Antibacterial activity of *Euphorbia damarana* extracts and the isolation of triterpenoids

by

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DECLARATION

I, Mmankeko Petunia Degashu, declare that the dissertation, which I hereby submit for the degree Master of Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:…………………………

DATE:…………………………………

ABSTRACT

The *Euphorbia* L. genus has over 2000 species and is regarded as the largest genus of flowering plants. *Euphorbia damarana* is a small tree belonging to the Euphorbiceae family which grows in the eastern parts of Namibia. It is a slender grey succulent round shrub. It is commonly known as Damara milk-bush or melkbos by the local community. *E. damarana* is believed to be one of the most poisonous plants in Namibia producing a milky latex that is capable of killing humans and animals. Poisonous plants occur in every garden worldwide thus forming an important part of the indigenous flora of southern African plants. They consist of a mixture of chemical compounds of which either a single or a collective number of compounds can be toxic. There is often variation of toxicity in different parts of the plants.

The stem extract of *E. damarana* yielded a rubbery material and the dried latex extract yielded a sticky yellow material. The hexane and methanol extracts of the stems of *E. damarana* and the dried latex extract were tested for antibacterial activity against *Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Proteus vulgaris, P. mirabilis, Salmonella typhimurium, Achromobacter xyloxidans* and unknown bacteria isolated from the soil of Namibia where the plant grows. Good antibacterial activity was obtained with both extracts using the TLC bio-autography method, however with the microtitre dilution method only the stem extract showed good antibacterial activity. The disc diffusion method did not show any inhibition. Toxicity studies of the stem and dried latex extracts on Vero cells did not show significant results.

The antibacterial activity of the stem extract was observed with the soil isolate 11 (0.312mg/ml), *P. aeruginosa* (1.25mg/ml), *B. subtilis* (1.25mg/ml), isolate 5 (2.5mg/ml) and isolate 10 (2.5mg/ml) bacteria. For dried latex extract antibacterial activity was observed with isolate 11 (5.00mg/ml), *P. aeruginosa* (5.00mg/ml) and *B. subtilis* (5.0mg/ml).

Bioassay guided fractionation of the stem extract of the plant led to the isolation of five pure compounds. The chemical structures of the isolated pure compounds was elucidated using NMR spectrometry as lupenone, euphol, lupeol, 20-hydroxy-lupan-3-one and 3-oxo-7,24E-tirucalladien-26-oic acid. All the isolated compounds belong to the family of triterpenoids.

E. damarana showed promising antibiotic potential in this study. Since this plant has many other antibacterial compounds as seen on TLC plates of the major fractions and has also shown significant activity against some bacteria in bioassays, further fractionation of the major fractions is recommended in order to obtain more pure compounds that could be tested against bacteria. Toxicity studies on the identified compounds and other compounds that are yet to be identified is in progress and will be reported in further studies. There is limited information published on the phytochemical analysis and properties of *E. damarana* and for this reason it is important to do further research on this plant as it has potential to be used as a natural ethnomedicinal product.

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LIST OF ABBREVIATIONS

WHO: World Health Organisation
LD: Lowest Dose
TLC: Thin Layer Chromatography
EtOAc: Ethyl acetate
MeOH: Methanol
mM: Millimolar
L: Litre
ml: Millilitre
µl: Microliter
g: Gram
mg: Milligram
µg: Microgram
cm: Centimetre
UV: Ultra violet
nm: Nanometre
NMR: Nuclear magnetic resonance
EM: Electromagnetic
H: Hydrogen
C: Carbon
$CDCl_3$: deuterated chloroform
Fig: Figure
MIC: Minimum inhibitory concentration
INT: Iodonitrotetrazolium violet
DMSO: Dimethyl sulfoxide
CFU: Colony forming unit
Cipro: Ciproflaxin
CO_2: Carbon dioxide
MEM: Minimum essential medium
XTT: 2,3-bis (2-methoxy-4- nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide

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1.1. INTRODUCTION

Poisonous plants occur in every garden worldwide thus forming an important part of the indigenous flora of southern African plants. These plants are important in a positive and in a negative way. It is not necessary to remove them from their natural habitat but rather to educate society on avoiding accidental poisoning fatalities in humans, wild animals and livestock. Most farmers lose their livestock annually due to plant poisoning during feeding on these plants. It is also important to increase awareness of the danger of these plants. Plant poisoning is most common since humans mistakenly ingest poisonous plants in place of food plants but low incidents have been reported (Van Koenen, 2001; Van Wyk et al., 2002). Some plants might not be poisonous when ingested but become toxic when they are absorbed by the skin or lungs (Van Wyk et al., 2002).

Plants consist of a mixture of chemical compounds of which either single or a collective number of compounds can be toxic. For one to determine which compounds are toxic; a number of tests need to be conducted. This includes extracting the compound from the plant material by separating it from the mixture of all other compounds present in the plant extract and determining its chemical structure. Once the toxic compound has been identified it will be put through specialized tests to determine its toxicity against living cells and evaluate the special form of toxicity i.e. carcinogenic, mutagenic or teratogenic (Van Wyk et al., 2002). It is important to know what kind of toxin/poison is found in the plant.

Toxins are chemicals which exerts negative effects on an organism and its metabolism. Toxicology is the sub-discipline of Pharmacology which is the study of toxins, their mode of action and treatment of intoxication (Van Wyk et al., 2002). Any substance can harm the body if given at a high dose. The toxic dose depends on:

- Route of administration (the intravenous or intraperitoneal application is usually more effective than the oral route).
- Solubility of poisons and toxins in body fluid.

- Frequency of intoxication (acute, sub-acute and chronic).
- Health and age of a person (sick people are more susceptible than healthy ones, babies and children more than adults; old more than younger people (Van Wyk et al., 2002).

In vivo acute toxicity data is obtained to compare the toxicity of different toxins. Laboratory animals such as rats, mice, rabbits and guinea pigs are used for acute toxicity studies and they provide a good indication of what to expect in humans although humans are usually more susceptible. Lethal dose (LD_{50}) values are often used in animal experiments, it indicates that dose which kills 50% of test animals (Van Wyk et al., 2002).

The World Health Organisation (WHO) list four toxicity classes,

- Class Ia: extremely hazardous
- Class Ib: highly hazardous
- Class II: moderate hazardous
- Class III: slightly hazardous

Classes Ia and Ib are highly poisonous, Class II is poisonous and Class III is less poisonous. Poisons from plants are subdivided into various groups (alkaloids, cyanogenic glycosides, coumarins, terpenoids, saponins, heart glycosides, lectins and oxalates) according to their chemical structure (Bruyns et al., 2011).

Factors that affect the severity of plant poisons include:

- Different soil types
- Different seasons
- Time of the day
- Different temperature (Bruyns et al., 2011)

There is variation of toxicity in different parts of plants. In some plants the leaves might be more toxic than the roots or the bark more toxic than the flowers (Van Koenen, 2001; Bruyns et al., 2011).

It is expected that a history of long-term use of plants by humans might have a low human toxicity; however some of these plants might be toxic to a given endemic culture which didn't have a reporting system to document the cases. Even though not reported, acute toxicity would have been noticed after the use of the plants and it would have been used cautiously or not used at all. Chronic toxicity on the other hand would not have been reported (Fabricant and Farnsworth, 2001).

The use of plants as medicine has been dated back to the middle Paleolithic ages about 60 000 years ago. According to the WHO almost 65% of the world's population have incorporated a traditional medical system incorporating the use of medicinal plants as a means of therapy into their primary health care system (Fabricant and Farnsworth, 2001).

Fabricant and Farnsworth (2001), has stated four objectives for using plants as source of therapeutic agents, namely:

"a) to isolate bioactive compounds for direct use as drugs, e.g. digoxin, digitoxin, morphine, reserpine, taxol, vinblastine and vincristine;

b) to produce bioactive compounds of novel or known structure as lead compounds for semi-synthesis to produce patentable entities of higher activity and/or lower toxicity, e.g. metformin, nabilone, oxycodone (and other narcotic analgesics), taxotene, tenposide, verapamil and amiodarone, which are based respectively on galegine, Δ^9-tetrahydrocannabinol, morphine, taxol, podophyllotoxin, khellin and khelling;

c) to use agents as pharmacologic tools, e.g. lysergic acid diethylamide, mescaline, yohimbine; and

d) to use whole plant or part of it as a herbal remedy, e.g. cranberry, Echinacea, feverfew, garlic, ginkgo biloba, St John's wort, saw palmetto".

1.2. Background and literature review on the *Euphorbia* genus

The variety of chemicals often present in a plant extract have different pharmacological activities such as anthelmintic, antimicrobial and anti-inflammatory, which may act in a synergistic manner resulting in the overall clinical effect. Therefore, the use of multiple bioassays in pharmacological testing is important as it gives a clearer indication of the effect of the extracts in relation to the disease state. In addition, it reduces the possibility of losing other potentially useful bioactive compounds present in the investigated plant extracts (Gurib-Fakim, 2006).

The genus *Euphorbia* L. has over 2000 species and is regarded as the largest genus of all flowering plants. It has different vegetation forms especially among the succulent species in Africa, the Arabian Peninsula and Peninsular India. In these areas they "range from enormous succulent trees reaching 30m height that may dominate the landscape (such as *E. ampliphylla* and *E. cussonioides*) to dwarf sphaeroid succulents that often do not exceed 50 mm in height at maturity (such as *E. gymnocalycioides* and *E. obesa*) to small geophytes (such as *E. acaulis* and *E. tuberosa*)". The larger members are the ones that form a dominant component of the vegetation in tropical, semi-arid areas of Africa, the Arabian Peninsular and Peninsular India and are also an important component in the semi-arid, temperate Greater Cape Flora area receiving winter-rainfall in southern Africa (Jassbi, 2006; Bruyns et al., 2011).

"The *Euphorbia* genus is unique in that succulents are found in all four major lineages of the it and in very different proportions in each of them. Succulence in *Euphorbia* species primarily takes the form of swollen, green, photosynthetic branches with retarded development of bark and with ephemeral, highly reduced leaves" (Bruyns et al., 2011).

Euphorbia damarana Leach was selected for this study because it is highly poisonous and its chemical composition, biological activity and mechanism of action is unknown (Van Koenen, 2001; Van Wyk et al., 2002; Joubert, 2008). For this reason it is of interest to isolate and identify the compounds that contributes towards its toxicity.

A study by Joubert (2008) identified two compounds, namely, lupeol and lup-20,29-ene-3β,11α-diol from *E. damarana*, toxicity for these compounds was not tested.

1.2.1. Lupeol

Lupeol is a triterpene that was first isolated from *Lupinus luteus* in 1889 (Joubert, 2008; Siddique and Saleem, 2011). It is found in many plants including mango, fig, red grape, strawberry, white cabbage, pepper, cucumber, tomato and olive. It is also found in the medicinal plants used in north America, Latin America, Japan, China, Africa and Carribean by native people: *Tamarindus indica, Allanblackia monticola, Himatanthus sucuuba, Celastrus paniculatus, Zanthoxylum riedelianum, Leptadenia hastate, Crataeva nurvala, Bombax ceiba and Sebastiania adenophora* (Chaturvedi et al., 2008; Joubert, 2008; Saleem, 2009; Saleem et al., 2009).

Lupeol has many biological activities which include antimicrobial, antiinflammatory, antiprotozoal, antiproliferative, antiinvasive, antiangiogenic, antidiabetic, antihepatoprotective, antinephroprotective, antiarthritis, antimutagenic, anticancernogenic, antimalarial and has blood cholesterol lowering activity (Chaturvedi et al., 2008; Saleem et al., 2009; Siddique and Saleem, 2011). It has also shown therapeutic efficiency against conditions such as wound healing, kidney disease, diabetes and heart disease (Saleem, 2009; Siddique and Saleem, 2011).

1.2.2. Lup-20,29-ene-3β,11α-diol

Lup-20,29-ene-3β,11α-diol commonly known as nepeticin is a rare triterpene which was first isolated from *Nepeta hindostana* and Salvia species. It has been reported to show antibiotic and blood cholesterol reducing activity (Mendes et al., 1989; Joubert, 2008).

1.2.3. ***Euphorbia damarana***

E. damarana is a small shrub (Fig 1.1) of great character which grows in the eastern parts of Namibia and it belongs to the family Euphorbiaceae. Common names are Damara milk-bush or melkbos. The plant is native to the Afromontane regions of southern African countries (www.biodiversity.org.na; www.namibian.org).

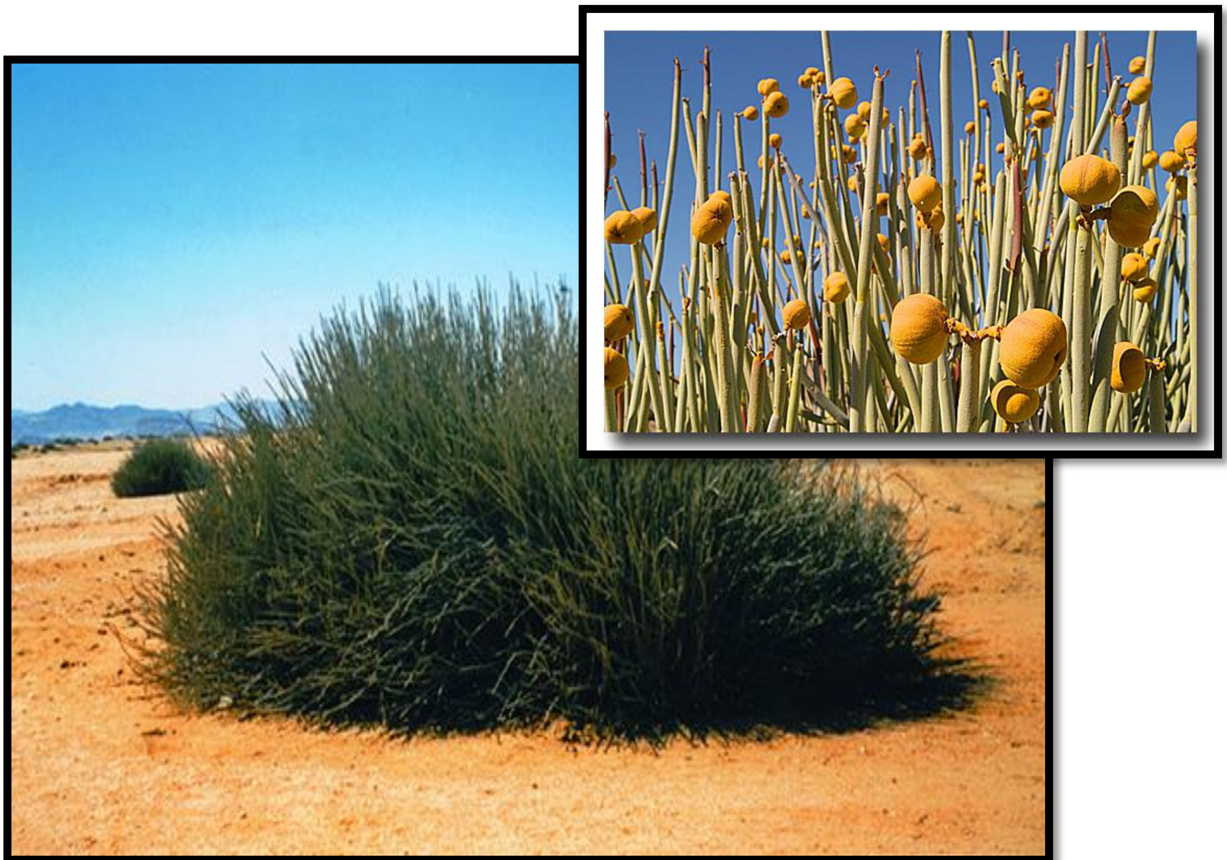

Figure 1.1: *Euphorbia damarana* in the Namib desert (www.namibian.com.na, www.fotoreisen.ch).

1.2.3.1. Morphology

The morphology of *E. damarana* was described by Leach (1975). *E. damarana* is a slender grey succulent round shrub growing up to 3 meters high and 6 meters in diameter. Plants of 11 meters in diameter has been observed (Meyer, personal comment). It is densely branched at the base and randomly rebranched at the top.

Branches: 6 - 12mm in diameter, straight and rod shaped, neither ribbed nor striated. They are fibrous and tough with succulent bark, yellowish green and slightly rough to the touch.

Leaves: about 9mm long and 3.5mm wide sub-acute. Can be erect and spreading with a dark gland on each side at the base.

Inflorescence: a cluster of pedunculatecymes on the branches or branchlets or on the lateral flowering spurs. Male and female are similar except that the female has less tomentose peduncles and cyme branches.

Ovary: ovoid, yellowish tomentose, stout and 1.5mm long.

Capsule: 20mm in diameter and 16mm high. They are yellow green, subspherical and an angular capsule at the tip of the plant branches. Capsules have a hard woody 4 - 6 septate endocarp with 4 - 6 locular. Not all plants bear fruits since the male and female flowers are produced on separate plants.

Seeds: 8mm long, 6.5mm wide and 5.5mm thick, varying depending on the number of seeds in the capsules. They are oblong-ovoid, brownish cream to pale brown (Leach, 1975; www.biodiversity.org.na; www.namibian.org).

1.2.3.2. Distribution

The plant grows in abundance usually in rocky slopes, river beds, river plains and also grows on sandy soil. It is restricted to northern Namibia and desert areas (Leach, 1975; Van Rooyen et al., 2004; Meyer et al. 2015; www.biodiversity.org.na; www.namibian.org).

1.2.3.3. Toxicity

E. damarana is believed to be one of the most toxic plants in Namibia producing toxic milky latex that is capable of killing humans and animals. Specific studies of its toxicity are unknown (www.biodiversity.org.na; www.namibian.org).

1.2.4. Traditional use of the *Euphorbia* genus

Species of the Euphorbiaceae are generally known to be toxic and to be skin irritants (Barla et al., 2007). The pharmacological and phytochemical information of *E. damarana* is not documented despite its frequent use by locals for hunting where the latex of the plant is applied to the arrow head (Wooding et al., 2016) and the fibres used by textile industries (www.namibian.org).

Species of the genus *Euphorbia* are known to produce a milky latex which has been used traditionally to treat cancers, tumours and warts in the olden days. *Euphorbia* latex is used in China to treat skin disease and used to treat warts in India and Africa. The latex composition includes toxic compounds and interesting potential bioactive compounds such as diterpenes and triterpenes (Fernandez-Ache et al., 2010).

E. damarana fibres have many applications in the textile and composite material (automotive) industries. It grows in abundance representing an opportunity for the sustainable harvesting of an existing natural resource and

the formation of a new textile industry in Namibia. The fibre is found in the stems of the plant and is known as the "bark fibre". The first person to identify and suggest commercialization of the *E. damarana* fibre is Mr J.J. van Zyl (www.godenefibre.com).

1.3. Aims and objectives

1.3.1. Aims

The aim of this study was to isolate, purify and identify antibacterial secondary metabolites from *E. damarana.*

1.3.2. Objectives

The objectives of this study were to:

- Use different solvents to prepare plant extracts
- Isolate and identify antibacterial compounds
- Do antibacterial tests on the plant extracts and compounds using several bacteria
- Test the plant extracts and semi-pure fractions for cytotoxic activity using Vero cell lines

1.3.3. Hypothesis

E. damarana contains antibacterial toxic compounds that might be responsible for its toxicity.

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CHAPTER 2: PLANT EXTRACTION, FRACTIONATION AND ISOLATION OF BIOACTIVE COMPOUNDS………………… 13

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2.1. Introduction

Freshly collected or dried plant material is usually used for preparation of plant extracts. There are different techniques that are used for extracting plant materials. The techniques include liquid solvent, steam distillation, supercritical fluid extraction and liquid gases under moderate pressure (Houghton and Raman, 1998).

Selective solvents and different extraction techniques are used to separate medicinally active fractions from inactive fractions in plant extracts. The solvent diffuses into the plant material which can be leaves, flowers, bark or roots to solubilise compounds with similar polarity. The choice of solvent is very important (Ncube et al., 2008; Shrivastava et al., 2010). For example, methanol will be used to solubilise polar compounds while hexane will be used to solubilise non-polar compounds.

When choosing a solvent to use for extraction the following properties should be taken into consideration: "low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate" (Ncube et al., 2008; Shrivastava et al., 2010).

For fractionation of plant material a chromatographic technique is widely used because it has made possible the isolation of many compounds from plants. There are two types of chromatography techniques; the planar and column methods (Houghton and Raman, 1998).

2.1.1. Principles of pressurised speed extraction

2.1.1.1. Theory of pressurised solvent extraction

The combination of elevated temperature and pressure results in faster extraction compared to other extraction technologies. This is the result of an improved mass transfer because of higher analyte solubility and enhanced penetration. An increase from normal pressure p_n to p_2 is

thereby necessary to keep the sample in the liquid state at T_2 (Fig 2.1) (www.buchi.com).

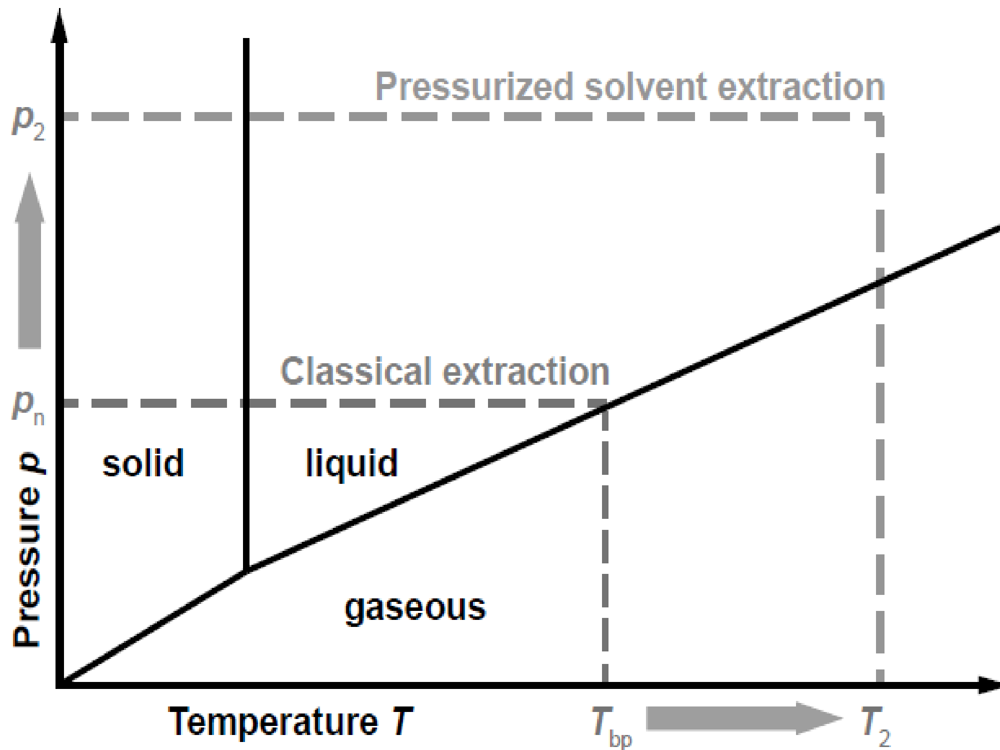

Figure 2.1: Principles of speed extraction (www.buchi.com).

2.1.1.2. Pressurised solvent extraction process

The speciality of the SpeedExtractor‘s heat up is a step by step increase to reach the pressure. Every extraction starts with a tightness test (1) as an inherent element of the method ensuring the presence of nitrogen and that all cells are in place on activated positions. After this quick initial check the extraction proceeds with multiple extraction cycles. Each extraction cycle consists of three steps - heating, hold and discharge (Fig 2.2 steps 2 - 4). Approaching the set pressure gradually guarantees a very consistent process avoiding overshooting the set pressure. After the final discharge of the last cycle the extraction cells are flushed with solvent and gas.

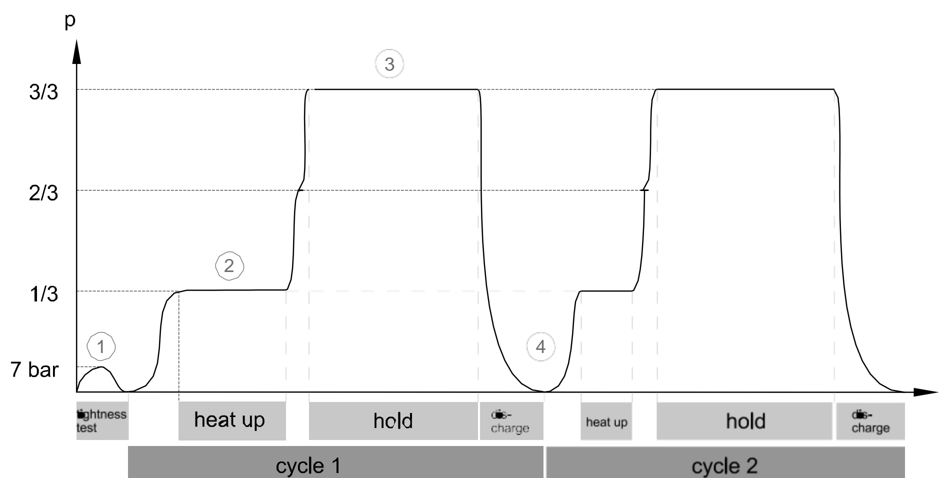

Figure 2.2: Graph showing the pressurised solvent extraction process (www.buchi.com).

2.1.2. Principles of silica gel column chromatography

Separation of the components in an extract (Fig 2.3) is achieved due to differences in their relative affinity for the stationary and mobile phases. The bands of similar compounds are then collected as fractions and concentrated with a rotary evaporator (Buchi rotavapor, Germany) or other similar apparatus under reduced pressure at set temperature. Collection of the fractions is continuous until the last fraction is collected, the column must never be left to dry while collecting so the addition of the solvent during collection is important. The fractions are analysed biologically (by bioassays) or chemically (by thin layer chromatography usually).

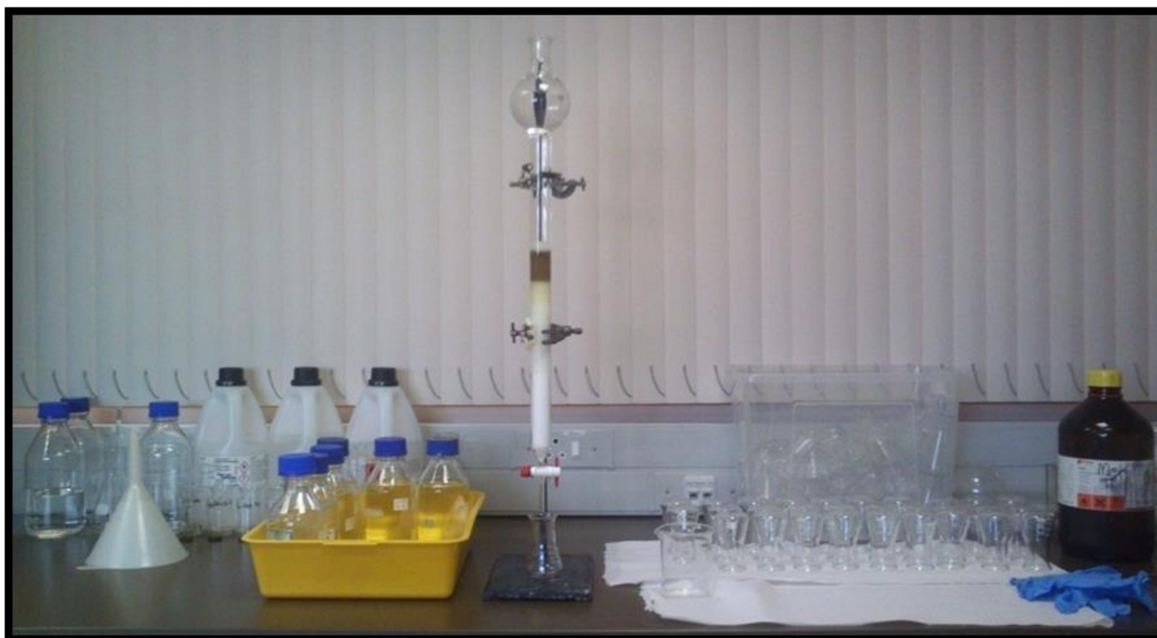

Figure 2.3: A 'running' silica column with different solvents and collecting flasks.

The aims of this chapter are to prepare plant extracts and use them to isolate pure compounds using column chromatography and thin layer chromatography methods.

2.2. Materials and methods

2.2.1. Plant material

Stems of *E. damarana* were randomly collected from mature plants growing in Giribis Plains (S19°11.416'/ E13°17.557) and Uis (S21°05.313'/ E14°51.207') in Namibia in May 2014. Voucher specimens were prepared, identified and preserved at the H.G.W.J Schwelcherdt Herbarium (PRU), University of Pretoria. PRU no: 122228 for sample collected in Uis and PRU no: 122229 for sample collected in Giribis Plains.

