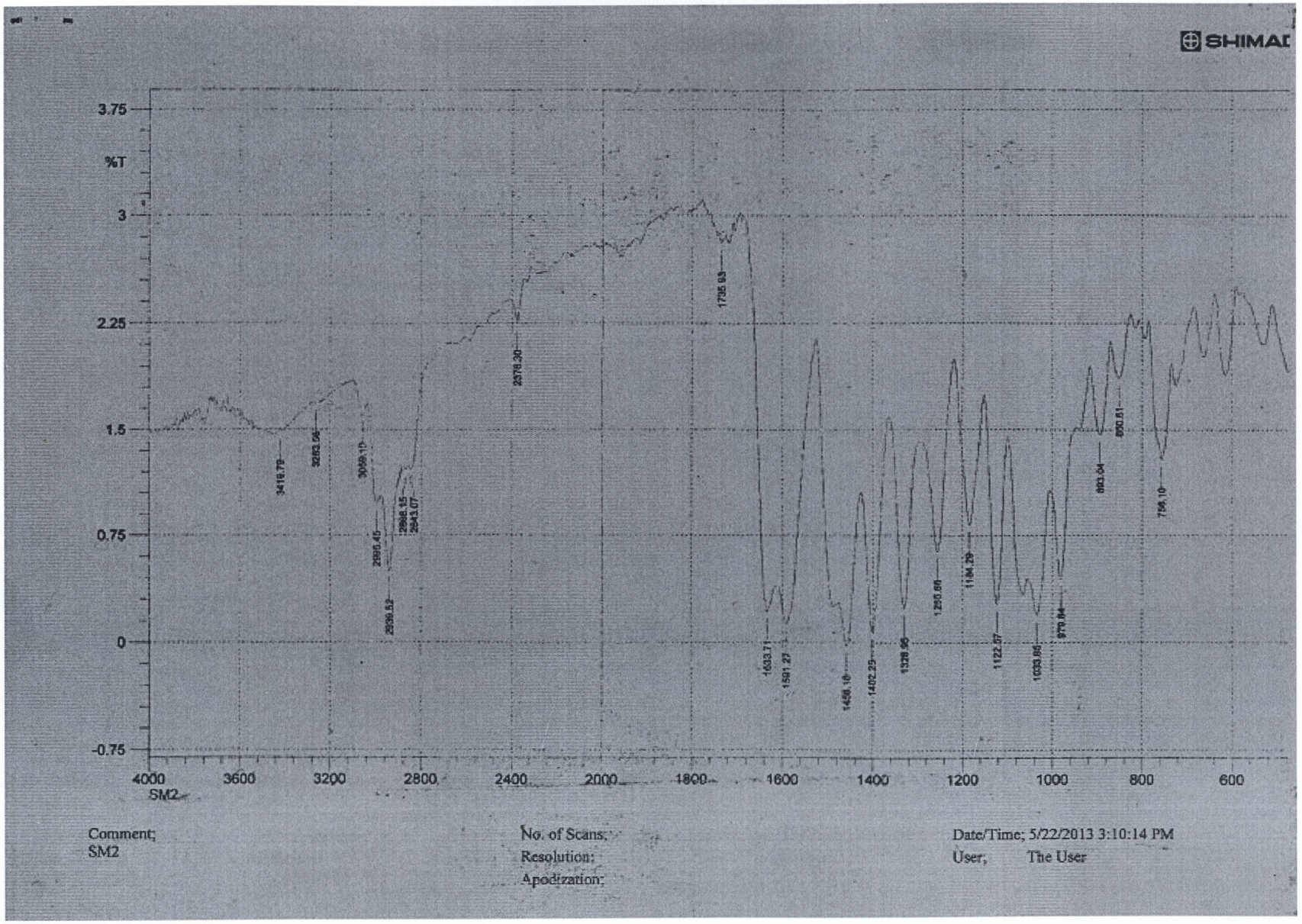
HPLC CHROMATOGRAM FOR SM2 USING HYPERSIL ODS2 5 MICROMETER 4.6MM X 250MM COLUMN AND ACETONITRILE: WATER (30:70) MOBILE PHASE AT 40 DEGREES CELCIUS AND 270NM, FLOW RATE: 1.3ML/MIN, OPERATED BY MR. NGUYO, ON 16TH TO 17TH MAY 2013



