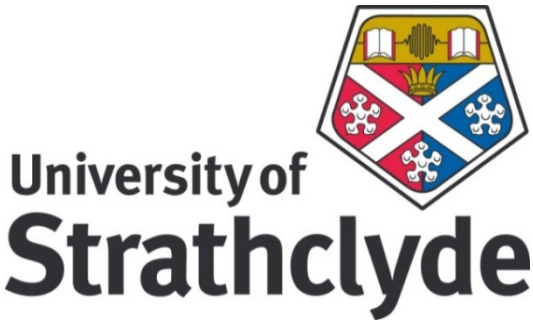


**Strathclyde Institute of Pharmacy and Biomedical Sciences**

# An ethno-pharmacological study of Egyptian Bedouin women's knowledge of medicinal plants

**By**

**Nabila Saleem**

**A thesis presented in fulfilment of the requirements for the degree of**

**Doctor of Philosophy**

**2012**

"This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree".



Signed:

Date:

**Dedicated to**

**Egypt protests and the Arab spring**

**Acknowledgements**

First of all, absolute and sincere thanks go to **Allah**, who gave me the grace to enjoy sound health, protection and guidance throughout my studies. To you all honour and glory.

All my greatest gratitude and deepest appreciation to the Citadel Capital Foundation for funding this project, without their funding this work would not have been possible.

I wish to express my deepest gratefulness to my supervisor Prof. Gray, his tremendous knowledge in NMR gave me the true essence of this technique; his father like affection encouraged me to carry on with my work. I am also very grateful to Dr. Val Ferro for being a secondary supervisor; her support and generosity were invaluable attributes to my overall PhD experience. Her invaluable instructions and advice helped me greatly to improve my research writing ability. I would like to express my sincere gratitude to my supervisor Dr. Ann Simpson for her kind supervision. I am also very thankful to Dr. Robert Drummond for supervising part of my research his methodological approach helped me greatly to improve my research ability and he was always happy to give me his advice and clarification whenever necessary. Especial thanks to Dr. Edward Rowan for offering me the opportunity to work in his lab and for his help. I am very thankful to Dr. RuAn for her help with NMR as well as for her availability, advice and support.

My endless thanks are extended to Dr. John Igoli (my best friend), who understood the pressure I was under and offered me his kind support and his valuable time throughout the period of my study and helped me to finish my work smoothly. I am indebted to Dr Jamil Shilpi for his help during my first year and I am very pleased to have shared this experience with him and all the postgraduate students in the Natural Product Research group from whom I have learned a lot, Jamal, Nouri, Andrea, Khanittha, LayAn, Giacomo, Ciarra, and Ahmed. Especial thanks to Ruby, Tome and Mohamed Sabar for their help and support. I would like to thank Carole Clements from SIDR for her help in some of the antioxidant work. I am also obliged to Alex

and Craig for their kind help in running MS and LC-MS experiments. I would further like to thank Martin and Mark for their contribution during the evaluation of the immunomodulatory properties. I acknowledge the University of Strathclyde technical and administrative staff for their kind assistance.

Thanks to my friends Ebtihaj, Azra, Adisa, Samar, Mirfate, Ayah and Aminah whose friendly help made my life easy and comfortable in Glasgow. I want to give credit to my friend whom special people in my life for their moral support and their company throughout my life and their assistance: Hoda, Haitham, Kassem, Ekramy, Amal, Hana and Samar. Special thanks to Adal and Anter, their communication and encouragement throughout my studies has been my source of strength. I am grateful to my colleagues and seniors at the unit of Environmental Studies and Development, Wadi Allaqi Biosphere reserve in Aswan, Saint Catherine and Siwa. Special thanks go out to Dr. Magdi Radi, his guidance, advice and help for suggesting the topics of this research led to the final successful completion of this work.

I would like to express special thanks to all the Bedouin people who generously gave me their time and shared their knowledge over the course of this study and for their help in collecting plant materials. Finally, my deepest gratitude and sincere deep thanks goes to my parents, brothers, sisters and family whose unconditional and amazing love and support made everything possible. My especial thanks are to my brother Mohamed for his moral support and tolerating the pressure with me. I would like to extend my warmest thanks to all those who helped me in one way or another in making my success pleasurable.

**Abstract**

This thesis presents the results of an ethnopharmacological survey conducted with Bedouin women in Egypt (South Eastern Desert, Siwa Oasis and North Sinai) to document their knowledge of using medicinal plants to treat women's health problems, such as dysmenorrhoea, perinatal problems, womb cleansing and urinary tract infections. Data collected revealed that the Bedouin women commonly use more than 45 different plant species. Four plant species (*Haloxylon salicornicum, Achillea fragrantissima, Mentha longifolia* and *Acacia nilotica*) were chosen for phytochemical and pharmacological investigations in this project.

Two flavonoids **AF18** (3',4',5-trihydroxy-3,6,7-trimethoxyflavone or chrysosplenol D) and **AF36** (5,7,4'-trihydroxy-3',6-dimethoxyflavone or Jaceosidin), which were recorded for the first time from *Achillea fragrantissima* together with two sesquiterpene lactones **AF17** (3-acetoxy-1-oxo-13-hydroxy-4,7(11),10(14)-germacratrien-12,6-olide) and **AF25** (13-O-Desacetyl-1α-hydroxy-afraglaucolide), were isolated from the leaf extract. **AF17**, **AF18** and **AF36** in a concentration of 7.5, 15 and 30 µg/ml, showed significant ($p<0.05$) inhibition of TNF-α production in lipopolysaccharide ( LPS) (500pg/ml) stimulated human monocyte cells, while the minimum inhibition concentration (MIC) for **AF18** and **AF36** against *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* was 62.5 µg/ml and 31.25 µg/ml for *Candida albicans*.

The analysis for *Mentha longifolia* showed the presence of monoterpenes **ML17** (3-methyl-6-(1-methylethylidene)-cyclohex-2-en-1-one or piperitenone) and the novel monoterpenes **ML19** (p-mentha-1,3,5-triene-3,8-diol or 8-hydroxy thymol). **ML17** and **ML19** showed antifungal activity with MIC of 62.5 and 125 µg/ml respectively. Five flavonoids **ML1** (acacetin-7-O-rutinoside or linarin), **ML2 (**5,7-Dihydroxy-4'-methoxyflavanone 7-O-[α-L-Rhamnopyranosyl-(1→6)-β-D-glucopyranoside] or didymin), **ML7**(5,6 -dihydroxy-3′,4′,7,8 tetramethoxyflavone), **ML10** (5,6,3'-trihydroxy-7,8,4'-trimethoxyflavone) and **ML16** (4′,5,6-trihydroxy-7,8-dimethoxyflavone or sorbifolin). In a concentration dependent manner (7.5, 15 and 30 µg/ml) they showed significant ($p<0.05$) inhibition of TNF-α production in LPS

(500pg/ml) stimulated cells. The majority of these flavonoids showed positive growth inhibition against *Escherichia coli*, *S. aureus*, and *C. albicans* with MIC ranging from 125 to 7.8 µg/ml. While their radical scavenging activity $IC_{50}$ was from 12.5 to 0.048µg/mL.

**AN4** (methyl gallate) and **AN5** (gallic acid) were the most active compounds isolated from *Acacia nilotica* with MIC ranging between 62.5 to 7.8 µg/ml. Their antioxidant activity in the DPPH assay was very potent with $IC_{50}$ of 0.02 and 0.195 µg/ml, while it was 1.56µg/mL for ascorbic acid as a standard.

**HS1** (Synephrine) and **HS4** (allantion) were isolated for the first time from *Haloxylon salicornicum*. These together with **HS3** (piperidine) and **HS2** (N-methyltyramine) were isolated from the chloroform phase of aqueous extracts. Synephrine and N-methyltyramine showed significant ($p<0.05$) relaxation effects on spontaneous uterine contractions in a concentration dependent manner. The relaxation activity of was attenuated in KCl contracted uteri and blocked by the addition of propranolol, a non-selective β-adrenceptor antagonist.

These finding justify the use of these plants by Bedouin women for treating (relief) of menstrual pain and perinatal problems. The results also validate the use of most of these plants as antimicrobial agents by the Bedouin women in Egypt to treat genital infection.

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**LIST OF ABBREVIATIONS**

| | |
|---|---|
| 1D | One Dimensional Nuclear magnetic Resonance Spectroscopy |
| 2D | Two Dimensional Nuclear magnetic Resonance Spectroscopy |
| ATCC | American Type Culture Collection |
| *brs* | Broad of singlet |
| cAMP | cyclic Adenosine MonoPhosphate |
| CC | Open Column Chromatography |
| $CDCL_3$ | Deuterated Chloroform |
| COSY | Correlation Spectroscopy |
| COX | Cyclooxygenase |
| *d* | Doublet |
| *dd* | Doublet of a doublet |
| *dm* | Doublet of multiplet |
| DMSO | Dimethyl sulfoxide |
| ELISA | Enzyme-linked Immunosorbent assay |
| FC | Flash Chromatography |
| FCS | Foetal calf serum |
| GF | Gel Filtration |
| GPCR | G-protein coupled receptor |
| HMBC | Heteronuclear Multiple Bond Coherence |
| HMQC | Heteronuclear Multiple Quantum Coherence |
| HRCI-MS | High Resolution Chemical Ionisation Mass Spectrometry |
| HREI-MS | High Resolution Electron Impact Mass Spectroscopy |
| Hz | Hertz |
| $IC_{50}$ | Inhibitory Concentration at 50% |
| IL-1β | Interleukin-1β |
| IL-6 | Interleukin-6 |
| IP3 | Inositol-trisphosphate |
| LPS | lipopolysaccharide |
| *m* | Multiple |

| | |
|---|---|
| MeOH | Deuterated methanol |
| MIC | Minimum Inhibitory Concentration |
| MLCK | Myosin Light-Chain Kinase |
| NF-κB | Nuclear factor kappa-B |
| NMR | Nuclear Magnetic Resonance |
| NSAID | Non-Steroidal Anti-Inflammatory Drugs |
| PLC | Phospholipase C |
| $R_f$ | Retardation Factor |
| Ry | ryanodine |
| *S* | Singlet |
| SD | Standard deviation |
| SR | Sarcoplasmic reticulum |
| TLC | Thin layer chromatography |
| TNF-α | Tumour necrosis factor |
| VLC | Vacuum Liquid Chromatography |

# CHAPTER ONE

# INTRODUCTION

# 1. Introduction

## 1.1 Research Background

The concept for this research project came from interaction with Bedouin women through work for the Unit of Environmental Studies and Development (UESD), Aswan, South Valley University. The unit has a field research centre in the Wadi Allaqi Biosphere Reserve (see the Map, page 15) in the southern part of the Eastern Desert and has carried out research (with and without Bedouin involvement) for more than 20 years (Sharp *et al.*, 2009). Visits were not considered intrusive or unusual due to the long history of contact. The first research project where women were consulted was about their own perspectives on resources and their roles in livestock production (Sharp *et al.*, 2003). One of the unit's key research areas is the study of medicinal plants and Bedouin knowledge of their uses and pharmacological applications. This research project follows on from a MSc. degree in Environmental Science, entitled "*Women and Indigenous Environmental Knowledge in the Wadi Allaqi Biosphere Reserve.*" (Saleem, 2004). The key aim of the MSc. thesis was to examine the ways in which Bedouin women in the Wadi Allaqi Biosphere Reserve use, classify and manage plant resources and their livelihood system to ensure sufficient household production in the context of long-term changing environmental resources. Subsequently, in this PhD Thesis the idea of how Bedouin women use natural resources, particularly plants to treat their reproductive health problems was conceived. Focus was on how women use medicinal plants to treat reproductive conditions such as painful menstruation, perinatal problems and genital infection.

## 1.2 Background Bedouin communities in Egypt

Low income, slow economic growth and difficulty in creating new jobs in Egypt have led to high rates of unemployment and poverty, environmental degradation and high illiteracy rates. Approximately 35% of the Egyptian population is illiterate, with a higher figure of 65% for women. Illiteracy occurs predominantly in rural areas among underprivileged women between the ages of 15 and 45 (UNFPA, 2009). Within Bedouin communities, the situation is even harder, since these people live in harsh conditions in the deserts of Egypt. Water is not available and the fundamental component of the Bedouin economy is livestock production of sheep and goats.

Bedouin have traditionally been a highly mobile group, moving all their belongings to follow seasonal pastures (Briggs *et al.*, 1993). The main problem with Bedouin desert inhabitants is water availability, because rainfall occurs only once every few years. Only their profound knowledge of the local environment and sustainable utilization of resources, help them to survive. Their knowledge helps them to use and manage their livelihoods to ensure sufficient household production in the context of long-term changing environmental resources (Briggs *et al.*, 1999).

### 1.2.1 Women in the Bedouin communities

In Bedouin communities, women avoid meeting men who are not from their family. According to Sharp *et al*., (2003) Bedouin communities have different social identities, roles, and responsibilities for men and women in their society. Women have greatest involvement in animal grazing activities while men concentrate on trading activities. Women are not mobile and they have to accompany by male relatives on their visits to town. Men have the power to take any decisions, which means women do not have easy access to financial resources. The differences in behaviour and the level of power between men and women can also have an important impact on the risk of contracting illness or infection. For example, women may have little choice in if, when, and with whom sexual activity occurs so they are unable to negotiate safer sexual practices (El-Kak *et al.*, 2009).

Women's reproductive health is an intimate and private affair that is traditionally only dealt with by women within these communities, especially when there is limited access to modern medical facilities and professional female practitioners. The nature of these women and of their community can prevent them from reporting their illness to outsiders. According to Bedouin custom, it regarded as shameful if a woman asks to visit a doctor, especially a male doctor, during illness. While the use of allopathic medicine might be increasing for certain types of illness, and for some members of the Bedouin community, others need to rely on traditional medicinal remedies. The aim of the present research was to examine the use of medicinal plants in the treatment of women’s illnesses and investigate the indigenous female knowledge of their reproductive health problems.

### 1.2.2 Women and reproductive health problems

Reproductive health problems cause significant morbidity and mortality for women in developing countries (Jamison *et al.*, 2006). The reality of such problems extend beyond a woman herself to her children and the family as a whole, with the consequence of negative socio-economic impact on the whole society (Roudi-Fahimi, 2003). Globally, ill health related to sex and reproduction accounted for 25% of the global disease burden in adult women (Jamison *et al.*, 2006). In sub-Saharan Africa, they account for over 40%. The catastrophic effects of reproductive ill health, however, are not limited to women; it extends to infants and adult men. Nearly 50% of the 10 million women who give birth every year in North Africa and the Middle East experience some kind of complications with more than one million of them suffering from serious injuries that could lead to long term illness (Christopher, 1998). Maternal mortality in Egypt is still relatively high, and the country faces challenges in reducing it further. Every year about 1,400 Egyptian women and half of their new-borns die from complications related to pregnancy and childbirth (Roudi-Fahimi, 2003). Furthermore, the majority experience other reproductive health issues such as: i) dysmenorrhoea or painful periods and premenstrual symptoms ii) perinatal and postpartum problems iii) reproductive tract infections (RTIs) and sexually transmitted infections (STIs).

#### i) Dysmenorrhoea

Dysmenorrhoea involves chronic, cyclic pelvic pain associated with menstruation. Typically, it consists of cramping, lower abdominal pain occurring just before and/or during menstruation, usually commencing soon after menarche once regular ovulation is established (Reddish, 2006). Dysmenorrhoea is a common gynaecological condition that is under diagnosed and undertreated. The severity of dysmenorrhoea is significantly associated with duration of menstrual flow and younger average menarche. High levels of stress can also greatly increase the incidence of dysmenorrhoea, as can depression, anxiety, and disruption of social networks. Primary dysmenorrhoea often improves in the third decade of a woman's reproductive life and after childbirth. The relationship between the prognoses of secondary dysmenorrhoea and the severity of underlying disease is unclear (Proctor

*et al.*, 2006). There is no one single treatment for dysmenorrhoea, however, over decades many practices such as Nonsteroidal anti-inflammatory drugs (NSAIDs) have been tried to relieve such symptoms.

### ii) Perinatal and postpartum problems

Complications of pregnancy and childbirth are the leading cause of death and disability among women of reproductive age in developing countries (Kurjak *et al.*, 2001). For example, anaemia, falls, blows or weakness can cause miscarriages. One of the most serious complications Bedouin midwifery have to deal with during birth is a retained placenta, which results from the failure to expel the placenta after birth. Haemorrhaging during pregnancy is the most common cause of maternal death in developing countries. It can cause by the lack of uterine contractions (relaxation of the muscle fibre) of the uterus or injury to the birth canal including uterine eruption. Infection is another leading cause of late bleeding postpartum (Chacko, 2009). Postpartum care includes dealing with issues such as abdominal pain, lactation, retained placenta, uterine cleansing, uterine haemorrhaging and uterine infection (Coe, 2008; Germain *et al.*, 1992).

### iii) Reproductive tract infection as a global burden

Reproductive tract infections (RTIs) recognized as a serious global health problem with negative impact on individual women, men, their families and communities. According to the WHO it is estimated that there are 340 million new cases of curable sexually transmitted Infections (STIs) such as, syphilis, gonorrhoea, chlamydia and trichomoniasis that occur annually throughout the world in adults (WHO, 2000). STIs results in severe consequences, including infertility, ectopic pregnancy, chronic pelvic pain inflammatory disease, miscarriage and increased risk of transmission of human immunodeficiency virus (Aral, 2001). RTIs are a broad definition that refers to three general types of infections that affect the reproductive tract; endogenous infections, iatrogenic infections and the more commonly known STIs (Germain *et al.*, 1992). Each has its own specific causes and symptoms, caused either by pathogens, transmitted sexually or on medical tools. Some of these are easily treatable and can cure, while others are more difficult. In women, overgrowth of

endogenous microorganisms (yeast infection, bacterial vaginosis) normally found in the vagina may cause RTI. Medical interventions may provoke iatrogenic infection in several ways. Viruses, bacteria, or parasitic microorganisms that transmitted through sexual activity with an infected partner cause sTIs (Table 1).

**Table 1** List of the most common STIs, their causes and other infections

| **Infection types** | **Organism name** |
|---|---|
| Common bacterial infections | *Neisseria gonorrhoea* (causes gonorrhoea or gonococcal infection)<br>*Chlamydia trachomatis* (causes chlamydial infections)<br>*Treponema pallidum* (causes syphilis)<br>*Haemophilus ducreyi* (causes chancroid)<br>*Klebsiella granulomatis* (causes granuloma inguinale or donovanosis) |
| Common viral infections | Human immunodeficiency virus (causes AIDS)<br>Herpes simplex virus type 2 (causes genital herpes)<br>Human papillomavirus (causes genital warts and certain subtypes lead to cervical cancer in women)<br>Hepatitis B virus (causes hepatitis and chronic cases may lead to cancer of the liver)<br>Cytomegalovirus (causes inflammation in a number of organs including the brain, the eye, and the bowel). |
| Parasitic organisms | *Trichomonas vaginalis* (causes vaginal trichomoniasis)<br>*Candida albicans* (causes vulvovaginitis in women) |

Bacterial infection initiates inflammation through several mechanisms (Philpott *et al.*, 2001). Infection and inflammation can have devastating consequences to fertility and pregnancy (Gotsch *et al.*, 2008). To date, most interventions directed at preventing and treating such, infections have focused on pathogen elimination using antibiotics. Unfortunately, these strategies have been disappointing in eradicating such infections, or decreasing the burden of related complications, such as fertility, ectopic pregnancy and preterm birth (Horne *et al.*, 2008). Women are much more vulnerable to infection because they are more biologically, culturally and socioeconomically susceptible (WHO, 2010). Common symptoms include vulvo-vaginitis (itching and pain in the external genital region and vagina), painful or uncomfortable sexual intercourse, and the presence of an abnormal discharge. The majority of affected women tend not to seek treatment, often due to busy family lives, financial difficulties and inability to access health care facilities (Marin *et al.*, 2002). For these reasons, Bedouin women have to apply their knowledge about plants and use traditional medicine (TM).

## 1.3 Traditional medicine

Traditional medicine (TM) refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (WHO, 2000). In general, TM practices vary greatly from country to country, and from region to region, as they are influenced by factors such as culture, history, personal attitudes and philosophy (WHO, 2002). Countries in Africa, Asia and Latin America use TM to help meet some of their primary health care needs. In Africa, up to 80% of the population uses TM for primary health care, while in industrialized countries, adaptations of TM are termed "Complementary" or "Alternative" medicine (CAM) (WHO, 2000).

### 1.3.1 Reasons for promoting the use of traditional medicines

Over one-third of the population in developing countries lack access to essential medicines. Almost 80% of the population in developing countries is dependent on plants as the first source for health care (Uniyal *et al.*, 2006). The documentation of traditional knowledge on the medicinal uses of plants has led to the development of numerous important drugs. According to the WHO (2002), the provision of safe and effective TM/CAM therapies could become a critical tool to increase access to health care. However, there is the risk that a growing herbal market and its great commercial benefit might pose a threat to biodiversity through the over harvesting of the raw material. These practices, if not controlled, may lead to the extinction of endangered species and the destruction of natural habitats and resources. Another related issue is that at present, the requirements for protection provided under international standards for patent law and by most national conventional patent laws are inadequate to protect traditional knowledge and biodiversity (WHO, 2000). TM plays an important role in health care in Bedouin communities. In fact, due to their availability and affordability, the traditional medicines and therapy systems of these communities provide health care to the vast majority of them (Barbir, 2010).