The stems of *E. damarana* were collected using pruning scissors and gloves. The stems with the latex were collected from several trees located in the

same area and combined. Following collection the stems were stored in brown paper bags in a freezer at -20°C.

2.2.2. Plant drying

A Virtis freeze drier (United Scientific (Pty) Ltd) (Fig 2.4) was used to dry the stems. The combined stems (345 g) were cut and placed in freeze drying bottles, a total of 5 bottles were filled with 10 to 15 stems each. They were freeze dried at -77 °C for 7 days.

Figure 2.4: Virtis freeze drier (United Scientific (Pty) Ltd) with flasks filled with *E. damarana* stems.

2.2.3. Plant extraction

Extraction of the plant was done using two separate methods since the plant after drying had two types of compositions; the stems and a yellow-white powder which will be referred to as “dried latex” (see section 2.3.1.).

2.2.3.1. Stem extraction

Two solvents were used for extraction, namely hexane and methanol (MeOH). A speed extrator E916 (Buchi, Switzerland) was used for this purpose. The dried stems (155 g) were cut into 10 to 15 cm long stems and placed in 40 ml pressure vessels (Fig 2.5). The vessels were packed with a maximum of 5 stems each. The heat-up temperature was at 50°C with a pressure of 100bar. A total of five batches of four cycles were run over a period of two days since it took about 2 hours to run a single batch.

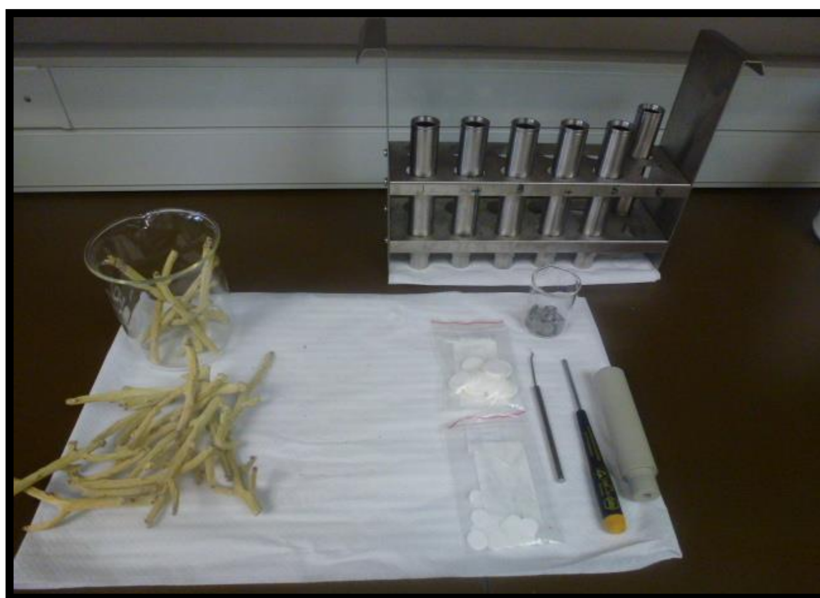

Figure 2.5: Stems, vessels and filter papers for the Speed Extractor E916.

The stem extract collected from the SpeedExtractor was concentrated using a rotatory evaporator (Buchi rotavapor, Germany) under reduced pressure at 40°C (Fig 2.6). The stem extract was left to dry at room temperature. The extracts were stored in the dark at 4°C until needed for further analysis

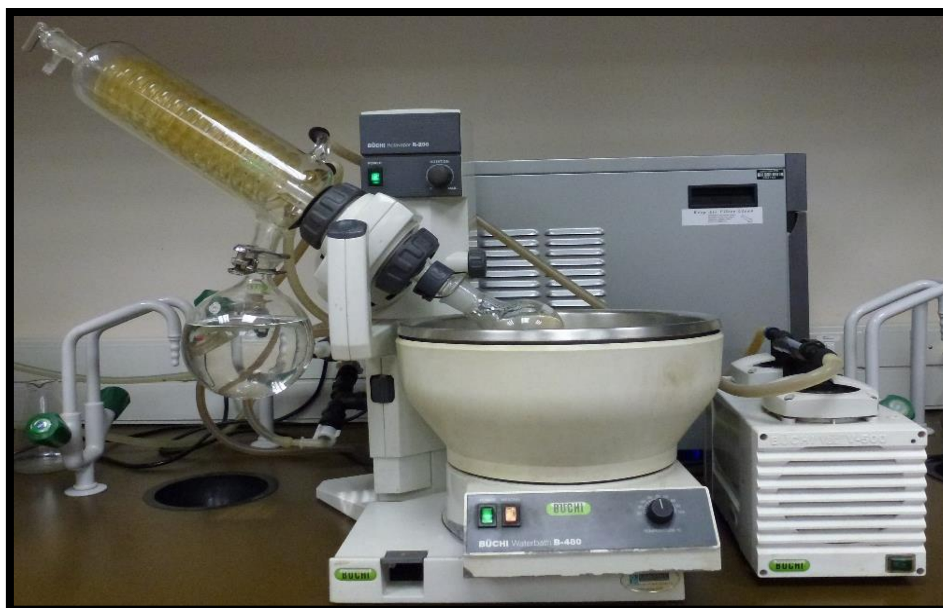

Figure 2.6: A rotary evaporator (Buchi rotavapor, Germany).

2.2.3.2. Dried latex extraction

The dried latex (30 g) was filtered under vacuum with methanol (Fig 2.7) through a Whattman No. 1 filter paper (Merck SA (Pty) Ltd.). It was washed 3 times with 200 ml of methanol to ensure maximum extraction of the compounds. The solvent extract was concentrated using a rotatory evaporator (Buchi rotavapor, Germany) under reduced pressure at 40°C to a volume of 100ml. The dried latex extract was left to dry at room temperature and complete dryness was done using the centrifugal evaporator (EZ 2 Plus, GeneVac, UK). The extracts were stored in the dark at 4°C until needed for further analysis.

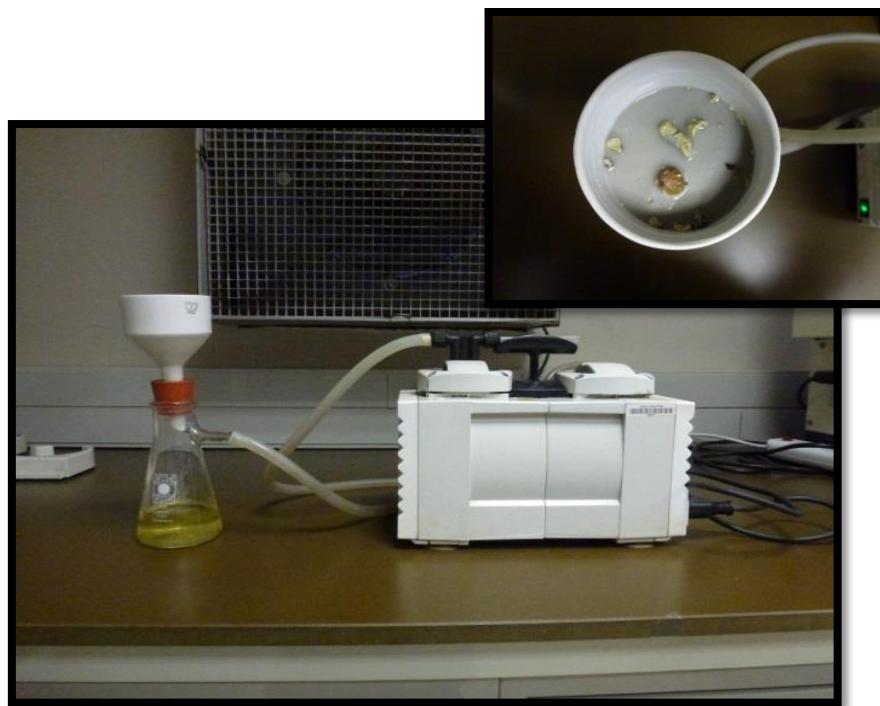

Figure 2.7: Extraction under vacuum of the dried latex material.

2.2.4. Fractionation and isolation of active compounds

Prior to eluting the first column, a series of TLC plates of the plant extracts were performed to determine which solvent concentration mixture to start with and also to determine which plant extract to use for further analysis. A solvent concentration mixture of hexane/ethyl acetate (EtOAc) followed by EtOAc/methanol with increasing polarity was used.

2.2.4.1. Silica gel column chromatography

2.2.4.1.1. Purification of methanol stem extract

The methanol stem extract (1.5 g) was chromatographed on 80 g silica gel (Merck, South Africa) in a column and applied as a solid mixture of 6g silica gel plus 1.5 g plant extract using hexane/EtOAc followed by EtOAc/MeOH mixtures of increasing polarity (Table 2.1) to yield 181 fractions. Similar fractions were combined and concentrated based on compounds which have similar polarities after TLC analysis. The same solvent mixture as the one used at the time of collection was used as an eluent to run the TLC plates. A

total of 52 combined fractions were obtained and were coded by roman numbers with Me for methanol (MeI – MeXXXXIII) as indicated in Table 2.2.

Table 2.1: Solvent mixtures (%) used to elute the methanol extract's column.

Hexane	(EtOAc)	MeOH	Volume used (L)	Fractions collected
60	40		0.5	Me1 – Me8
50	50		1.0	Me9 – Me20
40	60		1.0	Me21 – Me30
30	70		0.5	Me31 – Me40
20	80		1.0	Me41 – Me50
10	90		0.5	Me51 – Me58
	100		0.5	Me59 – Me66
	90	10	0.5	Me67 – Me77
	80	20	1.0	Me78 – Me89
	70	30	0.75	Me90 – Me98
	60	40	0.75	Me99 – Me108
	40	60	0.5	Me109 – Me118
	30	70	0.5	Me118 – Me127
	20	80	0.5	Me128 – Me135
	10	90	0.5	Me136 – Me144
		100	2.0	Me 145 – Me181

Table 2.2: Combined fractions obtained after fractionation of methanol extract.

Combined fractions	Designated number	Combined fractions	Designated number	Combined fractions	Designated number
1	MeI	57 – 60	MeXXI	118 – 120	MeXLI
2	MeII	61 – 63	MeXXII	121 – 130	MeXLII
3	MeIII	64 – 67	MeXXIII	131 – 139	MeXLIII
4	MeIV	68 – 69	MeXXIV	140	MeXLIV
5	MeV	70	MeXXV	141 – 151	MeXLV
6 – 7	MeVI	71 – 72	MeXXVI	152	MeXLVI
8 – 10	MeVII	73 – 74	MeXXVII	153 – 160	MeXLVII
11 – 15	MeVIII	75 – 76	MeXXVIII	161 – 170	MeXLVIII
16 – 20	MeIX	77 – 79	MeXXIX	171 – 175	MeXLIX
21 – 26	MeX	80 – 82	MeXXX	176	MeL
27	MeXI	83 – 86	MeXXXI	177 – 180	MeLI
28 – 31	MeXII	87 – 89	MeXXXII	181	MeLII
32 – 36	MeXIII	90 – 93	MeXXXIII		
37	MeXIV	94 – 96	MeXXXIV		
38 – 39	MeXV	97 – 100	MeXXXV		
40 – 44	MeXVI	101	MeXXXVI		
45 – 47	MeXVII	102 – 106	MeXXXVII		
48 – 50	MeXVIII	107 – 110	MeXXXVIII		
51 – 52	MeXIX	111 – 114	MeXXXIX		
53 - 56	MeXX	115 – 117	MeXL		

Combined fraction MeXXIV (110.6 mg) which showed good antibacterial activity (see section 3.3.2.1.) was further chromatographed on silica gel (11 g) using hexane/EtOAc followed by EtOAc/MeOH mixtures of increasing polarity to yield 122 fractions. Similar fractions were combined to obtain 27 combined fractions as indicated in Table 2.3. Fractions MeXXIV2 (0.8 mg), MeXXIV3

(0.9 mg), MeXXIV4 (4.1 mg), MeXXIV5 (1.7 mg) and MeXXIV6 (5.0 mg) were further analysed for antibacterial activity.

Table 2.3: Combined fractions from the column of fraction MeXXIV.

Combined fractions	**Designated number**	**Combined fractions**	**Designated number**	**Combined fractions**	**Designated number**
1	MeXXIV1	25 – 30	MeXXIV11	71 – 80	MeXXIV21
2	MeXXIV2	31 – 34	MeXXIV12	81 – 90	MeXXIV22
3	MeXXIV3	35 – 40	MeXXIV13	91 – 102	MeXXIV23
4	MeXXIV4	41 – 43	MeXXIV14	103 – 105	MeXXIV24
5	MeXXIV5	44 – 47	MeXXIV15	106	MeXXIV25
6-8	MeXXIV6	48 – 60	MeXXIV16	107 – 111	MeXXIV26
9	MeXXIV7	61	MeXXIV17	112 - 122	MeXXIV27
10 – 13	MeXXIV8	62	MeXXIV18		
14 – 20	MeXXIV9	63 – 65	MeXXIV19		
21 - 24	MeXXIV10	66 - 70	MeXXIV20		

Combined fraction MeXXV (84 mg) which showed good antibacterial activity (see section 3.3.2.1.) was further chromatographed on 8 g silica gel with hexane/EtOAc followed by EtOAc/MeOH mixtures of increasing polarity to yield 128 fractions which were also combined according to similarity and concentrated to obtain 22 combined fractions as indicated in Table 2.4. Fractions MXXV21 (8.5mg) and MXXV22 (7.2 mg) were further analysed for antibacterial activity and toxicity.

Table 2.4: Combined fractions from the column of fraction MeXXV.

Combined fractions	Designated number	Combined fractions	Designated number	Combined fractions	Designated number
1 – 10	MeXXV1	54 – 57	MeXXV11	111 – 120	MeXXV21
11 – 20	MeXXV2	58	MeXXV12	121 - 128	MeXXV22
21 – 27	MeXXV3	59 – 60	MeXXV13		
28 – 30	MeXXV4	61 – 65	MeXXV14		
31 – 40	MeXXV5	66 – 70	MeXXV15		
41 – 45	MeXXV6	71 – 78	MeXXV16		
46 – 48	MeXXV7	79 – 80	MeXXV17		
49	MeXXV8	81 – 90	MeXXV18		
50 – 51	MeXXV9	91 – 100	MeXXV19		
52 – 53	MeXXV10	101 - 110	MeXXV20		

2.2.4.1.2. Purification of hexane stem extract.

The hexane stem extract (8 g) was chromatographed on 250 g silica gel in a column with 23 g solid loading of a mixture of 15 g silica gel plus 8 g plant extract using hexane/EtOAc followed by EtOAc/MeOH mixtures of increasing polarity (Table 2.5) to yield 105 fractions. Similar fractions where combined and concentrated to obtain 28 combined fractions which were coded by roman numbers with He for hexane (HeI – HeXXVIII) as indicated in Table 2.6. Four fractions from the 28 combined fractions, namely, HeII (1.6 g), HeX (50mg), HeXI (550 mg) and HeXII (550 mg) were analysed further for antibacterial activity (see section 3.3.3. and 3.3.4.) and identified.

Table 2.5: Solvent mixtures (%) used to elute the hexane extract's column.

Hexane	**EtOAc**	**MeOH**	**Volume used (L)**	**Fractions collected**
98	2	0	2.0	He1 – He11
96	4	0	1.0	He12 – He25
94	6	0	2.0	He26 – He31
90	10	0	3.0	He32 – He56
85	15	0	1.0	He57 – He68
80	20	0	1.0	He69 – He77
70	30	0	1.0	He78 – He82
60	40	0	2.0	He83 – He88
50	50	0	2.0	He89 – He95
70	30	10	1.0	He96 – He97
40	40	20	1.0	He98 - He105

Table 2.6: Combined fractions obtained from fractionation of hexane extract.

Combined fractions	**Designated number**	**Combined fractions**	**Designated number**	**Combined fractions**	**Designated number**
3 – 4	HeI	49 – 51	HeXI	83 – 84	HeXXI
23 – 27	HeII	52 – 54	HeXII	85 – 86	HeXXII
28 – 32	HeIII	55 – 58	HeXIII	87	HeXXIII
33 – 35	HeIV	59 – 64	HeXIV	88 – 90	HeXXIV
36 – 38	HeV	65 – 68	HeXV	91 – 93	HeXXV
39 – 40	HeVI	69 – 73	HeXVI	94 – 96	HeXXVI
41 – 42	HeVII	74 – 77	HeXVII	97 – 99	HeXXVII
43 – 44	HeVIII	78 – 79	HeXVIII	100 - 101	HeXXVIII
45 – 46	HeIX	80 – 81	HeXIX		
47 - 48	HeX	82	HeXX		

Combined fractions HeXVIII and HeXIX which showed good antibacterial activity (see section 3.3.2.2.) were combined to yield a mass of 230 mg and further chromatographed on 23 g silica gel using hexane/EtOAc mixtures of increasing polarity to yield 71 fractions. Similar fractions were combined to obtain 14 combined fractions is indicated in Table 2.7. Fractions HeXVIII/HeXIX7 (16 mg) was analysed further for antibacterial activity (see section 3.3.3. and 3.3.4.) and identified.

Table 2.7: Combined fractions from the column of fractions HeXVIII/HeXIX.

Combined fraction	**Designated number**	**Combined fraction**	**Designated number**
1 – 5	HeXVIII/HeXIX1	40 – 42	HeXVIII/HeXIX8
6 – 8	HeXVIII/HeXIX2	43 – 48	HeXVIII/HeXIX9
9 – 13	HeXVIII/HeXIX3	49 – 55	HeXVIII/HeXIX10
14 – 21	HeXVIII/HeXIX4	56 – 60	HeXVIII/HeXIX11
22 – 28	HeXVIII/HeXIX5	61 – 66	HeXVIII/HeXIX12
29 – 34	HeXVIII/HeXIX6	67 – 69	HeXVIII/HeXIX13
35 – 39	HeXVIII/HeXIX7	70 - Finish	HeXVIII/HeXIX14

Combined fractions HeXXIV (180 mg) which showed good antibacterial activity (see section 3.3.2.2.) was further chromatographed on 18 g silica gel with hexane/EtOAc mixtures of increasing polarity to yield 51 fractions which were also combined according to similarity and concentrated to obtain 8 combined fractions as indicated in Table 2.8. Fraction HeXXIV3 (15 mg) was further analysed for antibacterial activity (see section 3.3.3. and 3.3.4.) and identified.

Table 2.8: Combined fractions from the column of fraction HeXXIV.

Combined fractions	Designated number	Combined fractions	Designated number
1 – 11	HeXXIV1	22 – 30	HeXXIV5
12 – 14	HeXXIV2	31 – 40	HeXXIV6
15 – 17	HeXXIV3	41 – 49	HeXXIV7
18 - 21	HeXXIV4	50	HeXXIV8

2.2.5. Thin layer chromatography (TLC)

Extracts and collected fractions were spotted on TLC plates (TLC Silica gel 60 F_{254}, Merck, 10 x 7 cm) and run with different solvent mixtures. Developed plates were visualised by UV light at 245 and 366 nm and a vanillin solution (250 ml ethanol, 5 ml sulphuric acid and 7.5 g vanillin powder) to visualise the compounds appearing as spots or bands. After analysis the fractions that showed bands were used for further fractionation and isolation.

Collected fractions were spotted on TLC plates with 10 fractions spotted on each plate. The spotted TLC plates were developed in the same solvent concentration mixture as the one used to elute a column at the time of collection. The plates were sprayed with vanillin after viewed under UV light.

2.3. Results and discussion

2.3.1 Plant extraction

For the purpose of this study a freeze drier was employed to dry the stems of *E. damarana* because freeze drying removes all excess water in succulent plants and the secondary metabolites are preserved much better that the other methods (Popp et al., 1996; Asami et al., 2003).

The combined plant stems collected from the two regions in Namibia weighed 345 g. Following drying with a freeze drier the weight was 185 g. It was noted

that the outer layer of the stems peeled off into small flakes that crumble into yellow-white powder (Fig 2.8), probably dried latex. The dried latex weighed 30 g while the dried stems weighed 155 g.

Figure 2.8: Stems inside freeze drying flasks with dried latex in powdered form.

2.3.2. Extraction of dried latex and stem extract

The preparation of the crude extracts was done using two methods: speed extraction and vacuum filtration. This was done because upon drying, it was noticed that there were residues of powder at the bottom of the flask. Since the plant produces latex, it was concluded that the powder is the dried latex that was produced after the plant was cut.

For the dried latex the vacuum filtration method was employed using methanol. When the extract was air dried to remove all the methanol there was a precipitation of yellow sticky material and a yellow liquid supernatant (Fig 2.9). The two parts were separated by decanting the supernatant into another container and dried separately. The final weight of the dried latex

extract was 9.45 g of which 1.10 g was the yellow supernatant and 8.35 g was the yellow sticky precipitation.

Figure 2.9: Dried latex extract of methanol with the yellow sticky precipitation.

For the stem extract the speed extractor was employed using hexane and methanol. When the extract was air dried to remove all the methanol there was a precipitation of green-brown rubbery material and a green liquid supernatant (Fig 2.10). The two parts were also separated by decanting the supernatant into another container and dried separately. The final weight of the stem extract was 13.48 g of which 3.57 g was the supernatant and 9.91 g was the precipitation.

Rubbery and waxy constituents were also isolated from *E. candelabrum, E. grantii, E. tirucalli* and *S. grantii* in a study by Uzabakiliho et al., 1987. They used different parts of the plants and used dichloromethane as their solvent.

Figure 2.10: Stem extract of methanol with the green-brown rubbery precipitation.

2.3.3. Fractionation and isolation of antibacterial compounds

The supernatant methanol stem and methanol dried latex extracts were spotted on the TLC plates in order to observe the difference in compounds composition. The stem extract was chosen for further analysis since it showed more compounds than the dried latex extract (Fig 2.11).

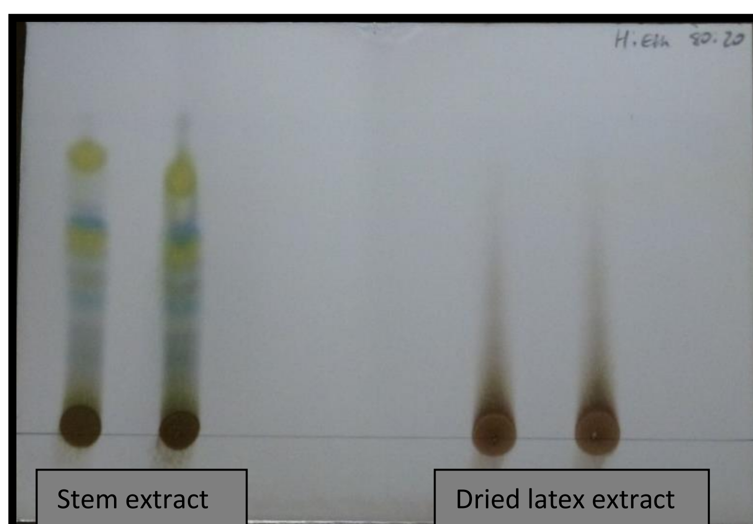

Figure 2.11: TLC plate of the dried latex and the stem extracts using hexane: MeOH (80:20).

To determine which solvent concentration mixture to use to develop a column, it was necessary to 'run' the stem extract on TLC plates using different solvent combinations. The hexane:EtOAc mixture at a concentration of 60:40 was chosen to start a column since this was the concentration were the stem extract started to show good separation of compounds.

When a column is properly packed and correct solvents are used, compounds can be observed separating on the column according to their polarity (Fig 2.12). The non-polar compounds were collected first then the polar compounds at the end. About 30ml of the fraction was collected in each polytop and the fractions were spotted on TLC plates.

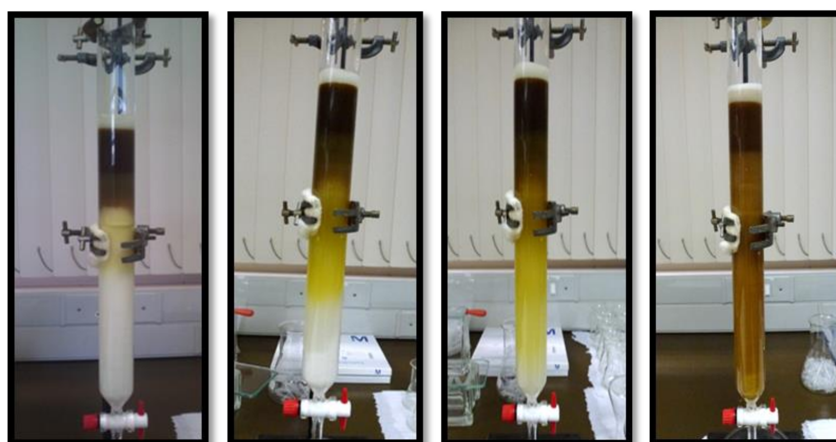

Figure 2.12: Silica gel column with separation of compounds.

2.3.3.1. Purification of methanol stem extract

A total of 181 fractions were isolated from the methanol stem extract. The fractions that looked similar were combined and concentrated to obtain 52 combined fractions. Combined fractions MeXXIV (110.6 mg) and MeXXV (84 mg) were further fractionated using hexane/EtOAc followed by EtOAc/MeOH mixtures of increasing polarity to obtain 122 and 128 fractions respectively. Similar fractions were combined to obtain 27 combined fractions from column MeXXIV and 22 combined fractions from column MeXXV. Fractions MeXXIV2 (0.8 mg), MeXXIV3 (0.9 mg), MeXXIV4 (4.1 mg), MeXXIV5 (1.7 mg) and MeXXIV6 (5.0 mg) from column MeXXIV were tested for antibacterial activity because these fractions showed to consist mainly of single compounds as per

the TLC analysis. Fractions MeXXV21 (8.5 mg) and MeXXV22 (7.2 mg) from column MeXXV were tested for cytotoxicity and antibacterial activity because these fractions showed to consist mainly of single compounds as per the TLC analysis.

TLC plates of the collected fractions are shown in Fig 2.13. The purity of the fractions can be determined by looking for fractions with only one or two compounds. From these plates, one can also decide which fractions need to be purified further with another column.

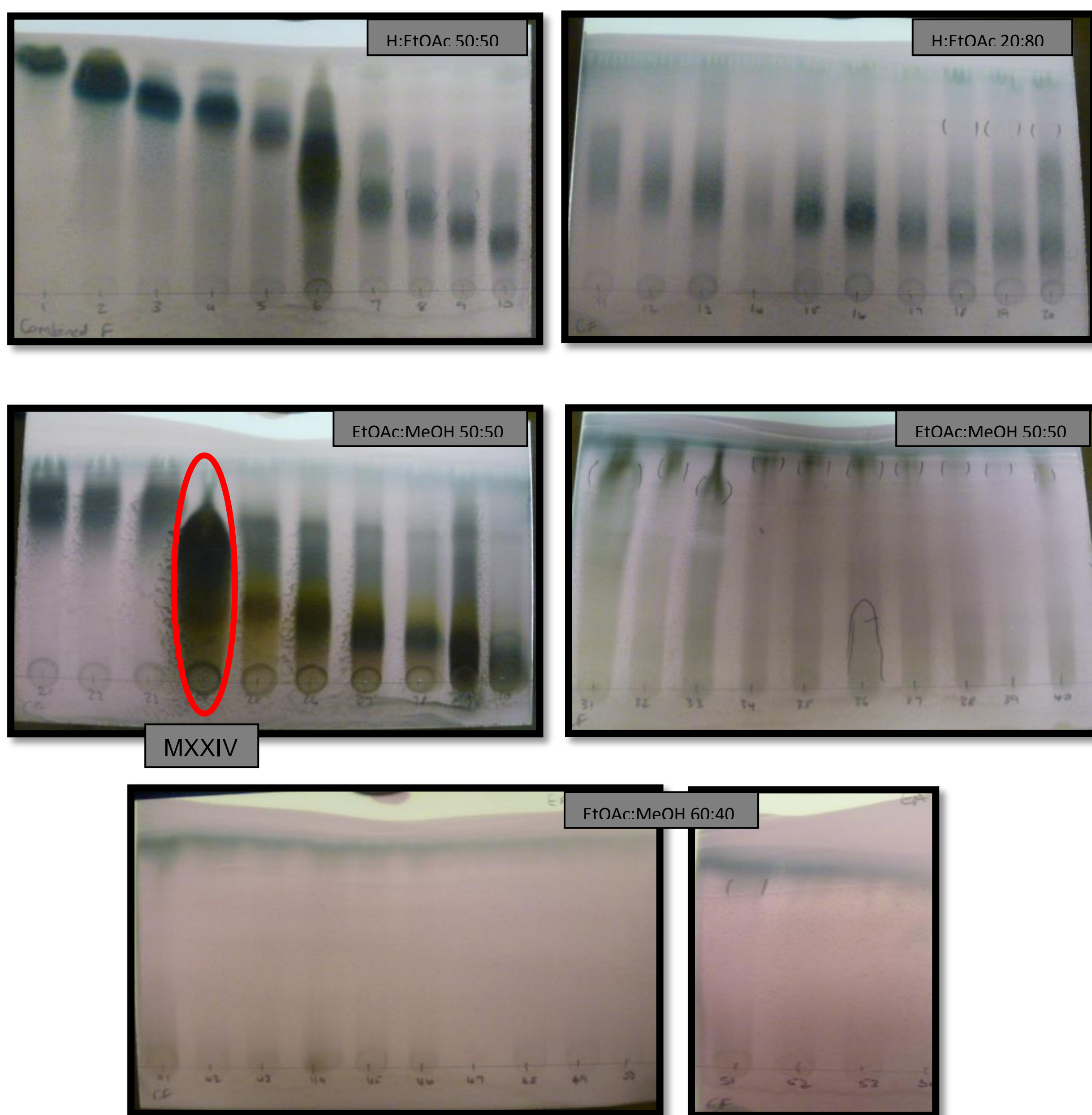

Figure 2.13: TLC plates of the combined similar fractions of methanol extract using hexane, EtOAc and methanol as eluents.