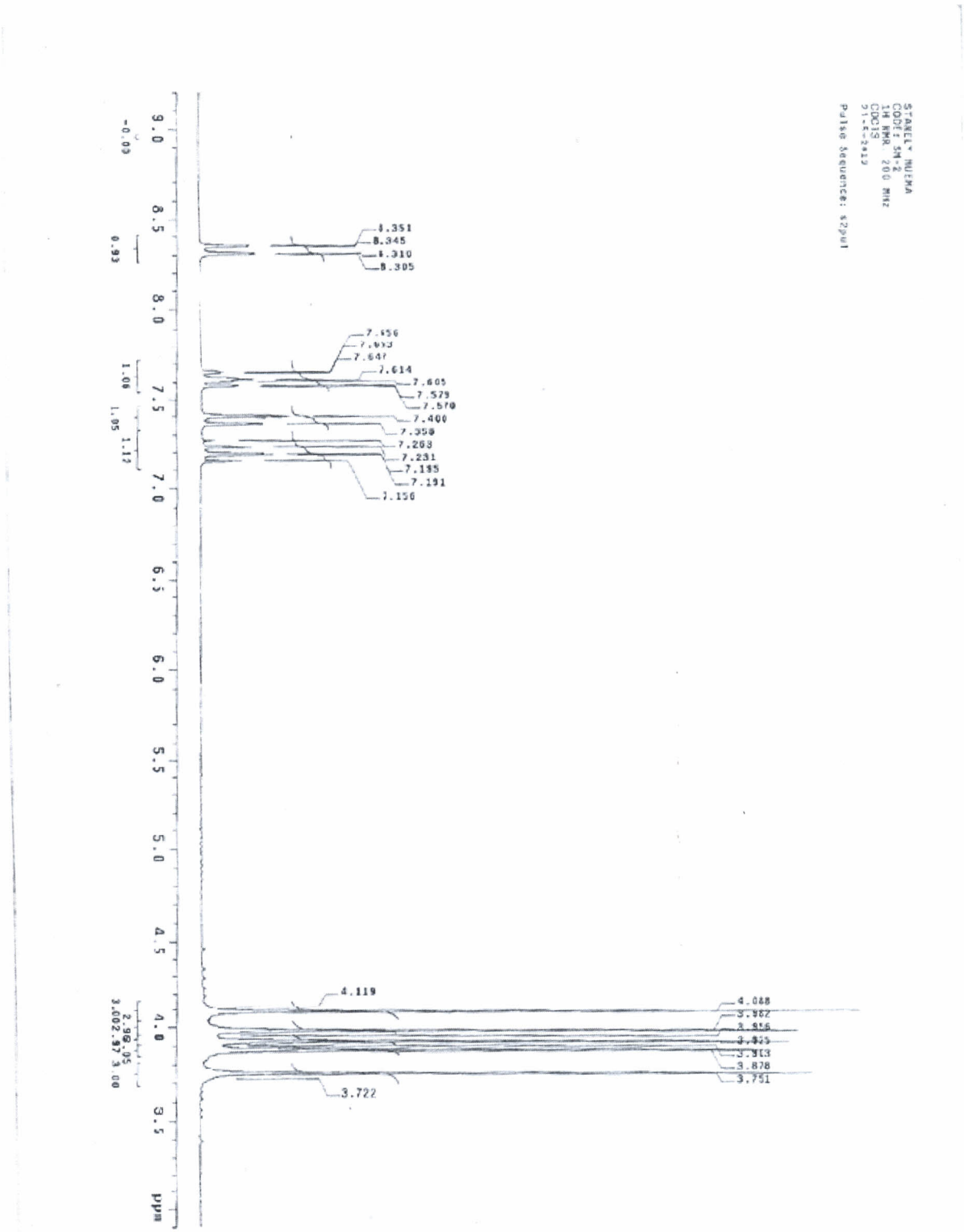
Appendix 8: UV spectrum of melicopicine



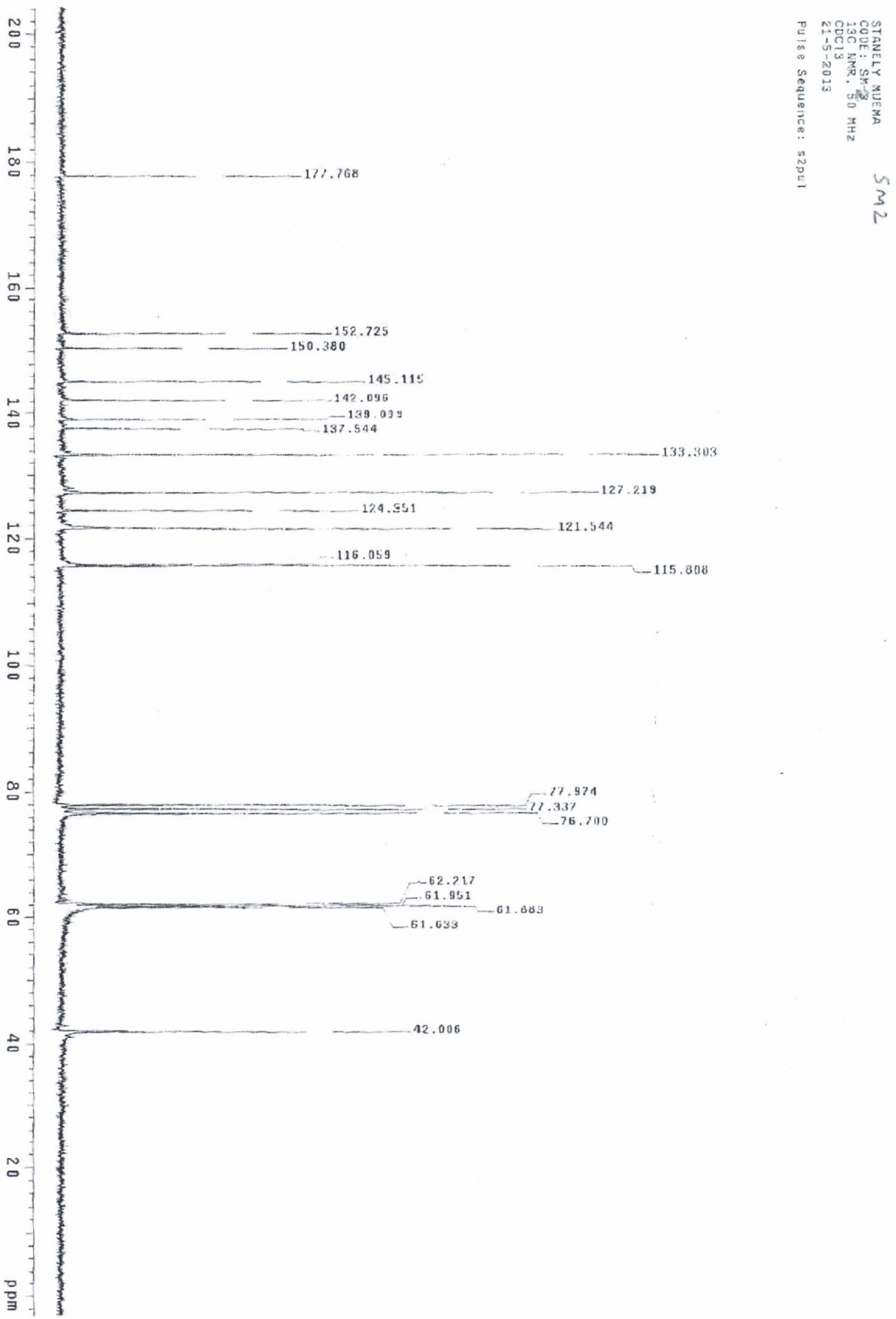
Appendix 9: FTIR spectrum of melicopicine