## 1.4 Ethnopharmacology

In the beginning of the 20th century, the study of the pharmacological qualities of traditional medicinal substances has been defined as ethnopharmacology. Ethnopharmacology is the scientific study of the use of plants by native cultures, including their use as medicines (Etkin, 1988). It can also defined as a multidisciplinary area of research concerned with the observation, description and experimental investigation of indigenous drugs and their biological activities, which have also been long used in TM. The term ethnopharmacology has undergone only slight evolution in meaning; its contemporary definition addresses the interdisciplinary study of the physiological actions of plants, animals and other substances used in indigenous medicines of past and present culture (ISE, 2005).

### 1.4.1 The role of the ethnopharmacology approach in drug discovery

Plants have used as the basis of many TM systems throughout the world for thousands of years. They continue to provide humankind with new remedies. The ancient Egyptians made the first description of plants in relation to their medicinal uses on Papyrus, approximately five thousand years ago (Kruger, 1993). They were followed by the Greeks (371-28 B.C) and the Christian era handed down their medical knowledge of plants to the Romans who subsequently passed it on to the Arabs (Sayed, 1980). However, it was not until the early 19th century that the first active substance was isolated from a plant (in a pure form) when morphine was isolated from opium (Kinghorn, 2001). This purification was followed by substances from other plants including quinine, nicotine, cocaine, artemisinin (Newman *et al.*, 2000) and taxol (Jennewein *et al.*, 2001). When the first synthesized drugs were introduced and their role steadily increased, there was a preference to synthesize compounds derived from natural sources, as the percentage of chemical constituents in plants can be low and vary depending on the seasons, climate and time of collection. Even though the medicinal world prefers synthetic drugs for practical reasons, approximately 40% of these are still obtained from herbs to treat cardiovascular conditions and cancer (Gurib-Fakim, 2006). Later in the 20th century, the documentation of traditional knowledge on the medicinal uses of plants has led to the development of numerous important drugs.

### 1.4.2 Examples of key drugs based on Ethnopharmacology knowledge

Natural compounds of pharmaceutical importance that were once obtained from higher plant sources, but which are now produced commercially largely by synthesis, include caffeine, theophylline, ephedrine, pseudoephedrine, papaverine, L-dopa, salicylic acid, and tetrahydrocannabinol (Balandrin Manuel *et al.*, 1993). A guanidine-type alkaloid, galegine, was found to be the active principle of *Galega officinalis* L., and is used clinically for the treatment of diabetes (Roth, 1986). Recently, several new medicinal plant-derived drugs have introduced to the market. Artemether is a potent semi-synthetic anti-malarial drug and derived from artemisinin, a sesquiterpene lactone isolated from *Artemisia annua* L. (Asteraceae), a plant used in traditional Chinese medicine. Other derivatives of artemisinin are in various stages of use or tested in clinical trials to treat malaria (van Agtmael *et al.*, 1999). Now, the artemisinin and its derivatives have become essential components of artemisinin-based combination therapies and are the recommended first-line treatments of malaria because they are effective against all four human malarias (Kano, 2010).

Galantamine is a natural product discovered through an ethnobotanical lead and first isolated from *Galanthus woronowii* Losinsk (Amaryllidaceae) in Russia in the early 1950s. Galantamine now approved for the treatment of Alzheimer's disease. It slows the process of neurological degeneration by inhibiting acetyl cholinesterase as well as binding to and modulating the nicotinic acetylcholine receptor (Heinrich *et al.*, 2004). Tiotropium has recently been released in the United States for treatment of chronic obstructive pulmonary disease (Barnes, 2002). It is an inhaled anticholinergic bronchodilator, based on ipratropium, a derivative of atropine isolated from *Atropa belladonna* L. and other members of the Solanaceae family (Barnes *et al.*, 1995). Tiotropium has shown increased efficacy and longer lasting effects when compared with other available chronic obstructive pulmonary disease medications (Barnes, 2002).

In addition, numerous other plant-derived drugs are currently undergoing clinical tests. Screening of more medicinal plants is important for finding potential new

compounds for therapeutic uses. Thus, researches based on ethnopharmacological information generally consider an appropriate approach in the discovery of new effective agents from higher plants (Kloucek *et al.*, 2005). It is widely acknowledged that extensive research still needs to be carried out and a cause of major concern is that some species are becoming extinct with their potential properties unknown (Jachak *et al.*, 2007). Bedouin women in Egypt are heavily involved in all aspects of medicinal plants, from collecting, processing and storing and their uses. They do not have access to western medicine or general practitioners; as a result medicinal plants are being used as TM to treat different ailments. Therefore, documentation of their traditional knowledge to treat common diseases that affect their livelihood could help in conserving these plants for future generations. Understanding the effectiveness natural products responsible for the actions of these medicinal plants would also be a good development.

Thus the main aims of this project are:

## 1.5 Project aims

- To investigate and document Bedouin women's knowledge and use of medicinal plants used to alleviate reproductive conditions.
- To select plants identified by Bedouin women for phytochemical study including isolation and characterization of potentially active compounds.
- To evaluate the isolated compounds as follows:
    i) To screen for anti-bacterial and anti-fungal activities.
    ii) To screen for anti-inflammatory properties.
    iii) To assess their effects (contraction & relaxation) on uterine mouse tissue.

The results of this project will be communicated to the Bedouin communities and Ministry of Environment in Egypt.

# CHAPTER TWO

# ETHNO-PHARMACOLOGICAL STUDIES

# 2. Ethnopharmacological study of Egyptian Bedouin women's knowledge of medicinal plants

## 2.1 Introduction

Egypt is endowed with a unique variety of ecosystems and a corresponding variety of wildlife that ranges from Eurasian to purely sub-Saharan species. Most of Egypt is either arid or hyper arid; however, due to its very varied eco-zones, the country is home to a wide diversity of terrestrial habitats, which although relatively low in species numbers and with few endemics, is extremely varied in composition. Out of the 5000 plant species growing in Egypt, about 20% are used in TM. Within Egypt, the Sinai Peninsula has a remarkably high inter-specific endemism for an arid environment, and this is particularly true in the mountainous region of the St Catherine's Protectorate.

In developing countries, people use medicinal plants to improve their state of health. TM remains the main source for a large majority of people for treating health problems. Approximately 70% of the population in Egypt use TM as their only type of medicine (Saleh, 2003). TM is a significant element within the Bedouin communities. The medical skills held by the Bedouin women, especially the older women, are generally valued and represent an important percentage of medical knowledge in the community. The knowledge of medicinal plants is transmitted from the older generation to the younger via word-of-mouth (Pilgrim, 2006) and most of this knowledge has not been documented. This thesis attempts to record Egyptian Bedouin women knowledge and collect information on herbal remedies traditionally used by Bedouin women for the treatment of female reproductive health issues such as dysmenorrhoea, pregnancy, perinatal problems, womb cleansing and the sensitive issue of genital tract infections.

## 2.2 The study areas and target groups

Egypt is located in North East Africa, with an area of approximately 1,001,449 $km^2$ for an estimated population of 85 million inhabitants. About 95% of that land is arid desert. The remaining 5% is the narrow valley of the Nile River, which bisects the country in a north-south direction. Living on only 3.5% of Egypt's land surface, almost all Egyptians have settled in the Nile Valley and Delta. Egypt demarcated by five regions, including Nile River Valley, Delta, Western Desert, Eastern Desert and Sinai Peninsula. A number of Bedouin tribes inhabit various regions of Egypt and are divided into clans or extended family units; they divided into three main groups, namely the Bedouin of Sinai, the Bedouin of the Eastern Desert and Red Sea and the Bedouin of the Western Desert (Siwa Oasis). The location of the main areas can see from the map in Figure 1.

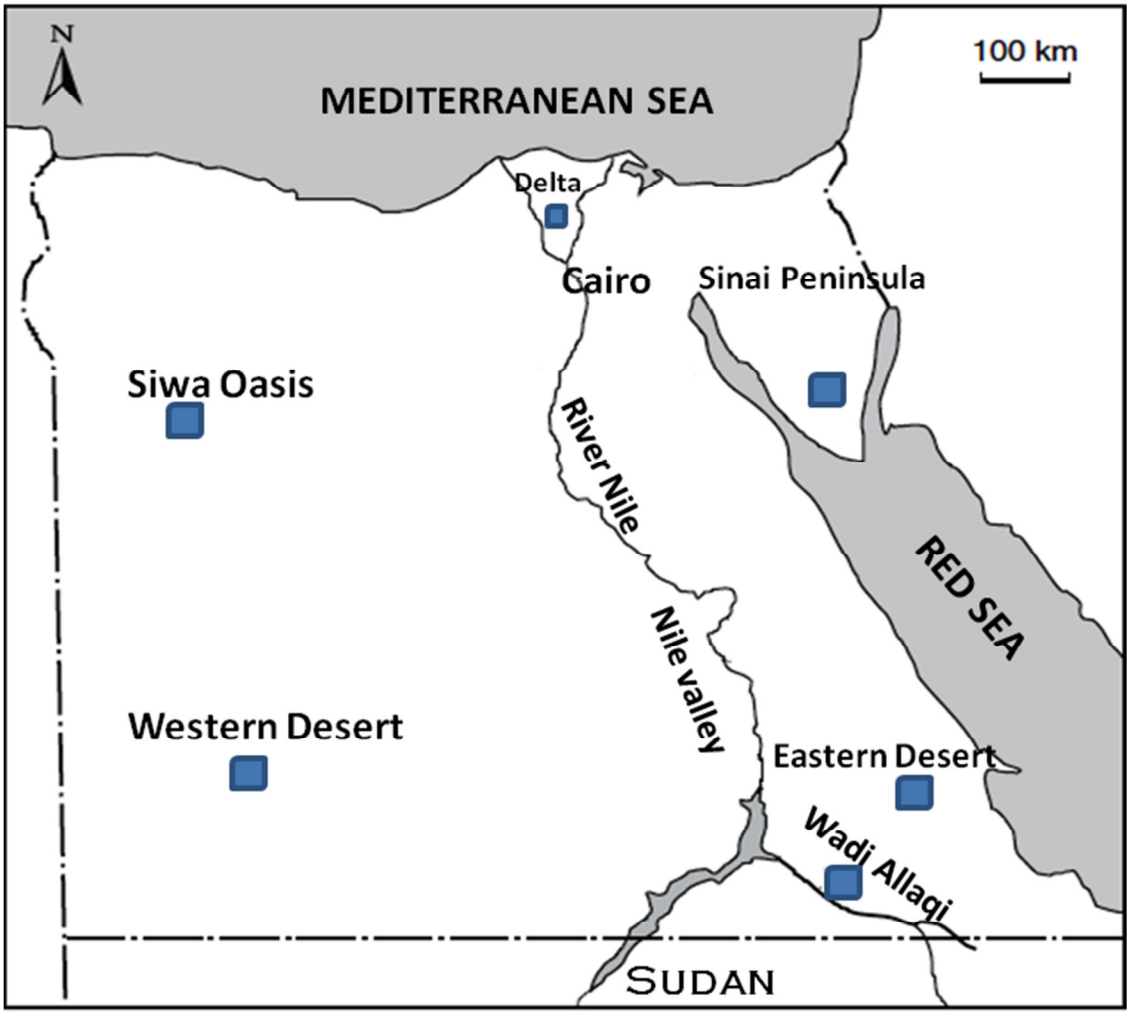



**Figure 1** Geographical locations of the Bedouin communities in Egypt.
Modified from Briggs *et.al*, (2007). = Main locations of Bedouin inhabitation.

### 2.2.1 Bedouin of Sinai

The Sinai Peninsula has geographical importance in that it is where the continents of Africa and Asia meet. St Catherine is located on the Southern part of the Sinai Mountains between 33$^{o}$ 570' to 34$^{o}$ 000' south, and 28$^{o}$ 260' to 28$^{o}$ 340' east reaching greater than 2600 meters above sea level (Danin, 1983) (Figure 2). The flora of the region is typically of Saharo-Arabian origin with Irano-Turanian elements. The variation in rock type, weather, moisture, temperature, light and soil conditions as well as altitude provide a wide range of habitat for various flora (Danin, 1986). Saint Catherine is the coldest area in Sinai due to its high elevation. Temperatures vary from 1.4 to 30.8$^{o}$ C, with relative humidity ranging from 24.9 to 49.8 % (Moustafa *et al.*, 1996). .

There are about 300 plant species, of which 33 are endemic to the Sinai. At least 47% have medicinal, aromatic, cosmetic or culinary uses in addition to their value as fodder or fuel (Ayyad *et al.*, 2000). The endemic species found in St Catherine represent over 60% of Egypt's floral endemism (Kamel *et al.*, 2002). St. Catherine's Protectorate was declared a protected area in 1996 due to its immense biological and cultural interest. It has been recognized by the IUCN as one of the most important regions for floral diversity in the Middle East, containing 30% of the entire flora of Egypt. Within the Protectorate, ten species are extremely endangered and 53 are endangered. Localized overgrazing, uprooting of plants for fuel or camel fodder, and over-collection of medicinal and herbal plants are greatly threatening the floral diversity of the Protectorate (UNDP, 2001). The global significance of species found in St Catherine's Protectorate is not only related to the uniqueness of the flora and habitat, but also the value of using them. At least 47% have medicinal, aromatic, cosmetic or culinary uses in addition to their value as fodder or fuel (UNDP, 2001).

The management of the Protectorate was established under a project funded by the European Union that concluded in December 2002. Arrangements for sustainable funding of the Protectorate activities were delayed, and have yet to be fully realised; this has caused a current funding gap. The management plan called for a number of

monitoring tasks, but with the reduction in support, the rangers are unable to achieve them without assistance in terms of manpower (Guenther, 2005).

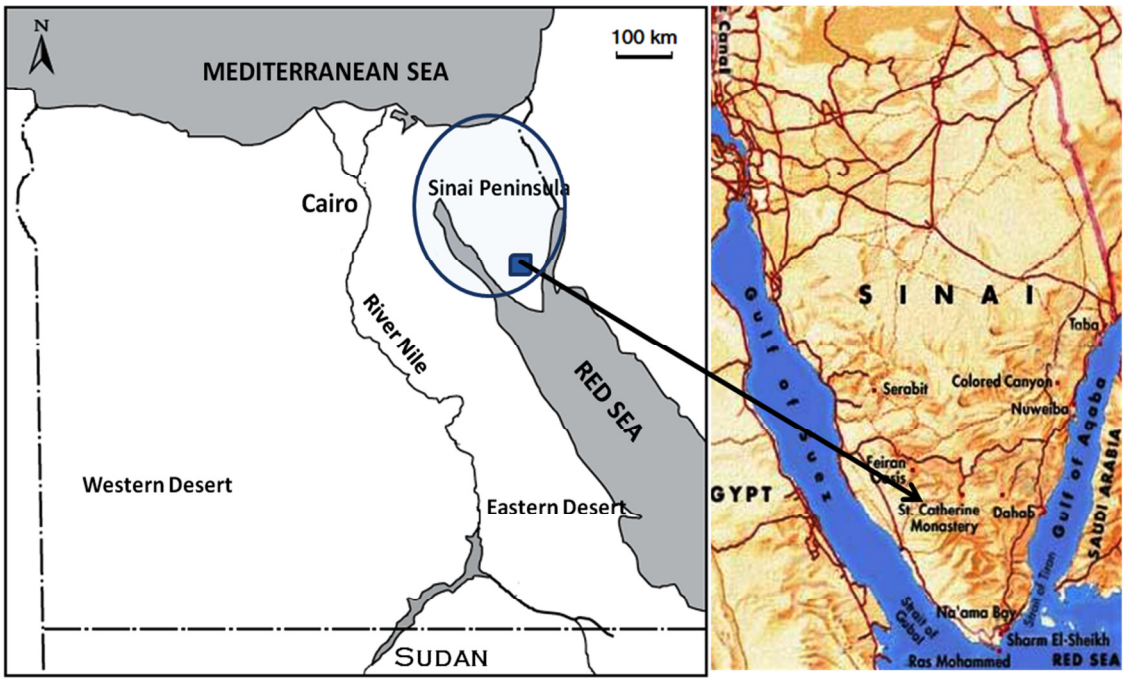



**Figure 2** Location of St. Catherine's Bedouin community.
modified from: http://www.google.co.uk/ima

Bedouins of south Sinai are split into seven tribes. The oldest tribes inhabiting the Sinai desert are the Aleigat and the Sawalha sharing a territory between Suez and Al Tor reaching into the high mountain region around Wadi Feiran and Sarabit el Khadem (Shoukir, 1948). For the last 500 years, the Muzeina tribe has occupied the territory from around St. Catherine to the Gulf of Suez and from Al Tor covering the southern Sinai from Sharm el Sheikh to Nuweiba. The Tarabin Bedouins are located just north of Nuweiba and arrived some 300 years ago (Abdel Rahman, 2009). An exception among the Bedouin tribes in Sinai is the Gebeliya tribe. Gebeliya literally means "mountaineers" and this tribe lives in the high mountains around St. Catherine. They originally came from Eastern Europe (most likely Yugoslavia, as they speak a different language besides Arabic) to serve in the monastery of St. Catherine. They converted to Islam and adapted to nomadic customs. They are often divided into small, tightly knit tribes or clans and their leaders are selected for their

wisdom and judgment. The family unit is the basis for domestic life. Strong family ties and taking responsibility for one's relatives is expected (UNDP, 2001).

The Bedouin who live in St Catherine have developed an extensive knowledge for the use of the different plant species, which is an integral part of their economy. Bedouin women have important roles in collecting plants for medicinal, aromatic and culinary use (Hobbs, 1985). The medicinal skills held by their women are generally valued in the community, especially for women's reproductive health problems. The over exploitation of medicinal plant resources to meet increased demand from local consumption, traditional healers (Hakim) and medicinal plant traders threaten the existence of many important species of commercial value (UNDP, 2001). Many Bedouin men work in tourism, although some continue to cultivate their walled mountain gardens or work in their oases. They grow vegetables, as well as almonds, pomegranates, apples, apricots, olives and figs. Small amounts of this produce are sold in the market, but this is mostly for local consumption. Women herd livestock and produce traditional craft items.

### 2.2.2 Eastern Desert Bedouin

The Eastern Desert, covers about 220,000 sq km (136,701 sq mi) and is dissected by wadis (dry riverbeds), rising to mountains near the Red Sea. Briggs *et al.*, (1993), identified, Wadi Allaqi as the largest wadi in the southern part of the Eastern Desert located about 180 km south of Aswan City between latitude 22°.00' and 23°.00' North and longitude 31°.01' and 32°.80' East (Figure 3). Wadi Allaqi is classified as a hyper-arid area which is characterized by extremely low, irregular rainfall which usually falls in winter (Briggs *et al.*, 1998).

The annual rainfall in this area rarely exceeds 5 mm, is highly variable and many years may pass without any rain. Temperatures in Wadi Allaqi fall into the category of mild winter with the coldest month ranging from 10 to 20˚C. The maximum temperature in summer can often reach above 45˚C, especially in August. The highest relative humidity values during the winter months range between 27.6 to 64.7% (Springuel *et al.*, 1995). Wadi Allaqi was declared a conservation area in 1989 (Belal, 1994) and has had protected status since then within the Egyptian

Environmental Affairs Agency (EEAA). The arid environment and combination of two ecosystems (extreme arid desert and the shores of Lake Nasser) and inhabitation by nomadic tribes, this area was designated a biosphere reserve in 1993 within the UNESCO Man and Biosphere Programme (MAP).

According to (Belal, 1997), Wadi Allaqi Biosphere Reserve is organized into three interrelated zones, known as the core areas, the buffer zones and the transition areas (Figure 3).

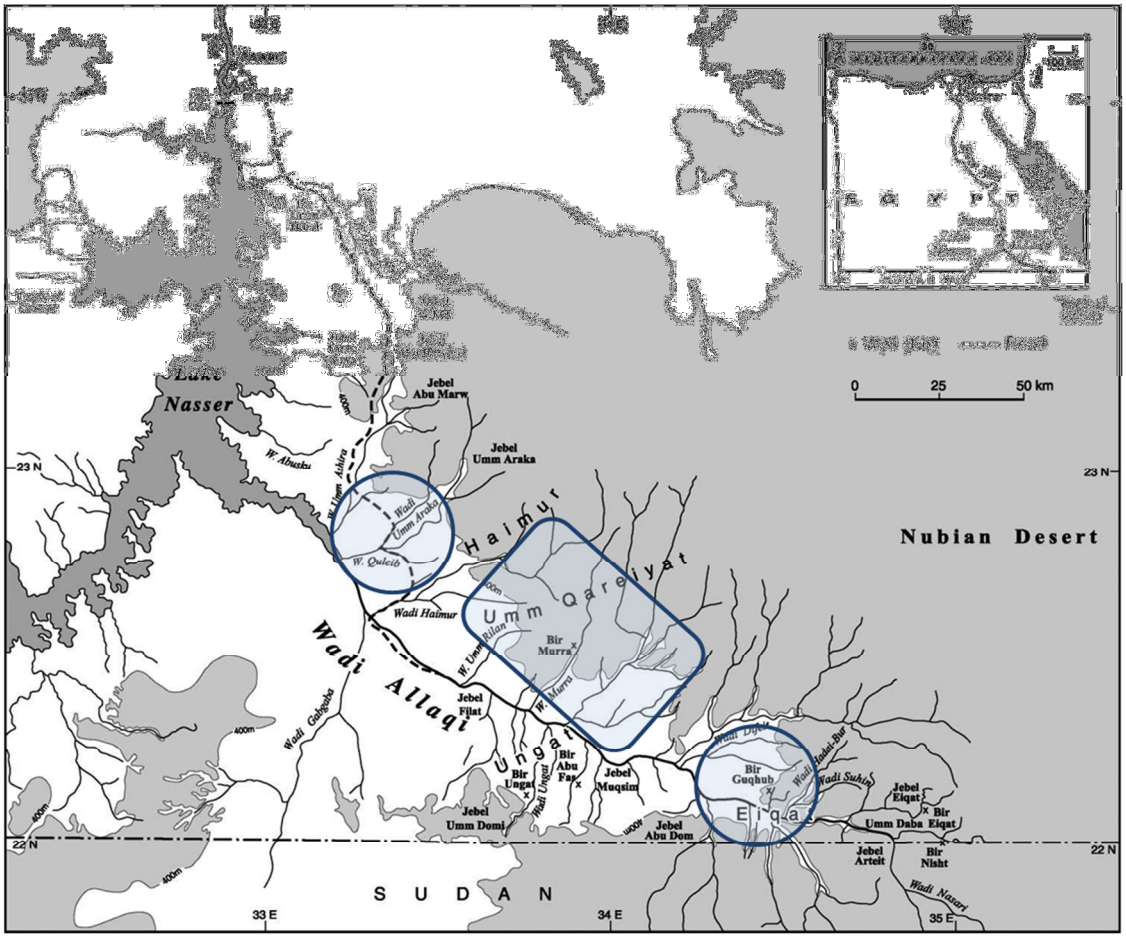



**Figure 3** Regions where the first and second core zones are located. Wadi Quleib and Egat on downstream and upstream tributaries of Wadi Allqi. Buffer zone indicated by O. Modified from Briggs and Sharp (2009).