Combined fraction MeXXIV was selected to run a column MeXXIV using a solvent mixture (EtOAc/MeOH) of increasing polarity because it showed interesting antibacterial activty of compounds which can be seperated further. Fig 2.14 shows combined fractions of the column of MeXXIV from which fractions MeXXIV2 to MeXXIV6 were further analysed for bioassay analysis.

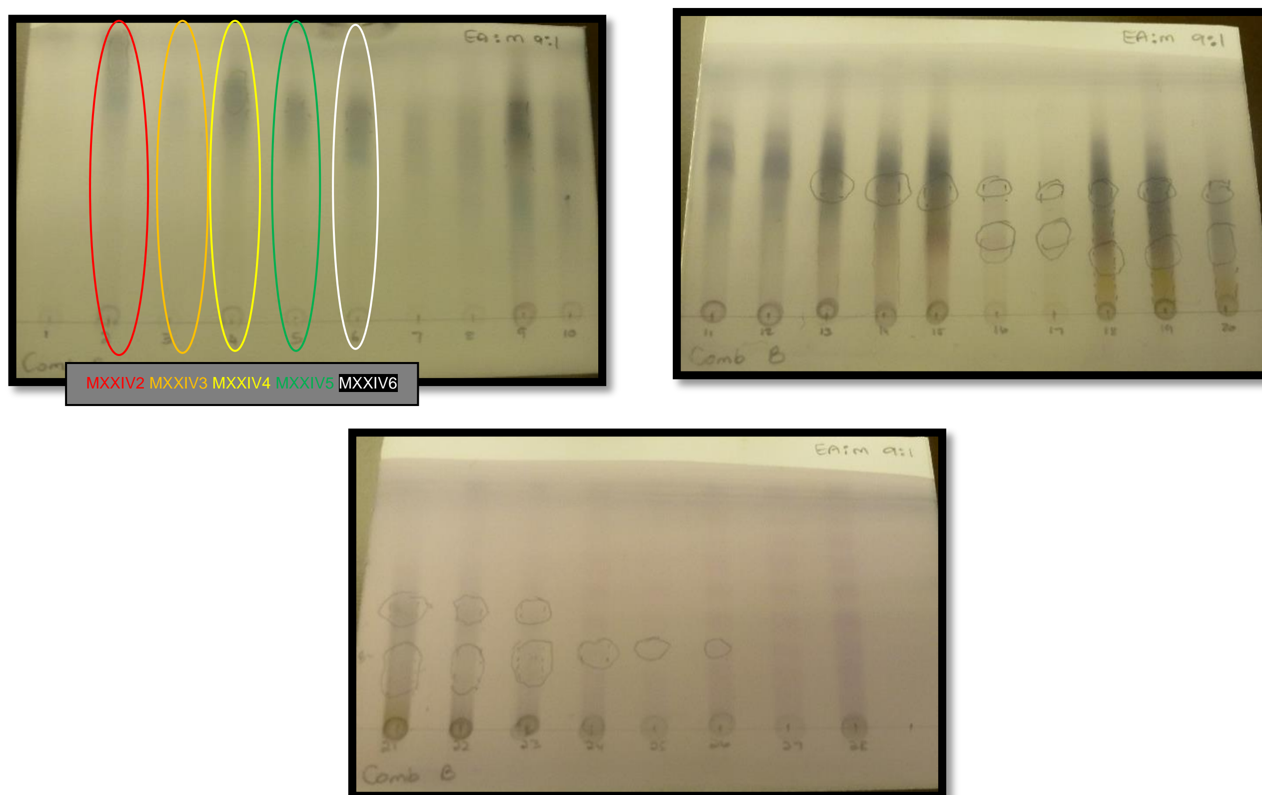

Figure 2.14: TLC plates of combined fractions for column MeXXIV using EtOAc: MeOH (90:10).

Combined fraction MeXXV was also selected for further fractionation. The same solvent mixture used to purify the column of MeXXIV was used for this column because it was a fraction that was collected immediately after combined fraction MeXXIV which also showed good antibacterial activity. Fig 2.15 shows combined fractions of column MeXXV where fraction MeXXV21 and MeXXV22 were further analysed for bioassay analysis.

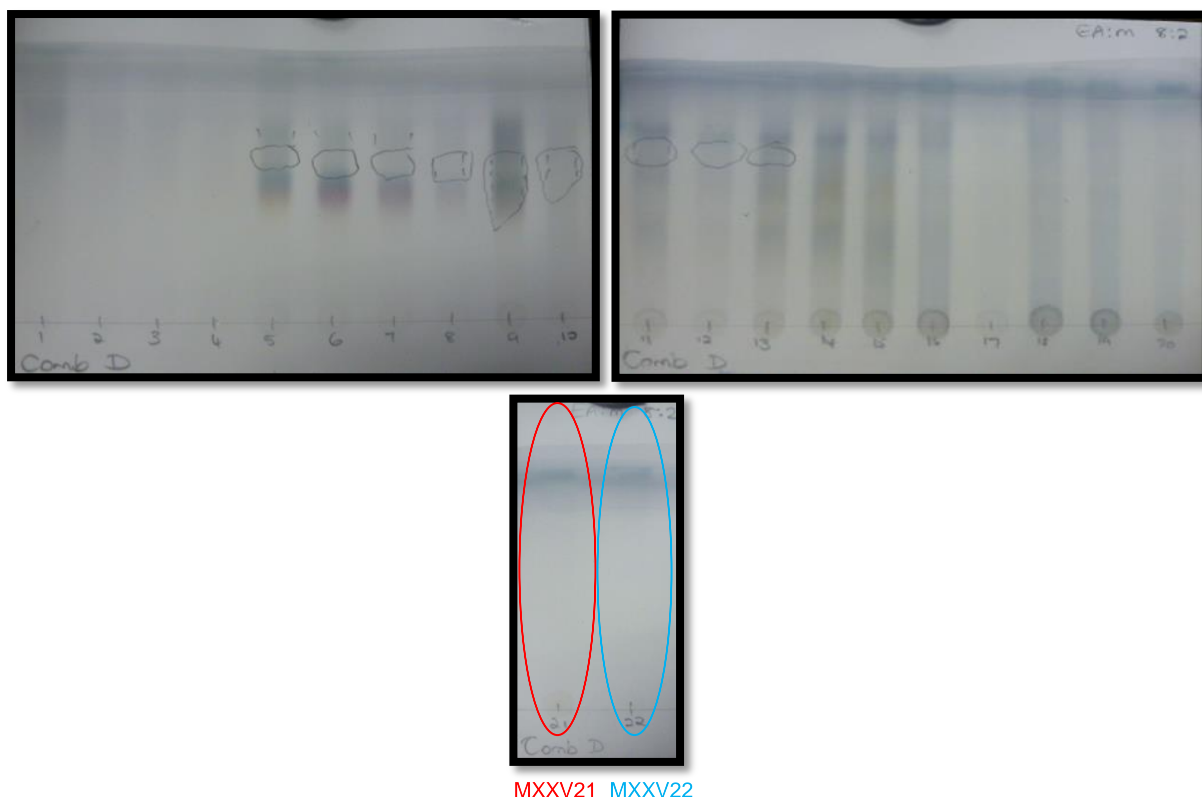

Figure 2.15: TLC plate of combined fractions for column MeXXV using EtOAc: MeOH (80:20).

2.3.3.2. Purification of hexane stem extract

A total of 105 fractions were isolated from the hexane stem extract (Fig 2.16). The fractions that looked similar were combined and concentrated to obtain 28 combined fractions. Combined fractions HeII (1.6 g) (**Compound I**), HeX (50 mg) (**Compound II**), HeXI (550 mg) (**Compound III**) and HeXII (550 mg) (**Compound IV**) from the combined fractions were analysed for purity and their chemical structure determined. Combined fractions HeXVIII and HeXIX were further combined and fractionated to obtain 71 fractions which were also combined according to similar fractions to obtain 14 combined fractions. Combined fraction HeXVIII/HeXIX7 (16 mg) (**Compound V**) was analysed for purity and its chemical structure determined. Combined fraction HeXXIV was further fractionated to obtain 51 fractions which were also combined according to similar fractions to obtain 8 combined fractions. Combined fraction

HeXXIV3 (15 mg) (**Compound VI**) was analysed for purity and its chemical structure determined.

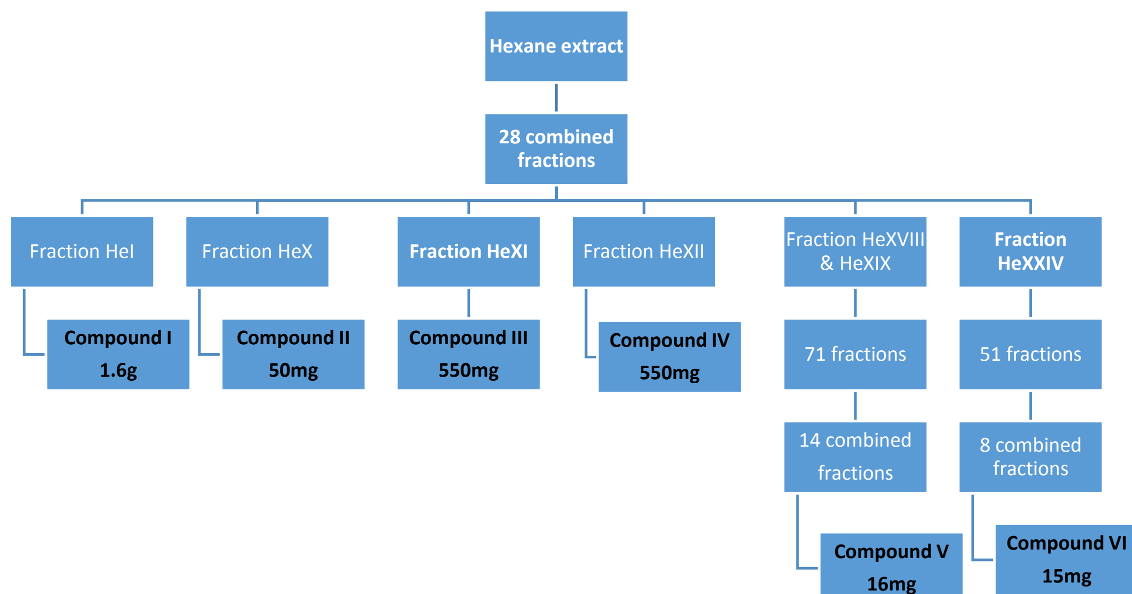

Figure 2.16: A flow diagram showing fractionation process of the hexane stem extract until pure compounds.

Collected fractions were combined and spotted on a TLC plate and their separation is shown if Fig 2.17. From this plate fractions were selected for further fractionation.

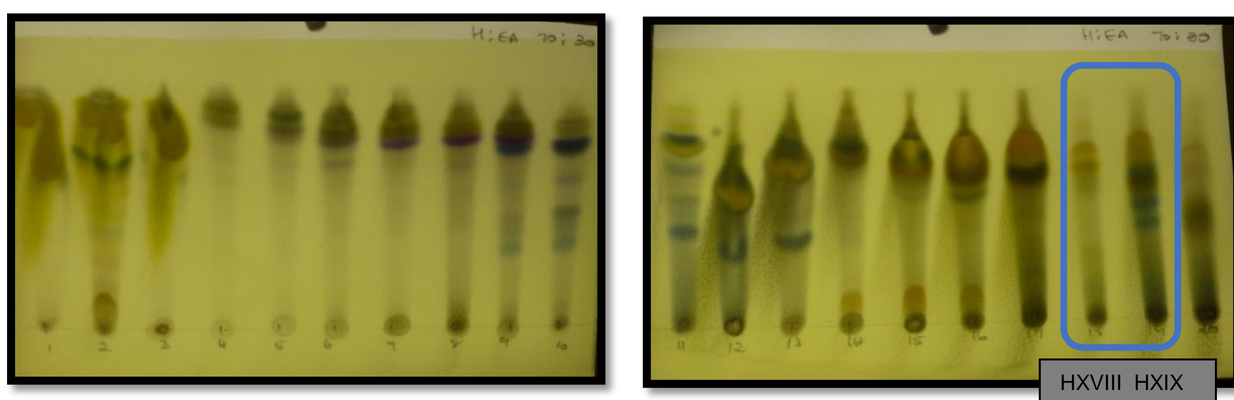

Figure 2.17: TLC plates of combined similar fractions for hexane extract using Hexane: EtOAc (70:30).

Combined fractions HeXVIII and HeXIX were selected to develop column HeXVIII/HeXIX and combined fraction HeXXIV was selected to develop column HeXXIV using solvent mixture (hexane/EtOAc) of increasing polarity because they showed good antibacterial activity. Fig 2.18 shows fractions of column HeXVIII/HeXIX and HeXXIV where combined fraction HXVIII/HeXIX7 and combined fraction HeXXIV3 were further analysed for bioassay.

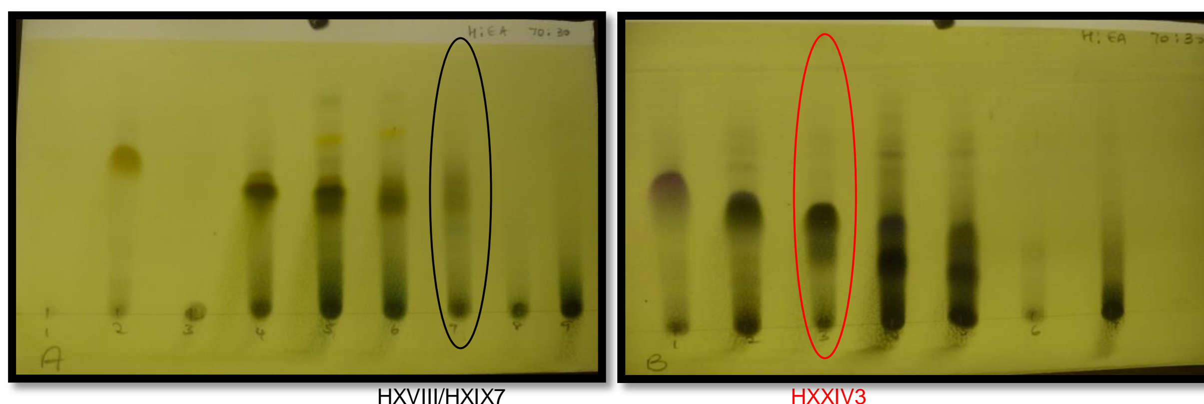

Figure 2.18: TLC plates of combined fractions for column HeXVIII/HeXIX and HeXXIV using Hexane: EtOAc (70:30).

2.4. Conclusions

The extraction of the stem produced a rubbery material which confirms the report that the plant is used in fibre textile industries and also in making rubber (www.godenefibre.com).

The dried latex extract didn't show interesting bands on TLC hence the stem extract was used for this study. The stem extracts showed different compounds with different polarities. A total of seven columns were run from the methanol stem extract. For this study only columns of MeXXIV and MeXXV fractions were discussed. The results of the other five columns will be used for future studies. From the hexane stem extract four columns were run and only column HeXVIII/HeXIX and HeXXIV are discussed.

The bioautography method (Chapter 3) was used to purify and isolated compounds of interest. Only fractions showing significant antibacterial activity

were further chromatographed. A total of five compounds (see Chapter 4) were isolated and their identity need to be confirmed with other methods such as the NMR and their biological activity determined.

2.5. References

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3.1. Introduction

Plants, fruits and vegetables are good sources of a number of bioactive compounds with therapeutic importance (Dewanjee et al., 2015). Natural occurring substances from the plant kingdom have proven to be the most used for treating many diseases since the earliest times known to man. Experiments conducted for drug discovery has succeeded in distinguishing plants that have beneficial effects from those that are toxic or show no effect. About 3 000 plant species are used medicinally in South Africa and they form part of the primary health system (Heyman et al., 2009).

Over 350 natural products, mainly from plant species, which have been used in traditional medicines, have been assessed for their antimicrobial activities. A number of them have been shown to demonstrate significant *in vitro* antimicrobial activities and active plant-derived compounds belonging to various chemical classes have been isolated (Newton et al., 2002).

The ability to treat infectious diseases is threatened by the evolution of antimicrobial resistance in hospitals and the community (Rice, 2009). Bacteria are becoming more resistant to available antibiotics, this is seen with the rapid emergence and spread of multidrug resistant Gram-positive and Gram-negative bacteria with the latter posing the greatest risk to public health worldwide (Hemaiswarya et al., 2008; Kumarasamy et al., 2010; Ventola, 2015). The resistance in Gram-negative bacteria is due to mobile genes on plasmids that can readily spread through bacterial populations and their outer membrane that acts as a barrier to many environmental substances (Aburjai et al., 2001; Palombo and Semple, 2001; Kumarasamy et al., 2010). Many Gram-negative bacteria exist as normal human flora and only become infectious when the immunity is compromised (Kumarasamy et al., 2010).

Bacterial resistance to antibiotics has increased due to the increased use of antibiotics and misuse by patients by overdosing themselves and also lack of new drug development by pharmaceutical companies (Kotze and Eloff, 2002, Ventola, 2015). According to Kotze and Eloff (2002) "this resistance may be unavoidable and is a result of the rate at which bacteria multiply and disperse,

as well as the ease with which their genetic material can change, thus inactivating the curative or remedial treatment, or changing the target area". For this reason there is a need to find new treatments and new antibiotics to fight infections.

Beneficial effects of antibiotics are seen worldwide. They have saved many lives by preventing and treating infections that occur in patients with chronic diseases (renal disease, diabetes, rheumatoid arthritis etc.) and patients who have undergone major surgeries (joint replacement, organ transplant, heart surgery) (Ventola, 2015). In a study by Ventola, 2015, the author listed the following as a cause of the antibiotic resistance crisis: overuse, inappropriate prescribing, extensive agricultural use, availability of few new antibiotics and regulatory barriers.

A large number of antimicrobial agents have been described and are available for use of treatment of many diseases but only a limited number of them are in use and prescribed due to the side effects produced by these agents (Table 3.1).

Table 3.1: Common antibacterial agents and their side effects (Slots and Ting, 2002; Samaranayake, 2006).

Antibiotics	**Side effects**
Clindamycin	Diarrhoea, pseudomembranous colitis
Metronidazole	Nausea, vomiting, transient rashes, metallic state in the mouth
Penicillin	Hypersensitivity (rash), fever, anaphylaxis occurs rarely, nausea, diarrhoea
Tetracycline (doxycycline)	Photosensitivity (sunscreen advised), nausea, diarrhoea, tooth-staining, prolonged use lead to hepatotoxic reaction. Not for children under the age of 13 when still developing teeth
Erythromycins	Nausea, diarrhoea, prolonged use lead to hepatotoxic reaction
Azithromycin	Diarrhoea
Clarithromycin	Diarrhoea, photosensitivity
Fluoroquinolone(ciprofloxacin)	Nausea/vomiting, photosensitivity

3.1.1. Selected bacteria

Gram-negative and Gram-positive bacteria which are commonly found in households, hospitals and the environment were selected for this study (Palombo and Semple, 2001, Natarajan et al., 2005; Parekh et al., 2005; Sudhakar et al., 2006; Ogbulie et al., 2007, Abubakar, 2009; Heyman et al., 2009). They are responsible for many disorders in humans. In recent years

these bacteria have become resistant to treatment using antibiotics. The selected bacteria include:

- *Klebsiella pneumonia*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*
- *Bacillus subtilis*
- *Proteus vulgaris*
- *Proteus mirabilis*
- *Salmonella typhimurium*
- *Achromobacter xyloxidans*

Bacteria are important components of the soil where they can be pathogenic or beneficial to the plants. They can actively colonize or inhabit the plant roots thereby enhancing plant growth and yield. This group of bacteria are known as plant growth promoting rhizobacteria (PGPRs) (Singh, 2003; Saharan and Nehra, 2011; Munees and Mulugeta, 2014). PGPR are beneficial plant bacteria which can be classified into extracellular (ePGPR) or intracellular (iPGPR) plant growth promoting rhizobacteria. The ePGPR are found in the soil around the roots known as the rhizosphere, on the rhizoplane or in the spaces between the cells of root cortex while iPGPR are found inside specialized nodular structures of the root cells (Bhattacharyya and Jha, 2012; Munees and Mulugeta, 2014). Bacterial genera belonging to the ePGPR are *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcus, Pseudomonas, Klebsiella, Enterobacter, Alcaligenes* and *Serratia* etc. and those belonging to the iPGPR are *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium* and *Rhizobium* of the family *Rhizobiaceae* (Singh, 2003; Saharan and Nehra, 2011; Bhattacharyya and Jha, 2012; Munees and Mulugeta, 2014).

PGPRs can also be classified according to their mode of action as biofertilizers, phytostimulators and biopesticides with several of them having overlapping modes of action (Bhattacharyya and Jha, 2012). PGPR promotes plant growth by either direct stimulation which includes nitrogen fixation,

phosphate solubilisation, siderophore production and phytohormone production or by indirect stimulation where PGPRs acts as biocontrol agents or exert antifungal activity (Singh, 2003; Saharan and Nehra, 2011; Bhattacharyya and Jha, 2012; Miransari, 2014; Munees and Mulugeta, 2014).

Several bacteria were previously isolated and identified by students in our Department and included in the study. The bacteria were isolated from soil samples of fairy circles located in Namibia where *E. damarana* grows. Soil samples were collected from inside and outside fairy circles.

Species of *Euphorbia* have been used to cure skin diseases, migraine, gonorrhoea, warts and intestinal parasites. They have been used in Turkish folk medicine for rheumatism, swelling and most importantly to remove warts (Hoe et al., 2006; Barla et al., 2007).

The aim of this chapter was to test for antibacterial activity of the extracts, fractions and compounds isolated (Chapter 2) using known bacteria that are commonly found in hospitals, household and working environment and unknown soil bacteria that were isolated from soil samples where the plant grows. For purpose of this study three methods are used for testing antibacterial activity, namely: bioautography, minimum inhibition concentration and disc diffusion method.

3.2. Materials and methods

Microorganisms were cultured and maintained in nutrient agar in petri dishes (Merck SA (Pty) Ltd.) under aerobic conditions in an incubator at 37°C for 24 to 48 hours. Microorganisms were sub-cultured once a month to keep them alive.

The microorganisms used for this study were:

- *Klebsiella pneumonia*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*
- *Bacillus subtilis*

- *Proteus vulgaris*
- *Proteus mirabilis*
- *Salmonella typhimurium*
- *Achromobacter xyloxidans*
- Soil isolates
  - Isolate 1
  - Isolate 2
  - Isolate 3
  - Isolate 5
  - Isolate 6
  - Isolate 8
  - Isolate 10
  - Isolate 11
  - Isolate 12

Nutrient broth was prepared as per container instruction; 16 g of nutrient broth mixed with 1000 ml of distilled water. The broth was placed in an autoclave at 121°C for 20 minutes. Once the broth has cooled to room temperature, a colony of a bacterium from a culture plate was streaked and inoculated in the broth. The broth was incubated in an incubator (Labotec) at 37°C for 24 hours.

3.2.1. Thin layer chromatography (TLC)

Prepared TLC plates were sprayed with the 24 hours old bacterial solution and incubated in an incubator at 37°C for 24 hours (Dewanjee et al., 2015). After 24 hours incubation the TLC plates were sprayed with INT which was prepared with distilled water using the following concentration: 0.2 mg/ml. The sprayed TLC plates were incubated in an incubator at 37°C for 1 hour (Dewanjee et al., 2015).

3.2.2. Minimum Inhibition Concentration (MIC)

The microplate method developed by Eloff (1998a) was used, with some modifications. Suspensions of bacteria in nutrient broth were standardized to 3 x 10^8 CFU prepared following a McFarland turbidity standard. Fractions were dissolved in 10% DMSO to a final concentration of 20 mg/ml for stem and dried latex extracts and 5 mg/ml for sub-fraction MeXXV21 and sub-fraction MeXXV22. Ciproflaxin at a concentration of 0.05 mg/ml was used as a positive control. The minimum inhibitory concentration (MIC) value of the extracts and fractions against micro-organisms under study were evaluated using the microdilution assay in 96-well microtitre plates. Nutrient broth (100 µl) and fractions or extracts (100 µl) were serially diluted in the 96-well micro-titre plates and 100µl of 24 hours old microorganisms (MacFarlen standard 1) grown at 37°C added to the microtitre plates. Ciproflaxin was added as a positive control. The final concentration of extracts, fractions and positive control ranged from 20.0 mg/ml to 0.156 mg/ml, 5.0mg/ml to 0.0390 mg/ml and 0.05 mg/ml to 0.00039 mg/ml respectively. Following incubation at 37°C for 24 hours, microbial growth was indicated by adding 50µl (0.2 mg/ml) of iodonitrotetrazolium violet (INT) to microtitre plate wells and incubated at 37°C for 1 hour. The MIC was defined as the lowest concentration that inhibited the colour change of INT (Eloff, 1998a).

3.2.3. Disc diffusion assay

The disc diffusion assay method as described by Natarajan et al. (2005) was used with some modifications to determine the growth inhibition of the stem extract, dried latex extract and isolated compounds. Stem and dried latex extracts were dissolved in methanol to a final concentration of 10 mg/ml while the isolated compounds were dissolved in ethyl acetate to a final concentration of 1 mg/ml. Ciproflaxin at a concentration of 1 mg/ml was used as a positive control. Filter paper discs were 5mm in diameter. The discs were each impregnated with 100µl of the stock concentrations. A total of 10 impregnated discs were made for the stem extract (1mg/ml per disc), dried

latex extract (1 mg/ml per disc), isolated compounds (0.1 mg/ml per disc) and Ciproflaxin (0.1 mg/ml per disc). Nutrient agar was prepared as per container instruction of 31 g of nutrient agar mixed with 1000 ml of distilled water. The agar was placed in an autoclave at 121°C for 20 minutes. Once the agar has cooled to lower temperature, it was poured into 90mm diameter petridishes. The petridishes were left to cool to room temperature. 100 µl of the prepared bacterial suspension was streaked on the agar plate with a sterile glass L-shaped rod to cover the whole plate. Impregnated disks were placed on the agar, four disks per petri dish. The agar plates were incubated in an incubator (Labotec (Pty) Ltd) at 37°C for 24 hours and the growth of inhibition zones were recorded.

3.3. Results and discussion

3.3.1. Antibacterial tests

Different microbiological methods are employed for various reasons. TLC bioautography is used often as a first method to screen for the presence or absence of active compounds in a plant extract. It is often more sensitive than other methods. TLC bioautography are widely preferred and employed due to being effective, rapid, cheap and time saving technique to identify bioactive compounds (Silva et al., 2005; Ncube et al., 2008; Shrivastava et al., 2010; Choma and Grzelak, 2011; Dewanjee et al., 2015). They also do not require sophisticated equipment to perform. Results are read visually as the lowest compound concentration that causes zones of inhibition of growth on the TLC plate (Choma and Grzelak, 2011). TLC bioautography is also employed as a preliminary phytochemical screening technique, by bioassay guided fractionation, to detect active components (Shrivastava et al., 2010).

The dilution and diffusion methods are used to estimate the MIC concentration of the compound in the agar or broth suspension (Choma and Grzelak, 2011). Of these two methods; the broth dilution method showed better results that the agar diffusion method.