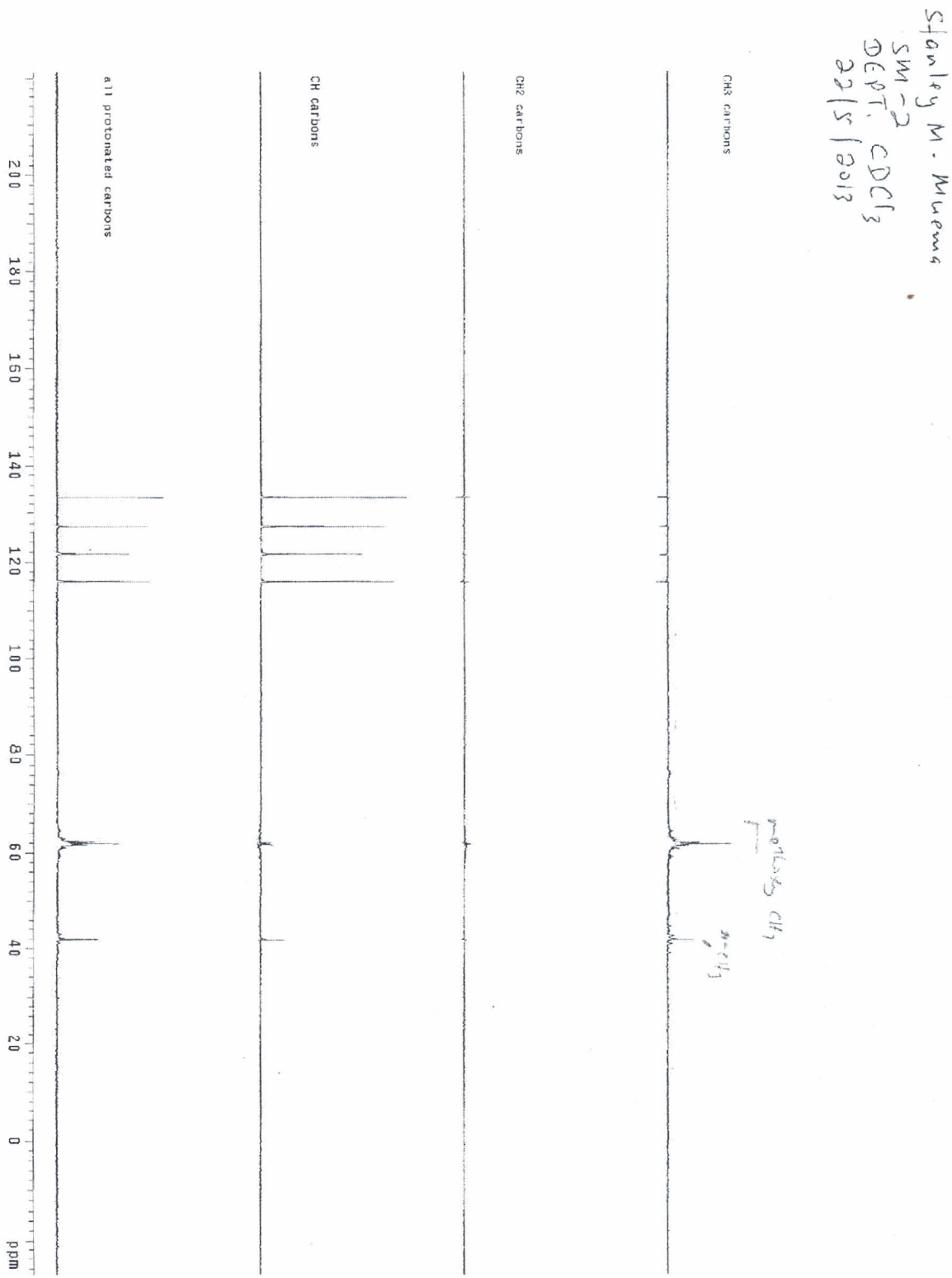
Appendix 10: ^{1}H-NMR spectrum of melicopicine