The two core areas and buffer zones located in the downstream and upstream parts of the wadi are separated by approximately 150 km. The first core area comprises the floor of Wadi Quleib, which is in a downstream tributary of Wadi Allaqi. The second core area lies in an upstream part of the Wadi Allaqi basin, named the Eigat area after Mount Eigat, which rises 1,400 m above sea level. The initial focus of this research was on the Wadi Allaqi downstream part.

The last documented assessments of Wadi Allaqi were described by Belal in 1994 and 1997. Around 127 species of higher plants were discribed. No endemic species were recorded, but large undisturbed populations of *Salvadora persica* (Siwak) and *Balanites egyptiaca* (Heglige) were found to occur in the upstream area of Wadi Allaqi, while these were threatened elsewhere in Egypt (Belal, 1994), the situation now is unknown. The vegetation of the main wadi is an open scrubland of sparse and scattered *Acacia* trees and a few perennial species during the rainless period. However, after the rain, annuals appear on the floor of the wadi. Numerous fossil hillocks with dead remnants of *Tamarix* and *Salvadora* are found in the midstream part of the wadi, which are obvious relicts of extensive thickets that grew in the past.

There are two main semi-settled ethnic groups living in the Eastern desert. One is the Bisharin, who came from the Egypt-Sudan border area and the Red Sea Hills and mostly live in the upstream part of the wadi. The other group is the Ababda, who have inhabited the southern part of the Eastern Desert for several centuries (Hobbs, 1989). The Bedouin have traditionally been a highly mobile group, moving all their belongings to follow seasonal pastures in the hyper-arid environment of the Eastern Desert. The livelihood of Wadi Allaqi residents is derived from sheep, goat and camel herding, cultivation of small farms, charcoal production and collection of medicinal herbs for trade purposes (Briggs *et al.*, 1993). The most important of these are the first three, as other activities are no longer used. The inhabitants of the downstream part live along the lakeshore and shift their settlements according to fluctuations in the level of the lake. Women no longer migrate with the men, but have settled near to the water supply of the lake (Sharp *et al.*, 2003). Bedouin here

have become more sedentary, in 1999 Briggs *et al.*, described that because of the successive droughts, the Bedouin ceased to move to the desert.

According to Briggs *et al.*, (1999) Bedouin women in Wadi Allaqi avoid meeting men who are not from their family. The place where women stay is separate from the area where guests are met. The traditional tents (*El bersh*) used by Bedouin in this area, of extended family groups are arranged between a few metres and a few hundred metres from one another. A senior patriarch who makes major household decisions, while day-to-day decision-making is left to the individual households dominates each extended household. In the past, the entire household would move to follow resources. Now, only men take the household herds to distant parts of the desert in search of grazing, and to trade in markets such as Aswan. Women are not mobile and must be accompanied by a male relative on their rare visits to town in case of illness or an important family occasion (Sharp *et al.*, 2003).

### 2.2.3 Bedouin of Siwa Oasis

Siwa Oasis is located between the Qattara Depression and the Egyptian Sand Sea in the Libyan Desert, nearly 50 km east of the Libyan border, and 560 km from Cairo. It is bounded by longitudes 25° 18'–26° 5' E and latitudes 29° 5'–29° 20' N, (
). The climate exhibits extreme aridity from April to November with very low rainfall occurring from December to March and high evaporation and highest summer temperatures 37.7 °C in July and August (Misak *et al.*, 1997). The region comprises mainly closed flat depressions bounded in the north by a limestone plateau and in the south by areas of mobile sand dunes. Saline lakes and cultivated land are the main habitats of the closed flat depressions, whose lowest point is 22m below sea level (Masoud AND Koike 2006).

UNESCO (2004) has defined Siwa as one of the world's last remaining pristine oases, home to spectacular natural landscapes, ancient historical ruins and unique cultural traditions. Famed as the location of the Oracle of Amon, whom Alexander the Great consulted before continuing his Persian conquest. Siwa is a place for different species and an island of biodiversity with a variety of endemic flora and

fauna. Date palm and olive groves are found wherever water is available at or near the surface. In the lower parts of Siwa, wetlands, salt marshes and a range of sand

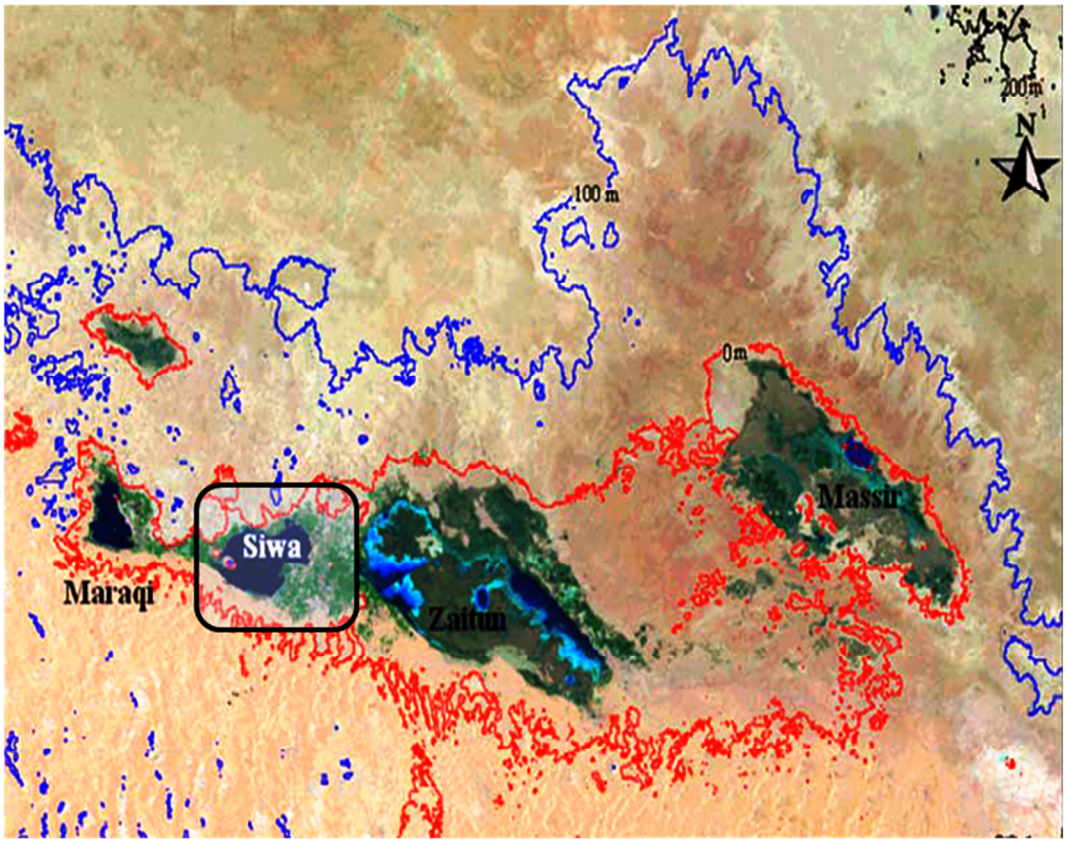



**Figure 4** map to show the location of the Bedouin in Siwa. Modified from Masoud *et al*., 2006.

formations have emerged. To the south, the dunes of the Great Sand Sea stretch endlessly into the landscape. In 2002, the Egyptian Government declared 7,800 square km in and around the Siwa Oasis a protected area, in recognition of Siwa's cultural, biological and environmental value (Masoud and Koike, 2004). The new status prohibits all activities that damage or deplete the natural environment, including indigenous flora and fauna, and has bolstered the movement to preserve Siwa's invaluable resources (UNESCO, 2004).

Agriculture particularly the cultivation of dates and olives is the main activity of the modern Siwian. All such agricultural activities depend completely on ground-water resources in the form of artesian wells of good quality water that permit intensive cultivation (Masoud *et al.*, 2006). Fakhry (1973) described the importance of

handicrafts like basketry anfd that tourism has in recent decades become a vital source of income. Much attention has been given to creating hotels that use local materials and play on local styles. In addition, Fakhry (1973) described how the number of years of isolation in the desert have allowed the Siwan community to develop unique cultural traditions, building techniques, styles of embroidery and systems of agricultural production that are remarkable for their beauty and harmony with the natural environment. Siwa Oasis has a population of around 27,000 spread out around the villages in the oasis, but mainly in the town of Siwa. There are 11 tribes in Siwa, all Berber (Siwans) except for one Bedouin tribe in the village of Baha El Din in the west of the oasis. Siwans are the descendents of the Berbers, or Imazighen, North Africa's original inhabitants, Siwans share more with cultures to its west than with Egypt. Siwa is the easternmost reach of Berber culture, and the oasis features rites, traditions, dress, tools, and a language distinct from the other oases of Egypt's Western Desert. While most Siwans speak Arabic, Siwan children first learn to speak the Siwan language, called Siwi. Siwi is a dialect of the northern branch of Tamazight (the Berber languages) and is closely related to dialects spoken in other Amazigh (Berber culture) communities in Libya, Algeria and Morocco (Fakhry, 1973). Siwa is similar to other Bedouin communities being a very conservative society. Traditionally, both married and unmarried women alike do not leave the home unaccompanied, though stronger restrictions are placed on the movement of married women. If and when they travel outside their home, they cover themselves from head to toe.

## 2.3 Research methods

### 2.3.1 Methodology development

A methodology of participative observation with the Bedouin, was followed which involved discussions and focus groups. The methodology used in this study was based on a series of monthly in-depth conversational interviews, and, as far as possible, the atmosphere of these meetings was one of informal conversation. This was particularly with Bedouin of Wadi the Allaqi who are a more remote community (Saleem, 2004). Before the present research work was commenced, frequent visits to Bedouin women of Wadi Allaqi area had already taken place. Previous studies were conducted in Wadi Allaqi area with Bedouin men from 1997 to 1998 (Briggs *et al.*, 1999) while with women from 2000 to 2004 (Sharp *et al.*, 2003;Saleem, 2004). It included eight families, six Ababda and two Bishari households. They were resident in the Wadi Quleib (tributory of Wadi Allaqi). The main objectives of the previous studies were to explore the value of Bedouin indigenous knowledge for understanding of the use and management of the environmental resources of some plant species in the downstream part of Wadi Allaqi. Another aim was to illustrate the potential role for women in sheep production and poverty alleviation.

All the interviews and conservations with the women were in Arabic, although each group spoke their own languge along with Arabic. The discussions were made with small focus groups consisting of five or six women. The research teams were divided into two; women researchers having discussions with Bedouin women and men researchers with Bedouin men. Prior to the field visits, the set of questions were prepared for the present study. Non-family males are not permitted to enter the women's area in the Bedouin settlements, although female visitors can enter male spaces. Female researchers sat with women in their tents, while the men stayed outside with the Bedouin men. Initially, Bedouin women were quite worried about the visits and some avoided speaking by doing something else. They did not like to have their voices recorded or notes to be taken in front of them, so researchers had to memorize the discussion with each focus group, and write up the results as soon as possible on returning to the field station. The research work was over a period of one year. There were mainly two field visits per month, sometimes more. Each visit

lasted a minimum of half an hour and a maximum of two hours. Through time consuming, these discussions helped to build up trust, and so reduced the emotional distance between Bedouin women and the researchers. After a number of visits and frequent discussions, the Bedouin women became used to the visits and became more friendly, relaxed and welcoming to researchers. They always made Gabana coffee for guests, as it is considered a way of showing hospitality. This familiarity encouraged other women to come and sit with the researchers as one group. At this point, the discussion between the women and researchers took a more natural form of communication. It alternated between listening and talking during the conversation to make the situation more natural than interviews, which impose their own logic on the respondent, or questionnaires, which are sometimes seen as being like an exam by the Bedouin. For Bedouin of St. Cathrine and Siwa this was not necessary as they are distributed close to tourist areas and are used to interacting with outsiders. It was quite easy to speak to them from the first visit.

### 2.3.2 Ethnobotanical survey

In the present study, ethnobotanical studies were conducted between November 2005 and February 2008. The number of visits varied depending on the area and group. For example, visits to Bedouin in Wadi Allaqi were frequent, every three or four weeks, while for the Bedouin in Siwa it was four times during the whole study period and more than six times for the Bedouin of St. Catherine. Ethics clearance was obtained from the EEAA before the onset of the study. Objectives of the study were explained in each area to each of the interviewees and before conducting the interview, a form of oral consent was agreed with the Bedouin because they cannot read or write and understandably they are suspicious of anyone asking them to put their finger prints on any papers. However, video recordings were used to document the Bedouin declaring agreement to share their knowledge. The consent stated that the project is for academic purposes only and is of no commercial value. It also informed the interviewee that the results from this study will be presented at conferences and published in academic journals.

The interviewee was also assured that he/she was under no obligation to share any information which he/she did not feel comfortable in sharing. According to Bedouin customs, women should not be either seen or heard by outsiders, so promises had to be made that their records or photographs will not be seen by foreigners especially males. Furthermore, the property rights of the community are protected by the Biodiversity Act and its associated Bio-prospecting, Access and Benefit Sharing (NEMBA, 2004). The information was collected using focus groups and semi-structured interviews with 90 persons (71 women, 19 men) whose ages ranged from 15 to 65 years, and who retain traditional knowledge. Five of them were midwives and two were traditional healers. Observation and participation in respondents' activities were the initial data gathering methods during the first weeks of the field study. In the first phase of the field study, Bedouin were asked to freely recall all medicinal plants and other natural remedies that they use or have used in the past. More specific information was recorded later by using structured interviews in which a specifically developed questionnaire was completed (Appendix I). The questionnaire was designed to obtain the following information; local plant names, plant parts used, preparation methods, dosage forms and quantities, use of plants in combination as well as related symptoms. They were asked to precisely describe the method of use and preparation of the medical remedies for each species. During the interviews, women were followed in the field to see how they collected the mentioned species. Each species recognised by the Bedouin to be used for medicinal aims (voucher specimens) was collected and deposited at the Herbarium (of the South Valley University and St. Catherine's Protectorate Herbarium) for identification. Dr. Magdi Radi and Mr. Ahmed Saleh helped in the identification of the plants.

## 2.4 Results of the ethnopharmacological survey

### 2.4.1 Collection methods of plants by the women

During the collection of the plants Bedouin women followed a certain strategy. For example, women in St. Catherine and Siwa collect the plants early in the morning. Specifically, if they collected during the flowering season they collected small quantities, including leaves. Sometimes they cut the whole plant leaving the roots

only. Then they placed the plants on rocks in shaded areas where they could be dried on their exposure to sun light. Then after two or three days they collected them. Sometimes they took the plants home to dry them in the same way. The plants were stored in cloth bags until used. In Wadi Allaqi where the plant materials come from trees, women used a stick to hit the tree or shake it until the pods and small leaves fall down so they could use them as medicine or to feed their goats (Figue 5)


**Figure 5** photograph of Bedouin women collecting pods from an *Acacia albida* tree. Permission was given to use this photograph; photo was taken by Prof. Irina Springuel.

### 2.4.2 Parts used, method of preparation, means of administration

The plant parts predominantly used were the leaves and flowers. Other parts included, bark, roots or seeds. The most common way of preparing these herbal medicines was decoction, mostly taken orally or applied externally on the skin, while others were used as an infusion. The length of the treatment was highly variable from one day to six months. The plants used in the treatments by the women were usually used alone, but in a few cases, two or three were used in combination. Figure 6 shows the preparation method for one of these plants.

**Figure 6** Bedouin women describing the preparation of *Cleome droserifolia* as tea. Permission was given to use this photograph.

### 2.4.3 Knowledge of the use of medicinal plants

In most interviews, it was found that their mother or other members of the family such as grandparents had passed down the Bedouin women's knowledge about medicinal plants to them. This knowledge had been orally transferred from one generation to the next. Most of the people being interviewed were female and although 98% of the interviewees had received no formal education, they had a good knowledge regarding their health and the plants that they were using; also their knowledge on conserving and management of the resources was high. For example, they knew that after flowering they should not collect the plants, so that seeds could be formed. Most of the plants cited were principally recorded based on information supplied by the women. These plants sometimes have no use for treatment of diseases experienced by men, but this is justified by the fact that women are more aware of their illnesses. In addition to herbal remedies, diet also plays an important role in the health of expecting mothers and their offspring.

Traditionally, after labour and delivery Bedouin mothers should have a rest period of 40 days as she is more fragile during this period (Nahas et al., 1999). They are

advised not to do any work to recover their health through bed rest, food supplements and herbal medicines, which act as health tonics. Rest has been adopted for '40 days' because according to many Muslim scholars the maximum period for post-natal bleeding is forty days.

### 2.4.4 Medicinal plants and their diversity

The number, names, sex, age and clans of the Bedouin are described in Appendix II. Table 2 summaries the plant species used in this study, for treating women's health problems. Data revealed that more than 40 different plant species in 22 plant families and 30 genera are commonly used. The largest number of species (four) was noted from the family Lamiaceae (Labiatae), three from Asteraceae (Compositae) and three species from Chenopodiaceae. Most of the plant species encountered in the study are indigenous, which is important with respect to the sustainable use of indigenous flora. Urinary tract infections (UTIs) were ranked the highest for the numbers of species used (65%), followed by dysmenorrhoea (30%), then postpartum (25%), and during birth (15%) as can be seen in Figure 7.

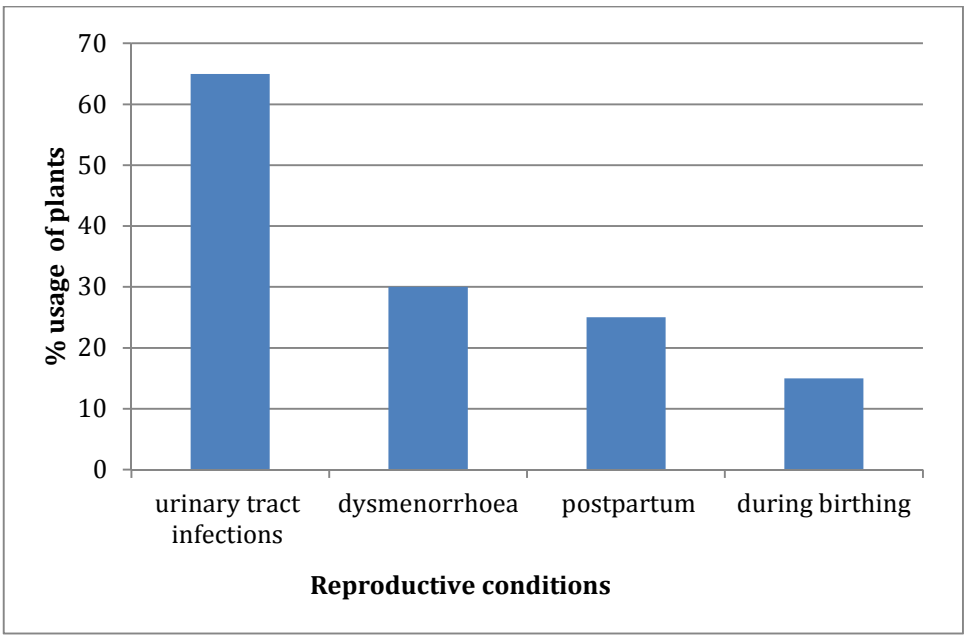



**Figure 7** the percentage use of plants against various female reproductive conditions within the three Bedouin communities.

### 2.4.5 Women's perinatal and other health problems experienced by Bedouin women

During the interviews, Bedouin women were asked to mention all the key female reproductive problems affecting them. The main problems mentioned were, postpartum, dysmenorrhoea and UTIs. They also identified the following symptoms associated with pregnancy: morning sickness or *nausea*; swelling of legs and ankles (oedema); muscular and abdominal pain during pregnancy; threatened miscarriage; speeding up of contractions and *anoxia* during labour; retained placenta; postpartum haemorrhage, womb cleansing and postpartum abdominal pain; lactation, in addition they mentioned eye discharge of the new born infant.