In a study by Silva et al. (2005), it was established that the TLC bioautography method was the best practical and easy method to perform. They reported that the agar dilution method was more versatile than the broth dilution method and does not present the problems encountered with the sample solution, contamination and MIC determination. They also reported that the agar diffusion method can be used only for pure compounds to get reliable results.

3.3.2. Thin layer chromatography (TLC) plates

Bacterial growth was shown after spraying with INT and incubating for an hour. The INT sprayed TLC plates turned from white to brown-pink with white spots 'appearing' where compounds inhibited bacterial growth on the TLC plates. The white spots indicated that the fractions killed or inhibited growth of the bacteria.

3.3.2.1. Methanol stem extract

In Fig 3.1, several white inhibition zones were observed on TLC plates indicating that the fractions are inhibiting growth of bacteria. *B. subtilis* and *P. vulgaris* were used for these fractions as a screen test to see if activity can be observed. It was noted that there was good inhibition with fractions that were collected with non-polar solvent mixtures (hexane/EtOAc) however with increased polarity there was no inhibition noted with these two solvents (see combined fractions MeXIII to MeXIX). Fractions collected with increased polarity of more polar solvents also showed good inhibition, this is observed in major fractions MeXXV to MeXXXII. All fractions collected after major fraction MeXXXII did not show significant inhibition of the bacteria.

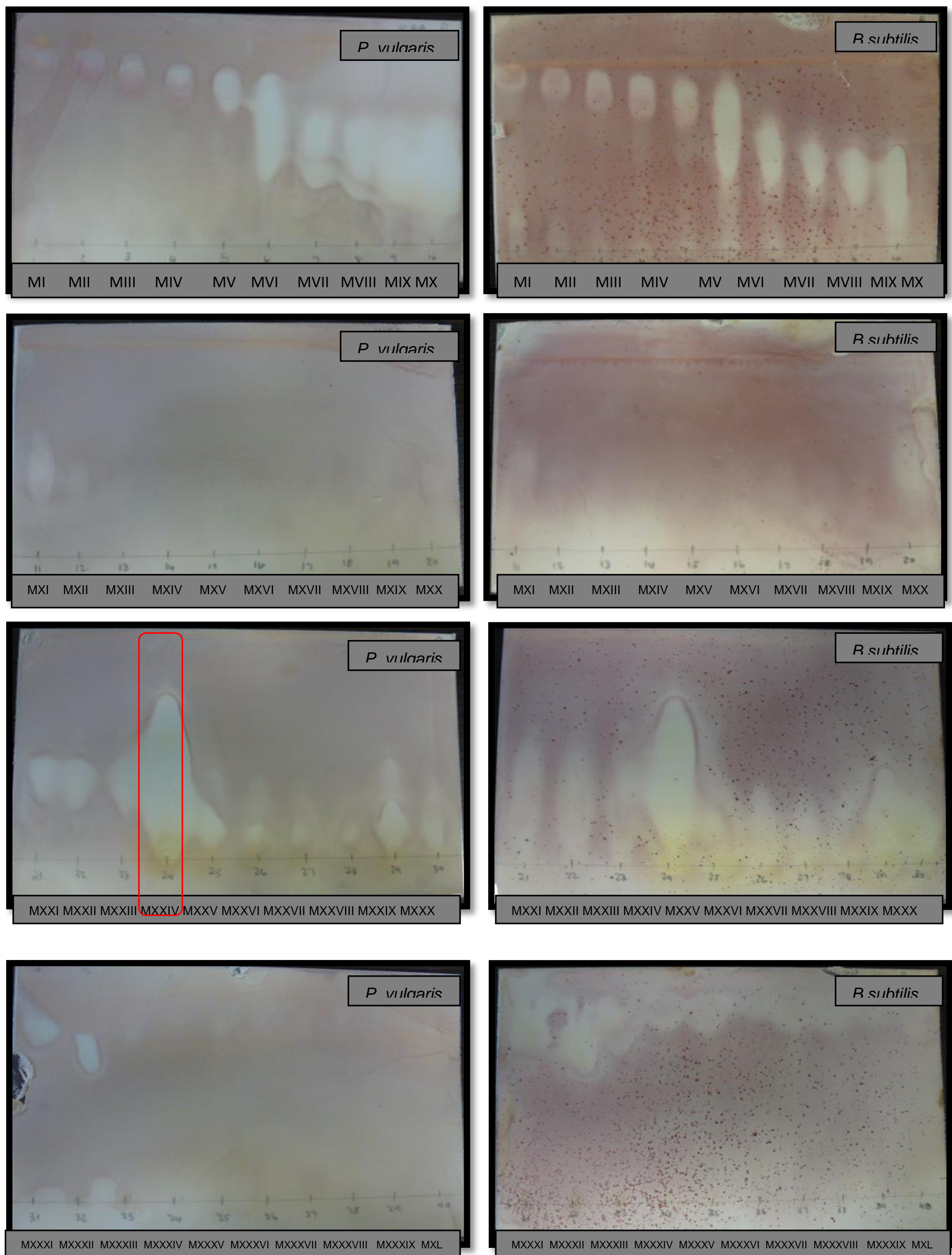

Figure 3.1: Selected TLC plates of fractions after spraying with INT using *P. vulgaris* and *B. subtilis* using hexane: EtOAc (80:20).

Combined fraction MeXXIV from first column which was collected when 100% ethyl acetate was applied to the column was spotted on TLC plates after combining similar fractions together. Fractions were spotted and sprayed with isolate 11 and isolate 12 (Fig 3.2). Combine fractions MeXXIV2, MeXXIV3, MeXXIV4, MeXXIV5 and MeXXIV6 were spotted and sprayed with *B. subtilis, P. vulgaris,* isolate 10 and isolate 11. This was done to see if white zones of inhibition will be observed with other bacteria and also see if the same compounds will inhibit different bacteria . White zones on TLC plate was observed on all fractions indicating that the fractions are good antibacterial agents (Fig 3.3). As mentioned before, the less polar the fractions showed more inhibition of the bacteria is observed. This was again observed on the TLC plates in Fig 3.2 and 3.3.

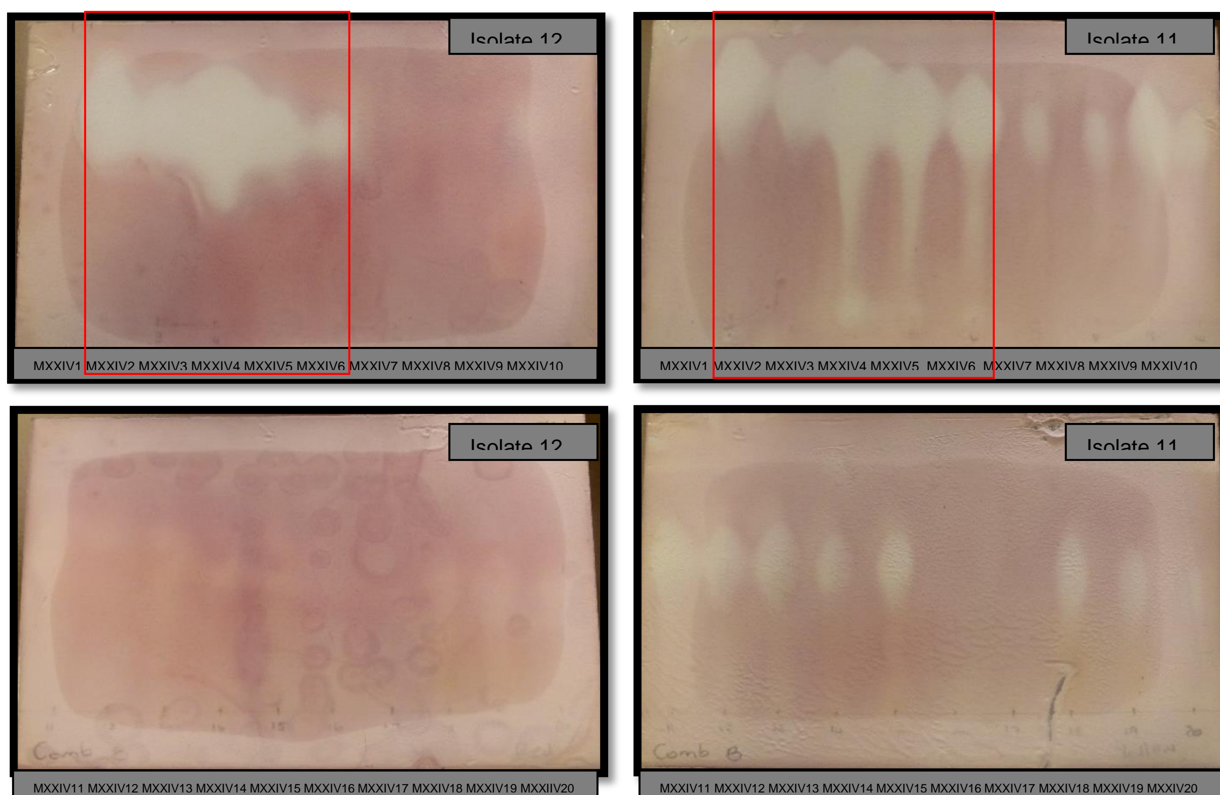

Figure 3.2: TLC plates of column MeXXIV fractions after spraying with INT using isolate 11 and isolate 12.

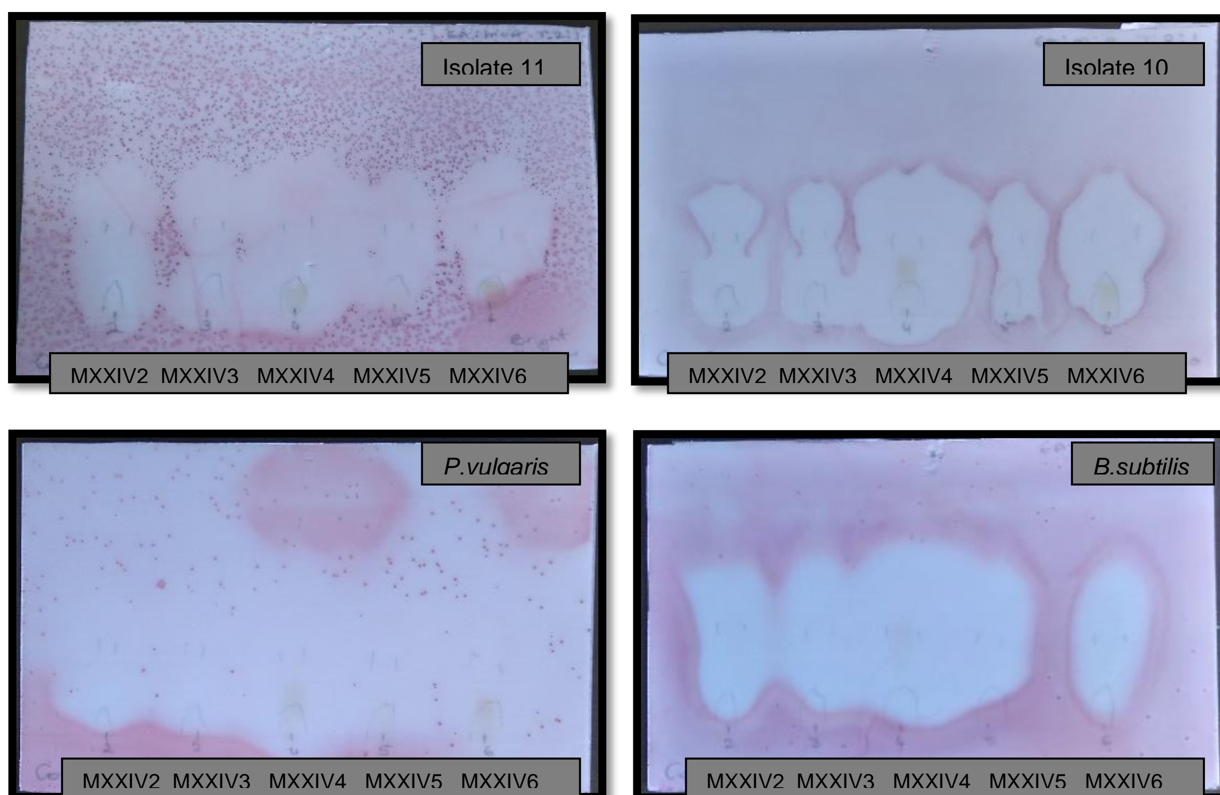

Figure 3.3: TLC plates of column MeXXIV combined fractions MeXXIV2 to MeXXIV6 after spraying with INT using *P. vulgaris, B. subtilis*, isolate 10 and isolate 11.

Combined fraction MeXXV from the first column which was collected when 100% ethyl acetate was applied to the column was spotted on TLC plates after combining similar fractions together. Only isolate 11 and isolate 12 were tested. White zones on the TLC plate was observed of fractions MeXXV21 and MeXXV22, indicating that the fractions have some antibacterial activity (Fig 3.4).

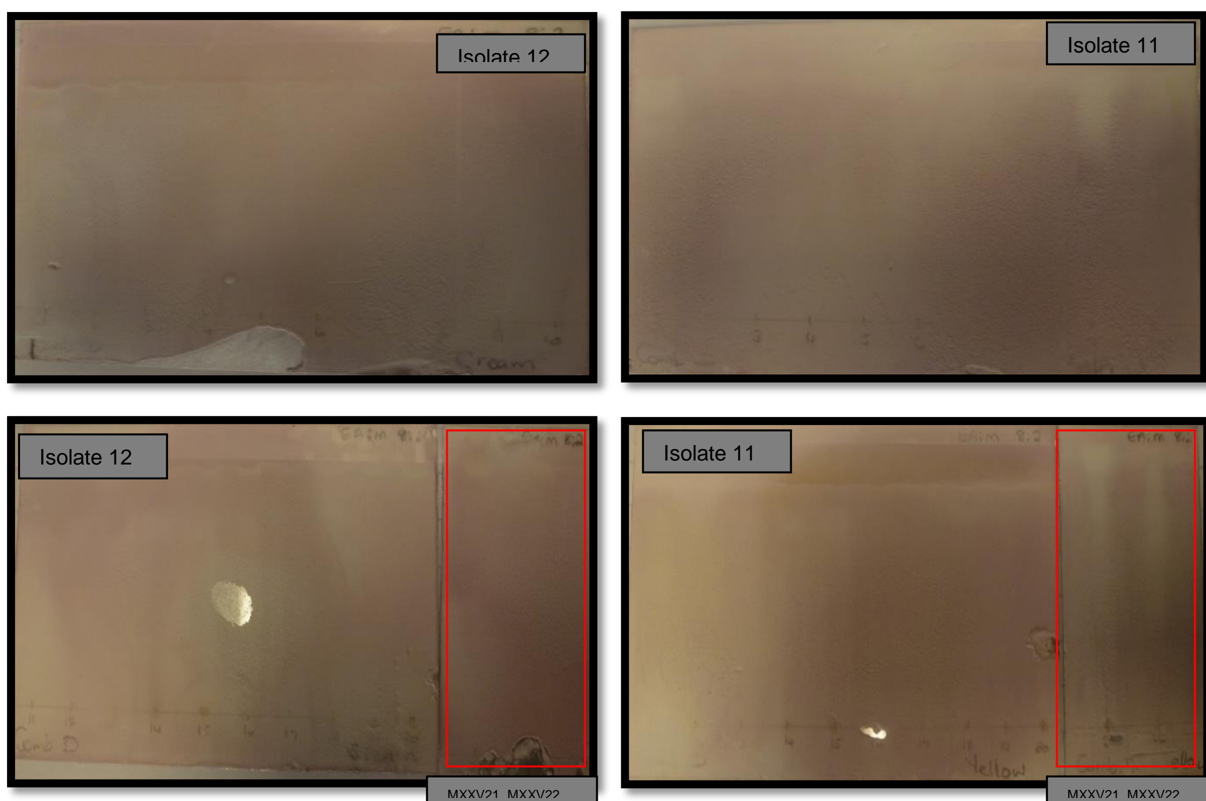

Figure 3.4: TLC plates of column MeXXV combined fractions MeXXV21 to MeXXV22 after spraying with INT using isolate 11 and isolate 12.

3.3.2.2. Hexane stem extract

In Fig 3.5, inhibition zones were observed on TLC plates indicating that the fractions are inhibiting growth of bacteria. In addition to *B. subtilis* and *P. vulgaris*, isolate 10 and isolate 11 were used to screen test the fractions. White zones of bacterial inhibition were observed with fractions collected with non-polar solvents mixtures (hexane/EtOAc). As mentioned earlier in this chapter, the less polar the solvents the more antibacterial activity is observed. The inhibition pattern was similar for all the bacteria.

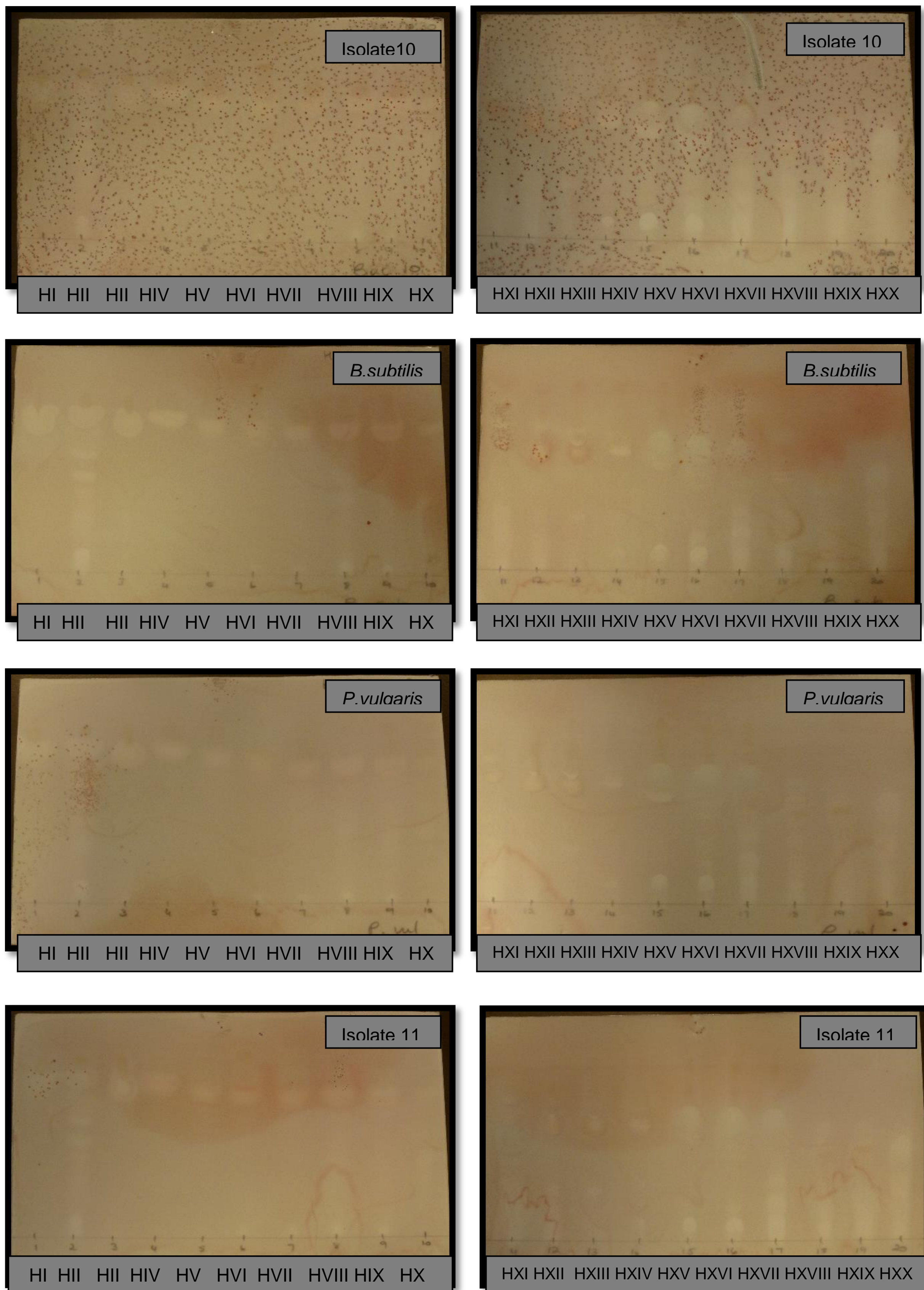

Figure 3.5: TLC plates of fractions HeI to HeXX after spraying with INT using *P. vulgaris, B. subtilis*, isolate 10 and isolate 11.

Combined fractions HeXVIII and HeXIX from the first column which was collected when 30% ethyl acetate and 70% hexane was applied to the column was spotted on TLC plates after combining similar fractions together. *B. subtilis* and *P. vulgaris*, isolate 10 and isolate 11 were used to test for activity. White zones on TLC plates were observed on combined fraction HeXVIII/HeXIX4, HeXVIII/HeXIX5 and HeXVIII/HeXIX6 indicating that the fractions have some antibacterial avtivity (Fig 3.6).

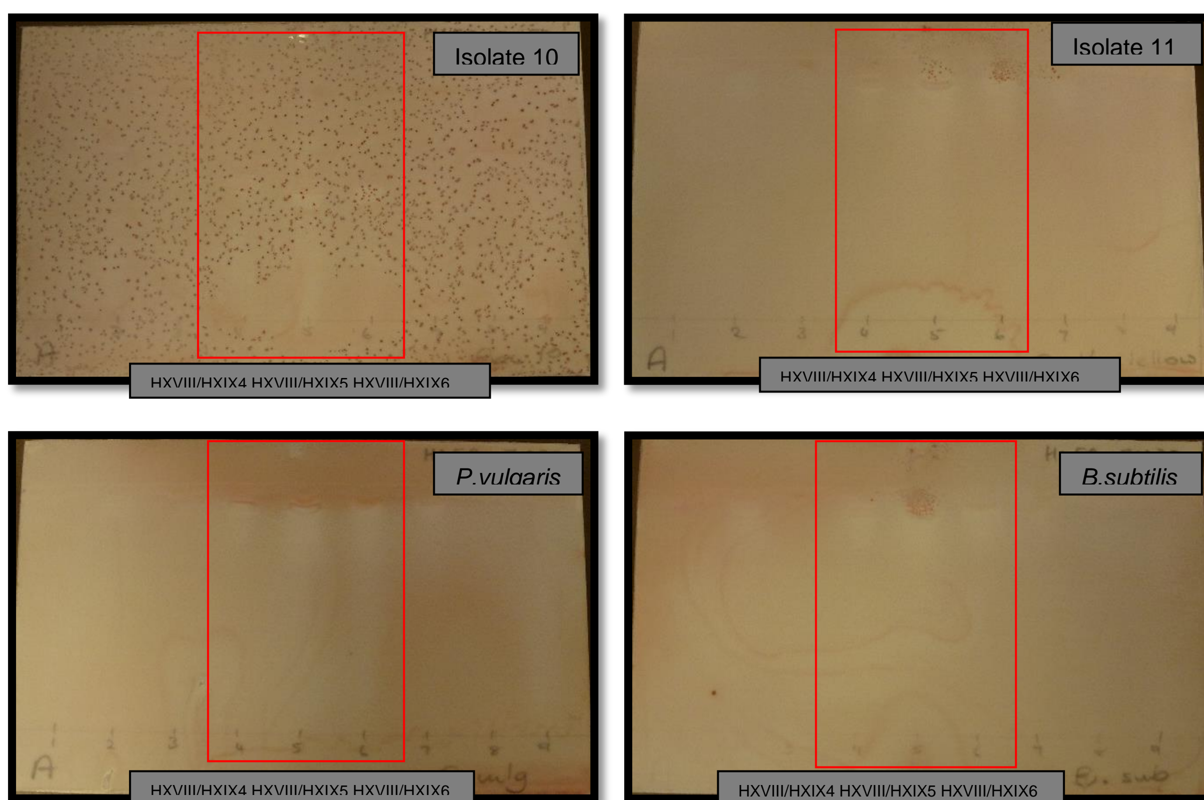

Figure 3.6: TLC plates of column HeXVIII/HeXIX combined fractions HeXVIII/HeXIX1 to HeXVIII/HeXIX9 after spraying with INT using *P. vulgaris, B. subtilis*, isolate 10 and isolate 11.

Combined fraction HeXXIV from the first column which was collected when 50% ethyl acetate and 50% hexane was applied to the column was spotted on TLC plates after combining similar fractions together. The same bacteria as in column HeXVIII/HeXIX were tested (*B. subtilis* and *P. vulgaris*, isolate 10 and

isolate 11) for activity. White zones on TLC plates were observed on all fractions indicating that the fractions have some antibacterial activity (Fig 3.7).

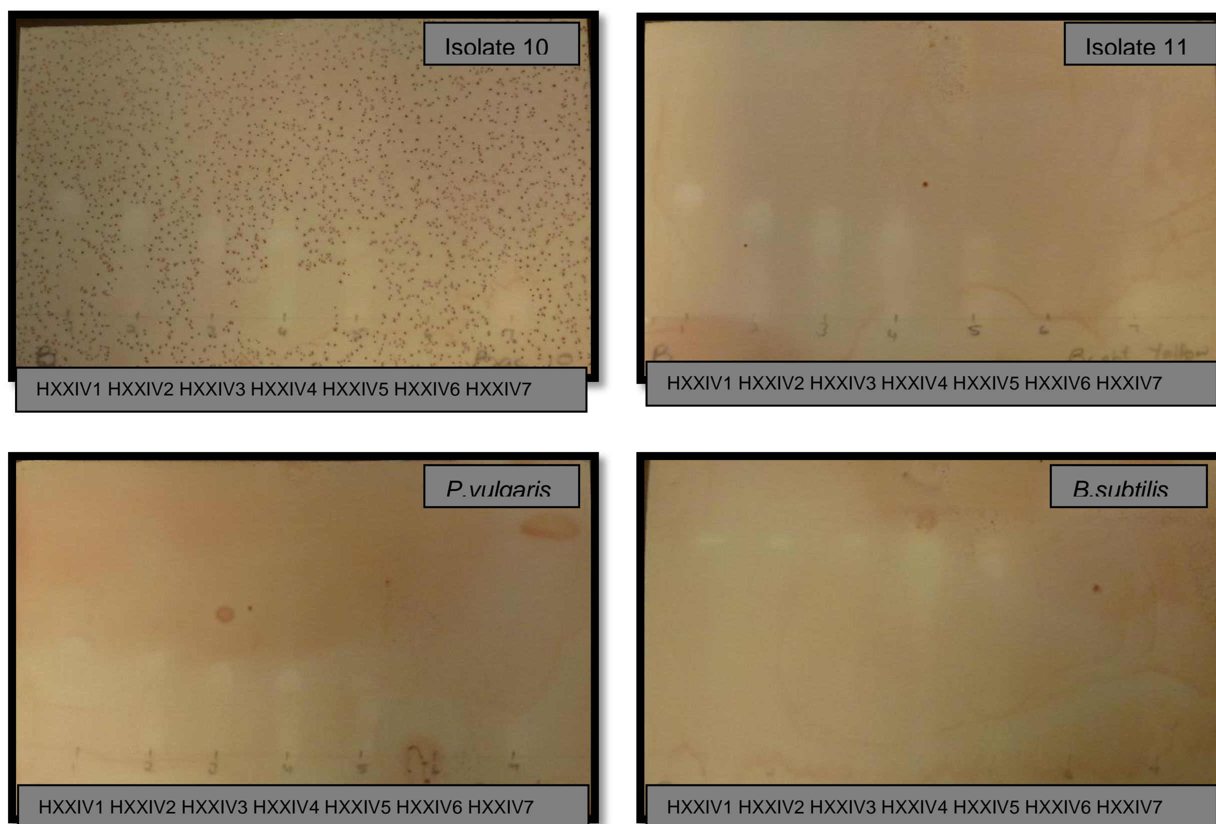

Figure 3.7: TLC plates of column HXXIV combine fractions HXXIV1 to HXXIV7 after spraying with INT using *P. vulgaris, B. subtilis*, isolate 10 and isolate 11.