Appendix 11: ^{13}C-NMR spectrum of melicopicine



Appendix 12: DEPT spectrum of melicopicine

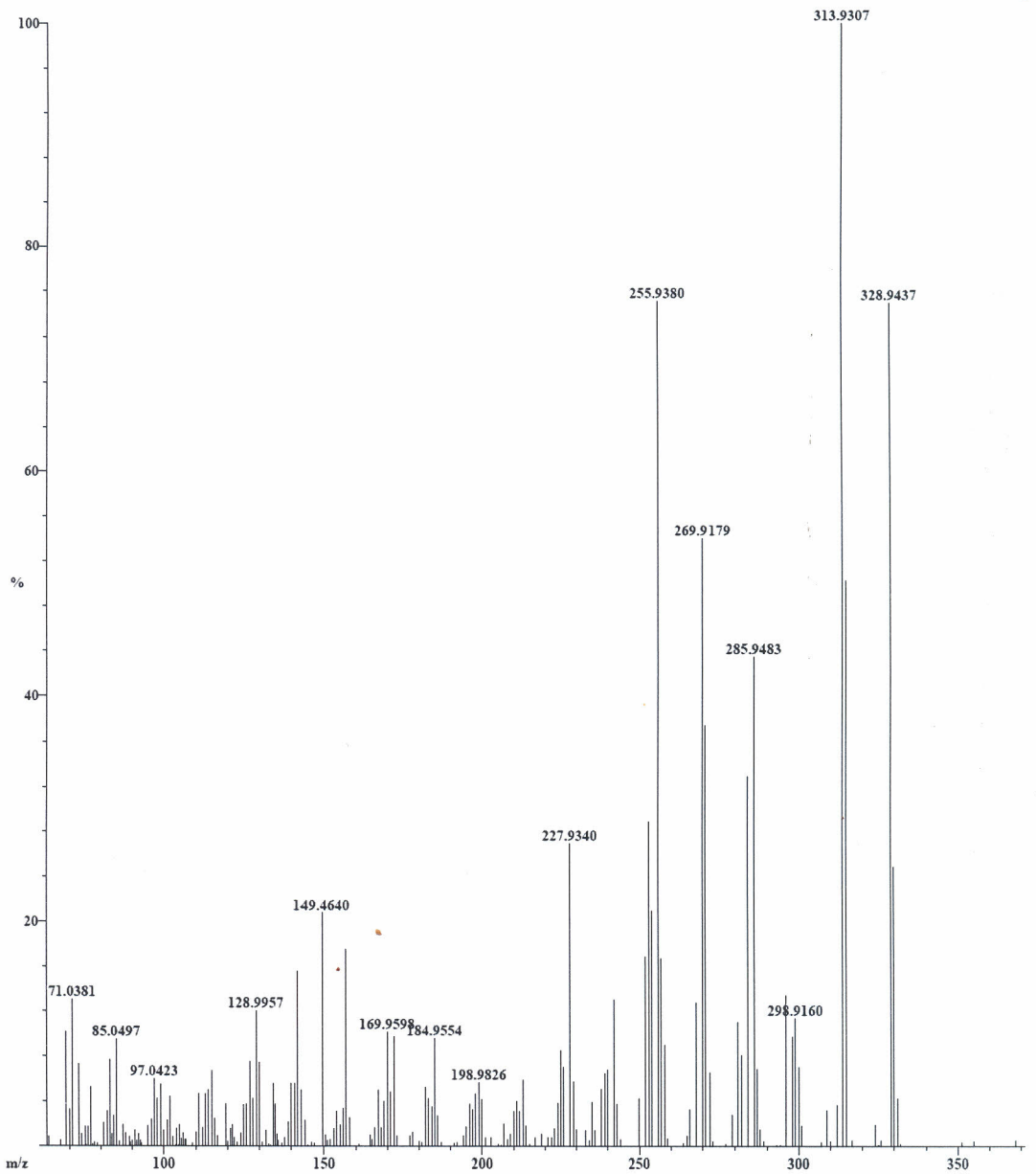


Appendix 13: MS spectrum of melicopicine

Univ CapeTown
6/21/2013 **Page 1**

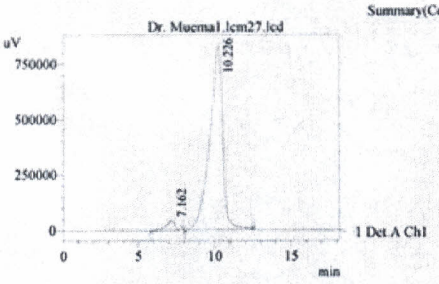
File: 13F21-SM2 **Date Run: 06-21-2013 (Time Run: 12:28:02)**
Sample:
Instrument: JEOL GCmateII
Inlet: Direct Probe **Ionization mode: EI+**