### 2.4.6 Plants used during birth for induction of uterine contractions

Bedouin women in St. Catherine used *Pistacia atlantica* and *Rhamnus disperma* to increase uterine contractions during birth and stop postpartum haemorrhage, while in Siwa Bedouin women used *Anastatica hierochuntica* and *Haplophyllum tuberculatum* for the same reasons. In Wadi Allaqi women were advised to eat well and have palm dates, butter, meat or honey as a means of nutrition to give them strength during the birth.

### 2.4.7 Plants used for post-partum problems

*Haloxylon salicornicum* (aerial parts), *Juncus rigides* and *Rosa Arabica* (flowers and leaves) are used as purgatives to cleanse the uterus and also to stop postpartum abdominal pain. They are used continuously during the postpartum period. To the best of my knowledge *Rosa arabica* and *Juncus rigides* are recorded for the first time globally as medicinal plants used for the treatment of postpartum problems. No record of any medicinal uses for *Rosa arabica* and *Juncus rigides* could be found in the literature. However, they were highly cited (99%) by Bedouin women for this uses. Due to that the conservation status for *Rosa arabica* (endemic to Sinai) is critically endangered species, *Haloxylon salicornicum* was by far the most frequently (90%) instead used plant species within Bedouin women in St. Catherine to cleanse the uterus or stop postpartum abdominal pain. Wadi Allaqi women drink tea made from *Cleome droserifolia, Solenostemma argel* and *Cymbopogon proximus* for

postnatal care; they take it three to five times a day and they also use herbal steam baths using these species. This is prepared by adding a handful of each plant to boiling water in an open bucket. The bucket is placed in front of the woman being treated so that the steam can reach the woman's buttocks and vulva. The woman is then wrapped herself in a blanket. The women believe that this contributes to their recuperation and body restoration, as well as in the prevention of any infection. In Siwa, they mix *Artemisia judaica* with *Haplophyllum tuberculatum* put on a small piece of cotton which is then inserted into the uterus overnight as a pessary after expelling the placenta. They also drink *Cinnamomum verum* and *Zingiber officinale* as tea, a spoonful in a cup of water.

### 2.4.8 Dysmenorrhoea and pelvic pain

Pelvic pain during menstruation is a problem for both women and girls. They use different plant species to alleviate menstrual pain, including species ranging from those used as antispasmodics to those used for postpartum. All the species used are taken as much as required ther are prepared as a tea, for example, in St. Catherine, *Achillea fragrantissima, Artemisia judaica, Cleome droserifolia, Mentha longifolia* and *Haloxylon salicornicum* are used, while in Allaqi they use *Cleome droserifolia, Solenostemma argel* and *Cymbopogon proximus*. In Siwa they prefer to drink *Cinnamomum verum*, *Zingiber officinale* or eat Palm dates with olive oil.

### 2.4.9 Urinary tract infections

Around 21 species are used for the treatment of vaginal infections which was the highest percentage of the total number of species. Water extracts were prepared by boiling the plants with water and filtering them for *Achillea fragrantissima, Artemisia judaica, Cleome droserifolia, Mentha longifolia* and *Solenostemma argel*. The solution was applied externally to wash the vagina (as a douche) and consumed as a tea for a few (3-6) days. The latex from *Citrullus colocynthis* fruit was applied externally to the vagina. The water extract of Acacia seeds were slightly warmed up and mixed with *Lawsonia inermis* then applied to the vagina. Alternatively, the extract was placed in a small basin in which, the woman can sit in the preparation for 10 to 20 minutes. It is sometimes administered by insertion in the birth canal (this is

for married women only). In addition, the bark and branches of *Balanites aeyptiaca, Leptadenia pyrotechnica Acacia nilotica, Acacia etbica*, roots of *Panicum turgidum* and rhizome of *Cymbopogon proximus* are used as a fume infusion; the plants are burned in front of the seated woman in such a manner that the fumes reach the woman's vaginal area. In Siwa, *Artemisia judaica* and *Allium cepa* L. is ground and then mixed with olive oil before applying to the vagina as a pessary. In all Bedouin the treatment is self administered in the woman's tent or space.

In general most of the common diseases for Bedouin women were urinary tract infections (Julie Cwikel, 2010), this explaining the high percentage cited for the number of the plants that used for this it. Common factors underlying these health conditions were poverty, high fertility rates, lack of access to sanitary waste disposal and hygiene (Alean Al-Krenawi, 2003). These environmental exposures serve to deepen the relative poverty already felt by these women. In some areas, toilets do not exist at all and women wait till the cover of darkness to relieve themselves in outhouses on the outskirts of their family compounds. This has been associated with the high rate of urinary tract infections. Furthermore, Bedouin women have problems of access to health services, finding them expensive and difficult to access without male accompaniment (Mazen zoabi, 2011).

**Table 2** List of the scientific and vernacular names of medicinal plants used by Bedouin women in Egypt, together with preparation and uses, listed in order of decreasing citations.

| Scientific name | Family | Local name | Voucher no. | Part used | Uses | Preparation |
|---|---|---|---|---|---|---|
| **Plants from St. Catherine** | | | | | | |
| *Achillea fragrantissima* (Forssk) SCH.BIP | Asteraceae | قيصوم | 1058 | Leaves and flowers | eye lotion, colic, indigestion, antiseptic, women infection, stomach pain | infusion or decoction |
| *Artemisia judaica* L. | Asteraceae | بعثران | 003993<br>003992 | Leaves and flowers | antispasmodic, anthelminthic, purgative, antiseptic | infusion or decoction |
| *Chiliadenus montanus* (Val)Brullo | Asteraceae | هنيده | | Leaves and flowers | renal colic and stones, antispasmodic, kidney diseases | decoction or maceration |
| *Citrullus colocynthis* L. | Cucurbitaceae | حنظل | 008597 | Fruit, leaves and oil from dry seeds | Joints pain, skin disease, fungal infection antirheumatic, antihelminthic. | external application |
| *Cleome droserifolia* Delile | Capparaceae | سموه | 007650 | Leaves and flowers | anti-diabetic, skin diseases, diuretic, renal colic, antispasmodic, genital infections, menstruation pain | boiled with water, taken on empty stomach |
| *Haloxylon Salicornicum* Bunge ex Boiss | Chenopodiaceae | رمث | 010874 | The whole plant | menstruation and birth pain, to cleanse womb after birth | decoction or infusion |
| *Juncus Spp.* | Juncaceae | ديس | 011305<br>011306 | Flowering parts | menstruation pain and after birth | boiled with water |
| *Mentha longifolia (L.) Huds. subsp. schimperi (Briq.) Briq* | Lamiaceae | حبق | 2415 | Leaves and flowers | antispasmodic, cold and menstruation pain | decoction or infusion |
| Origanum syriacum L. var. sinaicum (Boiss.) | Lamiaceae | زعتر | 004028<br>004026 | Leaves and flowers | spices, analgesic, cough, stomach pain | decoction |
| *Thymus decussatus* Benth | Lamiaceae | زعيتران | 007854 | The whole plant | antispasmodic, vomiting, abdominal colic | decoction |
| *Salvia multicaulis* vahl | Lamiaceae | بردقوش | 008141 | Leaves and flowers | cough, cold throat infection, analgesic | decoction |
| *Pistacia atlantica* Desf. | Anacardiaceae | بطم | 1124 | Leaves and flowers | accelerate contractions during labour, to stop bleeding- | decoction or infusion |
| *Rosa arabica* Crép. | Rosaceae | ورد بر | 004008<br>004087 | Leaves and flowers | for women after birth and to cleanse womb | decoction |
| *Rhamnus disperma* Ehrenb. ex Boiss | Rhamnaceae | عرن | 2135 | Leaves and flowers | accelerates contractions during labour and to stop bleeding | decoction |
| *Anabasis setifera* Moq. | Chenopodiaceae | جلو | 010932 | Leaves | bleeding | boil with water |
| *Anabasis articulata* Forssk. | Chenopodiaceae | عجرم | 010933 | Whole plant | skin diseases and as a soap and antiseptic | boil with water |

| | | | | | | |
|---|---|---|---|---|---|---|
| Peganum harmala L. | *Zygophyllaceae* | حرمل | 2456 | Seeds, roots | urine retention (seeds), dental pain (roots) | boiled with water |
| *Solenostemma argel* Hayne | Asclepiadaceae | حرجل | 008343 | Leaves and flowers | urinary tract infection, antiseptic, abdominal colic, gastric problems | boiling with water |
| *Teucrium polium* L. | Lamiaceae | جعدة | 008028 | Leaves and flowers | antispasmodic, antiseptic, skin allergy-diarrhoea and urinary tract infection | decoction or infusion |
| *Globularia arabica* Jaub. & Spach | Globulariaceae | حندقوق | 001153 | roots | Leather dying, burned skin and stomach diseases, urinary tract infection | boiled with water |
| *Retama raetem* (Forssk.) | Leguminosae | **الرتم** | 6532 | Leaves, whole branches and bark | For wounds and Antiemetic, infection | Boil with water |
| *Zilla spinosa* Prantl | Brassicaceae | سلة | 002196 | Flowers leaves | Urinary tract diseases | infusion or decoction |
| *Capparis spinosa L.* | Capparaceae | لصف | 010475 | Leaves, flowers and fruits | Antirheumatic, biles and dysmenorrhea | infusion or decoction |
| *Hyoscyamus muticus* L. | Solanaceae | سكران | 011192 | Leaves | Bronchial diseases | Wrapped leaves as cigarettes |
| *Acacia tortilis* Hayne | Leguminosae | سمر | 006928 | The whole branches | Fuel wood | Infusion or Burning |
| *Artemisia herba-alba* (Asso) Sojak | Asteraceae | شيح | 004290<br>010846 | The whole herb | Antispasmodic, Flatulent | Decoction or boiled |
| *Cassia senna* L. | Leguminosae | سنا | 008336 | Legumes | Laxative | Maceration or boiled |
| *Tanacetumsinaicum (Fresen.) Delile* | Asteraceae | مر | 4125 | The whole herb | Antispasmodic, Antiallergenic | Boiled or dry powder |
| *Tamarix aphylla* L. | Tamaricaceae | طرفه | 5421 | Roots | Digestion and Skin diseases | Boiled with water |
| *Ballota undulate* Benth. | Lamiaceae | رسا او | 6845 | The whole herb | Skin allergy | Boiled with water and wash the skin |
| **Plants from Wadi Allaqi** | | | | | | |
| *Acacia nilotica* L. | Mimosaceae | سنط | 005437 | Seeds | Genital infection, antispasmodic, vomiting and abdominal pain | Maceration or external application |
| *Leptadenia pyrotechnica* | Asclepiadaceae | مرخ | | Stems | Genital infection | Fume infusion |
| *Cymbopogon proximus* Stapf | Poaceae | حلف بر | 009845 | Leaves and flowers | Genital infection, pain during birth and menstruation | Fume infusion or boiling with water |
| *Panicum turgidum* | Poaceae | شوش | 004308 | Roots | Genital infection | Fume infusion |
| *Acacia etbaica* Schweinf. | Leguminosae | قرض | 007224 | Leaves and branches | Pain during birth and menstruation, cleanse womb after birth and dysentery | Fume infusion |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Balanites aeyptiaca* | Zygophyllaceae | الوب | 10587<br>10586 | Fruits, Leaves, shoots and roots | Diabetes, constipation and genital infection | Fruits eaten, decoction for leaves fume infusion for roots and branches |
| *Lawsonia inermis* | Lythraceae | حنة | 007181<br>10646 | Leaves | Genital infection, skin and hair dyeing | Maceration and external application |
| *Salvadora persica L.* | Salvadoraceae | اراك | 005207 | Bark and shoots | oral hygiene, chewing stick (toothbrush), antiseptics and genital infection | Infusion |
| *Zizyphus spina-christi* | Rhamnaceae | نبق | 008413 | Leaves and shoots | Sores as wound powder and antiseptics | Maceration or infusion |
| **Plants from Siwa** | | | | | | |
| *Anastatica hierochuntica L.* | Brassicaceae | كف مريم | 002761 | The whole plant | accelerate contractions during labor and stop bleeding | Boil with water |
| *Haplophyllum tuberculatum* | Rutaceae | شجر ريح | 001007<br>010228 | The whole plant | Accelerate contractions during labor and stop bleeding | Boiled with water |
| *Cinnamomum verum* | Lauraceae | | | bark | Pain during menstruation and birth | Boiled with water |
| *Phoenix dactylifera* | Palmae | رطب | 011209 | fruits | Accelerate contractions during labor | Eaten fresh or boiled with water |
| *Olea europaea L.* | Oleaceae | زيتون | 006738 | Oil from fruits | Oil used for genital infection mixed with artemisia | External use |
| *Allium cepa L.* | Alliaceae | بصل | 3541 | tuber | Genital infection | External use |
| *Zingiber officinale* Roscoe | Zingiberaceae | زنجبيل | 6845 | Rhizome | Pain during menstruation and birth | Boiled with water |

# CHAPTER THREE
# PHYTOCHEMICAL STUDIES

## 3. Phytochemical evaluation

This Chapter will give some information about the plants have been collected for further phytochemical investigation. Information will include the way of collection, time, date, place and the reason for chosen these species (3.1). Review for the previous phytochemical and pharmacological studies on these species as well as their geographical distribution and the worldwide traditional uses (3.1.1 to 3.1.4). Then the phytochemical methods (3.2) and results of investigation will be presented (3.3).

### 3.1 Plants chosen for the phytochemical and pharmacological study

Four plants species (*Mentha longifolia, Acacia nilotica*, *Achillea fragrantissima,* and *Haloxylon salicornicum)* were selected for analysis based on the plants listed in the general survey within the Bedouin communities (Table 3). Plants were collected under guidance of the Bedouin women. Only the highly cited plant species for the same pathology were collected. Although many of other species received a high percentage of citation their unavailability was the reason for not collecting them. The collection method and preparation for analysis for these plants was possible with help from Bedouin women. Bedouin women helped and taught the author how to collect the plants. The botanical information name, family, voucher number, location and time of collection for the four species are listed in Table 3. During the collection trips Bedouin women were accompanied at all times to observe how they collect the plants, the exact location and method of collections. Plants were left to dry according to Bedouin advice and were kept in the shade for a few days at home and sometimes above the smooth rocks in the field. The dried plants were stored in cloth bags for use whenever needed.

***Mentha longifolia***: Used by Bedouin women as an antispasmodic, to treat common cold and menstruation pain. The leaves and flowers were collected from Mulgah, St. Catherine on 18/8/2007 at 07.45 in the morning, with help from Salma, about 2km from her house. The plant was exposed to the sun and growing in a wet area. The plants are prepared as a decoction. A spoonful of the dried leaves were ground and boiled with water for five to seven minutes, then filtered. The solution was mixed

with sugar and drunk while warm. Depending on the complaints, the mixture was drunk three to five times during the day.

***Acacia nilotica***: A hot water extract from the seeds used by Bedouin women for genital infections as an external application. Sometimes the extract is taken as antispasmodic, for vomiting, inflammation and abdominal pain. The Author was collected the plant pods in June 2007 from Saluga and Jazal protectorate in Aswan near Wadi Allaqi.

***Achillea fragrantissima*:** The leaves and flowers were collected from Wadi Arbaeen; St. Catherine on 17/8/2007 at 07.30 in the morning with help from Gama, from an area not far from her home. The plant was exposed to the sun. Bedouin women used it for eye treatment as a lotion, for colic, indigestion, antiseptic, women's genital infections and stomach pain. The plant prepared as an infusion and decoction; a spoonful of the dried leaves were ground and boiled with water for 5-7 minutes, then the solution mixed with sugar and drunk while still warm. Depending on the complaints the tea was drink three to five times during the day.

***Haloxylon salicornicum*:** used by Bedouin women for menstruation, postnatal problems and to cleanse the womb after birth. The areal parts were collected from Wadi Selaf, St. Catherine 18/8/2007 at 15.45 hrs in the afternoon with help from Halah. They used it as infusion or decoctions, for which a spoonful of the dried leaves ground and boiled with water for five to seven minutes, then filtered, the filtrates was mixed with sugar and drink it while it is warm. Depending on the complaints they can drink it from three to five times during the day.

**Table 3** List of plants collected for phytochemical and pharmacological study together with location and voucher number.

| **Species** | **Family** | **Plant part** | **Location & time of collection** | **Voucher no.** |
|---|---|---|---|---|
| *Mentha longifolia* | Lamiaceae | Leaves & flowers | St.Catherine, August 2007 | 2415 |
| *Acacia nilotica* | Mimosaceae | Pods & seeds | Saluga and Jazal, November 2007 | 005437 |
| *Achillea fragrantissima* | Asteraceae | Leaves & flowers | St.Catherine, August 2007 | 1058 |
| *Haloxylon Salicornicum* | Chenopodiaceae | Leaves & flowers | St.Catherine, August 2007 | 10874 |

The following section, will give a brief review for the previous phytochemical and pharmacological studies for these species.

### 3.1.1 *Mentha longifolia* (Schimper) L.

**Family:** Lamiaceae

**Genus:** Mentha

**Common name(s):** Horse Mint, Biblical mint, Wild mint, Silver mint and Habak (Arabic).

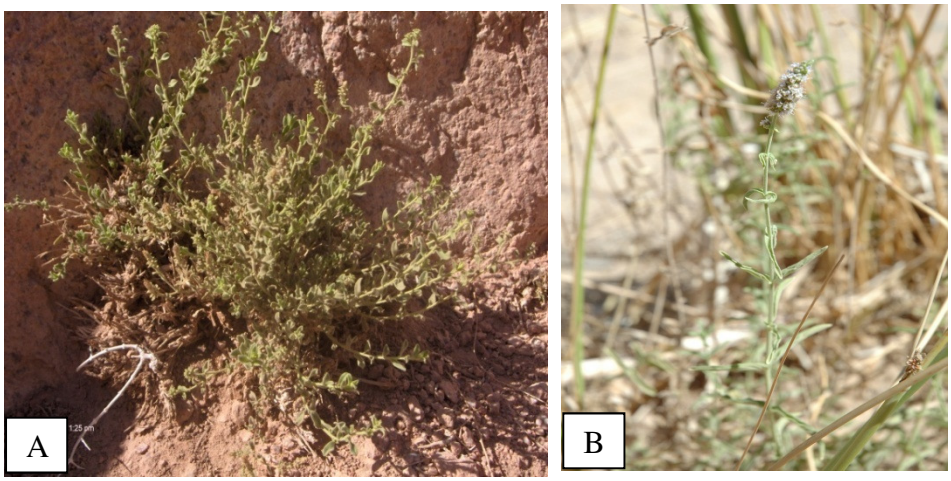



**Figure 8** *Mentha longifolia* (A) and the flower (B).

#### 3.1.1.1 Geographical distribution and habitat

*Mentha longifolia* is an aromatic perennial herb that creeps along an underground rootstock. It can reach up to 1.5 m high in favourable conditions, but is usually between 0.5-1 m high and even shorter in dry conditions. Strongly aromatic with a mint smell, the leaves are usually coarsely hairy and vary from light and dark green to grey (Boulos, 1983). The small flowers of *M. longifolia* (Figure 10) vary in colour from white to mauve. *M. longifolia* grows in marshes and along streams and abundant water resources and is found in Palestine, Jordan, Iran, Syria, Lebanon, Arabian Peninsula and Egypt (Boulos, 2000).

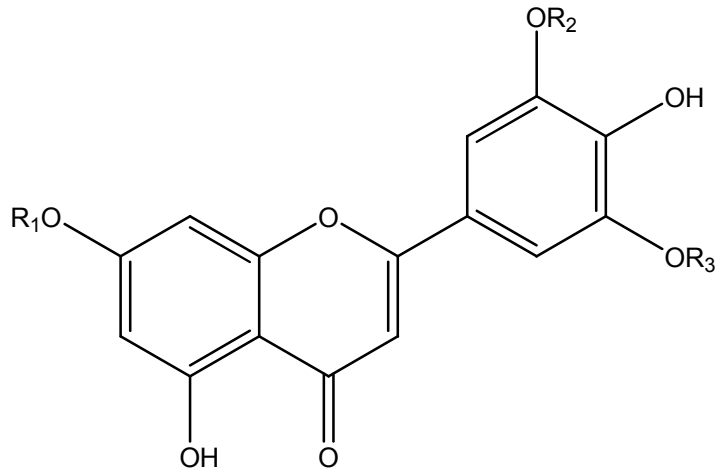
#### 3.1.1.2 Traditional uses

Members of the Mentha genus are some of the most important sources of essential oil production in the world. *M. longifolia*, like many other members of this genus, is often used as a domestic herbal remedy, being valued especially for its antiseptic properties and its beneficial effect on the digestion. The leaves and flowering stems are antiasthmatic, antispasmodic, carminative and used as a stimulant. A tea made

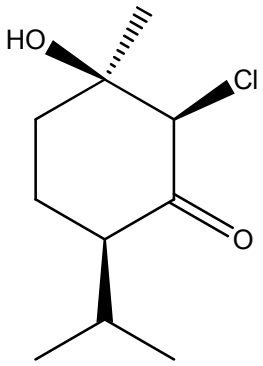
from the leaves has traditionally been used in the treatment of fevers, headaches, digestive disorders (Boulos, 1983). The leaves are used as an antiseptic, in diarrhoea and gut spasm (Amini, 1997) and a choleretic, carminative in indigestion and to relieveflatulence (Duke, 2002). *M. longifolia* is commonly used in the Mediterranean diet as herbal teas and spices (Conforti *et al.*, 2008).