3.3.3. Minimum Inhibition Concentration (MIC)

The control plates (Figs 3.8 and 3.13) gave the expected results (although the bacteria didn't grow well as seen in Fig 3.13 with isolate 8 indicating that the test results are reliable and accurate. Ciproflaxin, a positive control was used at a starting concentration 0.2 µg/ml (Fig 3.13) and it inhibited bacteria in all the wells. It was reduced to a starting concentration of 0.05 µg/ml (Fig 3.8) with the testing of other fractions and compounds. Fig 3.9 to 3.12 and Fig 3.14 show microtiter plates of bacteria tested with plant extracts and fractions. The results are summarized in table Table 3.2. The antibacterial activity of the

plant extracts and the fractions tested against the bacteria revealed good antibacterial activity. The minimum inhibitory concentration of the plant extracts and fractions was determined. The stem extract showed significant activity against isolate 11 (0.312 mg/ml) and good activity against the following bacteria: *P. aeruginosa* (1.25 mg/ml), *B. subtilis* (1.25 mg/ml), isolate 5 (2.5 mg/ml) and isolate 10 (2.5 mg/ml). The dried latex extract showed moderate activity with isolate 11 (5.00 mg/ml), *P. aeruginosa* (5.00 mg/ml) and *B. subtilis* (5.0 mg/ml). Combined fractions MeXXV21 and MeXXV22 good activity was obtained with isolate 11 (2.5 mg/ml). There was no activity with the other bacteria.

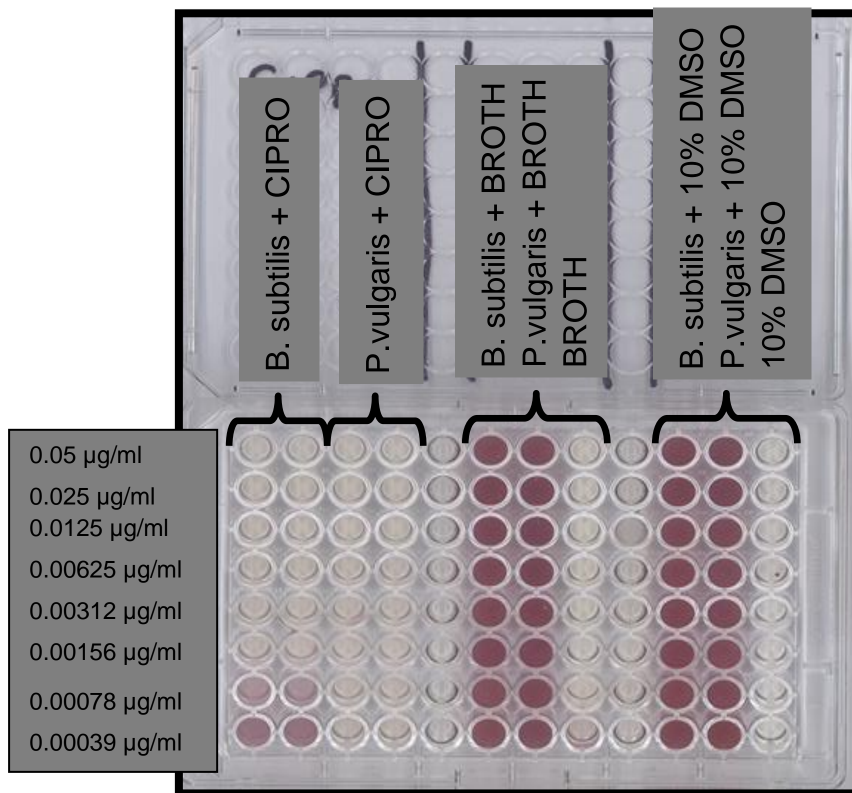

Figure 3.8: MIC of control plate.

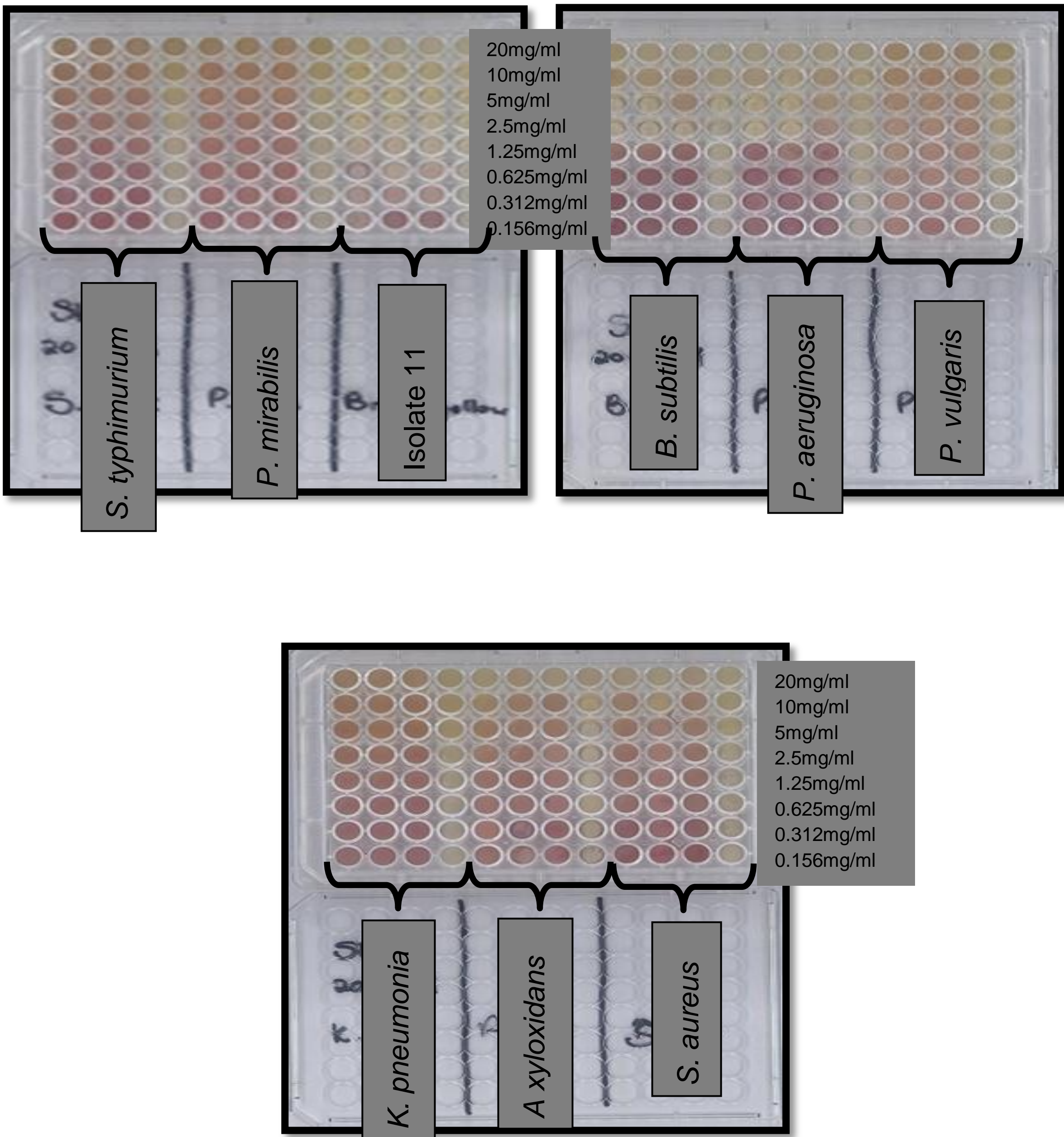

Figure 3.9: MIC plates of methanolic stem extract with the following bacteria: *S. typhimurium, P. mirabilis*, isolate 11, *B. subtilis, P. aeruginosa, P. vulgaris*, *K. pneumonia, A. xyloxidans* and *S. aureus.*

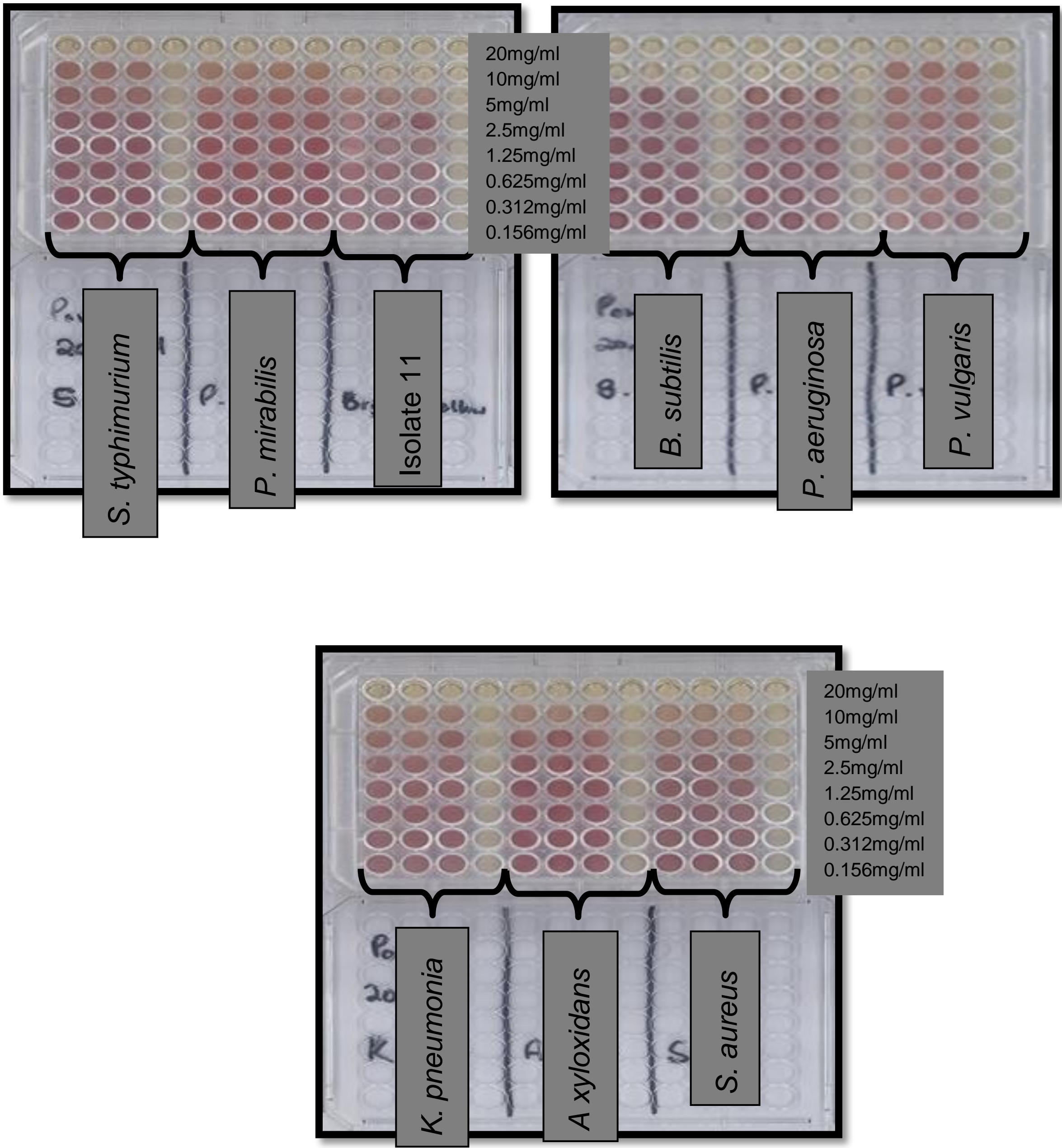

Figure 3.10: MIC plates of methanolic dried latex extract with the following bacteria: *S. typhimurium, P. mirabilis*, isolate 11, *B. subtilis, P. aeruginosa, P. vulgaris*, *K. pneumonia, A. xyloxidans* and *S. aureus.*

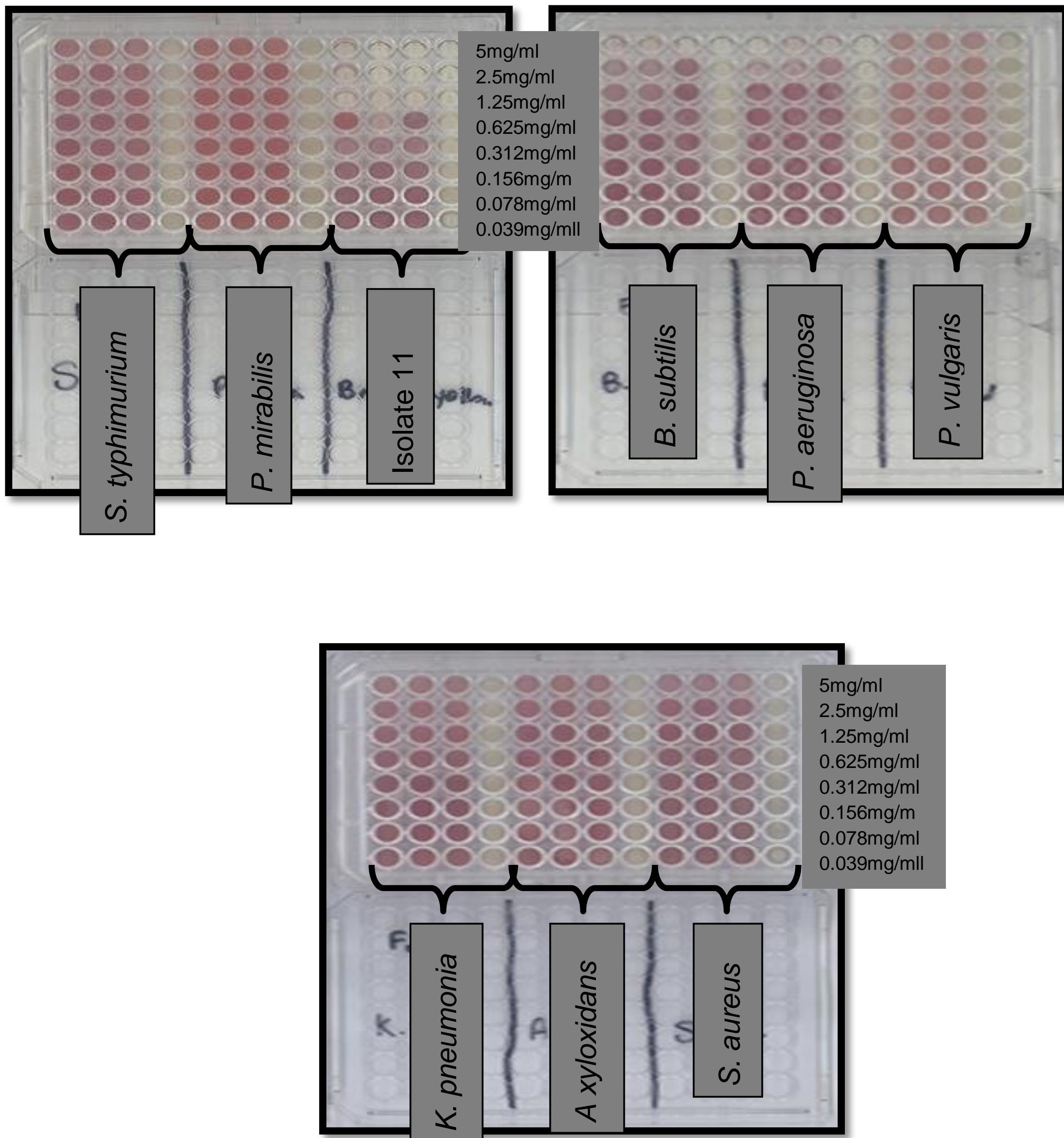

Figure 3.11: MIC plates of combined fraction HeXXV21 (F21) with the following bacteria: *S. typhimurium, P. mirabilis*, isolate 11, *B. subtilis, P. aeruginosa, P. vulgaris*, *K. pneumonia, A xyloxidans* and *S. aureus.*

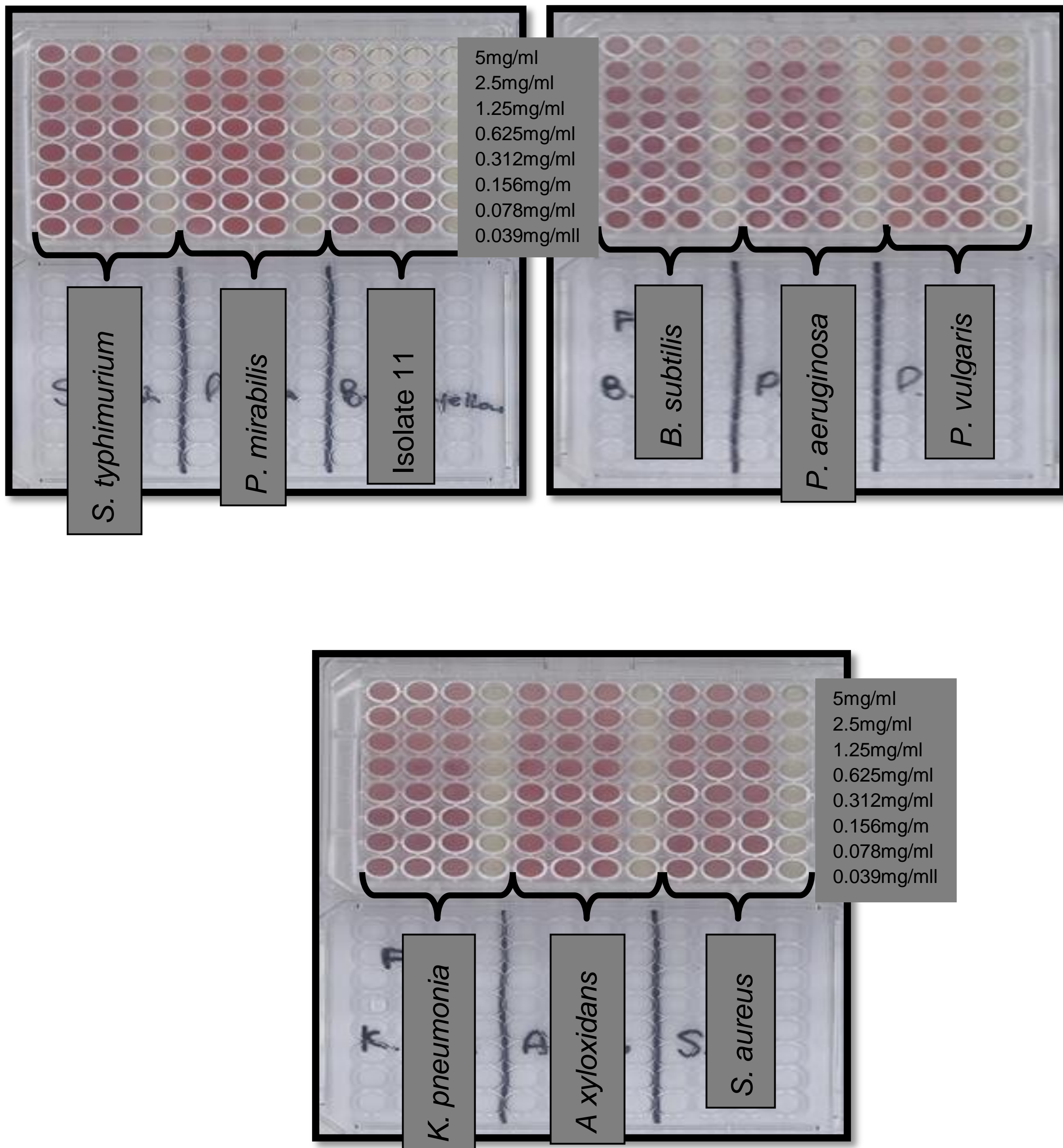

Figure 3.12: MIC plate of combined fraction HeXXV22 (F22) with the following bacteria: *S. typhimurium, P. mirabilis*, isolate 11, *B. subtilis, P. aeruginosa, P. vulgaris*, *K. pneumonia, A xyloxidans* and *S. aureus.*

The number of bacteria was reduced when testing the purified compounds isolated from the hexane stem extract. This was done because when testing for antibacterial activity against the stem and dried latex extract some of the soil bacteria didn't grow properly in the broth medium since the nutrient broth medium used did not support their growth, see isolate 6 and isolate 8 (Fig 3.14). Purified isolated compounds were tested against *B. subtilis, S. aureus, P. vulgaris, P. aeruginosa*, isolate 1, isolate 2, isolate 3, isolate 5, isolate 10 and isolate 11. The selected known bacteria are commonly found in households, hospitals, schools and many other places. Many studies have been conducted against them (Palombo and Semple, 2001, Natarajan et al., 2005; Parekh et al., 2005; Sudhakar et al., 2006; Ogbulie et al., 2007, Abubakar, 2009; Heyman et al., 2009).

The compounds were first tested at a starting concentration of 1mg/ml and repeated at a starting concentration of 10mg/ml. No activity was observed for all the compounds against the bacteria tested. The compounds are non-polar; this makes them hydrophobic and they do not dissolve completely in a polar broth medium. The disc diffusion assay was used to further determine the growth inhibition of the compounds since no activity was observed with the dilution assay.

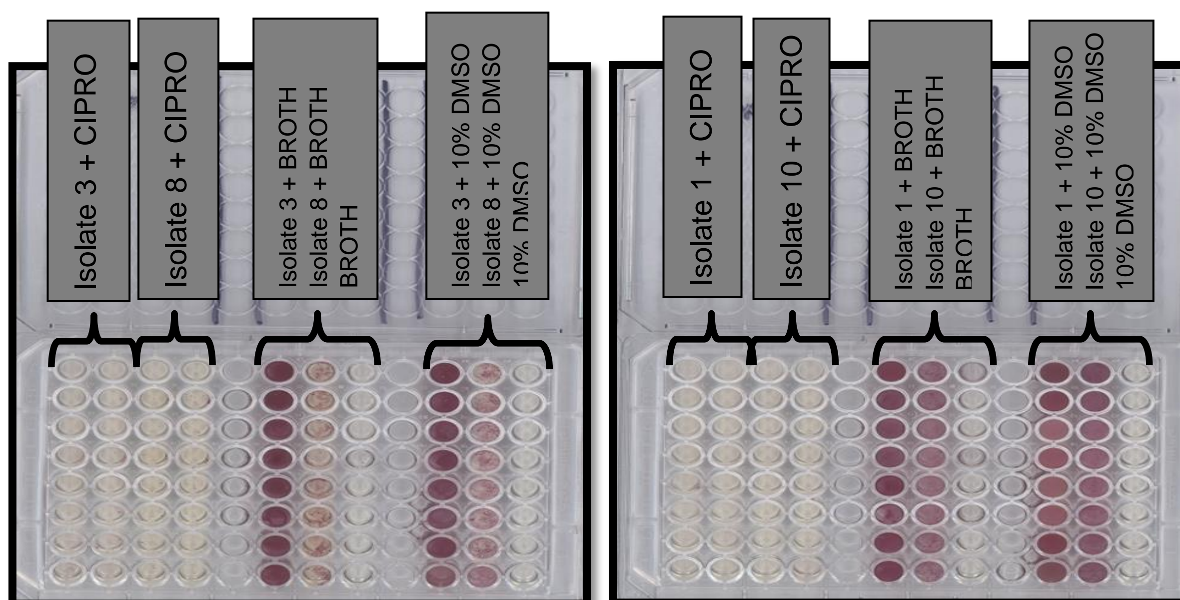

Figure 3.13: MIC of control plate.

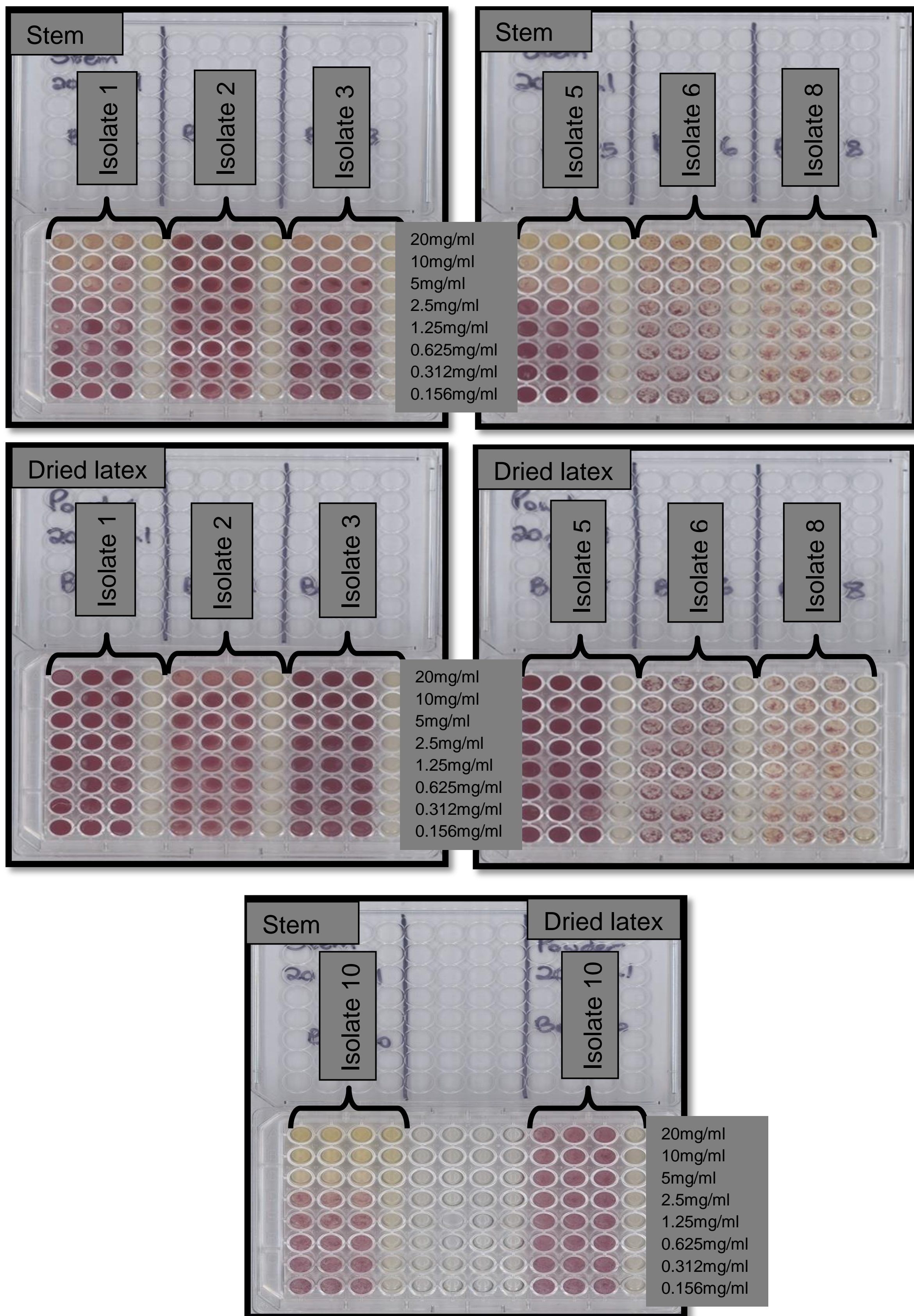

Figure 3.14: MIC plates of stem and dried latex extracts of isolate 1, isolate 2, isolate 3, isolate 5, isolate 6, isolate 8 and isolate 10.

Table 3.2: MIC of stem and dried latex extracts of combined fractions MeXXV21 and MeXXV22 against 16 bacteria

BACTERIA	MIC (mg/ml)			
	Stem extract	**Dried latex extract**	**Fraction MeXXV21 (F21)**	**Fraction MeXXV22 (F22)**
Klebsiella pneumonia	>20.00	10.00	> 20.00	> 20.00
Pseudomona aeruginosa	1.25	5.00	10.00	>20.00
Staphylococcus aureus	10.00	10.00	> 20.00	> 20.00
Bacillus subtilis	1.25	5.00	10.00	> 20.00
Proteus vulgaris	5.00	10.00	> 20.00	> 20.00
Proteus mirabilis	10.00	10.00	> 20.00	> 20.00
Salmonella typhimurium	10.00	10.00	> 20.00	> 20.00
Achromobacter xyloxidans	10.00	10.00	> 20.00	> 20.00
Isolate 1	10.00	> 20.00	> 20.00	> 20.00
Isolate 2	> 20.00	> 20.00	> 20.00	> 20.00
Isolate 3	10.00	> 20.00	> 20.00	> 20.00
Isolate 5	2.50	> 20.00	> 20.00	> 20.00
Isolate 6	> 20.00	> 20.00	> 20.00	> 20.00
Isolate 8	> 20.00	> 20.00	> 20.00	> 20.00
Isolate 10	2.50	> 20.00	> 20.00	> 20.00
Isolate 11	0.3125	5.00	2.50	2.50

In this study, the results obtained showed that the methanol stem extract of *E. damarana* inhibited the growth of the tested bacteria, *B. subtilis, P. aeruginosa*, isolate 5, isolate 10 and isolate 11. This shows that the extract contains substances that can inhibit the growth of some bacteria. Heyman et al. (2009) tested the ethanol extract of *E. damarana* and other plants against methicillin sensitive *S. aureus* and reported that *E. damarana* inhibited the bacterium at an MIC of 6.25mg/ml. While Ogbulie et al. (2007) reported on the MIC assay method of ethanol extract of *E. hirta* against *B. subtilis, S. aureus* and *P. aeruginosa*. The MIC of the bacteria was reported as 74.61mg/ml for *B. subtilis*, 57.64mg/ml for *P. aeruginosa* and 22.55mg/ml for *S. aureus*. Other studies reported on the antibacterial activity of methanol leaf extract of *E. hirta* against *S. aureus* and *P. mirabilis* with an MIC value of 12.50mg/ml and 50.00mg/ml respectively (Rajeh et al., 2010).