Scan: 48 **R.T.: .95**
Base: m/z 314; 25.3%FS TIC: 3237732 **#Ions: 230**



Appendix 14: HPLC spectrum of 6-methoxytecleanthine

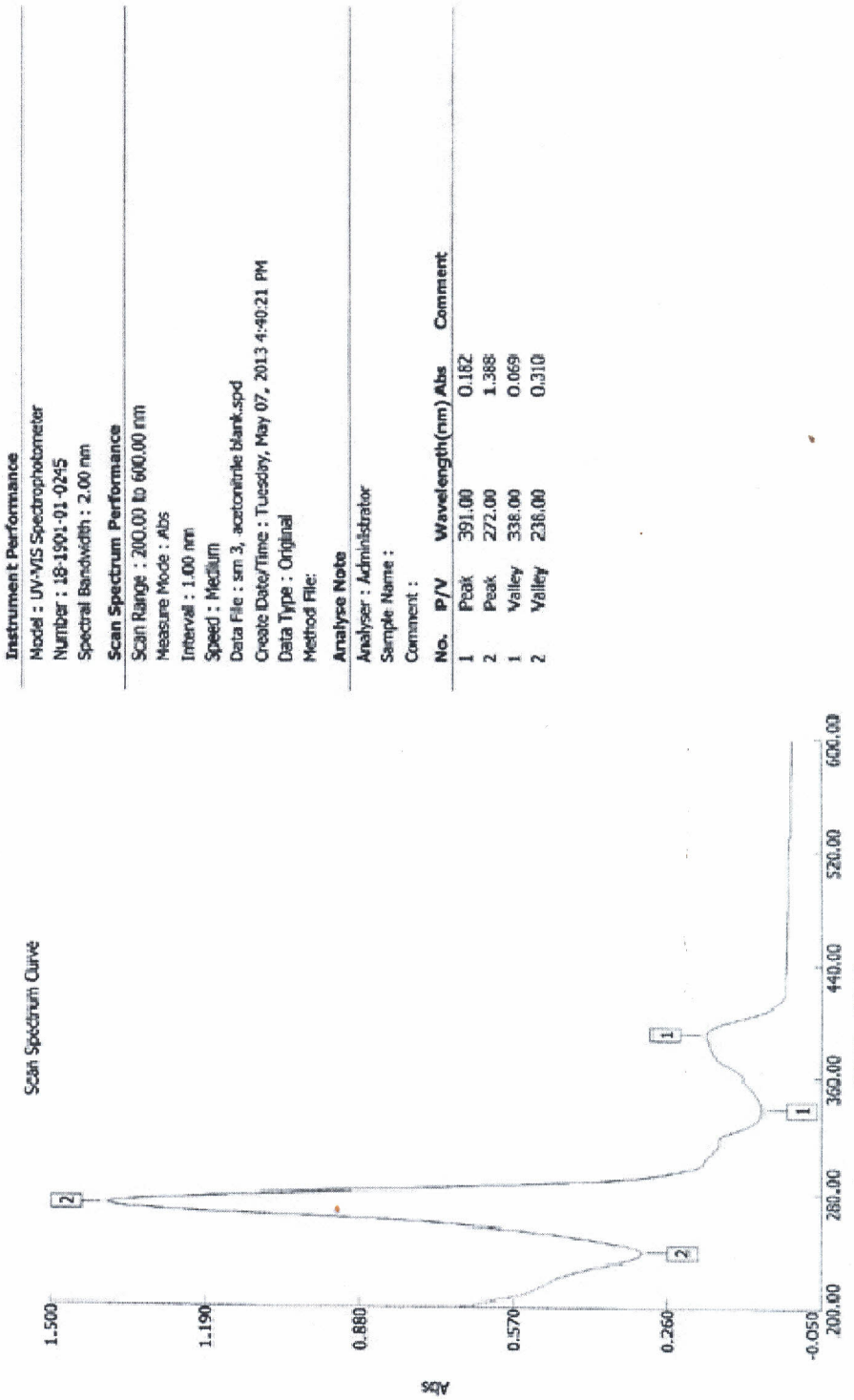
HPLC CHROMATOGRAM FOR SM3 USING HYPERSIL COLUMN AND ACETONITRILE: WATER (30:70) MOBILE PHASE AT 40 DEGREES CELCIUS AND 270NM