### 3.1.1.3 Phytochemical reports on *Mentha longifolia*

Most of the literature surveys of the genus *M. longifolia* describe the chemical composition of essential oils. Constituents of essential oils and their glycosides have been reported by several groups from various species of this genus and include: menthone, menthol, menthyl acetate and neo-isomenthyl acetate (Shaiq Ali *et al.*, 2002). GC-MS analysis of leaf extract contained pulegone, isomenthone 1,8-cineole borneol, piperitone and piperitenone oxide (Mkaddem *et al.*, 2009). Other than the essential oils, various species of this genus have afforded steroidal glycosides (saponins), terpenoids, flavones, flavone glycosides (see structure **7** below) (Sharaf *et al.*, 1999), tannins, reducing sugars and longifone (see **8** below) (Amabeoku *et al.*, 2009; Ghoulami *et al.*, 2001; Shaiq Ali *et al.*, 2002).




$R_1 = CH_3$, $R_2$=Glucosyl, $R_3$=Rhamnosyl
(**7**) $R_1$= H, $R_2$=glucosyl, $R_3$=rhamnosyl
$R_1 = R_3$= H, $R_2$=dirhamnosyl,




(**8**) longifone

### 3.1.1.4 Pharmacological reports on *Mentha longifolia*

The essential oil from *M. Longifolia* shows an inhibitory effect on bacterial growth (Pajohi *et al.*, 2011). Flavones of *M. longifolia,* represent a potential anti-HIV agent (Amzazi *et al.*, 2003). Piperitenone oxide was found to have highly larvicidal activity (Koliopoulos *et al.*, 2010). *M. longifolia* extract protects human keratinocytes from short-term chemically-induced oxidative stress (Shahverdi *et al.*, 2004). Cellular viability was significantly protected by *M. longifolia* extract, which decreased lipid peroxidation (Berselli *et al.*, 2010). The petroleum extract blocks calcium channels, which is effective in diarrhoea and spasm (Shah *et al.*, 2010). Aqueous leaf extract has antipyretic and antinociceptive properties (Amabeoku *et al.*, 2009). Study of the central nervous system activities of Mentha species exhibited antioxidant inhibitory activities with high affinity to GABA-receptor assay (López *et al.*, 2010). Some *Mentha spp* have anti-inflammatory and antioxidant activities (Conforti *et al.*, 2008). The essential oil showed strong antimicrobial activity against 30 microorganisms tested including *Candida albicans* while the methanol extract showed antioxidant activity (Sarac *et al.*, 2009); (Gulluce *et al.*, 2007; Gursoy *et al.*, 2009).

### 3.1.2 *Acacia nilotica (Linn)*

**Family:** Mimosaceae

**Genus:** Acacia

**Common names:** Acacia, Egyptian mimosa, Egyptian thorn and *Sont* (Arabic).

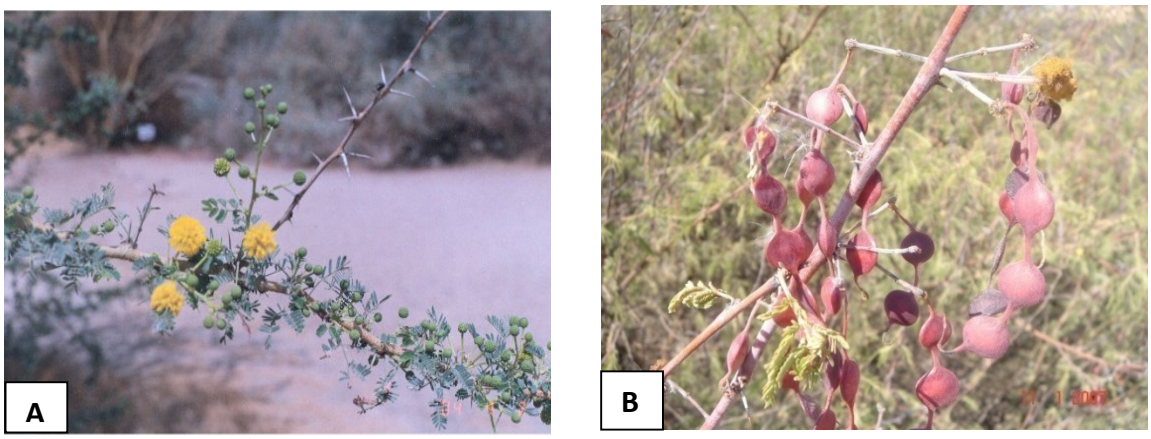



**Figure 9** *Acacia nilotica* tree flowers (A) and seeds (B).

### 3.1.2.1 Geographical distribution and habitat

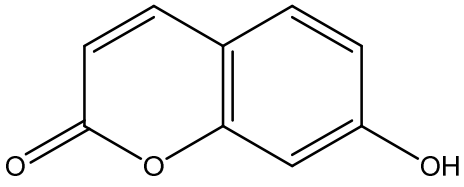
This species can withstand extremely dry environments and floods; it thrives under irrigation (Boulos, 1983). *A. nilotica* is widely spread in subtropical and tropical Africa from Egypt to South Africa, and in Asia eastwards to Pakistan and India. It has been introduced in China, the Northern Territory and Queensland in Australia, in the Caribbean, Indian Ocean islands, Mauritius, United States, Central America, South America and the Galápagos Islands (Chowdhury *et al.*, 1972). *Acacia nilotica* is a small to medium tree Boulos (2000), 7 to 13 m tall, with a stem diameter of 20 to 30 cm. The bark is very dark brown to black. The leaves are bi-pinnate, 4.5 to 7 cm long, with bright yellow flowers in round heads (Figure 9). The trees generally lose their leaves during the dry season. *A. nilotica* is a pioneer species, easily regenerated from seed, and it is not under threat. However, a wide range of pests and diseases affect *A. nilotica*. In Africa, fungal rot attacks the seeds, destroying up to 70 % of them. Many wild mammals feed on the seed pods and a large number of insect species attack the mature seeds (Sheik, 1989).

### 3.1.2.2 Traditional uses

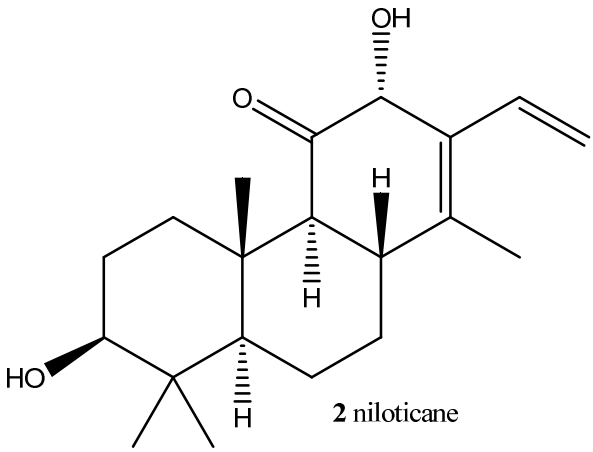
*A. nilotica* has a wealth of medicinal uses. Boulos (1983) described the use for stomach pain; the bark is chewed to protect against scurvy and an infusion is taken for dysentery and diarrhoea. In Egypt, the fruit infusion is used for diarrhoea and reported to be useful against diabetes and urinary tract infection and for tanning leather (Boulos, 1983). It has also been used to eliminate stomach worms, as an antiseptic for open wounds and as an expectorant for treating coughs (Solomon-Wisdom *et al.*, 2010). The pods and leaves are desirable as fodder for sheep and goats and are eaten by a wide range of herbivores (Springuel *et al.*, 1995). The wood is highly valued as a fuel in regions of semi-arid Africa and India. It is used as a pioneer species in land rehabilitation and as a barrier to desertification. It is also considered as a useful nitrogen fixing tree around the world (Fagg, 2001). *A. nilotica* also produces the Gum Acacia which is a demulcent and serves by the viscosity of its solution to cover and sheath inflamed surfaces.

### 3.1.2.3 Phytochemical reports on *Acacia nilotica:*

*A. nilotica* leaves have been reported to contain phenols (catechol and hydrolysable tannins), triterpenes (saponin glycosides), flavonoids, alkaloid, l-arabinose, and coumarin derivatives (Singh *et al.*, 2010), galactan, galactoaraban, galactose, N-acetyldjenkolic acid and sulphoxides pentosan (Banso, 2009; Solomon-Wisdom *et al.*, 2010). HPLC analysis of the bark methanol extract has been shown to contain protocatechuic, caffeic, ellagic acids, quercetin, gallic acid, epicatechin and rutin (Singh *et al.*, 2009a). Recently the composition of essential oils obtained from the stem bark of *A. nilotica* was found to contain menthol (34.9%) and limonene (15.3%) (Ogunbinu *et al.*, 2010). Two new peltogynoids, acanilol A and acanilol B were also isolated from the stem bark of *Acacia nilotica*, together with the triterpene lupenone (Ahmadu *et al.*, 2010), a cassane diterpene [niloticane] (see **2** below) (Eldeen *et al.*, 2010) and umbelliferone (see **1** below) (Singh *et al.*, 2010).




**1** umbelliferone




**2** niloticane

### 3.1.2.4 Pharmacological reports on *Acacia nilotica:*

A methanol extract of the bark of *A. nilotica* has been shown to have antimicrobial activity against *Streptococcus viridans*, *S. aureus*, *E. coli*, *B. subtilis*, *Shigella sonnei* and *Campylobacter coli* (Banso, 2009; Pai *et al.*, 2010) (Solomon-Wisdom *et al.*, 2010). Niloticane from an ethyl acetate bark extract showed antibacterial (Eldeen *et al.*, 2010), antitrypanosomal (Mann *et al.*, 2011) and antiplasmodial activity (Jigam *et al.*, 2010). *A. nilotica* ethanol extract represents a source of antioxidants that could be used in pharmaceutical and food preparations while umbelliferone and kaempferol from *A. nilotica* showed antioxidative effects (Singh *et al.*, 2010). It also prevents

lipid peroxidation (Singh *et al.*, 2009b; Singh *et al.*, 2009c). Tannins were the major compounds detected in an ethyl acetate bark extract and showed antidiarrhoea potential (Etuk *et al.*, 2009; Omwenga *et al.*, 2009) and anthelmintic activity (Bachaya *et al.*, 2009). Recently, *A. nilotica* extract was found to reduce non-fasting glucose levels and decreased non-fasting insulin levels (Babish *et al.*, 2010). The proanthocyanidins from *A. nilotica* have been shown to modulate insulin signalling *in vitro* (Minich *et al.*, 2010). A bark ethanol extract showed anti-cholinesterase activity (Crowch *et al.*, 2009). The methanol extracts from flowers was used to evaluate the protective effect against liver and kidney toxicity induced by cadmium chloride (El-Toumy *et al.*, 2009) and showed antihyperglycaemic effect.

### 3.1.3 *Achillea fragrantissma* (Forssk.) Sch. Bip.

**Family:** Asteraceae

**Genus:** Achillea

**Common names***:* Lavender cotton; santoline and Qaysūm (Arabic).

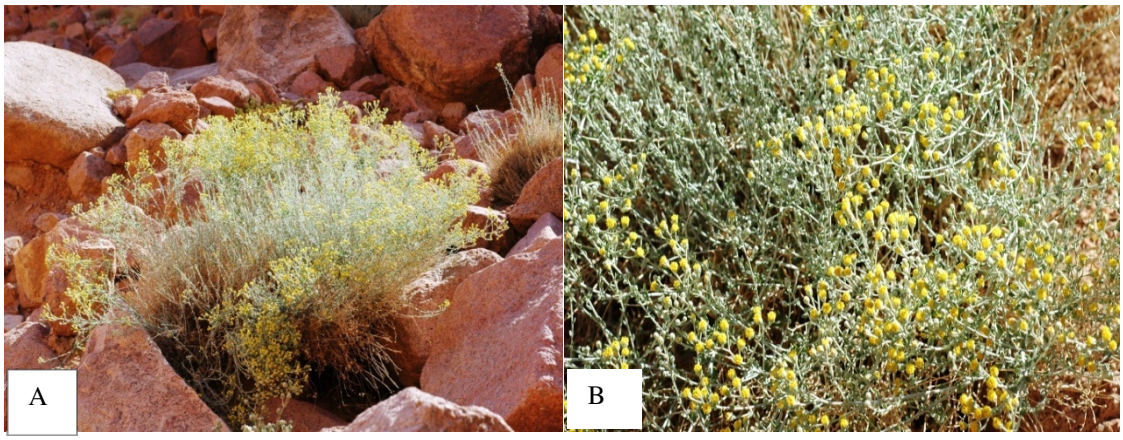



**Figure 10** *Achillea fragrantissima* the whole plant (A) and the flowers (B).

#### 3.1.3.1 Geographical Distribution and habitat of *Achillea fragrantissma*

*Achillea fragrantissima* is a perennial herb, white woolly, with erect stems up to 1m high. The leaves are white to greyish green, small and thick (Batanouny, 1999). Flower heads are yellow and very short (Figure 9) with a fragrant odour. The plant flowers from June to September and found in Syria, Lebanon, Jordan, Palestine, Arabian Peninsula and Egypt (Batanouny, 1999; Boulos, 2000). In Egypt the plant

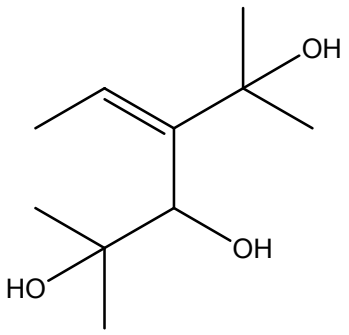
grows in limestone wadis of the North Eastern Desert and Sinai. The plant is over exploited by collection for folk medicinal uses. The rate of exploitation exceeds that of regeneration, therefore in 1999 the plant was considered threatened (Batanouny, 1999).

### 3.1.3.2 Traditional uses

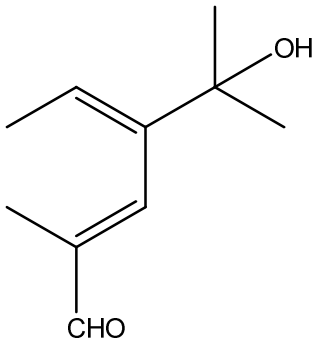
*A. fragrantissima* is widely used in folk medicine for the preparation of herbal teas with antiphlogostic and spasmolytic activity. An infusion of the dry or fresh flowering herb is used by local people for the treatment of coughs; stomach, as an anthelmintic (Boulos, 1983) and to treat diabetes (Al-Gaby *et al.*, 2000). *A. fragrantissima* is also used as an antiseptic; it is used to cover cuts and sores and hasten scar tissue formation, as an anti-inflammatory agent (Maswadeh *et al.*, 2006) and as an insect repellent.

### 3.1.3.3 Phytochemical investigation of *Achillea fragrantissima*

*A. fragrantissima* contains essential oil of which α and β-thujone, α-pinene, β-pinene, 1,8-cineole, carvacrol, eugenol, artemisia ketone, palustrol, sabinene hydrate, α-terpineol and santolina alcohol are the major constituents. Its tannin content reaches 8% and includes compounds such as resorcin, phloroglucin, methyl phloroglucin, and pyrocatechol (Barel *et al.*, 1991; El-Shazly *et al.*, 2004; Shalaby *et al.*, 1964).



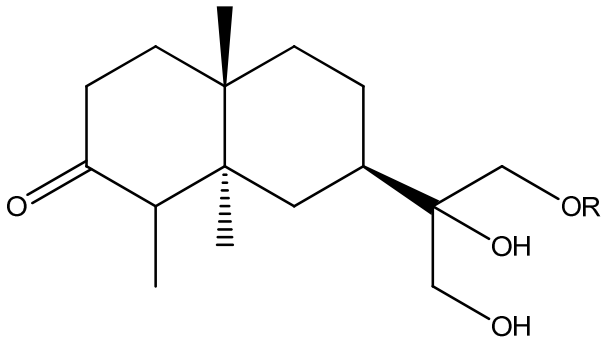

**(3)** 4,5,8-Trihydroxy-santolin-1-ene



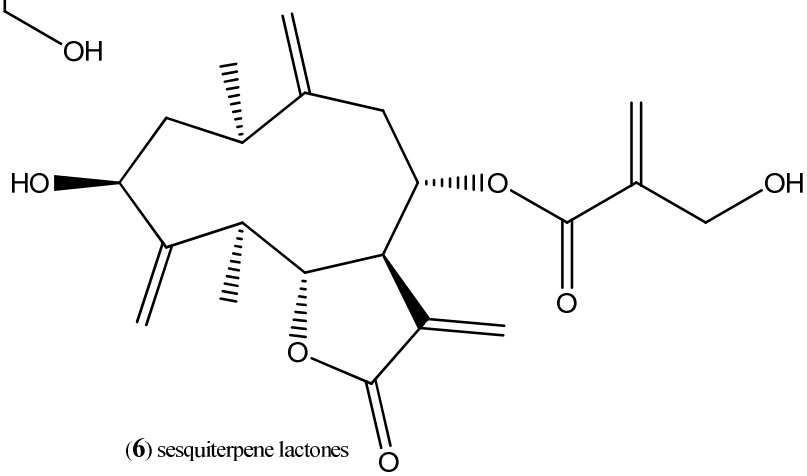

**(4)** 6-Oxo-8-hydroxy-santolina- 1,4 diene

*A. fragrantissima* also contains a high percentage of flavonoids, such as afroside, cirsimartin, chrysoplenol, cirsiliol and swertisin 2-arabinoside (Ahmed *et al.*, 1988), methylated aglycones, mono and di-C-glycosyl-flavones (Ahmed *et al.*, 1990). The

areal parts contain santoline (see **3** above) and santoline aldehyde (see **4** above) derivatives (Ahmed *et al.*, 1990), highly oxygenated eudesmanes, lignans (see **5** below) (Ahmed *et al.*, 2002), fatty acids, lauric, myristic, palmitic, stearic, oleic, linoleic and linolenic as well as sesquiterpene lactones (see **6** below) as achilloide A, taraxasterol and pseudotaraxasterol acetates, tannins, volatile oils, sterols and triterpenes (Maswadeh *et al.*, 2006).




(**5**) R=H
R=Ac




(**6**) sesquiterpene lactones

### 3.1.3.4 Pharmacological investigations of *Achillea fragrantissima*

*A. fragrantissima* has an inhibitory effect on rat isolated ileum smooth muscle, while the flavones isolated from it causes inhibition of the phasic contractions of rat isolated ileum and pre-contracted aorta and increased the phasic contractions of isolated urinary bladder (Mustafa *et al.*, 1992a; Mustafa *et al.*, 1992b). Some flavonoids from *A. fragrantissima* exhibited high spasmolytic effects on isolated guinea-pig ilea, whereas a dicaffeoylquinic acid showed choleretic effects in isolated rat liver (Lemmens-Gruber *et al.*, 2006). The volatile oil from *A. fragrantissim* showed anti-inflammatory activity in a carrageenan-induced paw oedema model (Maswadeh *et al.*, 2006), antiviral activity (Soltan *et al.*, 2009) and antimicrobial activities (Unlu *et al.*, 2002).

### 3.1.4 *Haloxylon salicornicum* (Moq.) Bunge ex Boiss

**Family**: Chenopodiaceae

**Genus**: Haloxylon

**Common name**: Rimth

#### 3.1.4.1 Geographical distribution and habitat:

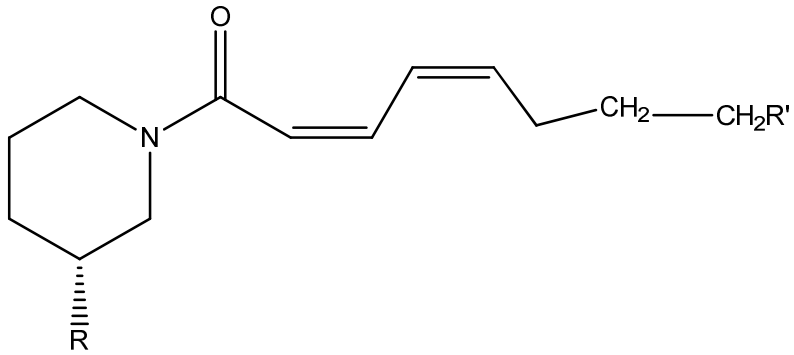
*Haloxylon salicornicum* is widely distributed in Egypt, Iran, Iraq, Jordan, Kuwait, Palestine and Pakistan. The genus *Haloxylon* consists of approximately 25 species and is widely distributed in Egypt where it is represented by four species (Tackholm, 1974). *H. salicornicum* is a diffuse shrub, pale, much branched, almost leafless, 25-60 cm tall, with woody stems and shoots ascending rigid, light coloured to white or waxy yellowish when dried; it is commonly found in sandy and stony deserts (Boulos, 1999).

#### 3.1.4.2 Traditional uses

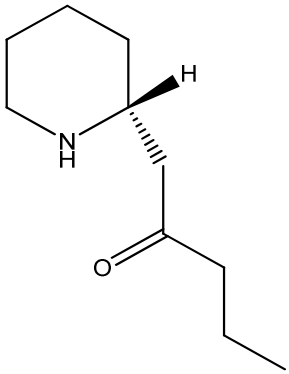
*H. salicornicum* is a good source of fuel and used as a fodder for camels. The plant is traditionally reported for its toxicity and applied topically on insect stings (Chopra *et al.*, 1956). The ash of the plant is used for internal ulcers (Sastri, 1959) and also mixed with salt for use as a tooth powder.