3.3.4. Disc diffusion test

The test showed inhibition of the stem and dried latex extracts against the bacteria tested. The inhibition zones were not significant as compared to that of the positive control (Ciproflaxin) (Fig 3.15 to 3.17). The test did not show significant antibacterial activity of isolated compounds and was only active against two of the ten bacteria tested. It could be that because of the hydrophobicity of the compounds they didn't diffuse completely in the medium. A small zone of inhibition around fractions HeXI (49 – 51) and HeXII (52 – 54) against *P. aeruginosa* (Fig 3.18) was observed while a poor zone of inhibition around fraction HeXI (49 – 51) and HeXII (52 – 54) was observed for isolate 3 (Fig 3.19). This indicates that the fractions are inhibiting the growth of the bacterium.

Although activity with *E. damarana* was not significant with the disc diffusion method several reports of activity were obtained from other *Euphorbia* species. In a study by Natarajan et al. (2005), they assayed the antibacterial activity of *E. fusiformis* extracts using different solvents with the disc diffusion method. They reported that the methanol and aqueous extract showed no

activity against *B. subtilis, K. pneumonia* and *P. vulgaris*. The ethanol extract showed poor inhibition against *P. vulgaris* and no activity against *B. subtilis, P. aeruginosa* and *S. aureus*. Interestingly, chloroform extract showed activity against the *P. aeruginosa* and *S. aureus*. In another study using the disc diffusion method the methanol extracts of *E. hirta* and *E. tirucalli* showed activity against *S. epidermidis, B. subtilis, P. pseudoalcaligenes, S. typhi* and *P. vulgaris* while the aqueous extract of *E. hirta* showed activity against *P. vulgaris, S. typhi* and *P. pseudoalcaligenes* and that of *E. tirucalli* showed activity against *P. pseudoalcaligenes* only (Parekh et al., 2005).

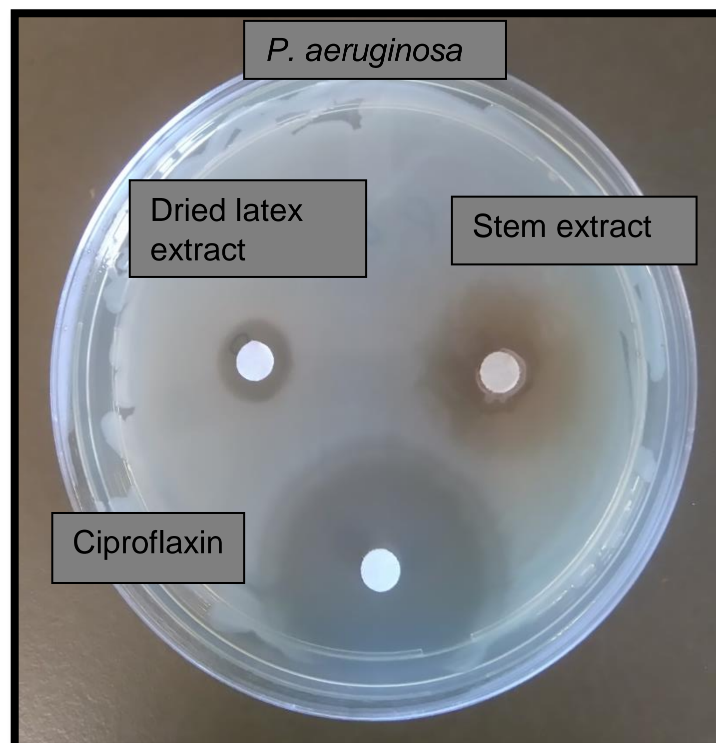

Figure 3.15: Disc diffusion test of the stem and dried latex extracts against *P. aeruginosa.*

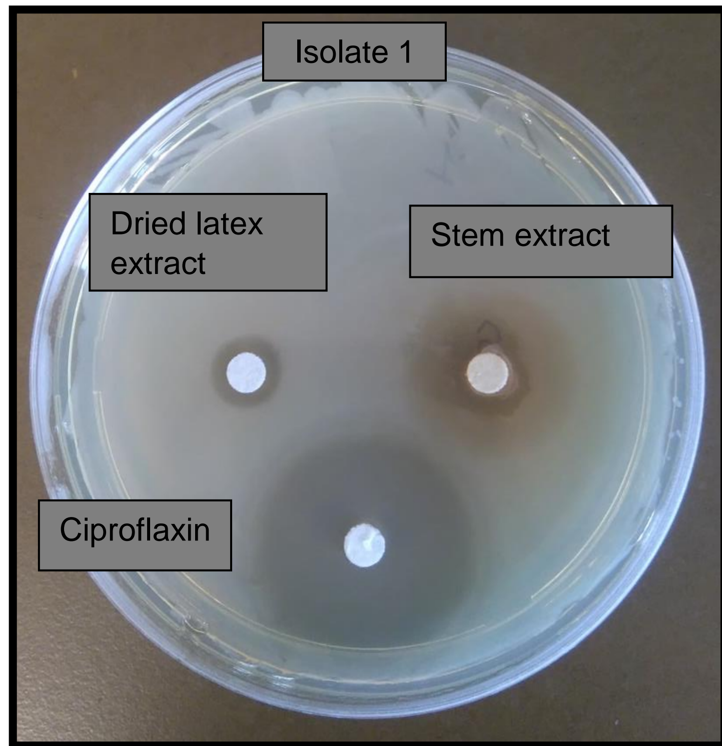

Figure 3.16: Disc diffusion test of the stem and dried latex extracts against isolate 1.

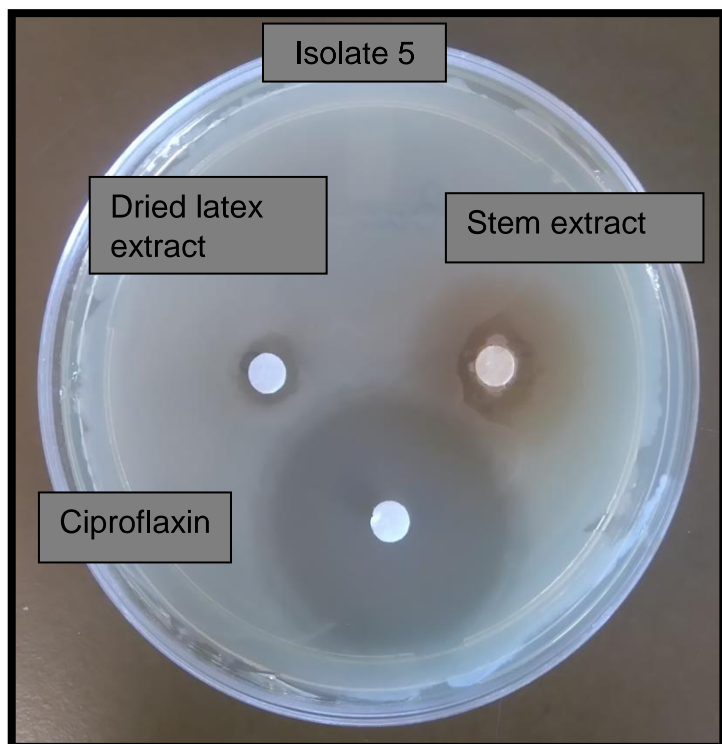

Figure 3.17: Disc diffusion test of the stem and dried latex extracts against isolate 5.

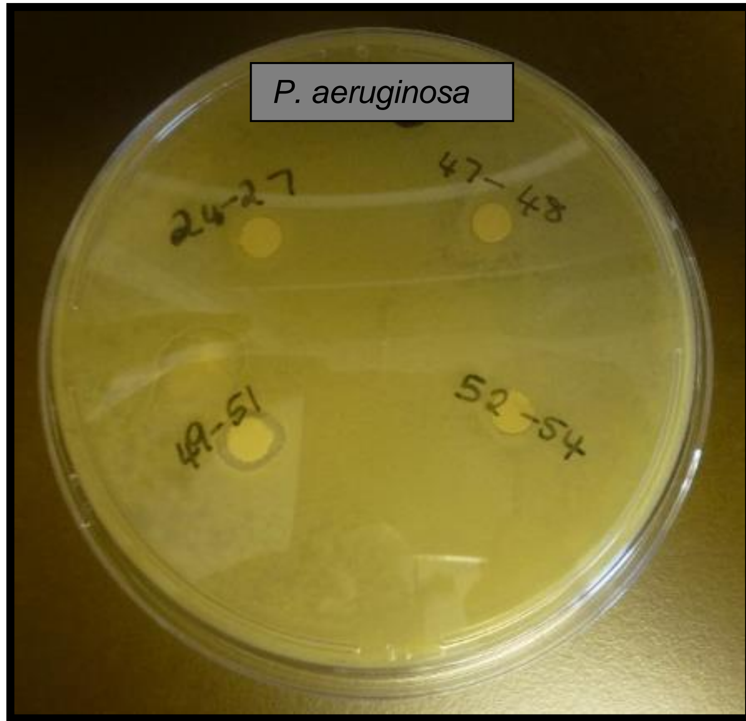

Figure 3.18: Disc diffusion test of isolated fractions against *P. aeruginosa.*

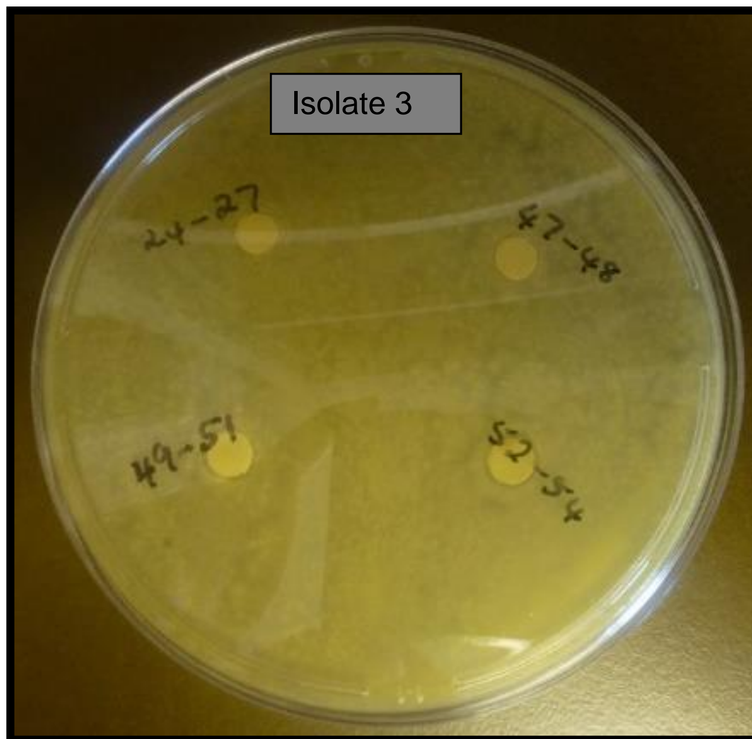

Figure 3.19: Disc diffusion test of isolated fractions against isolate 3.

There are several reports of antibacterial activity of other *Euphorbia* species using the agar well diffusion method. Madureira et al. (2003) did a study on screening antibacterial activity for *E. segetalis* using the bioautographic agar overlay assay, with *S. aureus*. They found that the crude extract was active at a concentration of 0.7mg/ml, with a starting concentration of 6mg/ml. The pure compounds which they isolated did not reveal significant activity. Palombo and Semple (2001), reported activity against *E. coli, K. pneumonia, P. aeruginosa* and *S. typhimurium* and no activity against *B. cereus, E. faecalis, S. aureus* and *S. pyogenes* using the ethanol extract of *E. australis*. The hexane, methanol, ethanol and aqueous extracts of *E. hirta* showed activity against *E. coli, P. mirabilis, S. dysentriae, S. typhi* and *K. pneumonia* with the aqueous extract displaying larger zones of growth inhibition than other extracts (Abubakar, 2009) and activity against *P. vulgaris, S, aureus, P. aeriginosa* and *B. subtilis* (Sudhakar et al., 2006).

The results obtained with the three antibacterial methods indicate that *E. damarana* possess compounds which have shown good to moderate antibacterial activity. Soil bacteria have been used to test for antibacterial activity, it was shown that the PGPRs found in the soil where *E. damarana* was growing were inhibited by the plant. In this regard it can be hypothesized that the plant inhibited the beneficial bacteria that are needed for the survival of grass species in the desert. In this study, small concentrations of the plant extract were tested and in the desert huge amount of different compounds are released by the dying plants into the surrounding soil. The plant grows in a region with low rain fall which also affect the survival of the plant when the beneficial bacteria are absent.

3.4. Conclusions

A large number of compounds have been isolated from *Euphorbia* species, these include skin irritants, proinflammatory and tumour promoting compounds (Gewali et al., 1990; Barla et al., 2007; Baloch et al., 2007; Prachayasittikul et al., 2010).

The TLC bioautography screening method was the first method applied to detect the antibacterial activity using the plant extracts and fractions. The results obtained indicate that the TLC bioautography method is a more sensitive, easy to perform and a reproducible test while the broth dilution method can be unreliable since the sample solution containing different compounds with different polarity does not mix well in broth and it is time consuming.

The low MIC results of the stem extract against the isolate 11, *B. subtilis* and *P. aeruginosa* bacteria could be of significance for survival of grass species in the desert conditions. *B. subtilis* is one of the most significant PGPR bacteria (Ryu et al., 2003; Zhang et al., 2008; Bhattacharyya and Jha, 2012).

The minimum inhibition observed around one of the compounds against *P. aeruginosa* indicates that the fraction can be a good antibacterial agent but this need to be confirmed with a solution that can dissolve the non-polar compound completely. Diffusion methods are less suitable to determine the MIC values than dilution ones, because it is impossible to measure the amount of the compound diffused into the agar.

The development of new antimicrobial compounds to control resistant organisms is needed due to the increased resistant of organisms and the identification of new pathogenic drug resistant organisms. This study was not only done to show the antibacterial activity of *E. damarana* for purposes of finding potential new antibiotics but was also to indicate the importance of PGPRs which are needed by plants to enhance plant growth and yield. The results obtained with the MIC assay method are important and significant in indicating the relationship between the fairy circle of Namibia and *E. damarana* where the plant grows. Observing the low MIC values obtained for *B. subtilis, P. aeruginosa* and some soil isolates, it can be hypothesised that *E. damarana* kills the PGPRs and this might lead to the cause of fairy circles.

3.5. References

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CHAPTER 4: STRUCTURE ELUCIDATION OF ISOLATED COMPOUNDS……………………………………………………….75

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3.3.1. Nuclear Magnetic Resonance (NMR)…………………….78

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4.1. Introduction

Nuclear magnetic resonance (NMR) is a valuable technique preferred by organic chemists to determine the chemical structure of compounds and in general for drug discovery and development (Fig 4.1). It is a property that magnetic nuclei have in a magnetic field and applied electromagnetic (EM) pulses, which cause the nuclei to absorb energy from the EM pulse and radiate this energy back. The energy radiated back is at a specific resonance frequency which depends on the strength of the magnetic field and other factors. All stable isotopes that contain an odd number of protons and/or of neutrons have an intrinsic magnetic moment and angular momentum. The most commonly studied nuclei are ^{1}H and ^{13}C (Houghton and Raman, 1998; Ward et al., 2007; Günther, 2013; Everett, 2015).

The NMR produces a signal for every proton in ^{1}H NMR. The compound of interest is dissolved in deuterated solvents, it is then poured in a tube and placed inside the magnet. The result will be displayed in a form of a spectrum.

Figure 4.1: NMR apparatus (www.pharmacy.arizona.edu)

By studying the peaks of nuclear magnetic resonance spectra (Fig 4.2), chemists can determine the structure of many compounds, determine the distribution of isotopes and do quantitative analysis of chemical mixtures. It can be a very selective technique, distinguishing among many atoms within a molecule or collection of molecules of the same type but which differ only in terms of their local chemical environment (Roberts, 1984; Everett, 2015).

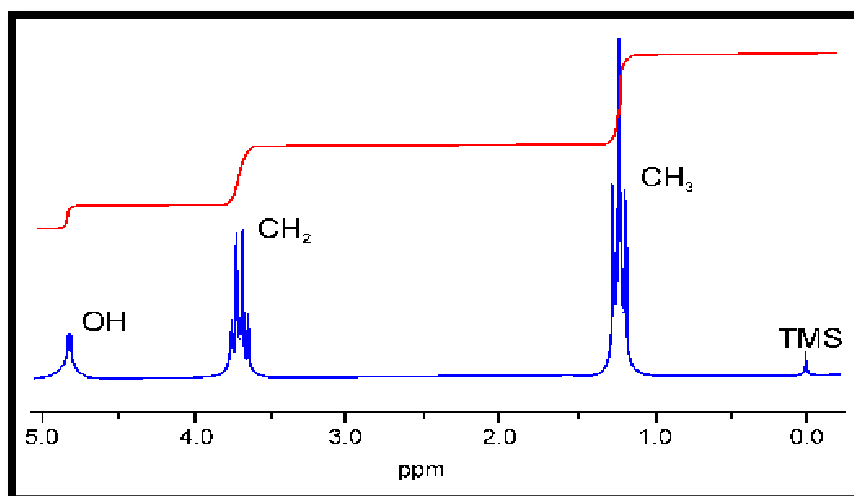

Figure 4.2: Picture of ethanol NMR spectrum (www.ibchem.com)

In this chapter our aim is to identify the isolated pure compounds and determine their structures.

4.2. Materials and methods

4.2.1. Nuclear Magnetic Resonance (NMR)

NMR analysis was done on a Varian 200MHz (University of Pretoria, Department of Plant and Soil Sciences). The compounds were dissolved in 1ml of $CDCl_3$ to yield a concentration of 20mg/ml for each compound. The compounds were transferred to 5mm tubes (700µl). For each spectrum, 1000 scans were recorded for the (^1H) proton and 10 000 scans for the (^{13}C) carbon NMR. Temperature was kept constant for each run at 25°C and all the spectra were referenced to their respective internal standards. They were also manually phased and baseline corrected.

4.3. Results and discussion

4.3.1. Nuclear Magnetic Resonance (NMR)

Compounds were identified based on NMR data (^{1}H and ^{13}C NMR) obtained and compared with literature data.

There were few differences in the chemical shift and resolution of peaks obtained due to the lower resonance of the 200 MHz spectrometer. All literature quoted used a 300 MHz spectrometer to obtain their spectra. The following triterpenoids were identified from the hexane stem extracts.

- **<u>Compound I (Fraction HII (23 – 27), Column 1), lupenone</u>:**

Compound I was obtained as a white powder with a mass of 1.6g. It was identified as lupenone with a chemical structure as shown in Fig 4.3 by using the NMR data obtained and comparing it with literature (Table 4.1). Fig 7.1 and 7.2 (Chapter 7: Appendix) shows the proton and carbon spectra of compound I.

It was previously isolated from air-dried plant material which was extracted with acetone from *E. segetalis* L. (Madureira et. al., 2003) and the hexane plant extract of *Diospyros rubra* Lec. (Ebenaceae) (Prachayasittikul et al., 2010).

Lupenone inhibit protein tyrosine phosphatase 1B (PTP 1B) which is used in developing new drugs for type 2 diabetes and obesity. It is also reported to possess anticancer, antiviral, antimicrobial and antiinflammatory activities (Prachayasittikul et al., 2010).

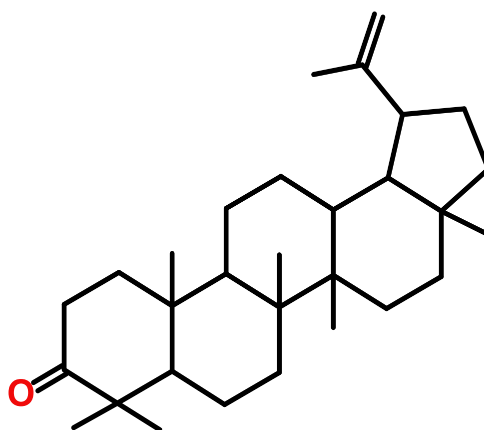

Figure 4.3: The chemical structure of lupenone.

Table 4.1: The ^{1}H-NMR and ^{13}C-NMR spectral data (δ/ppm) for compound I in $CDCl_3$.

Position (Proton)	^{1}H-NMR	^{1}H-NMR (Prachayasittikul et al., 2010)	Position (Carbon)	^{13}C-NMR	^{13}C-NMR (Prachayasittikul et al., 2010)
19	2.24–2.52m	2.2 –2.52m	1	39.5	39.6
21	1.84–1.96m	1.84–1.97m	2	34.2	34.1
23	1.06 s	1.04 s	3	218.4	218.2
24	1.02 s	1.00 s	4	47.4	47.3
25	0.87 s	0.90 s	5	55.0	54.9
26	1.25 s	1.22 s	6	19.4	19.2
27	0.93 s	0.93 s	7	33.5	33.5
28	0.74 s	0.77 s	8	40.8	40.7
29		4.44 br s	9	50.0	49.7
29		4.66 br s	10	36.8	36.8
30	1.65 s	1.66 s	11	21.1	21.4
			12	25.3	25.1
			13	38.3	38.1
			14	42.0	42.8
			15	27.6	27.4
			16	35.7	35.5
			17	42.4	42.9
			18	48.1	48.2
			19	47.6	47.9
			20	151.0	150.8
			21	29.4	29.6
			22	39.9	39.3
			23	26.7	26.6
			24	21.0	21.0
			25	15.9	15.9
			26	15.6	15.7
			27	14.6	14.4
			28	17.6	17.9
			29	109.6	109.3
			30	19.8	19.6

- **<u>Compound II (Fraction HX (47 – 48), Column 1), euphol:</u>**

Compound II was obtained as a white powder with a mass of 50mg. It was identified as euphol with a chemical structure as shown in Fig 4.4 by using the NMR data obtained and comparing it with literature (Table 4.2). Fig 7.3 and 7.4 (Chapter 7: Appendix) shows the proton and carbon spectra of compound II.

It was previously isolated from the following plants, ethyl acetate latex extract of *E. antiquorum* L. as the major fraction in hexane latex of *E. broteri* (Gewali et al., 1990, Lin et al., 2000), dried root of *E. kansui* (Lin et al., 2000, Zhang et al., 2012) and the leaves and roots of *E. fusiformis* (Natarajan et a., 2005)

Studies of euphol have proved it to be a good antiinflammatory and to have anticancer activity amongst others (Yasukawa et al., 2000, Dutra et al., 2011).

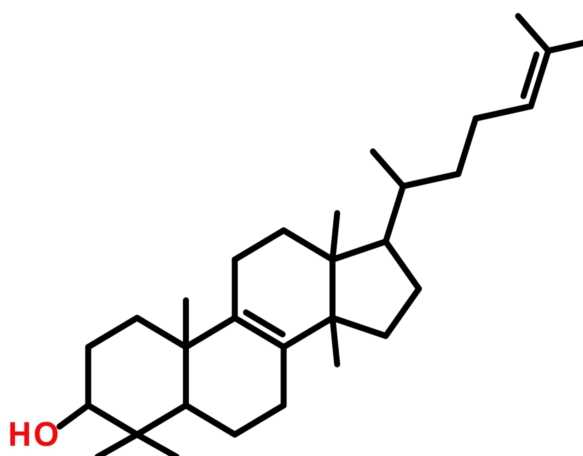

Figure 4.4: The chemical structure of euphol.

Table 4.2: The ^{1}H-NMR and ^{13}C-NMR spectral data (δ/ppm) for compound **II** in $CDCl_3$.

Position (Proton)	**^{1}H-NMR**	**^{1}H-NMR (Lin et al., 2000)**	**Position (Carbon)**	**^{13}C-NMR**	**^{13}C-NMR (Lin et al., 2000)**
3	3.23dd	3.22dd	1	34.57	35.23
18	0.73s	0.73s	2	27.63	27.65
19	0.98s	0.98s	3	79.20	79.00
21	0.86s	0.86s	4	39.10	38.92
24	5.07t	5.07t	5	51.11	50.94
26	1.59s	1.59s	6	18.92	18.93
27	1.66s	1.66s	7	27.87	27.92
28	0.78s	0.78s	8	133.71	133.53
29	0.93s	0.93s	9	134.22	134.00
30	0.83d	0.84d	10	37.46	37.25
			11	21.74	21.51
			12	28.13	28.13
			13	44.30	44.09
			14	50.23	50.01
			15	31.00	30.88
			16	29.93	29.74
			17	49.81	49.61
			18	15.59	15.52
			19	20.35	20.13
			20	35.61	35.86
			21	18.90	18.91
			22	35.45	35.40
			23	24.96	24.74
			24	125.41	125.20
			25	131.01	130.87
			26	17.90	17.68
			27	25.97	25.74
			28	24.69	24.46
			29	28.05	28.04
			30	15.63	15.60

- **<u>Compound III (Fraction HXI (49 – 51), Column 1) and compound IV (Fraction HXII (52 – 55), Column 1), lupeol</u>:**

Compounds III and IV were obtained as a white powder with a total mass of 1.1g. They were both identified as lupeol (with traces of euphol) with a chemical structure as shown in Fig 4.5 by using the NMR data obtained and comparing it with literature (Table 4.3). Fig 7.5 and 7.6 (Chapter 7: Appendix) shows the proton and carbon spectra of compound III.

Lupeol is found across many taxonomical diverse genera of plants including edible and medicinal plants (Chartevedi et al., 2008, Saleem et al., 2009, Siddique & Saleem, 2011). It was also isolated in the following plants, namely; hexane plant extract of *Diospyros rubra* Lec. (Ebenaceae) (Prachayasittikul et al., 2010), hexane and ethyl acetate leaf sheath, stems and roots of *Vellozia pusilla* Pohl (Pinto et al., 1988).

Anticancer and antiarthritic activity of lupeol was reported. Like lupenone it also inhibit protein tyrosine phosphatase 1B (PTP 1B) which is used in developing new drugs for type 2 diabetes and obesity (Prachayasittikul et al., 2010). It is also reported to possess anticancer, antiviral, antimicrobial, apoptosis inducing, antimutagenic, antiproliferative, antiinvasive, antiprotozoal, antioxidant and antiinflammatory activities among others (Saleem, 2009, Saleem et al., 2009, Prachayasittikul et al., 2010, Siddique & Saleem, 2011).

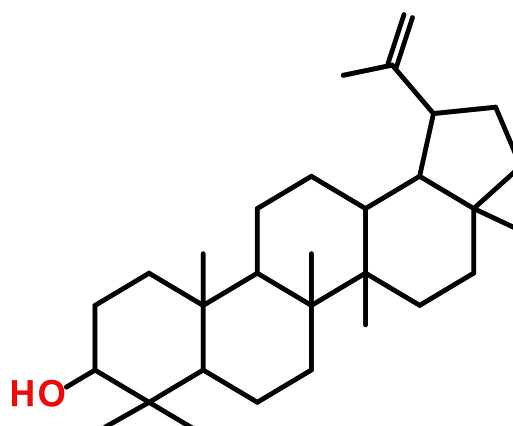

Figure 4.5: The chemical structure of lupeol.

Table 4.3: The ^{1}H-NMR and ^{13}C-NMR spectral data (δ/ppm) for compound III in $CDCl_3$.

Position (Proton)	**^{1}H-NMR**	**^{1}H-NMR (Prachayasittikul et al., 2010)**	**Position (Carbon)**	**^{13}C-NMR**	**^{13}C-NMR (Prachayasittikul et al., 2010)**
3	3.16 dd	3.16 dd	1	38.6	38.7
5	0.67 d	0.66 d	2	27.4	27.4
19	2.36 dt	2.35 dt	3	79.1	79.0
21	1.81–1.98 m	1.82–1.96 m	4	38.9	38.8
23	0.94 s	0.94 s	6	18.5	18.3
24	0.73 s	0.73 s	7	34.5	34.3
25	0.80 s	0.80 s	8	40.9	40.8
26	0.99 s	1.00 s	9	50.6	50.4
27	0.91 s	0.92 s	10	37.0	37.1
28	0.76 s	0.76 s	11	21.1	20.9
29	4.55 br s	4.55 br s	12	25.3	25.1
29	4.65 br s	4.65 br s	13	37.9	38.0
30	1.65 s	1.65 s	14	43.0	42.8
			16	35.4	35.6
			15	27.4	27.4
			17	43.0	43.0
			18	48.1	48.0
			19	48.5	48.3
			20	151.0	150.9
			21	29.6	29.7
			22	40.2	40.0
			23	28.2	28.0
			24	15.6	15.3
			25	15.9	15.9
			26	16.2	16.1
			27	14.4	14.5
			28	18.2	18.0
			29	109.6	109.3
			30	19.1	19.3

- **Compound V (Fraction HXVIII/HXIX7 (35 – 39), Column HXVIII/HXIX), 20-hydroxy-lupane-3-one :**

Compound V was obtained as colourless crystals with a mass of 15mg. It was deduced as 20-hydroxy-lupane-3-one with a chemical structure as shown in Fig 4.6 by using the NMR data obtained and comparing it with literature (Table 4.4). Only two articles were found that can be used as NMR reference for this compound. The ^{1}H-NMR data from these articles was not documented (Dantanarayana et al., 1982). Fig 7.7 and 7.8 (Chapter 7: Appendix) shows the proton and carbon spectra of compound V.