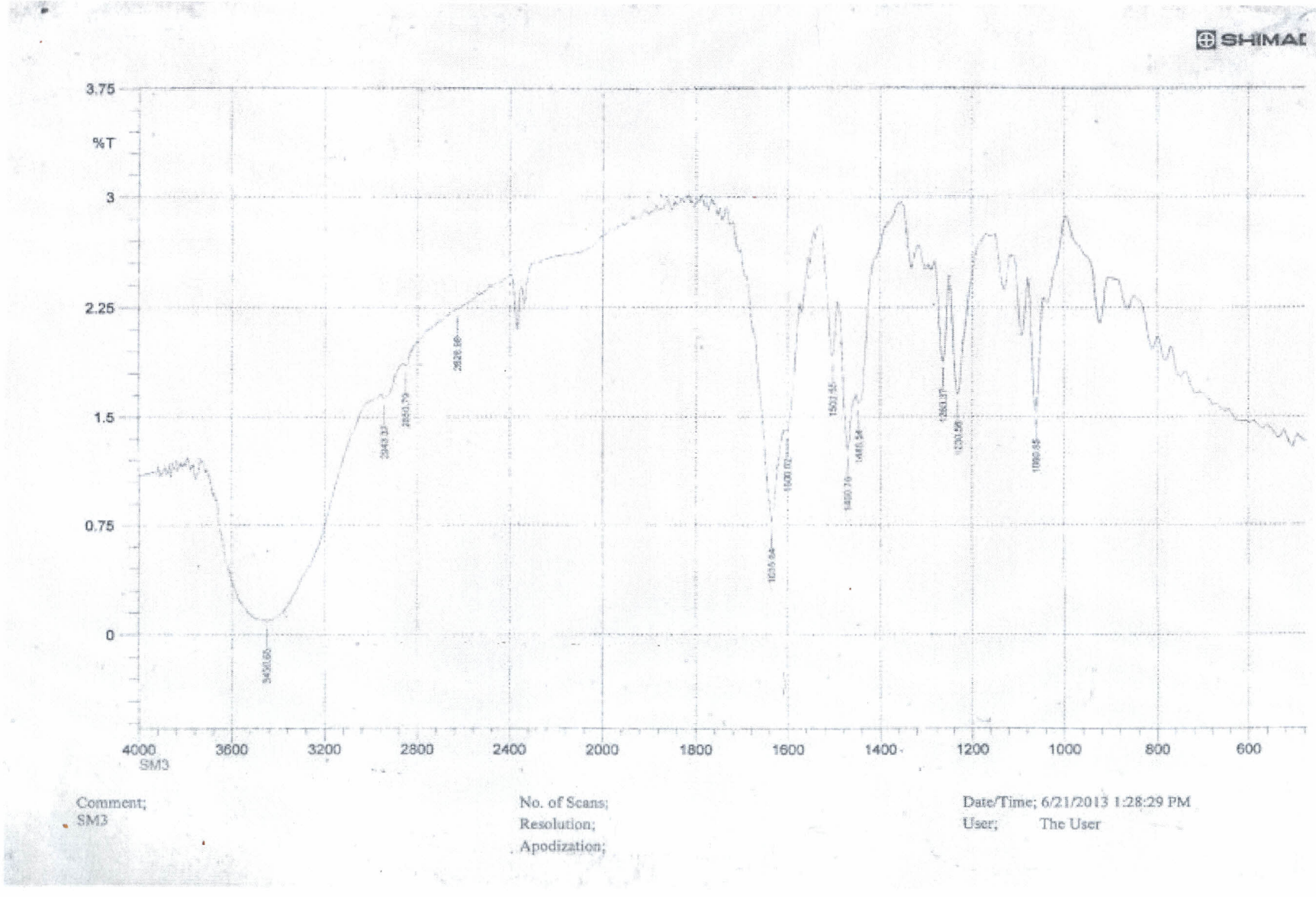
<< Detector A >>
ID#1 Compound Name: SM3 MP

Title	Sample Name	Sample ID	Ret. Time	Area	Area %	Tailing Factor
Dr. Muema1.lcm27.lcd	SM3	SM3	7.162	1634037	3.238	0.731
Dr. Muema1.lcm28.lcd	SM3	SM3	7.201	1380077	1.941	0.708
Average			7.181	1507057	2.589	0.719
%RSD			0.385	11.916	35.436	2.263

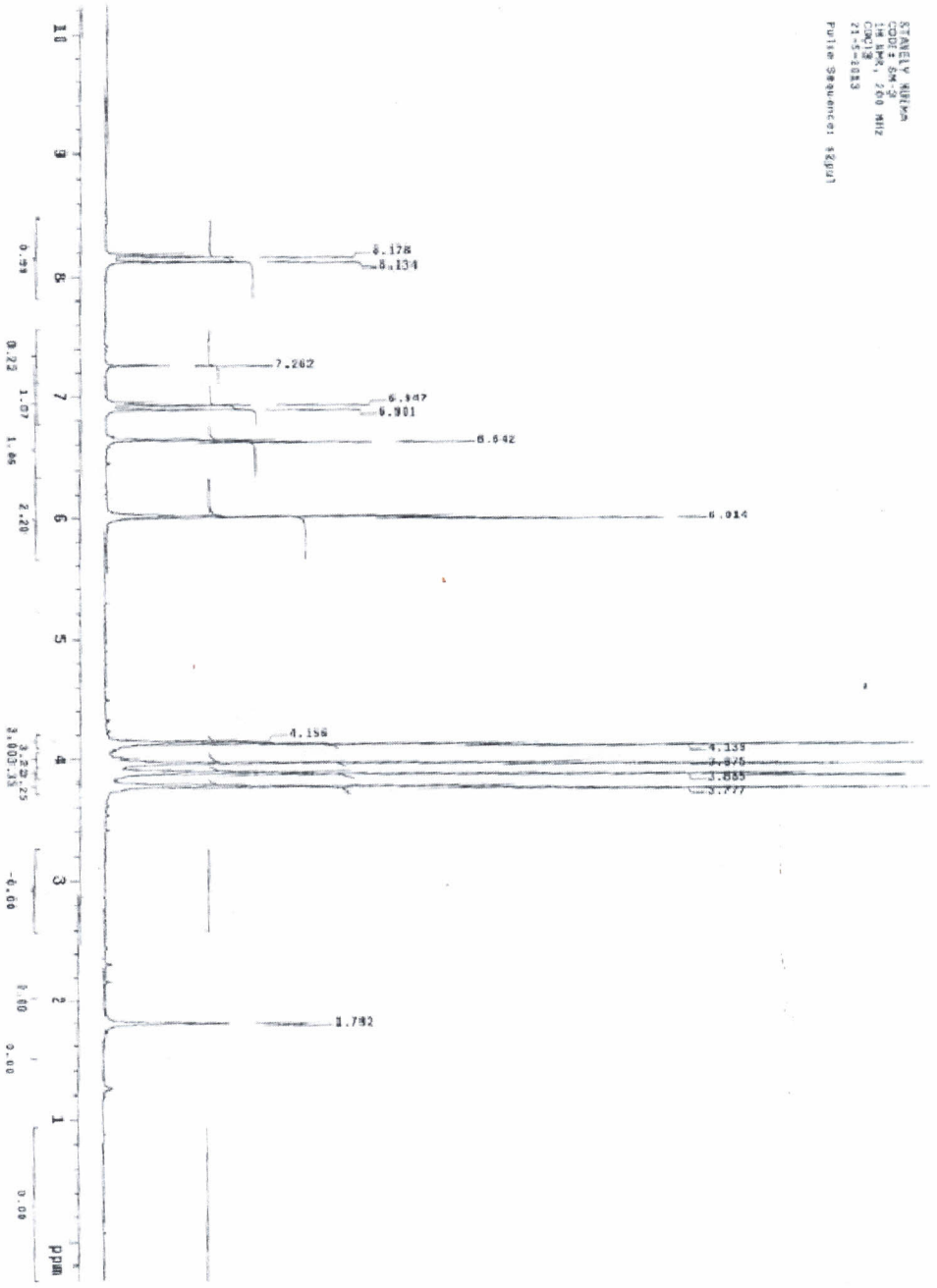
Appendix 15: UV spectrum of 6-methoxytecleanthine