#### 3.1.4.3 Phytochemical reports on *Haloxylon salicornicum*

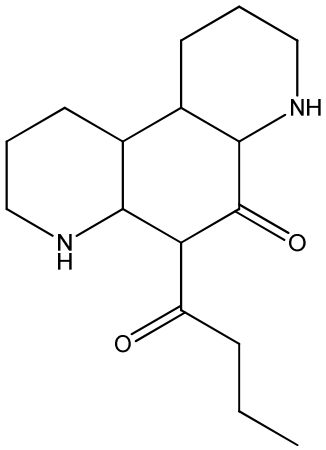
The phytochemical analysis of the aerial parts of the plant revealed the presence of alkaloids, cardiac glycosides, anthraquinones, flavonoids, saponins, coumarins, sterols, tannins, volatile oils and volatile bases (Ajabnoor, 1984). Simple quinolines (Michel *et al.*, 1967) tyramine derivatives (Michel *et al.*, 1968) a 5-hydroxy-3-methoxy-4*H*-pyran-4-one (**18**) (Gibbons *et al.*, 2000) and piperidine alkaloids [Haloxylines A and B (**9**)] have been reported from this species. Recently, (El-Shazly *et al.*, 2005) reported the isolation of a new piperidine alkaloid, haloxynine (**10**) and additional 17 known alkaloids including, haloxine (**11**), hordenine (**12**), anabasine (**13**), piperidine (14), aldotripiperideine (**15**), 3,4-dihydro-5-(2-piperidinyl)-1(2H)pyridine carboxaldehyde (**16**) and ammodendrine (**17**); (see structures 9-18 below).



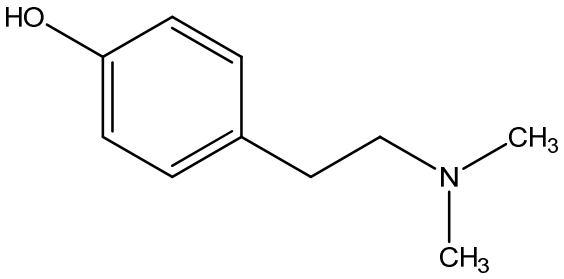

(**9**) Haloxylines (A) R=H, R'=OH
Haloxylines (B) R=R'=OH



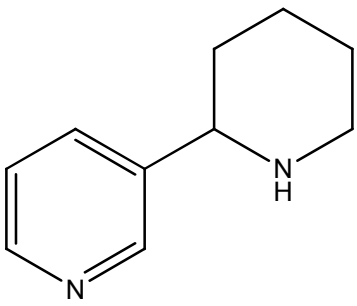

(**10**) Haloxynine



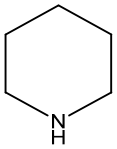

(**11**) Haloxine



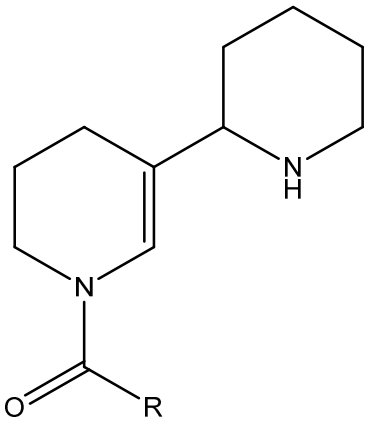

(**12**) Hordenine



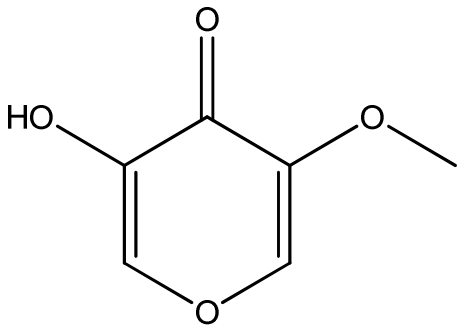

(**13**) Anabasine



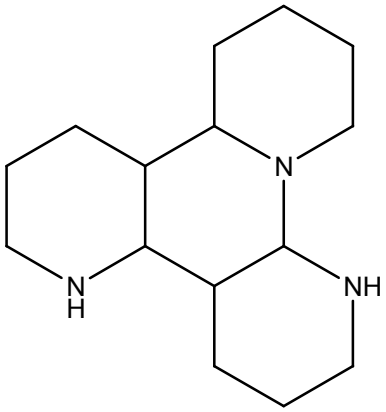

(**14**) Piperidine




(**16**) R=H, 3,4-Dihydro-5-(2-piperidinyl)-1(2H)pyridine carboxaldehyde
(**17**) R=Me, Ammodendrine




(**18**) 5-hydroxy-3-methoxy-4H-pyran-4-one




(**15**) Aldotripiperideine

### 3.1.4.4 Pharmacological reports on *Haloxylon salicornicum*

*H. salicornicum e*xhibits remarkable biological activities; an ethanol extract of *H. salicornicum* was found to have anti-diabetic and anticoagulant activity (Awaad *et al.*, 2001). An aqueous extract has been found to show anticancer and antiplasmodial activity (Sathiyamoorthy *et al.*, 1999). Furthermore, a volatile oil exhibits antimicrobial activities against *B. subtilis* and *S. aureus*. Piperidine showed antifungal activity and cholinesterase inhibition, which is an effective tool for treatment of Alzheimer (Ferheen *et al.*, 2005). Some of alkaloids such as haloxynine, haloxine and hordenine are known to be strong agonists at nicotinic acetylcholine receptors (El-Shazly *et al.*, 2005).

## 3.2 Material and methods

### 3.2.1 Collection and extraction of plant material

Plant materials were collected as described in section 3.1.The dried plant materials were ground into a fine powder using a Buchi mixer (Labotec®) grinder and kept ready for extraction.

### 3.2.2 Solvents for analytical and chromatographic purposes

The following HPLC grade solvents were used for extraction, chromatographic separation, analytical TLC and crystallisation:

Diethyl ether, *n*-pentane, *n*-hexane, chloroform, acetonitrile, ethanol, methanol, acetic acid, water and acetone. Solvents were obtained from Fisher Scientific Ltd, UK and VWR Ltd, UK.

#### 3.2.2.1 Solvents for NMR analysis

Deuterated (99.9%) solvents were used for all the NMR analyses including, $CDCl_3$, DMSO-$d_6$, $CD_3OD$ and $D_2O$. The solvents used were obtained from Sigma-Aldrich Ltd, UK.

#### 3.2.2.2 Reagents and chemicals

- *P*-Anisaldehyde reagent (VWR Ltd, UK)
- TLC grade silica gel coated aluminium sheet (Merck Precoated Silica gel PF254)
- Normal phase TLC grade silica gel (60H, Merck, Germany)
- Column grade silica gel (Silica gel 60, mesh size 20-200µm (Merck, Germany)
- Anti-bumping granules (BDH, UK)
- C-18 silica gel columns (Phenomenex to use on a Flashmaster Personal®, Biotage, UK).
- Sephadex® LH20, Sigma-Aldrich, bead size 25-100 µm)

### 3.2.3 Extraction and partitioning

Various methods of extraction were used, all extracts were evaporated at 40$^{o}$C under vacuum using a rotary evaporator and stored at -20$^{o}$C untill used.

#### 3.2.3.1 Soxhlet extraction

Plant materials were successively extracted with *n*-hexane, chloroform (or ethyl acetate) and methanol for 72 hours; the extracts were prepared using a Soxhlet apparatus.

#### 3.2.3.2 Liquid-liquid partition

Methanol extracts (unless otherwise stated) were dissolved in 2.5% (v/v) ethanol in water and partitioned with chloroform followed by *n*-butanol using a separating funnel. Chloroform and *n*-butanol phases were first dried over anhydrous sodium sulphate and then evaporated using a rotary evaporator. The remaining aqueous phase was freeze-dried.

#### 3.2.3.3 Maceration

Plant material was soaked in hot water and left over night, filtered, then the filtrate freeze-dried and stored until used.

### 3.2.4 Chromatographic techniques

#### 3.2.4.1 Thin layer chromatography (TLC)

Plant extracts, fractions or pure compounds were dissolved in the suitable solvent (see section 3.2.4.2) and spotted approximately 1 cm above the bottom edge of a TLC grade silica gel coated aluminium sheet. Spots were applied as bands to facilitate easy and accurate observation and the bands were kept as narrow as possible to reduce the overlapping of compounds. Based on the expected polarity of the extract or compound, a suitable solvent mixture was added to the TLC tank and left for a while to saturate the tank environment. Filter paper was put inside the tank to aid the saturation. Spotted TLC plates were then placed in the TLC tank; care was taken to ensure that the solvent remained below the sample. Upon the mobile phase reaching approximately 1cm from the top, the TLC plates were taken out of the tank, the solvent front was marked in pencil and the plates air dried. They were then examined visually under UV light and sprayed with chemical reagents as described by (Stahl, 1975).

### 3.2.4.2 Selection of mobile phase

Different solvent systems for the mobile phase were tried until a desirable resolution between the spots was obtained. For the analysis of fractions or pure compounds, retardation factor ($R_f$ values) in the range of 0.2 to 0.5 were desirable when choosing the starting solvent system for open column or flash chromatography.

### 3.2.4.3 Detection

#### i) Detection by UV light

Developed TLC plates were observed under a UV lamp using short (254 nm) and long (366 nm) wavelengths. Short UV light is useful for detecting aromatic C=C compounds whereas compounds that fluoresce are visible under long UV light.

#### ii) Detection by spray reagents

Anisaldehyde-sulphuric acid spray reagent was used routinely. Vanillin-sulphuric acid was also used where necessary. Anisaldehyde-sulphuric acid reagent consisted of: 0.5 mL of *p*-anisaldehyde was mixed with a mixture of 10 mL glacial acetic acid and 85 mL of methanol. Sulphuric acid (5 mL) was added to it. The latter reagent was sprayed over the dried developed TLC plates and the plates were then heated to 105-110$^{o}$ C for approximately 1 min with a hot air gun.

### 3.2.4.4 Vacuum liquid chromatography (VLC)

VLC is a form of column chromatography, which offers as high a resolution as that of TLC. A sintered glass fitted funnel Büchner with a suction outlet was packed with TLC grade silica gel under vacuum to afford a layer of 5 to 6 cm thick with some space above to accommodate the sample and a volume of solvent. The starting solvent was allowed to pass through the column under vacuum to check the uniformity of the column. The sample to be fractionated was mixed with column grade silica gel to get a free flowing powder, which was applied as a thin layer on to the top of the packed VLC column. Elution was carried out with a non-polar solvent and then with solvent mixtures of increasing polarity. A solvent mixture was added to the top of the column each time and vacuum was applied until the column dried up. Each fraction was collected and evaporated to dryness at 40$^{o}$ C under vacuum

using a rotary evaporator. The fractions were analysed by TLC and pooled according to similar chemical profiles (Pelletier, 1986).

#### 3.2.4.5 Flash chromatography (FC)

Flash chromatography is a simple and rapid chromatographic technique that can be used as a substitute for open column chromatography. In FC, the stationary phase consists of a smaller and narrower range of particle size to get a more efficient separation. Pressure is applied to overcome the reduced flow rate caused by the use of a smaller particle size of the stationary phase. Thus, with a flow rate of 2 ml/min, 0.1 to 10 g of sample mixtures differing in their $R_f$ value by 0.15 can be separated in 15 min, provided that TLC-mesh silica gel is used as the stationary phase (Braithwaite, 1996).

#### 3.2.4.6 Open column chromatography

**i) Silica gel column chromatography (CC)**

Glass columns of suitable sizes with porous plates at the bottom were used. Slurry of column grade silica gel was prepared with the starting solvent system. One third of the column was filled with the same solvent and the slurry was applied as a thin stream, with a gradual flow of solvent. Once the column was packed with silica gel, the solvent was allowed to flow to get a homogeneous packing. Care was taken to prevent the inclusion of any air bubbles or drying of the column. The sample to be fractionated was dissolved in a suitable solvent, mixed with silica gel grade and dried to get a free flowing powder. This was then applied to the top of the packed column. A cotton plug was applied over it to prevent any distortion while the solvent passed from the reservoir to the top of the column. Elution was carried out either isocratically or in a gradient manner. The collected fractions were checked by TLC and pooled according to similar chemical profiles (Ravindranath, 1989).

**ii) Size exclusion chromatography or gel filtration (GF)**

Gel filtration (GF) or molecular sieve chromatography is a form of liquid chromatography in which molecules can be separated according to their molecular size. In the present phytochemical work, a cross-linked dextran based resin was used

(Sephadex® LH20) to get an effective separation between molecules differing in their molecular size. When solvent passes through the cross-linked dextran molecules, smaller molecules have a greater tendency to diffuse into the porous gel particles and are thus eluted after the bigger molecules. Gel filtration is not only useful for removing chlorophyll or pigments; it also gives good separation between any molecules that differ in molecular size. For non-polar fractions, Sephadex® slurry was prepared in 5% (v/v) *n*-hexane in chloroform and kept overnight. A glass column with a sintered glass or cotton at the bottom was packed in such a way that the slurry properly settles in the column but no solvent remains over the surface of the packed Sephadex®. The sample to be fractionated was dissolved in the same solvent system as the one used for the column and applied onto the top of the column. Once the sample diffused into the column, a cotton plug was inserted to prevent the escape of swollen gel in to the solvent above the column bed. The column was run isocratically first and then with 100% chloroform. Afterwards a small proportion of methanol was added to the chloroform to elute more polar compounds. For mixtures of relatively polar compounds, the Sephadex® was soaked in chloroform, and the column was run with chloroform first and then the polarity was increased using methanol (Kremmer, 1979).

**iii) Normal-phase medium pressure liquid chromatography (NP-MPLC)**

Chromatography has recently trend towards smaller particle size to improve resolution and reduce run time. Medium Pressure Liquid Chromatography has been slower to adapt smaller particle sizes due to pump and back pressure limitations. The single use columns are made of is inexpensive material with limited pressure capability. The pumps also need to deliver a high volume while maintaining an accurate gradient and appropriate backpressure. Spherical silica allows the use of smaller particles with a narrower particle size range which improves resolution with a minimal increase in back pressure with improved and can be critical when the desire is to save time or solvent. Fractions were chromatographed by MPLC utilising a gradient of EtOAc and MeOH from 100:0 to 90:10 at a flow rate of 20 mL/min for 35 min to afford 5 ml each (Hwu *et al.*, 1987).

### 3.2.5 Structure elucidation

Characterisation of the isolated compounds was carried out primarily using spectroscopy and mass spectrometry (MS), NMR. IR and UV spectroscopy, melting point determination, optical rotation or crystallographic analysis were adopted depending on the need.

#### 3.2.5.1 Mass spectrometry

Positive ion and negative ion mode ESI experiments were carried out on a ThremoFinnigan LCQ-Decaiontrap or Orbitrap HRESI mass spectrometer (mass analyser set up at 100,000 ppm, externally calibrated at 3 ppm). Based on the polarity, samples were dissolved in acetonitrile, methanol or water (HPLC grade) or in a binary mixture of these solvents to get a concentration of 100 μg/mL. Sample solution (10-20 μL) was injected along with a direct infusion of 0.1% (v/v) formic acid in acetonitrile: water (90:10) at a flow rate of 200 μL/min.

#### 3.2.5.2 NMR spectroscopy

All NMR experiments were carried out on a JEOL (JNM LA400) FT spectrometer operating at 400MHz ($^{1}$H) and 100MHz ($^{13}$C) or Bruker (Avance) 500 MHz instrument. NMR tubes (5 mm) obtained from VWR were used for routine NMR experiments. Samples were dissolved in 750 μL of suitable deuterated solvent and placed in the NMR tube. All spectral data obtained were processed with MestReNova software (version 7). ChemBioDraw Ultra, Version 12, was used to draw compound structures.

#### 3.2.5.3 $^{1}$H NMR

A $^{1}$HNMR experiment was carried out for all compounds isolated and was used as the primary means of structural identification. In the $^{1}$H NMR, the chemical shifts and integration indicate the number of each type of protons present in the molecule. The multiplicity and extent of coupling constant gives an idea of the adjacent protons and their spatial proximity. The purity of the compounds was also shown where possible. $^{1}$H NMR was used successfully in determining the molar ratio of the components present in a mixture of two or more compounds.

### 3.2.5.4 $^{13}$C NMR and Distortionless Enhancement of Polarisation Transfer (DEPT)

Broad band decoupled $^{13}$C NMR was used to determine the number of carbons and where necessary DEPT experiments were obtained to distinguish the carbons according to their proton attachments. DEPT spectrum is a pulse sequenced experiment that transforms the information of the CH signal multiplicity and spin-spin coupling into phase relationship. In a DEPT 135 spectrum, $CH_3$ and CH are directed towards the positive phase of the spectrum while $CH_2$ facing the negative phase. The advantage of a DEPT spectrum over a carbon spectrum is that it is 4 times more sensitive as it uses $^{1}$H-$^{13}$C polarisation transfer.

### 3.2.5.5 $^{1}$H-$^{1}$H Correlation SpectroscopY (COSY)

COSY experiment shows $^{1}$H-$^{1}$H connectivity. The proton shifts are plotted on both axes the contour plot along the diagonal of the square. Results observed as cross peaks are arranged in the square symmetrically about the diagonal. Thus the cross peaks refer to the spin-spin coupled protons. The correlations observed are often due to geminal ($^{2}J$) and vicinal ($^{3}J$) couplings, but some smaller $^{4}J$ and $^{5}J$ couplings e.g., 'W', zigzag or allylic couplings can also sometimes be observed in a COSY spectrum.

### 3.2.5.6 Heteronuclear Multiple Quantum Coherence (HMQC)

HMQC experiment is $^{1}$H-$^{13}$C experiment identifies one-bond (1$J$) connectivity. The pulse sequence used in this experiment uses a time delay set to 1/2$J$ where $J$ is the value in the range to that of one-bond $^{1}$H-$^{13}$C coupling. In a HMQC spectrum, the $^{1}$H and $^{13}$C (or DEPT) spectrum is plotted along the abscissa and ordinate, respectively. Cross-peaks show protons and carbons that are directly connected to each other.

### 3.2.5.7 Heteronuclear Multiple Bond Coherence (HMBC)

HMBC is another 2D $^{1}$H-$^{13}$C experiment, the time delay (1/2$J$) used in the pulse sequence is kept such that the $J$ value is in the range of $^{3}J$CH and $^{2}J$CH. Unless otherwise stated, the HMBC experiments carried out for different samples in the present study used a time delay of 0.07s (*i.e.*, $J$CH = 7 Hz). For highly-substituted

compounds which lack sufficient protons to 'track' the carbons, a time delay of 0.25 second was used which enabled 4*J*CH correlations (*i.e.*, *J*CH = 2 Hz) to be found.

#### 3.2.5.8 Nuclear Overhauser Enhancement SpectroscopY (NOESY)

NOESY records the $^{1}$H-$^{1}$H NOE correlations occurring in a molecule. The spectrum is similar to a COSY spectrum, but in a correctly phased NOESY spectrum cross peaks represent NOE correlations between the respective protons. The NOESY experiment is of importance to establish the relative stereochemistry of a molecule.

## 3.3 Results

### 3.3.1 Selection of plant material for further fractionation

Selection of plant materials for further structure elucidation was made on the basis of:

i) The information gathered through results from the interviews with the Bedouin communities (Chapter 2)
ii) The preliminary cytotoxicity assessment of the plant crude extracts (Chapter 4)
iii) Previously reported work and the availability of material (section 3.1).

The following plant extracts were selected for further bioassay-guided fractionation:

- *n*-hexane, ethyl acetate and methanol extract of *Mentha longifolia*
- Water extract of *Acacia nilotica*
- Methanol extract of *Achillea fragrantissima*
- Methanol extract of *Haloxylon salicornicum*

Fractionation schemes of the selected extracts are presented in Appendix III.

## 3.4 Isolation and structure elucidation of compounds isolated from *Mentha longifolia*

A total of eight compounds were isolated from *Mentha longifolia*; the majority were flavonoids. The procedure of isolation of these compounds is presented in the fractionation Scheme 1 (Appendix III). While an extended description will be provided under the characterisation of each extract from which each compound was isolated.

### 3.4.1 Fractionation of *n*-hexane extract of *Mentha longifolia*

The hexane extract (28g) of *Mentha longifolia* was treated with *n*-pentane while gave a residue after filtration. The filtrate after removal of the solvent was subjected to sephadex LH-20. The initial greenish coloured fractions 1-16 (15ml each) were discarded as they were chlorophyll (TLC spot was red under UV) and the later fractions 17-25 were combined. After removal of the solvent, a whitish solid was obtained, this was further subjected to column chromatography, eluted with hexane

and diethyl ether gradient as the mobile phase, affording **ML17** and **ML19**. The residue part was subjected to silica gel chromatography affording **ML38**.

### 3.4.1.1 Characterisation of ML17 as Piperitenone

This compound was isolated from the hexane extract of *Mentha longifolia* as a colourless oil. On TLC it gave a dark spot under UV. After spraying with *p*-anisaldehyde sulphuric acid reagent it turned pink (with an $R_f$ 0.54).

The high resolution positive mode HRESI-MS of **ML17** gave an ion $[M+H]^+$ at 151.114 suggesting a molecular formula of $C_{10}H_{14}O$.