This triterpenoid was previously isolated from methanol and benzene stem bark extract of *Pleurostylia opposita* (Wall) Alston (Celastraceae) (Dantanarayana et al., 1981, Dantanarayana et al., 1982), hexane and ethyl acetate of the stems, roots and leaf sheaths of *Vellozia pusilla* Pohl (Pinto et al., 1988).

This compound is not well known, information about its uses was not found.

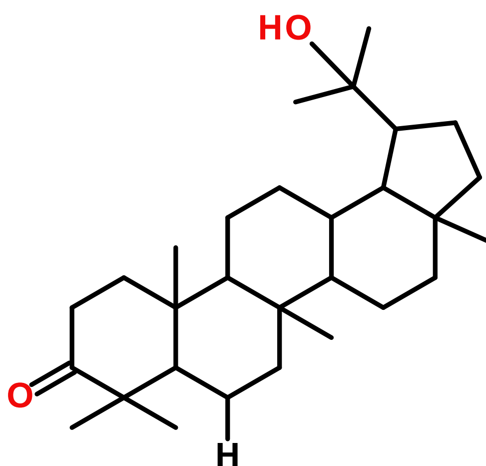

Figure 4.6: The chemical structure of 20-hydroxy-lupane-3-one.

Table 4.4: The ^{1}H-NMR and ^{13}C-NMR spectral data (δ/ppm) for compound V in $CDCl_3$.

Position (Proton)	**^{1}H-NMR**	**^{1}H-NMR**	**Position (Carbon)**	**^{13}C-NMR**	**^{13}C-NMR (Dantanarayana et al., 1982)**
	0.81 s		1	39.7	39.6
	0.93 s		2	34.3	34.6
	0.95 s		4	47.5	47.2
	1.02 s		5	55.0	54.9
	1.07 s		6	19.9	19.7
	1.09 s		7	33.9	33.9
	1.12 s		8	41.5	41.4
	1.22 s		9	50.1	50.0
	1.25 s		10	36.9	36.8
	1.36 s		11	22.1	22.0
	1.45 s		12	28.9	28.7
	7.26 s		13	37.7	37.7
			14	43.8	43.6
			15	27.7	27.6
			16	35.7	35.6
			17	44.8	44.6
			18	48.4	48.3
			19	49.8	49.7
			20	73.7	73.4
			21	29.2	29.7
			22	40.3	40.2
			23	26.9	26.7
			24	21.2	21.0
			25	16.2	16.0
			27	14.9	14.8
			28	19.3	19.2
			29	31.8	31.6
			30	24.9	24.8

- **<u>Compound VI (Fraction HXXIV3 (15 – 17), Column B), 3-oxo-7,24E-tirucalladien-26-oic acid:</u>**

Compound VII was obtained as white powder with a mass of 12mg. It was identified as 3-oxo-7,24E-tirucalladien-26-oic acid with a chemical structure as shown in Fig 4.7 by using the NMR data obtained and comparing it with literature (Table 4.5). No assignments for the chemical shifts in the proton and carbon atoms were published in the literature. Fig 7.9 and 7.10 (Chapter 7: Appendix) shows the proton and carbon spectra of compound VI.

It was previously isolated from the wood and bark of *Dysoxylum pettigrewianum* (Mulholland and Nair, 1994). Limited information about the compound is available. The other families of the genus *Dysoxylum* spp. are used as insecticides.

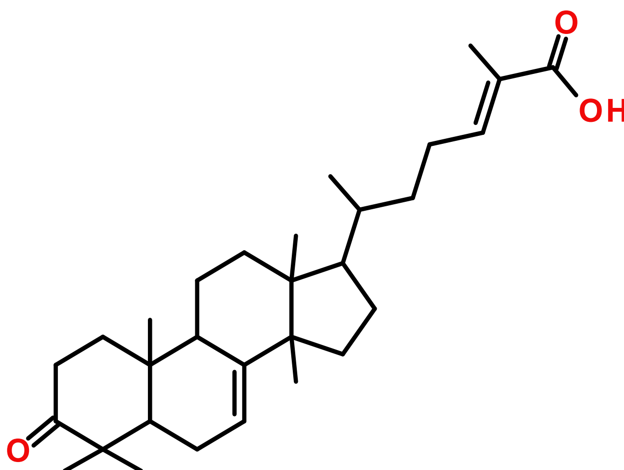

Figure 4.7: The chemical structure of 3-oxo-7,24E-tirucalladien-26-oic acid.

Table 4.5: The ^{1}H-NMR and ^{13}C-NMR spectral data (δ/ppm) for compound VI in $CDCl_3$.

^{1}H-NMR	^{1}H-NMR (Mulholland and Nair, 1994)	^{13}C-NMR	^{13}C-NMR (Mulholland and Nair, 1994)
0.81 s	0.81 s	12.1	11.9
0.96 d	0.90 d	13.0	12.7
1.02 s	1.01 s	18.4	18.1
1.07 s	1.01 s	18.5	18.2
1.09 s	1.05 s	19.0	21.6
1.12 s	1.12 s	21.8	21.9
1.84 d	1.84 d	24.5	24.3
5.00 br s	5.31 br s	24.7	24.5
7.26 s	6.90 t	26.2	26.0
		27.6	27.4
		28.4	28.2
		33.8	33.6
		34.2	34.0
		34.8	34.6
		35.1	34.9
		35.2	35.0
		36.2	36.0
		38.7	38.5
		43.7	43.5
		48.1	47.8
		48.6	48.4
		51.4	51.1
		52.5	52.2
		53.1	52.8

4.4. Conclusions

The isolated compounds all belong to the class of triterpenoids. Most triterpenes contain 29 or 30 carbons atoms and one or two carbon–carbon double bonds, typically one in the sterol nucleus and sometimes a second in the alkyl side chain. Natural triterpenoids are also known as phytosterols due to their wide spectrum of biological activities. Triterpenoids are natural components which are found in most human diets, they are derived from fruits, vegetables, cereals and oils derived from plants (Saleem et al., 2009; Siddique and Saleem, 2011). Triterpenoids have many therapeutic and preventative beneficial effects such as antiarthritic, antiprotozoal, antimicrobial, anticancerous, antidiabetic, antiinflammatory, cardioprotective, skin protective, hepatoprotective and nephroprotective (Chaturvedi et al., 2008, Siddique and Saleem, 2010). They are found in high amounts in the non-polar fractions of the plant (Jassbi, 2006). The five compounds isolated were identified as:

- Lupenone
- Euphol
- Lupeol
- 20-hydroxy-lupan-3-one
- 3-oxo-7,24E-tirucalladien-26-oic acid

The compounds were isolated with the non-polar solvents hexane and EtOAc. The molecular weight, chemical structure and formula of these compounds were confirmed with that of PubMed, ChemSpider and the NIH library. All five compounds are known and have been isolated in several plants however the last two of the five, 20-hydroxy-lupan-3-one and 3-oxo-7,24E-tirucalladien-26-oic acid have been poorly documented with limited information. This is the first report of the bio-assay guided isolation of different fractions and compounds from the stems of the *E. damarana* extract.

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CHAPTER 5: CYTOTOXICITY……………………………….92

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5.1. Introduction

Species of the genus *Euphorbia* have been used traditionally as medication to treat various human diseases. Several species have been tested for their activity as antimicrobial, antiviral and antitumor agents. There have been reports of antiviral activity of the genus *Euphorbia* against polio, rhinoviruses, coxsakie, antibacterial against several bacteria and antitumor activity against sarcoma 180 ascites and several tumours including leukaemia and cytotoxic activity against cancer cell lines (Betancur-Galvis et al., 2002).

Plants consists of a mixture of different compounds and therefore the toxicity of plants can be linked to a single active compounds or to a mixture of several compounds (Houghton and Raman, 1998). Experiments conducted for drug discovery has succeeded in distinguishing plants that have beneficial effects from those that are toxic or show no effect (Heyman et al., 2009). The effective dose of a drug used for treatment in humans can be ascertained by performing cell culture studies on animal models (Vijayaa et al., 1995)

The discovery of biological activity among plants with previously recorded medicinal use rather than from plants with unknown records of medicinal use which are randomly selected, is increasing. Once the biological active compound is identified it needs to be tested for cytotoxicity before it can be considered for use in drug development.

In this study two combined fractions with good antimicrobial activity were chosen together with the stem and dried latex extract to test for toxicity of the plant extracts and the fractions.

5.2. Materials and methods

5.2.1. Vero cells preparation

Vero cells, from African green monkey kidney cells (ATCC CCL-81) were grown in culture flasks in Minimum Essential Medium Eagle (MEM) (Highveld Biological (Pty) Ltd, Kelvin, South Africa) containing 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 µg/ml penicillin, 10 µg/ml streptomycin and 10% bovine serum at 37°C in a humidified atmosphere with 5% CO_2 for about a week. Subculturing was done every 2-3 days using phosphate buffer saline (PBS) to remove the media and 0.25% trypsin containing 0.01% EDTA to release the cells that have attached to the culture flasks. Trypsinization of the cells was done for 20 min at 37˚C in an atmosphere with 5% CO_2 and 95% humidity; following incubation the MEM was added to stop the process of trypsin. Viable cells were counted and about 1 x 10^5 of cells were re-suspended in complete medium. Medium (200 µL) was added to the outer wells of a 96 well culture plates to avoid evaporation. Viable cell suspension (100µl) was added to the inner wells. Incubated the 96 well culture plates for 24 hours at 37˚C in an atmosphere with 5% CO_2 and 95% humidity (Zheng et al., 2001).

5.2.2. Cytotoxicity assay

Plant extracts and fractions (1 mg/ml) were weighed and dissolved in 10% DMSO. The plant extracts and fractions were both tested at 50 µg/ml and 25 µg/ml concentrations and incubated for 48 hours at 37˚C in an atmosphere with 5% CO_2 and 95% humidity. Actinomycin-D was used as a positive control throughout the testing at a concentration of 0.05 µg/ml. Cytotoxicity was measured by the XTT method using the Cell Proliferation Kit II (Roche Diagnostics GmbH). The XTT reagent at a final concentration of 0.3 mg/ml was added to the wells and incubated for 1-2 h. After incubation the absorbance of the colour complex was quantified using an ELISA plate reader, which measured the optical density at 490 nm with a reference wavelength of 690 nm (Zheng et al., 2001; Lall et al., 2013).

5.3. Results and discussion

The test was done to preliminary screen the toxicity of the plants extracts and the selected fractions collected from different columns. The selected fractions tested showed good antibacterial activity and were obtained from the first methanol stem extract and MeXXV column. Since this was a screening test only visual observations were done to obtain results. A colour change of pink is observed in the wells where cells are still alive while absence of colour indicates that the cells are dead.

Fig 5.1 shows results of the methanol stem and dried latex extracts and selected fractions tested at concentrations of 50 µg/ml and 25 µg/ml. The tests were done in duplicate. Both the stem and dried latex extracts were not toxic to the Vero cells. Fraction MeXXV21 and MeXXV22 were the only two fractions that showed to be toxic to the cells at a concentration of 25 µg/ml. This was indicated by the light pink colour of the wells as compared to the dark pink/orange colour of the rest of the wells including the Actinomycin-D which acted as a positive control.

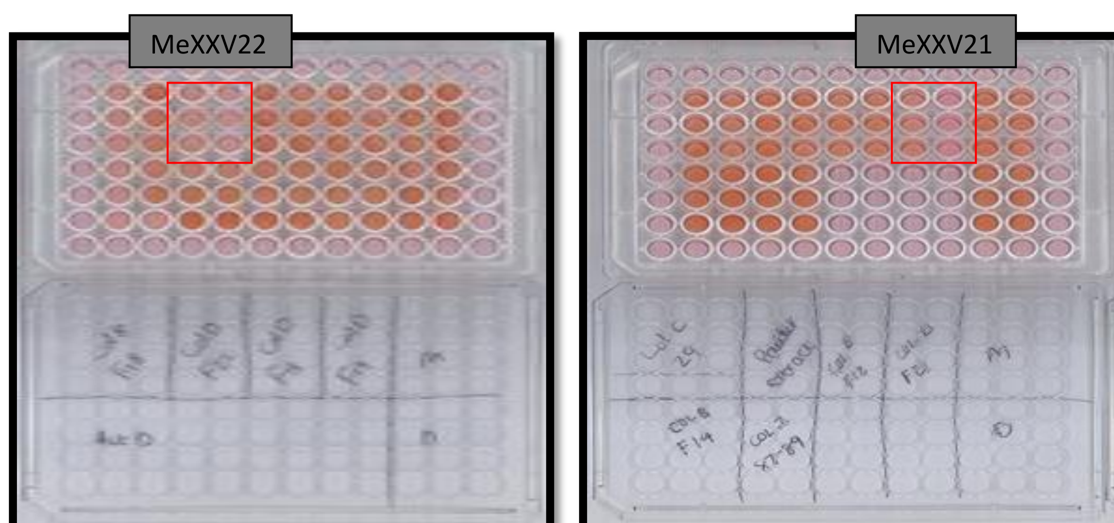

Figure 5.1: Microtiter plate of selected fractions, dried latex and stem extracts.

Euphorbiaceae are widely known to be toxic and with exposure they are known to show skin irritancy, antitumor and tumour promoting effects (Barla et al., 2007)

In a study by Vijayaa et al. (1995) on methanol extract of *E. hirta* it was reported that the plant shown to be non-cytotoxic at the concentration of 1.56 mg/ml, with a starting concentration of 25 mg/ml.

5.4. Conclusions

Testing for cytotoxicity is an important part of drug discovery and development. The toxicity of the two plant extracts could not be verified positively due to the concentration used. For future studies several concentrations of the plant extracts will be tested. Fraction MeXXV21 and MeXXV22 was shown to be toxic to Vero cells. The fractions consist of a mixture of compounds of which one or all of them are toxic to the cells. Further purification of these fractions is required in order to isolate the pure compound or compounds that are toxic. It was reported in literature that most plants which are toxic to human cells can be used for cancer treatment.

5.5. References

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CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

6.1. General discussion and conclusion……………………………99

6.2. References…………………………………………………….101

6.1. General discussion and conclusion

It is evident that there is a need for development of new antimicrobial agents that have the ability to treat infections without causing side effects. A number of biological active compounds have been isolated from other *Euphorbia* species (Gewali et al., 1990; Baloch et al., 2007; Fernandez et al., 2010; Prachayasittikul et al, 2010) and they have shown good pharmacological activities hence they are used in folk medicine to cure infections (Bani et al., 2006; Sudhakar et al., 2006; Barla et al., 2007; Fernandez et al., 2010). The ability to treat infectious diseases is threatened by the evolution of antimicrobial resistance in the hospitals and community (Rice, 2009).

In this study the stem and dried latex methanol extracts as well as different fractions from the hexane and methanol stem extracts were tested against several known and unknown bacteria by using different bioassays. The bioassay results showed good antibacterial activity of the stem extract, fractions and the compounds against the bacteria.

Bioassay guided isolation of hexane extract of *E. damarana* stems has led to the isolation of five known compounds. The structure of the new compounds isolated was elucidated by the NMR spectroscopy and confirmed with published literature and they were found to be:

- Lupenone
- Euphol
- Lupeol
- 20-hydroxy-lupan-3-one
- 3-oxo-7,24E-tirucalladien-26-oic acid

Antibacterial activity of the stem extracts yielded good results making *E. damarana* a good candidate to be used as a natural ethnomedicinal product depending on further testing and toxicity results. During the study it was established that *E. damarana* inhibited growth of soil bacteria that were isolated from the soil collected in regions where the plant grows in Namibia.

The bacteria used were PGPRs that are essential to promote and enhance plant growth and yield (Singh, 2003; Saharan and Nehra, 2011; Munees and Mulugeta, 2014). The results obtained with the MIC assay method are important and significant to indicate the relationship between the fairy circles of Namibia and *E. damarana* where the plant grows. This observation might add some input on the occurrences of fairy circles of Namibia which is a research question under intensive study by scientists (Theron, 1979; Van Rooyen et al., 2014; Naude et al., 2011; Picker et al., 2012; Juergens, 2013; Getzin et al., 2015).

In future more plant material will be collected and extracted to increase the yield in order to perform more bioassays. More plant material will also be collected to increase the yield of bioactive fractions, this will allow for pure compounds to be investigated with enough plant extracts to test for bioactivity.

Diterpenoids such as phorbol esters have been isolated from Euphorbia plants (Fürstenberger and Hecker, 1986; Jassbi, 2006; Haba et al., 2007; Baloch et al., 2008; Popplewell et al., 2010). Phorbol esters are naturally occurring compounds that have a tigliane skeleton. They have negative effects on humans and livestock because of the toxicity but also possess beneficial effects e.g. being tumor inhibitors and having antileukaemic activity (Goel et al., 2007; Baloch et al., 2008). Isolation of these compounds will be done in future studies.

In this study it was not proven beyond doubt that *E. damarana* is toxic because low concentrations of the stem and dried latex extracts and fractions were used and the test was done on one cell line only.

E. damarana showed promising antibacterial potential in this study and since the plant has shown many other compounds on TLC plates of the original fractions and has also shown activity against bacteria on bioassays, further fractionation of the original fractions is recommended to obtain more pure compounds. Some of the pure compounds that might be identified might be new since there was few scientific studies done and published on this species. Toxicity studies on the identified compounds and other compounds

that are yet to be identified is in progress and it will be reported in further studies.

There is limited information published on the phytochemical analysis and properties of *E. damarana*, for this reason it is important to conduct further research on this plant as it has potential to be used as a natural ethnomedicinal product.

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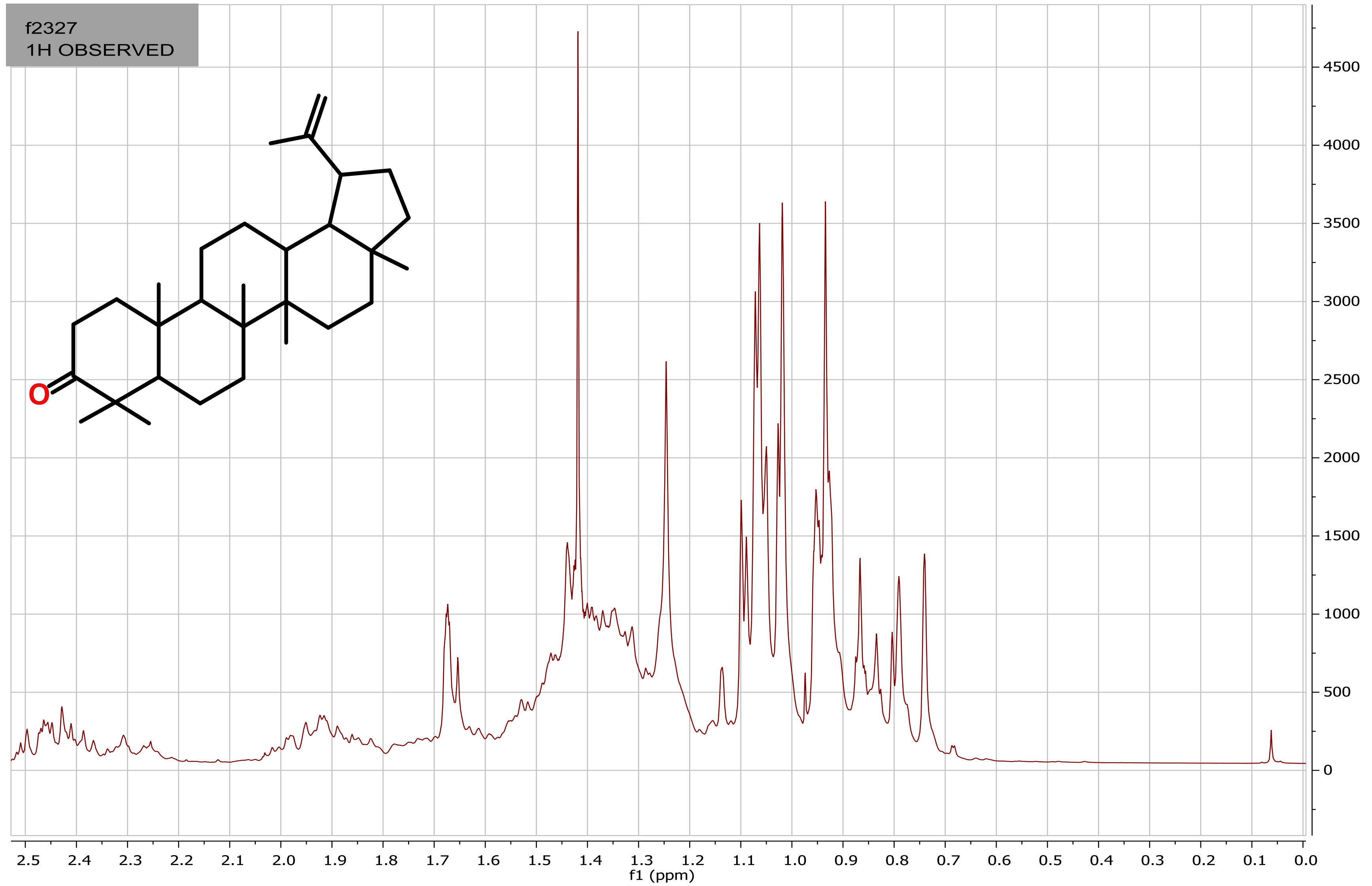

Figure 7.1: ^{1}H NMR spectrum of lupenone from first column isolated from the hexane stem extract

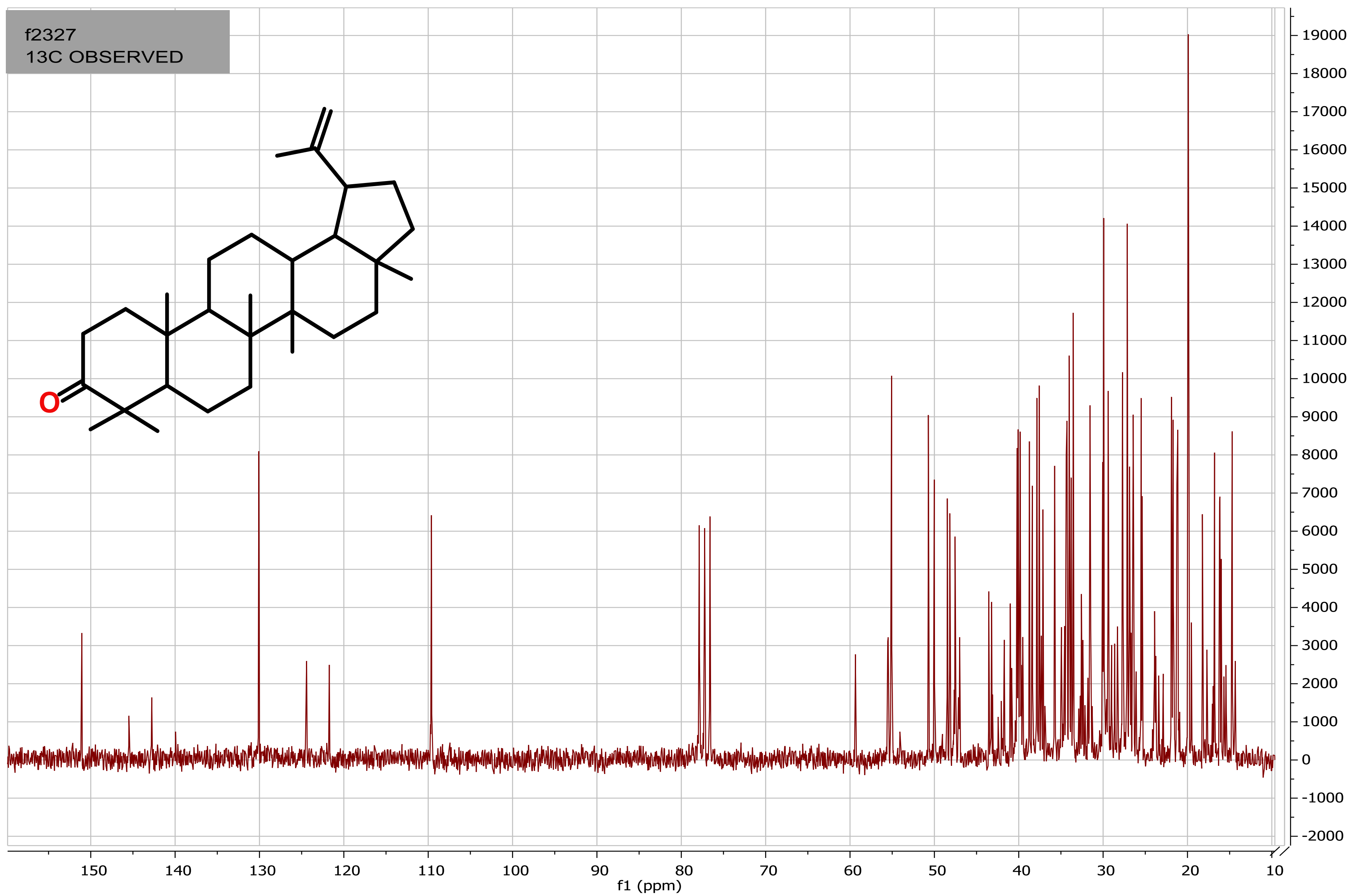

Figure 7.2: ^{13}C NMR spectrum of lupenone from first column isolated from the hexane stem extract

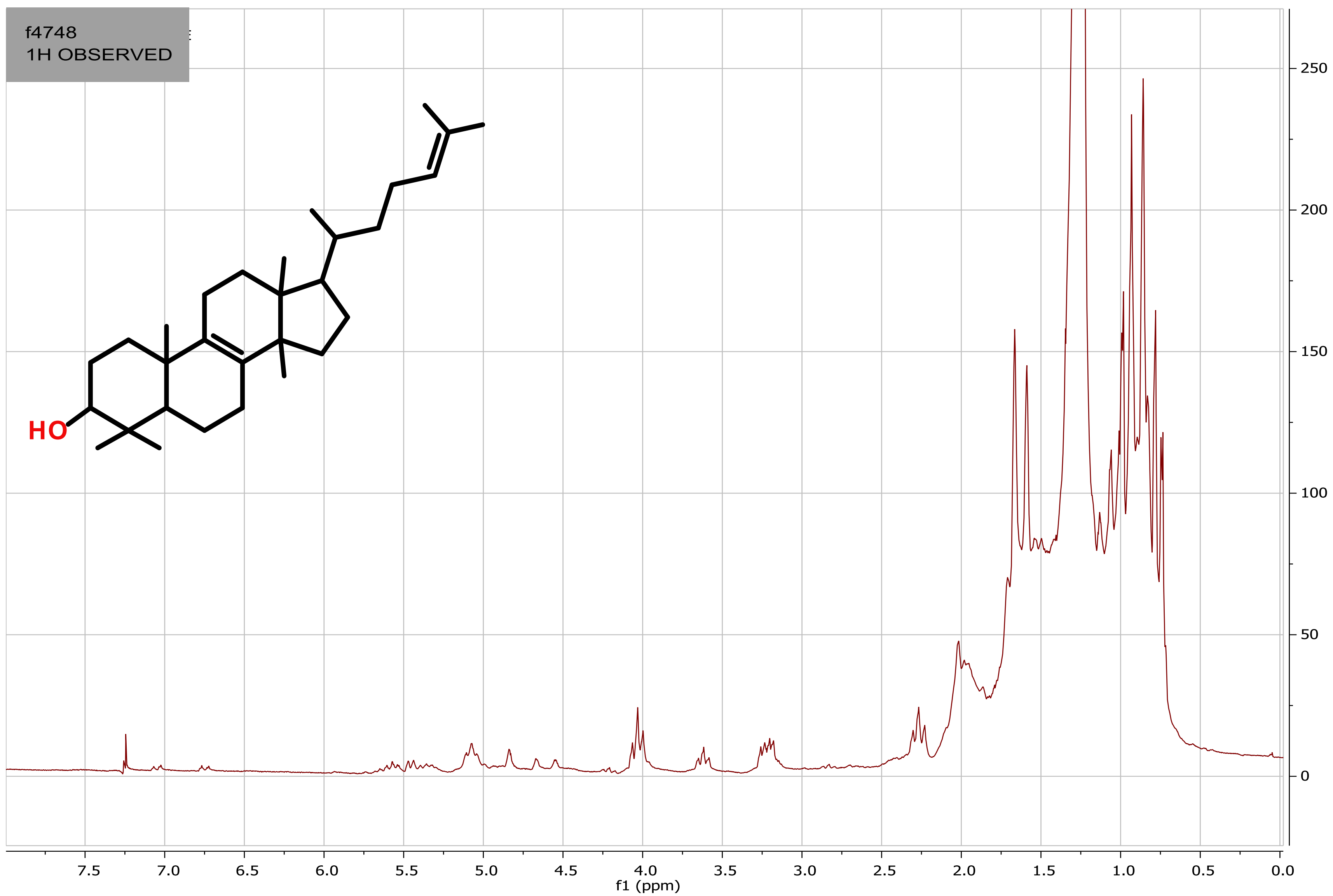

Figure 7.3: ^{1}H NMR spectrum of euphol from first column isolated from the hexane stem extract