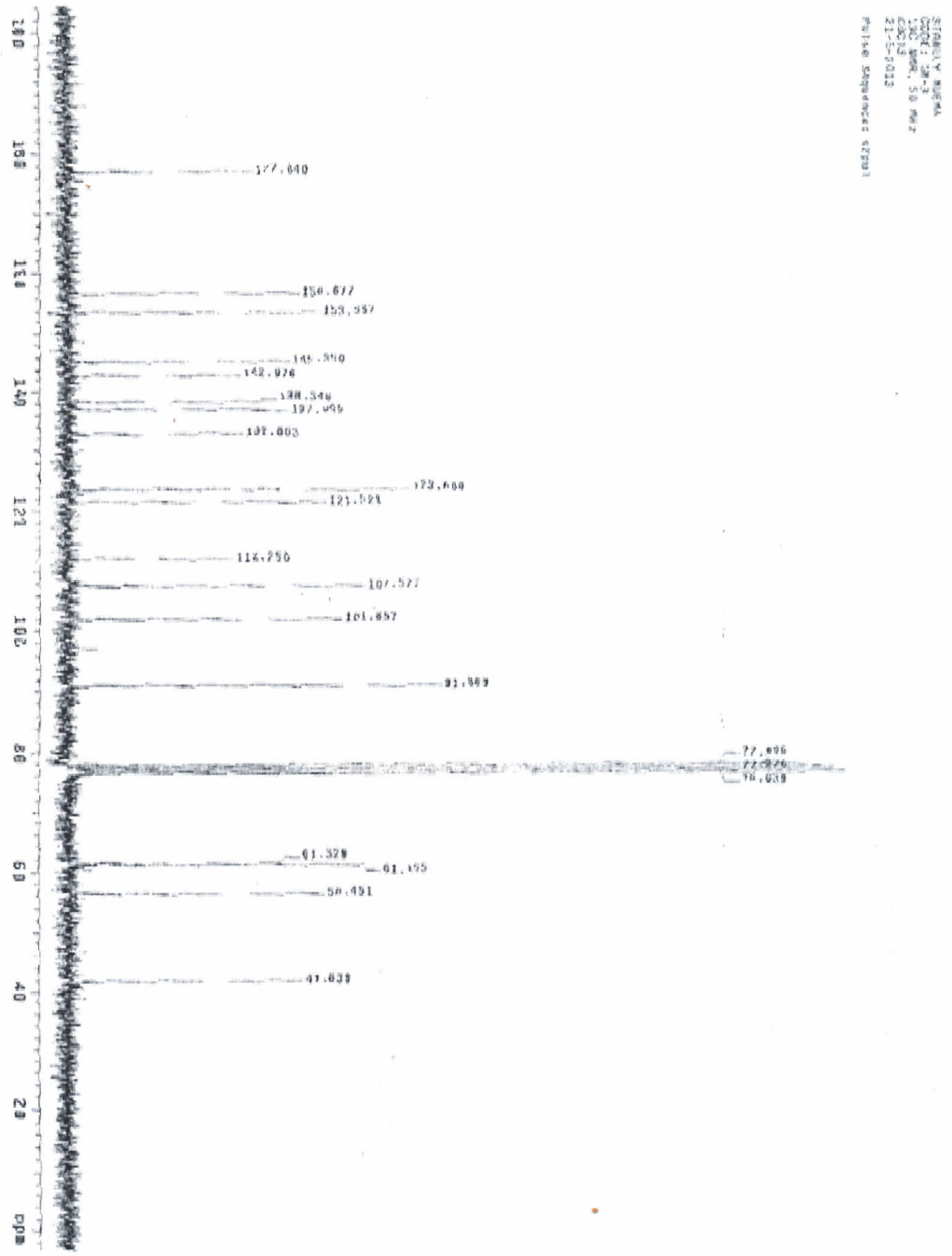
Appendix 16: FTIR spectrum of 6-methoxytecleanthine