The $^1H$ NMR spectrum (Figure 11) showed three methyl signals at δ 1.79, 1.86 and 2.02 ppm. Two methylene signals at 2.23 and 2.59 ppm, exhibiting long-range coupling to one of the methine signal, and a methine proton at 5.81 ppm. The $^{13}C$ NMR spectrum showed the presence of three methyl quartets at 22.6, 23.0 and 23.9, two methylenes at 28.0 and 32.0, and quaternary carbons at 128.9, 142.5 and 159.8 and a carbonyl signal at 191.8 ppm (Table 4). The analysis of the spectral data for the compound was identified as 3-methyl-6-(1-methylethylidene)-cyclohex-2-en-1-one or piperitenone and confirmed by comparison with previously published data (Manenzhe *et al.*, 2004).

**Table 4** $^1H$ (400 MHz) and $^{13}C$ (100 MHz) NMR data of ML17 (piperitenone) in $CDCL_3$.

| Position | $\delta_H$ | $\delta_C$ |
|---|---|---|
| 1 | - | 191.8 |
| 2 | 5.81(1H, m) | 128.7 |
| 3 | - | 142.6 |
| 4 | 2.59 (2H, t, *J*= 6.15 Hz) | 28.0 |
| 5 | 2.23 (2H, t, *J*= 6.16 Hz) | 31.9 |
| 6 | - | 159.8 |
| 7 | - | 128.9 |
| 8 | 1.02(3H, s) | 22.8 |
| 9 | 1.86(3H, s) | 22.6 |
| 10 | 1.79(3H, s) | 23.9 |

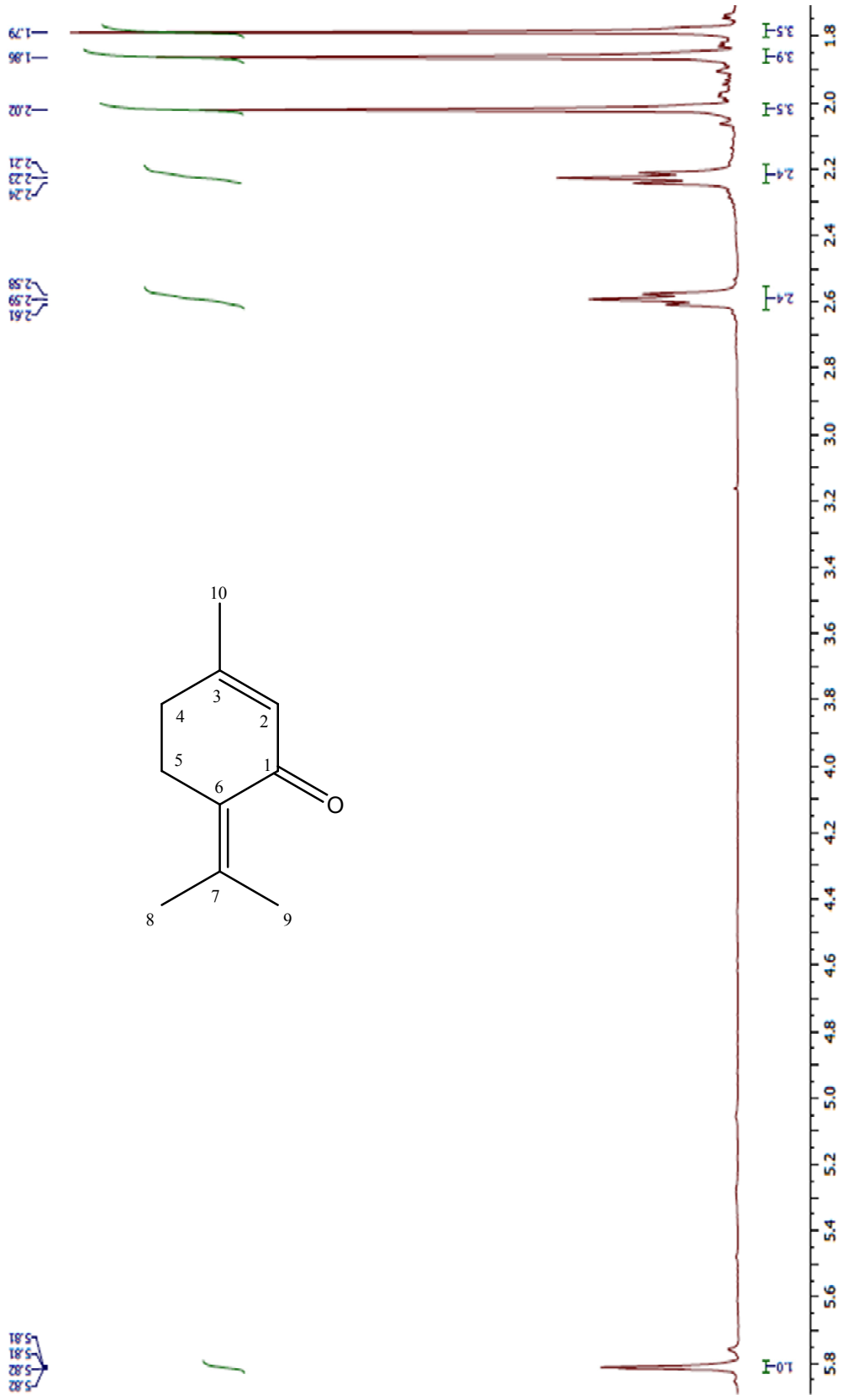



**Figure 11** $^{1}$HNMR spectrum and structure of ML17 (piperitenone) (400 MHz, $CDCl_3$)

### 3.4.1.2 Characterisation of ML19 as p-mentha-1,3,5-triene-3,8-diol (8-hydroxy thymol)

This compound was isolated from the hexane extract of *Mentha longifolia* as a colourless crystal On TLC it gave a dark spot under UV. After spraying with *p*-anisaldehyde sulphuric acid reagent they turned orange (with an $R_f$ 0.51).

The high resolution positive mode HRESI-MS of **ML19** (Figure 12) gave an ion $[M+H]^+$ at 167.162 suggesting a molecular formula of $C_{10}H_{14}O_2$.

$^1$HNMR spectrum (Figure 13) suggested the presence of ABX spin aromatic system at δ 6.70 (d, *J* = 0.4 Hz, 1H) 6.95 (d, *J* = 7.9 Hz, 1H) and 6.63 (dd, *J* = 7.9, 0.4 Hz, 1H). Three methyls at t δ 2.27 (s, 3H) and 1.65(s, 6H). The $^{13}$CNMR spectrum showed 9 signals including three methyl quartets at 30.5 and 29.7(2 methoxy) showing that the compound had 10 carbons of monoterpene (Table 5).

In the HMBC spectrum (Figure 14) the protons at $\delta_H$ 6.95 (H-5) and the methyl group at $\delta_H$ 2.27showed $^3J$ correlation to the carbon at $\delta_c$ 139.7. The methyl group at $\delta_H$ 1.6 showed $^3J$ correlation to the carbon at $\delta_c$ 76.2(C-8) and (C-4) at $\delta_c$ 128.2. While the proton at 6.70 (H-2) showed $^3J$ correlation to the carbon at $\delta_c$ 120.9 and 128.2 (C-4). The proton at $\delta_H$ 6.63 $^3J$ (H-6) correlation to the carbon at $\delta_c$ 118.5 and 128.2 (C-4) this led to the consideration of the presence of the hydroxyl group at (C-3) position. The correlation for two methyls at $\delta_H$ 1.65 to the carbon at $\delta_c$ 128.2 (C-4) and 76.2 assigned as C-9 and 10. With these data **ML19** was identified as p-mentha-1,3,5-triene-3,8-diol or 8-hydroxy thymol. This is a novel compound.

**Table 5** $^{1}H$ (400 MHz) and $^{13}C$ (100 MHz) NMR data of ML19 (8-hydroxy thymol) in $CDCl_3$.

| Position | $\delta_H$ | $\delta_C$ |
|---|---|---|
| 1 | - | 139.7 |
| **2** | **6.70 (1H, d, *J* = 0.4 Hz)** | **118.4** |
| 3/OH | - | 156.2 |
| 4 | - | 128.2 |
| 5 | 6.95 (1H, d, *J* = 7.9 Hz) | 125.3 |
| 6 | 6.63 (1H, dd, *J* = 7.9, 0.4 Hz) | 120.9 |
| 7 | 2.27 (3H, s) | 29.7 |
| 8 | - | 76.2 |
| 9/10 | 1.65(6H, s) | 30.6 |

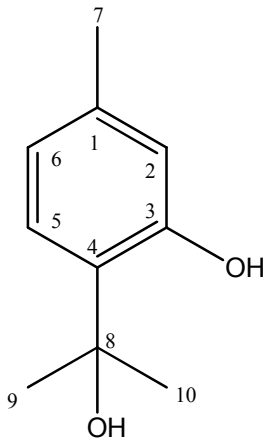



**Figure12** structure of ML19 (8-hydroxy thymol)

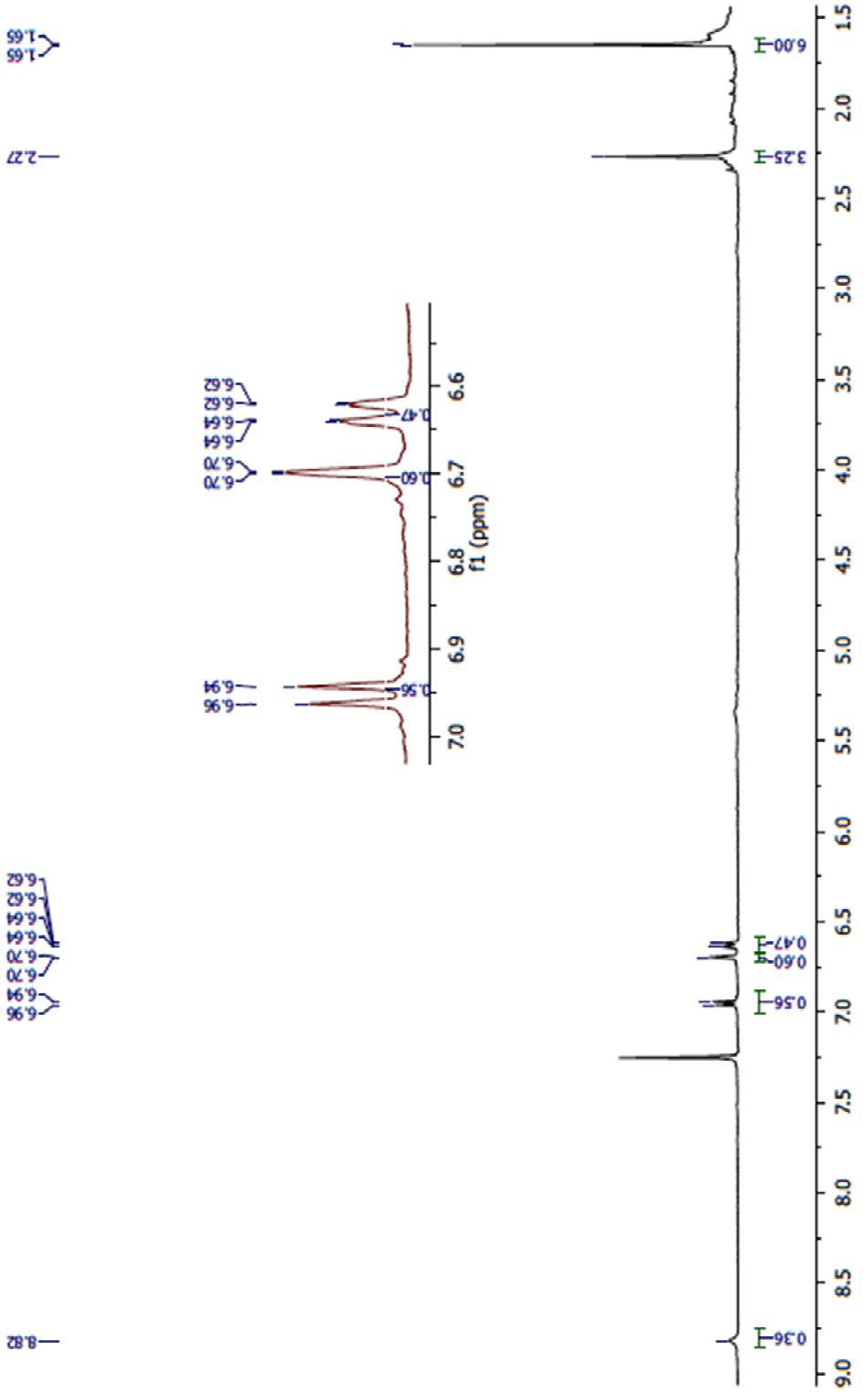



**Figure 13** $^{1}$HNMR spectrum of ML19 (8-hydroxy thymol) (400 MHz, $CDCl_3$)

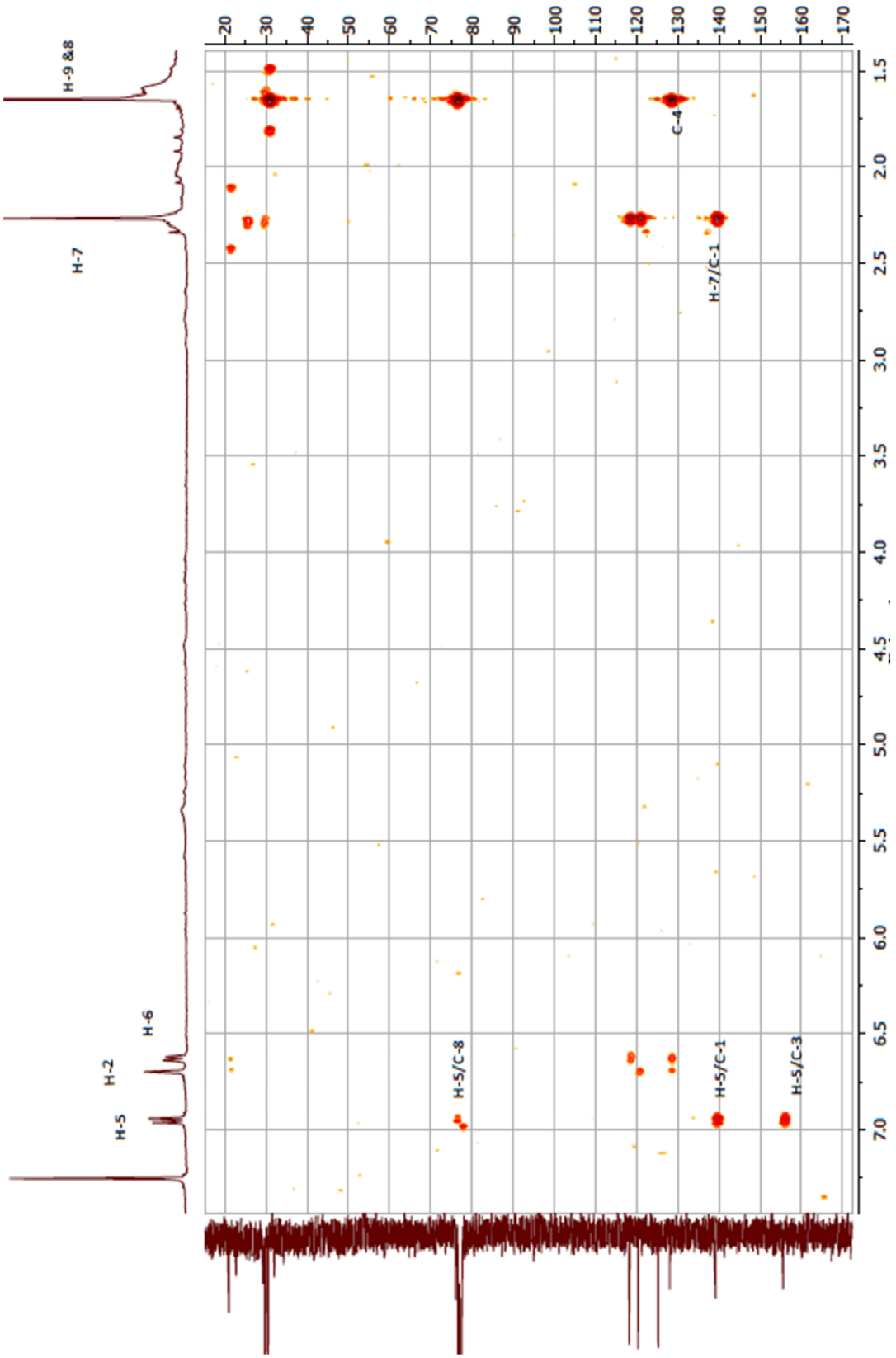



**Figure 14** HMBC spectrum of ML19 (8-hydroxy thymol) (400 MHz, $CDCl_3$)

### 3.4.1.3 Characterisation of ML38 as 5-hydroxy-3′,4′,6,7 tetramethoxyflavone

The residue from the *n*-pentane treatment of the hexane extract of *Mentha longifolia* was subjected to column chromatography, eluted with hexane and 30% ethyl acetate. This yielded 30 mg of a yellow crystal. On TLC, the compound showed as a dark spot under UV light. After treatment with *p*-anisaldehyde-sulphuric acid reagent and heating at 105$^{o}$C, the compound turned yellow with an $R_f$ 0.33 value on 40% ethyl acetate.

The high resolution positive mode HRESI-MS of **ML38** (Figure 15) gave an ion $[M+H]^+$ at 359.1125 suggesting a a molecular formula of $C_{19}H_{18}O_7$.

$^1$HNMR spectra (Figure 16) showed the presence of a hydroxyl signal at $\delta_H$ 12.75ppm (OH-5), a three spin aromatic system at δ 7.35(1H, d, *J*= 2.2 Hz) δ6.99 (1H, d, *J*=8.4 Hz) and δ 7.54(1H, dd, *J*= 1.84, 8.76Hz). Two other aromatic singlets were observed at δ6.56 and 6.61(Table 6).

In the HMBC spectrum (Figure 17) the 5-hydroxy proton showed $^3J$ correlation to the carbons at δ 134.1(C-6) and 107.4(C-10). One methoxy at δ 3.93attached to C-6 also showed $^3J$ correlation to the carbon at δ 134.1 (C-6) and thus was assigned as 6-OMe. The proton at δ 6.56(H-8) showed a strong $^3J$ correlation to C-6 and showed a weak $^4J$ "W" coupling with the carbonyl at δ 184.0 (C-4). The proton at δ 6.61(H-3) showed a weak $^2J$ correlation to the carbonyl and therefore is attached at C-2. Direct correlation were observed for (H-2) with their respective carbon. H-2 showed a $^3J$ correlation to (C-1). The protons at δ 7.54 (H-6′), 7.35 (H-2′) and the methoxy at δ 3.99 showed $^3J$ correlations to the carbon at δ 160.4 and thus the methoxy is attached to C-4′. The proton at δ 6.99 and the methoxy at δ 4.00 showed $^3J$ correlations to the carbon at δ 150.6 and the methoxy was thus assigned as 3′-OME. With the above spectral data, **ML38** was identified as 5-hydroxy-'3,4',6,7 tetramethoxyflavone and this agrees with the data from Djermanovic *et al.* (1975).