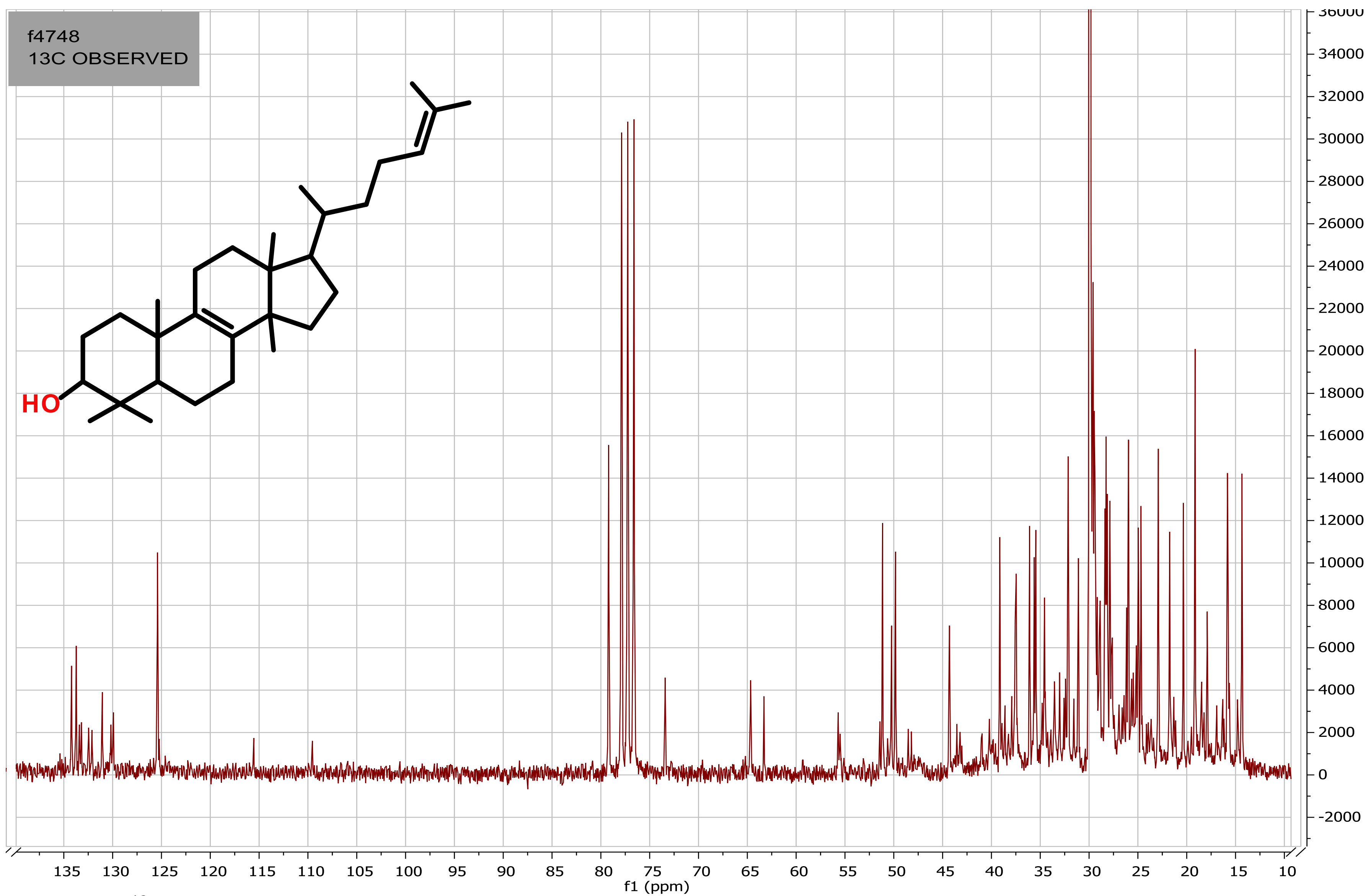

Figure 7.4: ^{13}C NMR spectrum of euphol from first column isolated from the hexane stem extract

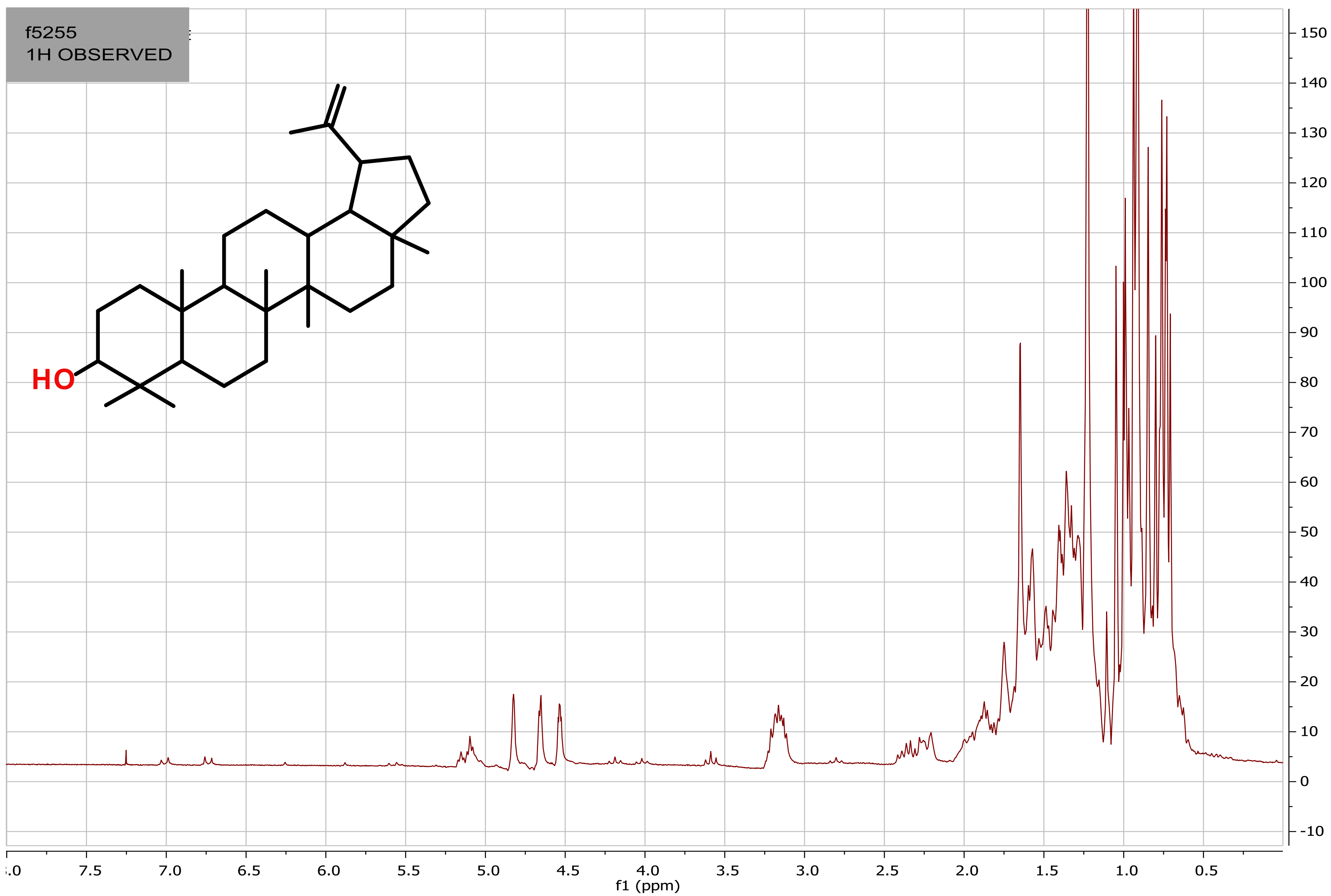

Figure 7.5: ^{1}H NMR spectrum of lupeol from first column isolated from the hexane stem extract

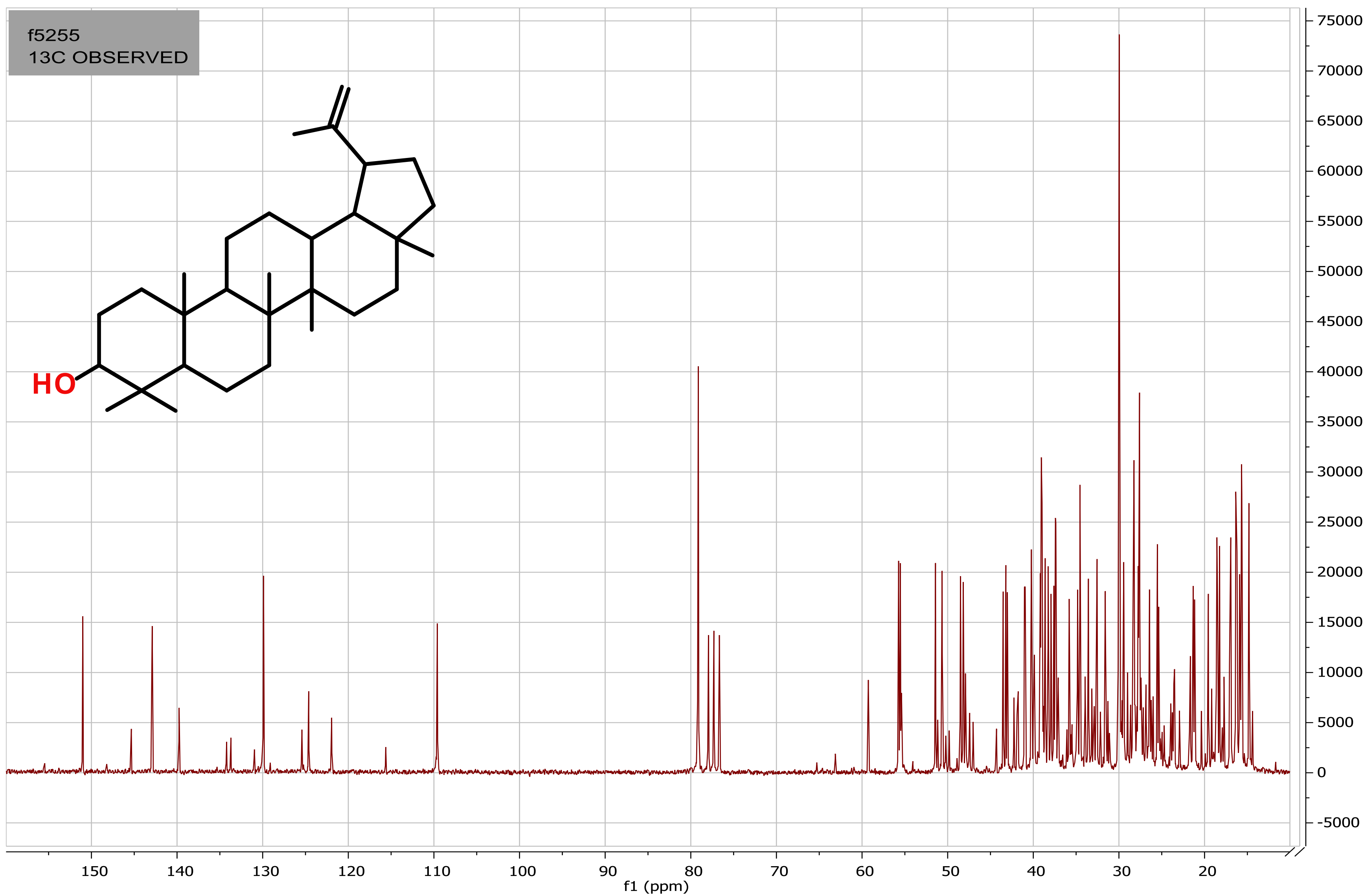

Figure 7.6: ^{13}C NMR spectrum of lupeol from first column isolated from the hexane stem extract

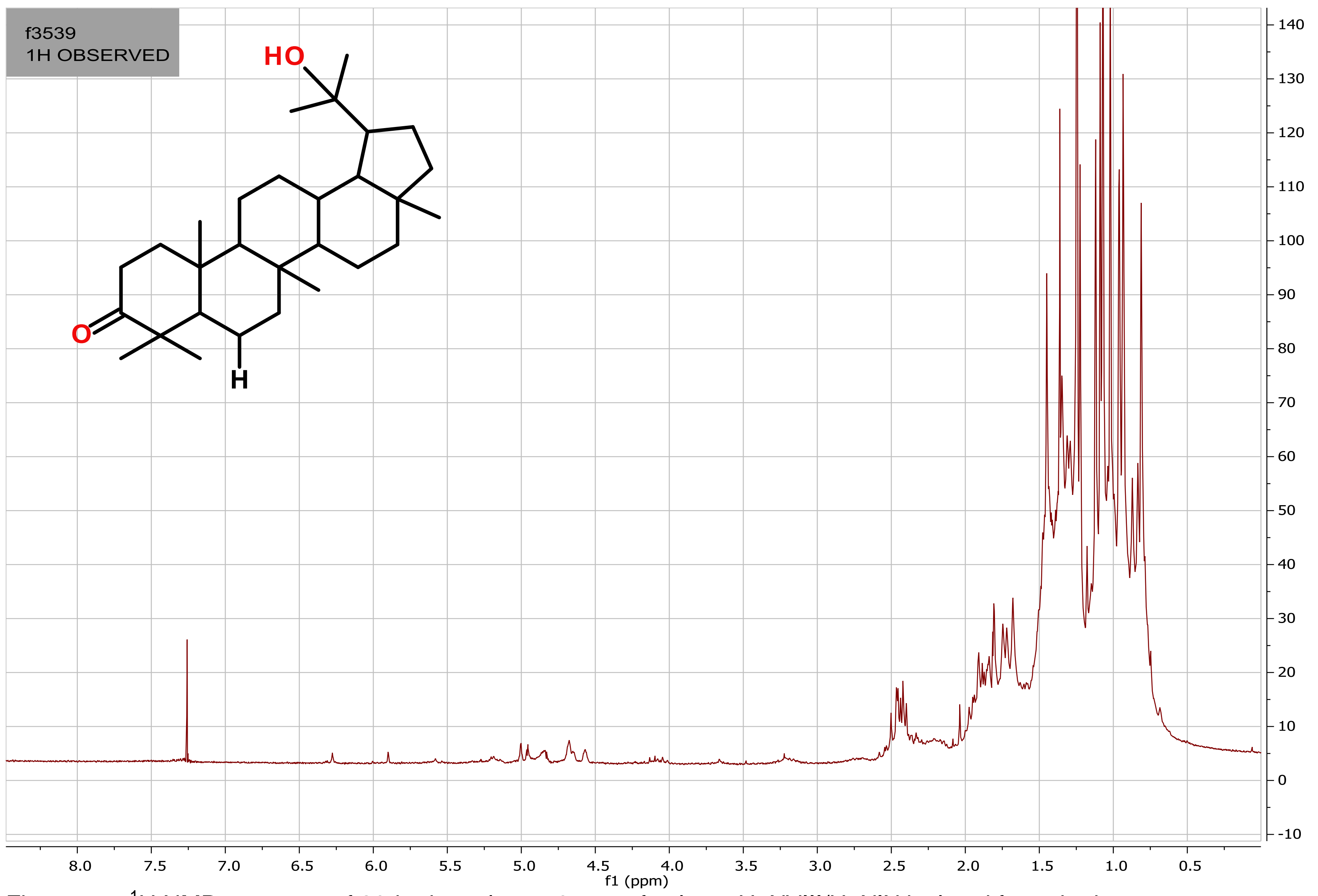

Figure 7.7: ^{1}H NMR spectrum of 20-hydroxy-lupan-3-one of column HeXVIII/HeXIX isolated from the hexane stem extract

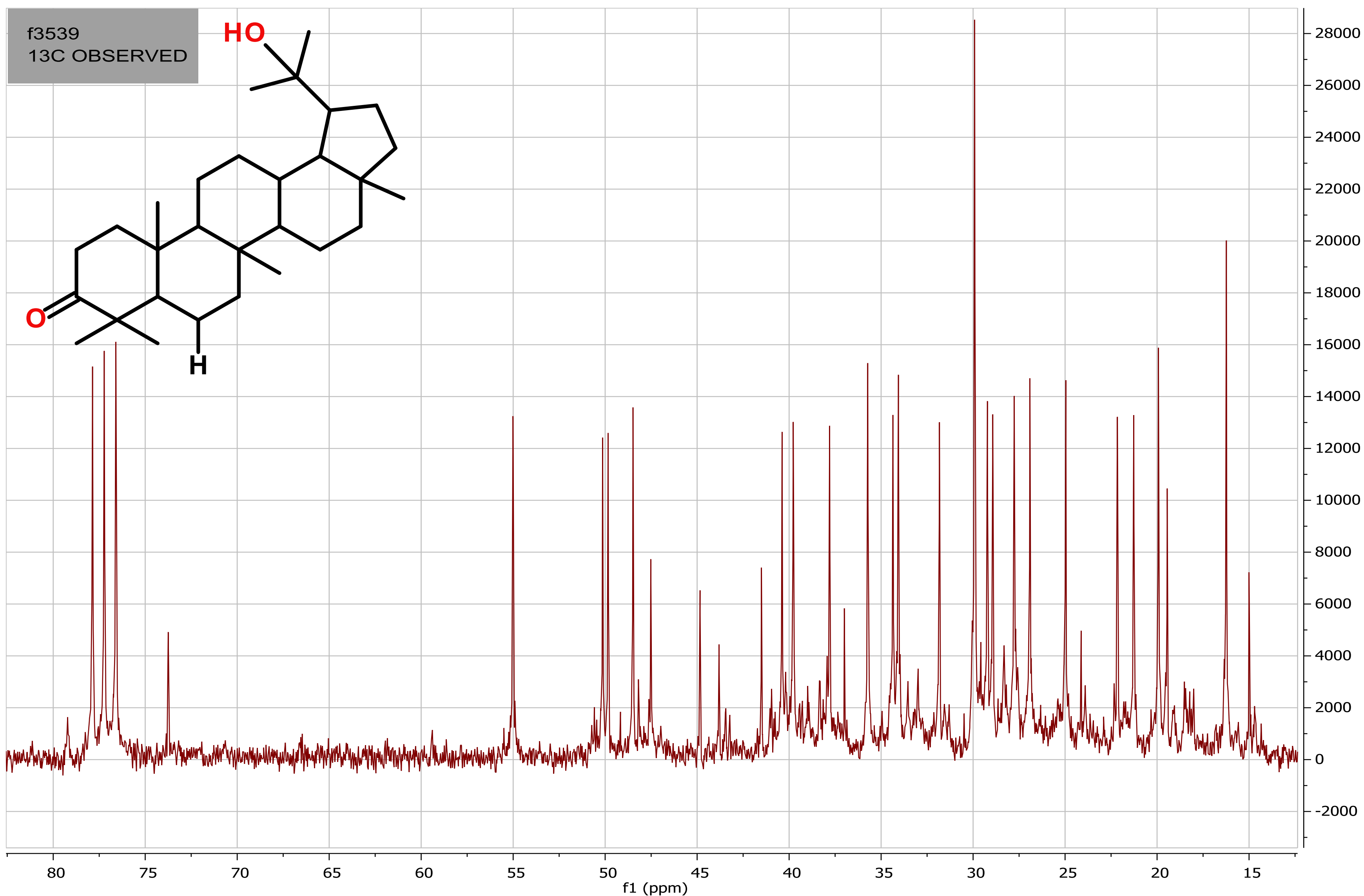

Figure 7.8: ^{13}C NMR spectrum of 20-hydroxy-lupan-3-one of column HeXVIII/HeXIX isolated from the hexane stem extract

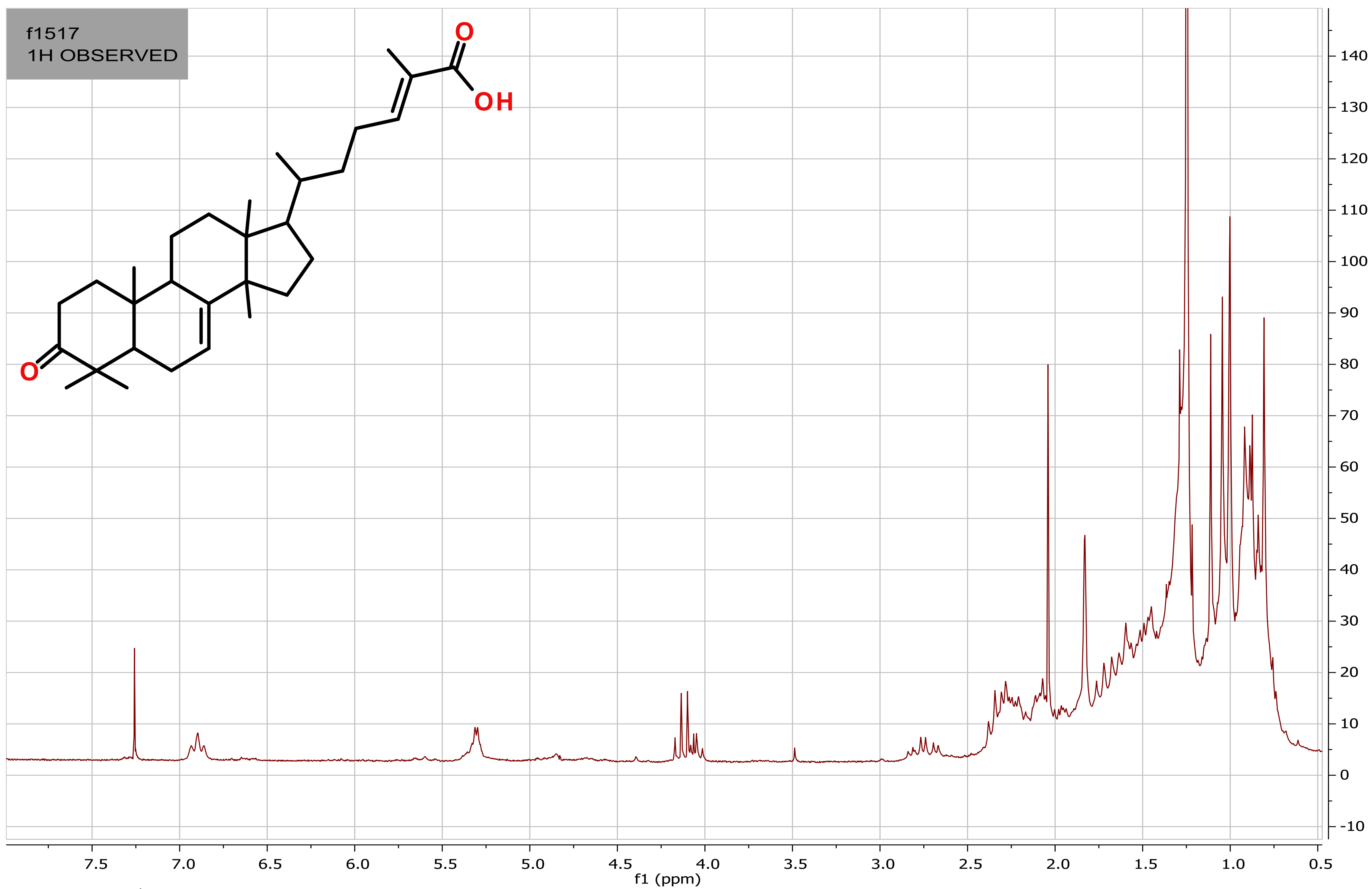

Figure 7.9: ^{1}H NMR spectrum of 3-oxo-7,24E-tirucalladien-26-oic acid of column HeXXIV isolated from the hexane stem extract

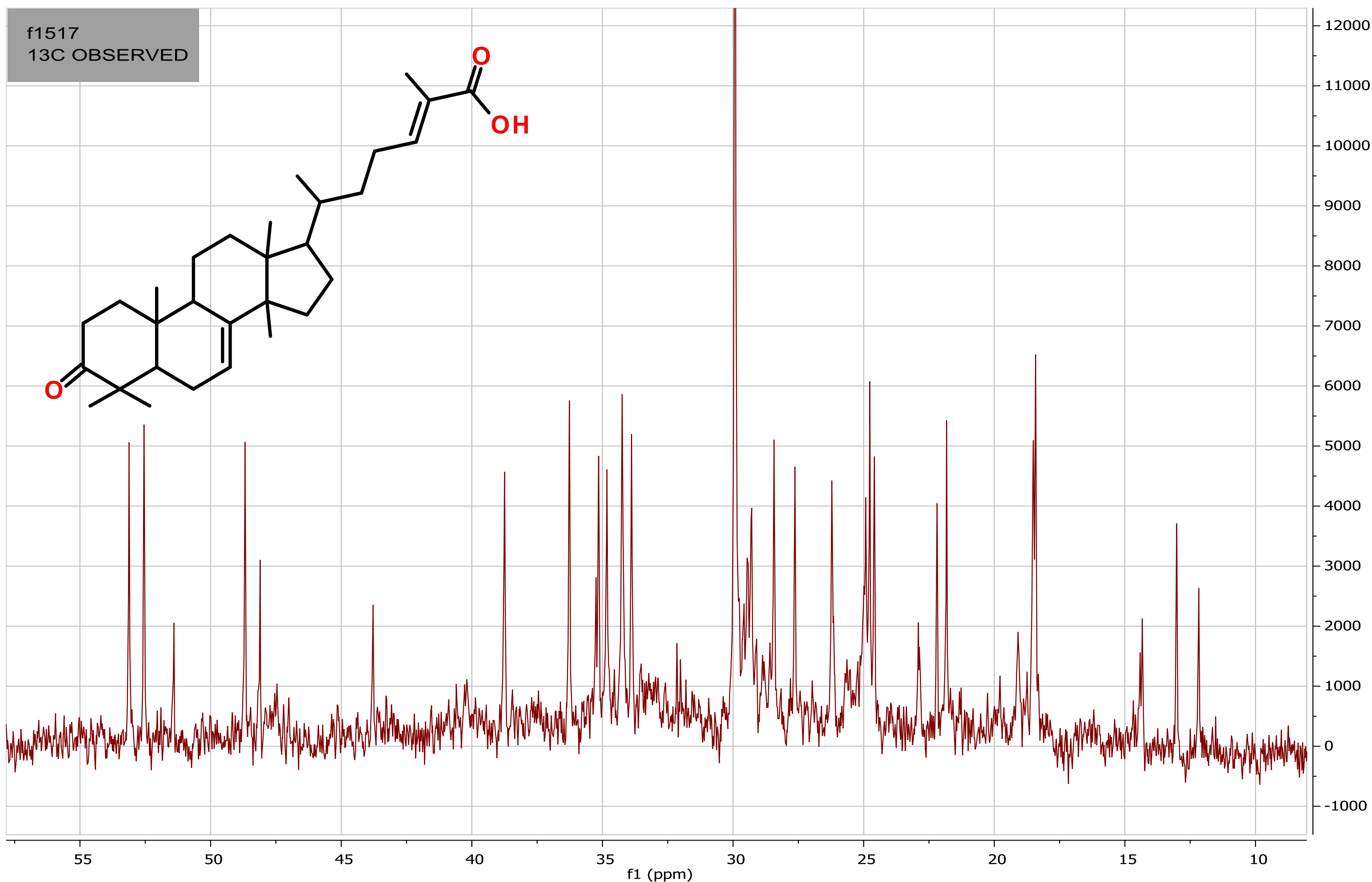

Figure 7.10: ^{13}C NMR spectrum of 3-oxo-7,24E-tirucalladien-26-oic acid of column HeXXIV isolated from the hexane stem extract