Appendix 17: ^{1}H-NMR spectrum of 6-methoxytecleanthine



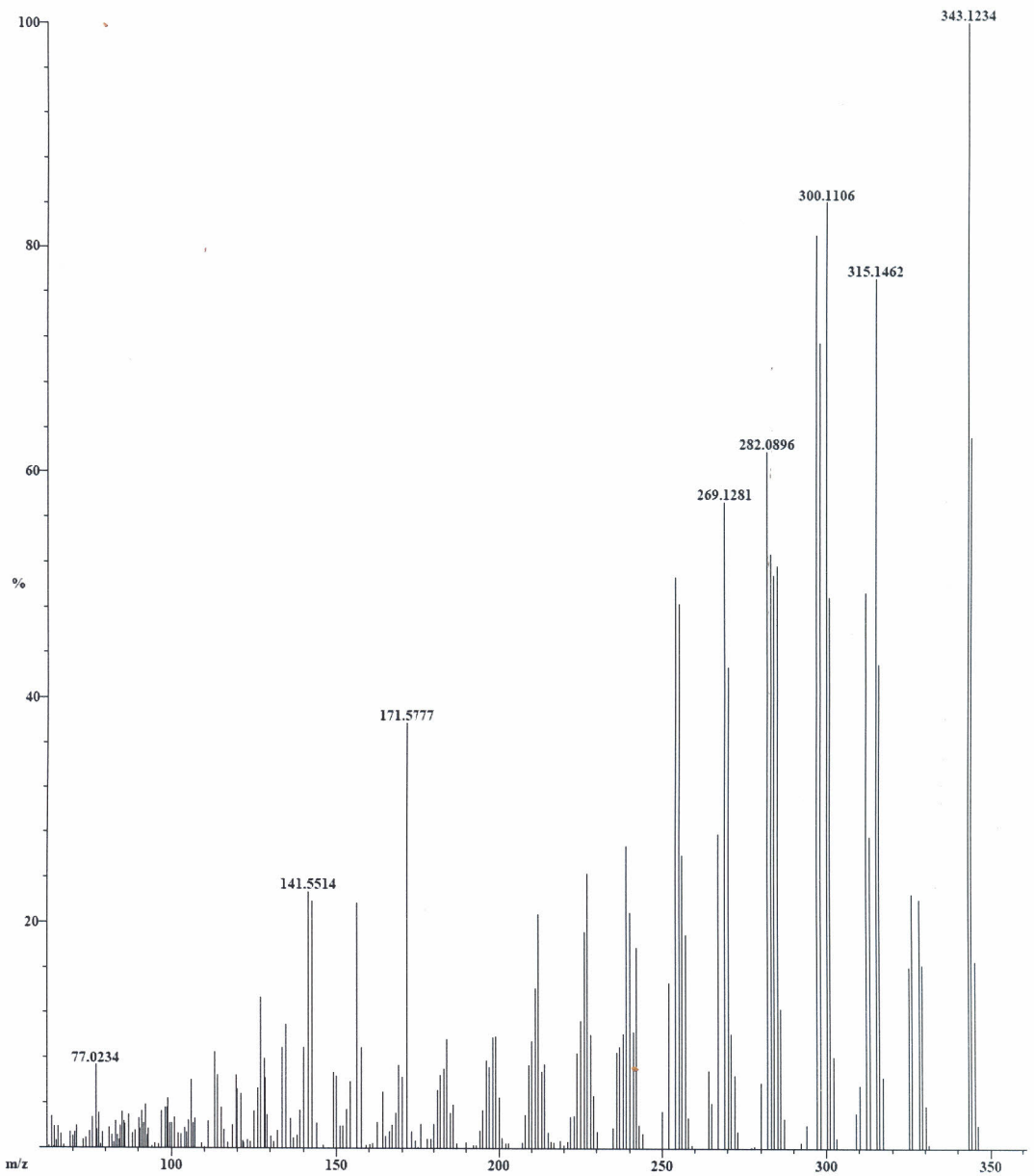
Appendix 18: ^{13}C-NMR spectrum of 6-methoxytecleanthine



Appendix 19: MS spectrum of 6-methoxytecleanthine

File: 13F21-SM 3 **Date Run: 06-21-2013 (Time Run: 14:11:42)**
Sample: Description
Instrument: JEOL GCmateII
Inlet: Direct Probe **Ionization mode: EI+**

Scan: 80 **R.T.: 1.6**
Base: m/z 343; 30.8%FS TIC: 6787754 **#Ions: 231**



Appendix 20: Anthelmintic and toxicity study data

Percentage inhibition of hatching of strongyle eggs

	Percentage inhibition (%)				
Concentration (µg/ml)	**Compound 1**	**Compound 2**	**Compound 3**	**Crude RB extract**	**DMSO**
5000	28	99	100	100	21
2500	22	78	97	100	18
1250	30	62	84	95	19
625.0	24	59	66	81	22
312.5	18	41	46	69	18
156.3	21	28	30	53	20
78.1	24	8	12	24	17
39.1	26	3	5	13	19

Percentage inhibition of development of strongyle larvae

	Percentage inhibition (%)				
Concentration (µg/ml)	**Compound 1**	**Compound 2**	**Compound 3**	**Crude RB extract**	**DMSO**
5000	0	0	34	48	0
2500	0	0	10	17	0
1250	0	0	0	4	0
625.0	0	0	0	0	0
312.5	0	0	0	0	0
156.3	0	0	0	0	0
78.1	0	0	0	0	0
39.1	0	0	0	0	0

Number of brine shrimps dead after 24 h of incubation

Concentration (µg/ml)	**Tube 1**	**Tube 2**	**Tube 3**
1000	10	10	10
100	10	10	7
10	5	6	6