**Table 6** $^{1}H$ (400 MHz) and $^{13}C$ (100MHz) NMR data of (ML38) 5-hydroxy-3,4,6,7 tetramethoxyflavone in $CDCl_3$

| Position | $\delta_H$ | $\delta_C$ |
|---|---|---|
| 2 | - | 165.7 |
| 3 | 6.61 (1H, *s*) | 106.0 |
| 4 | - | 184.0 |
| 5 | - | 154.5 |
| 6 | - | 134.1 |
| 7 | - | 153.8 |
| 8 | 6.56 (1H, *s*) | 92.5 |
| 9 | - | 159.5 |
| 10 | - | 107.4 |
| 1' | - | 125.3 |
| 2' | 7.35 (1H, *d* ,*J*= 2.2Hz) | 110.4 |
| 3' | - | 150.6 |
| 4' | - | 160.4 |
| 5' | 6.99 (1H, *d, J*= 8.4Hz) | 113.0 |
| 6' | 7.54 (1H, *dd, J*= 8.4, 2.2Hz) | 121.8 |
| 5-OH | 12.75(1H,s) | 154.5 |
| 6-OMe | 3.93 (3H, *s*) | 62.5 |
| 7-OMe | 3.98 (3H, *s*) | 57.8 |
| 3'-OMe | 4.00 (3H, *s*) | 57.8 |
| 4'-OMe | 3.99 (3H, *s*) | 57.8 |

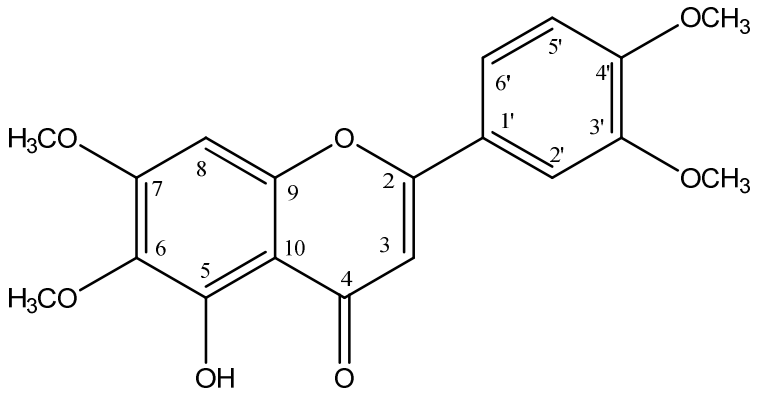



**Figure 15** Structure of ML38 (5-hydroxy-3′,4′,6,7-tetramethoxyflavone)

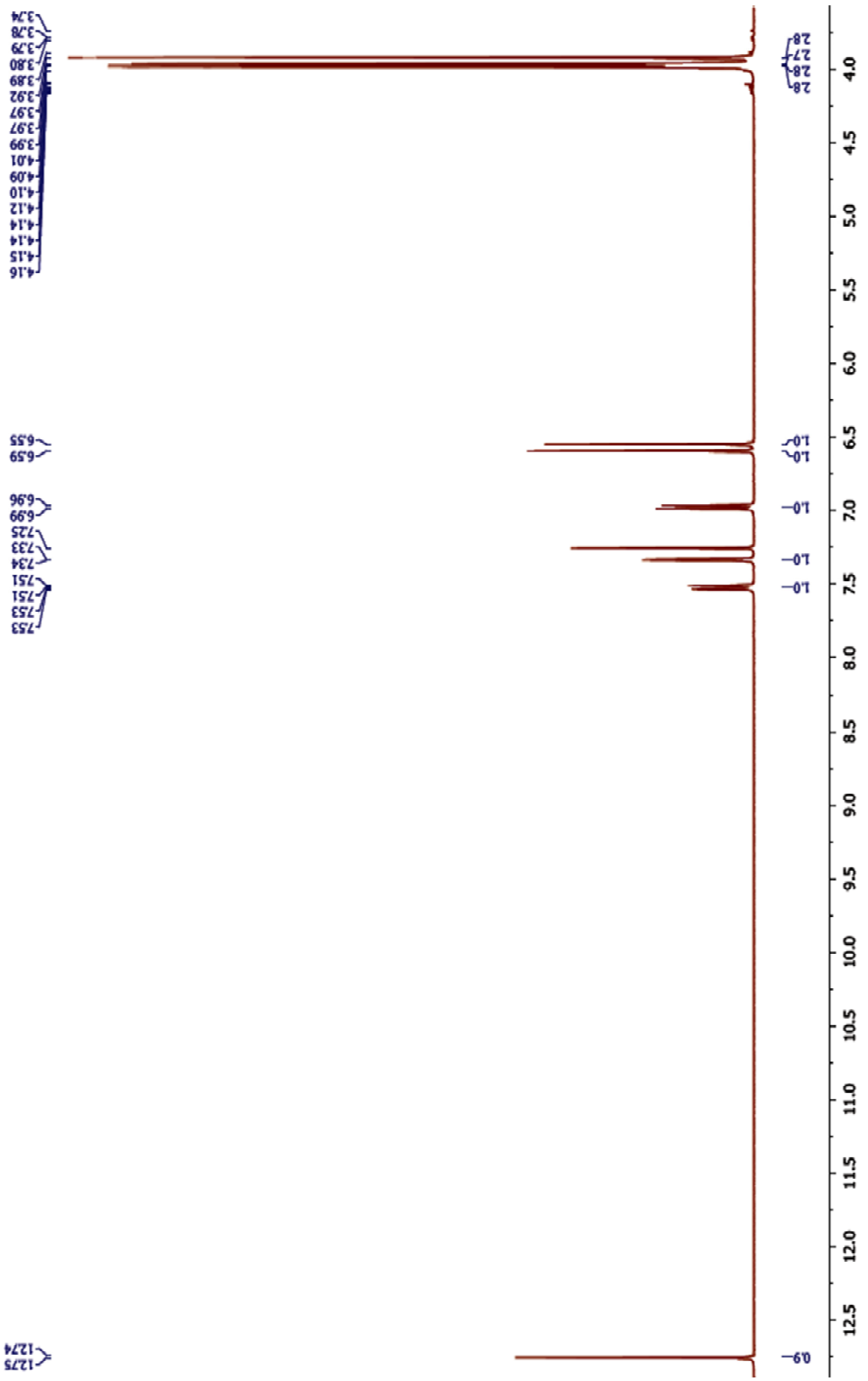


**Figure 16** $^{1}$HNMR spectra of ML38 (5-hydroxy-3′,4′,6,7-tetramethoxyflavone) (400 MHz, $CDCl_3$)

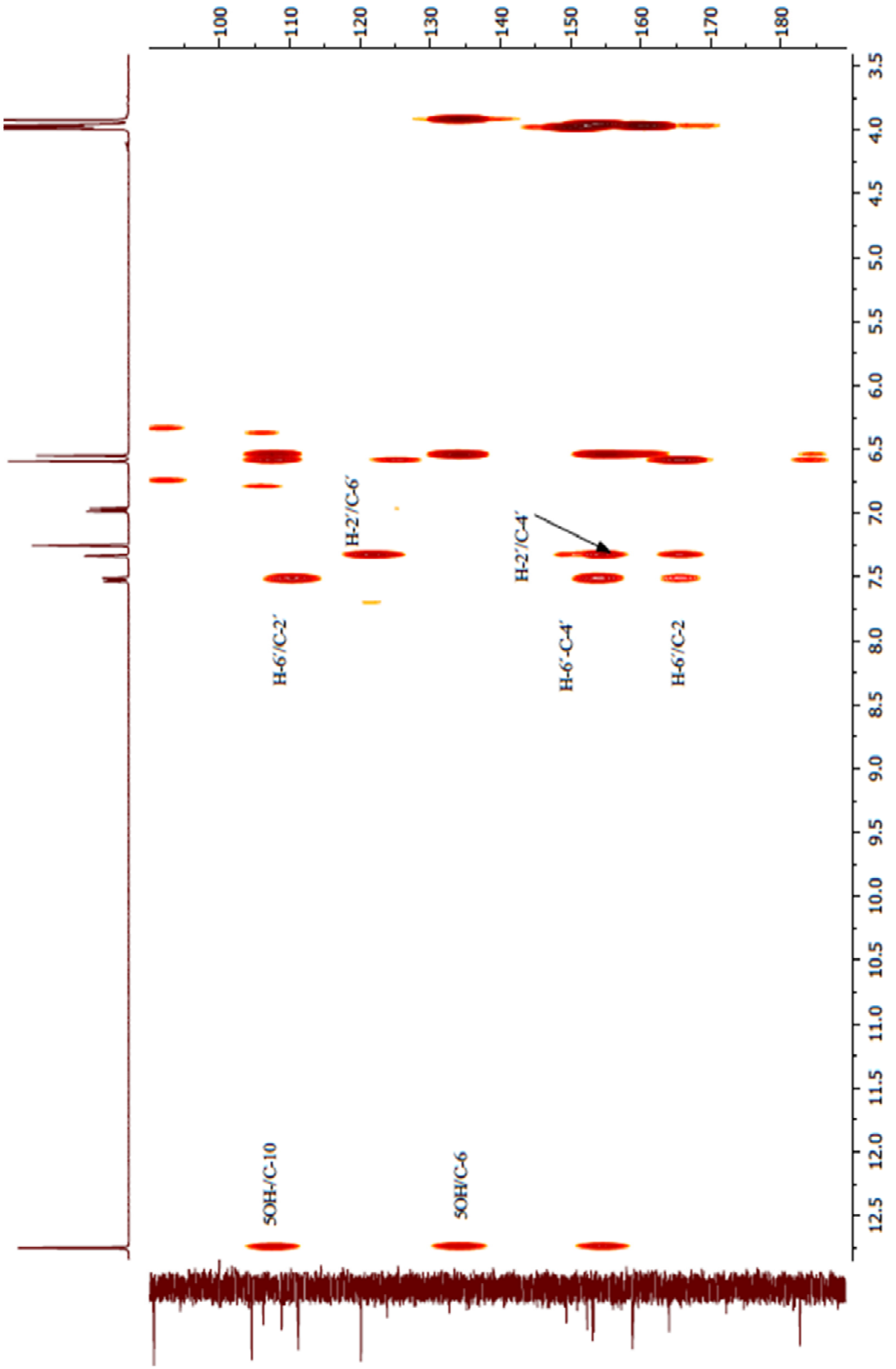



**Figure 17** HMBC spectrum of ML38 (5-hydroxy-3′,4′,6,7-tetramethoxyflavone) (400 MHz, $CDCl_3$)

### 3.4.2 Fractionation of ethyl acetate extract of *Mentha longifolia*

The ethyl acetate extract (26g) was subjected to VLC eluted with hexane increasing the polarity with ethyl acetate and up to 40% methanol. Fractions 9-14 combined and were further fractionated by Sephadex LH-20. A total of three compounds **ML7, ML10 and ML16** were isolated from this extract. These compounds were isolated as a yellow amorphous solid. On TLC they gave a dark spot under UV and a yellow fluorescence under long UV. After spraying with *p*-anisaldehyde sulphuric acid reagent they turned yellow with an $R_f$ 0.8, $R_f$ 0.77 and $R_f$ 0.75 values (on 60% ethyl acetate) for ML7, M-10 and M-16.

#### 3.4.2.1 Characterisation Of ML7 as 5,6-dihydroxy-3',4',7,8-tetramethoxyflavone

The positive mode HRESI-MS of **ML7** (Figure 18) gave an ion $[M+H]^+$ at 375.1071 suggesting a molecular formula of $C_{19}H_{18}O_8$.

The $^1$HNMR spectrum (Figure 19) showed the presence of a hydroxyl signal at $\delta_H$ 12.4ppm and ABX spin aromatic system (at δ 7.5d, *J*= 1.84, δ7.16d, *J*=8.76 and δ 7.67dd, *J*= 1.84, 8.76) and an aromatic singlet at δ6.95. The $^{13}$CNMR spectrum showed 19 signals including a carbonyl $\delta_c$ at (183.2ppm), six other oxygenated aromatic quaternary carbons at $\delta_c$ (133-183) as well as four methoxy groups' $\delta_c$ (56-62) and four quaternary carbons at $\delta_c$ (123.5, 164.5, 142.4 and 106.6).

In the HMBC spectrum (Figure 20) the singlet proton at δ6.95 (H-3) showed a weak $^2J$ correlation with the carbonyl carbon (C-4) and $^3J$ correlations with (C-1′) and (C-10). The 5-hydroxy proton showed $^3J$ correlation to the carbons at δ 106.6(C-10) and δ 134.7(C-6) and a weak $^2J$ correlation to (C-5) at 143.5. The proton at δ 7.16 (H-5′) showed a strong $^3J$ correlation to C-3′and C-1′. The proton at δ 7.53(H-2′) showed $^3J$ correlation to C-4′, C-6′ and C-2 and $^2J$ correlation with C-3′. While the proton H-6′ showed a $^3J$ correlation to δ 109.6 (C-2′), δ 152.7(C-4′) and $\delta_c$ 164.0(C-2). On the basis of these results and by comparison with previously published data, **ML7** was identified as 5,6 -dihydroxy- 3′,4′,7, 8 tetramethoxyflavone (Jullien *et al.*, 1984).

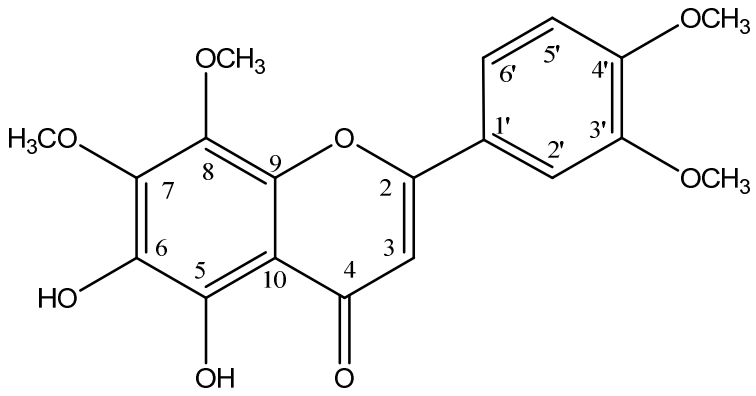



**Figure 18** Structure of ML7 (5,6-dihydroxy-3′,4′,7,8-tetramethoxyflavone)

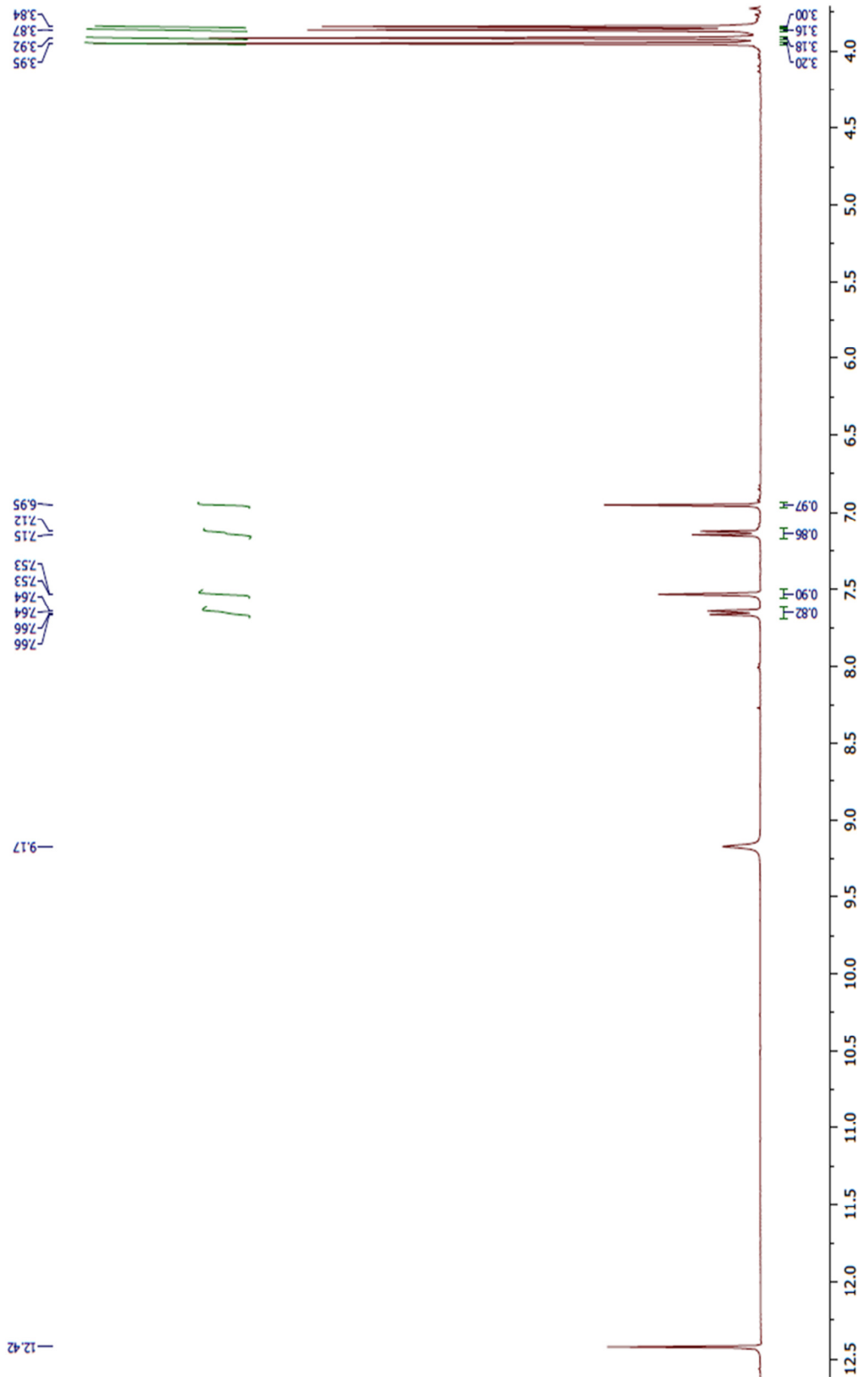



**Figure 19** $^{1}$HNMR spectrum of ML7 (5,6-dihydroxy- 3′ ,4′ ,7, 8-tetramethoxyflavone) with expansion (400 MHz, DMSO)

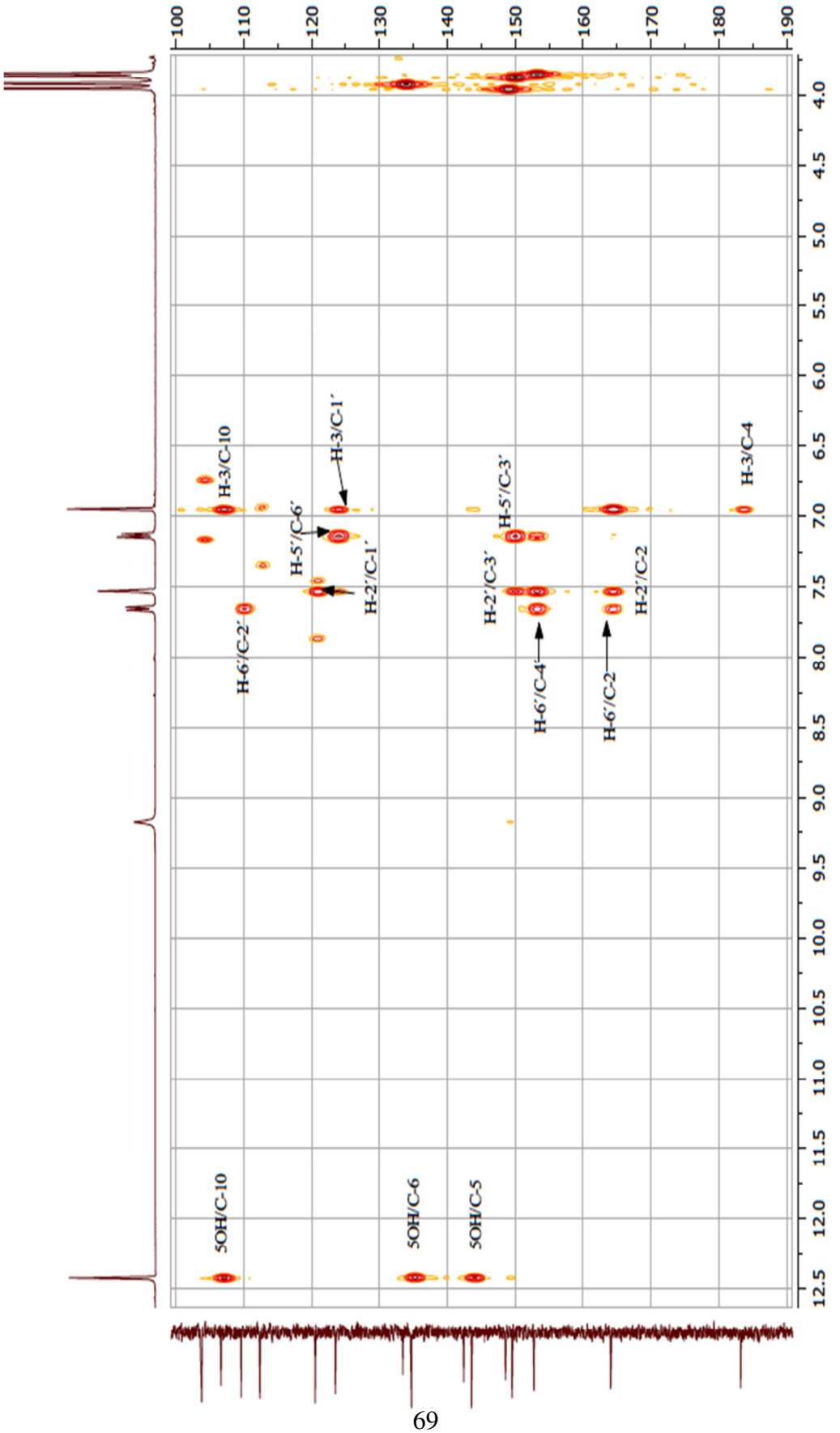



**Figure 20** HMBC spectrum of ML7 (5,6 -dihydroxy- 3′,4′,7, 8-tetramethoxyflavone) (400 MHz, DMSO)

### 3.4.2.2 Characterisation of ML10 as 5,6,3´-trihydroxy-7,8,4'-trimethoxyflavone

The high resolution positive mode HRESI-MS of **ML10** (Figure 21) gave an ion $[M+H]^+$ at 361.0914 suggesting a molecular formula of $C_{18}H_{16}O_8$.

Comparison of the $^1$HNMR spectrum (Figure 22) of **ML10** with **ML7** (3.4.2.1) showed they were analogues, the only difference being one less methoxy in **ML10**, which replaced by hydroxyl group at $\delta_H$ 10.0 and showed $^3J$ correlation to the carbon at $\delta_c$ 148.5 and $^2J$ correlation to the carbon at δc151.4, this led to the consideration of the presence of the hydroxyl groups at C-3´ positions (Figure 23). Thus the compound was identified as 5,6,3'-trihydroxy-7,8,4'-trimethoxyflavone (Gohari *et al.*, 2009).

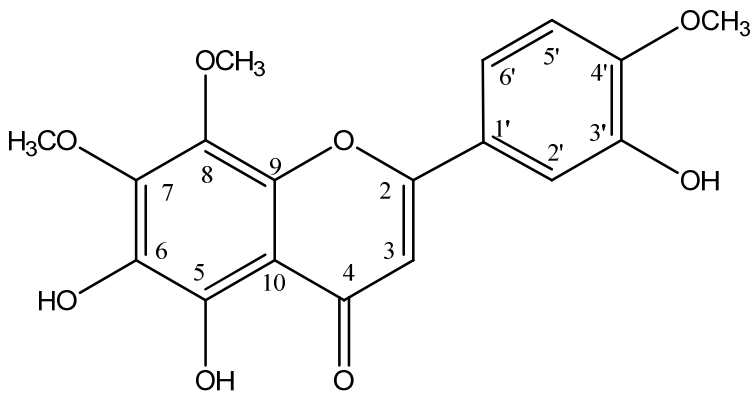



**Figure 21** structure of ML10 (5,6,3'- trihydroxy-7,8,4'- trimethoxyflavones)

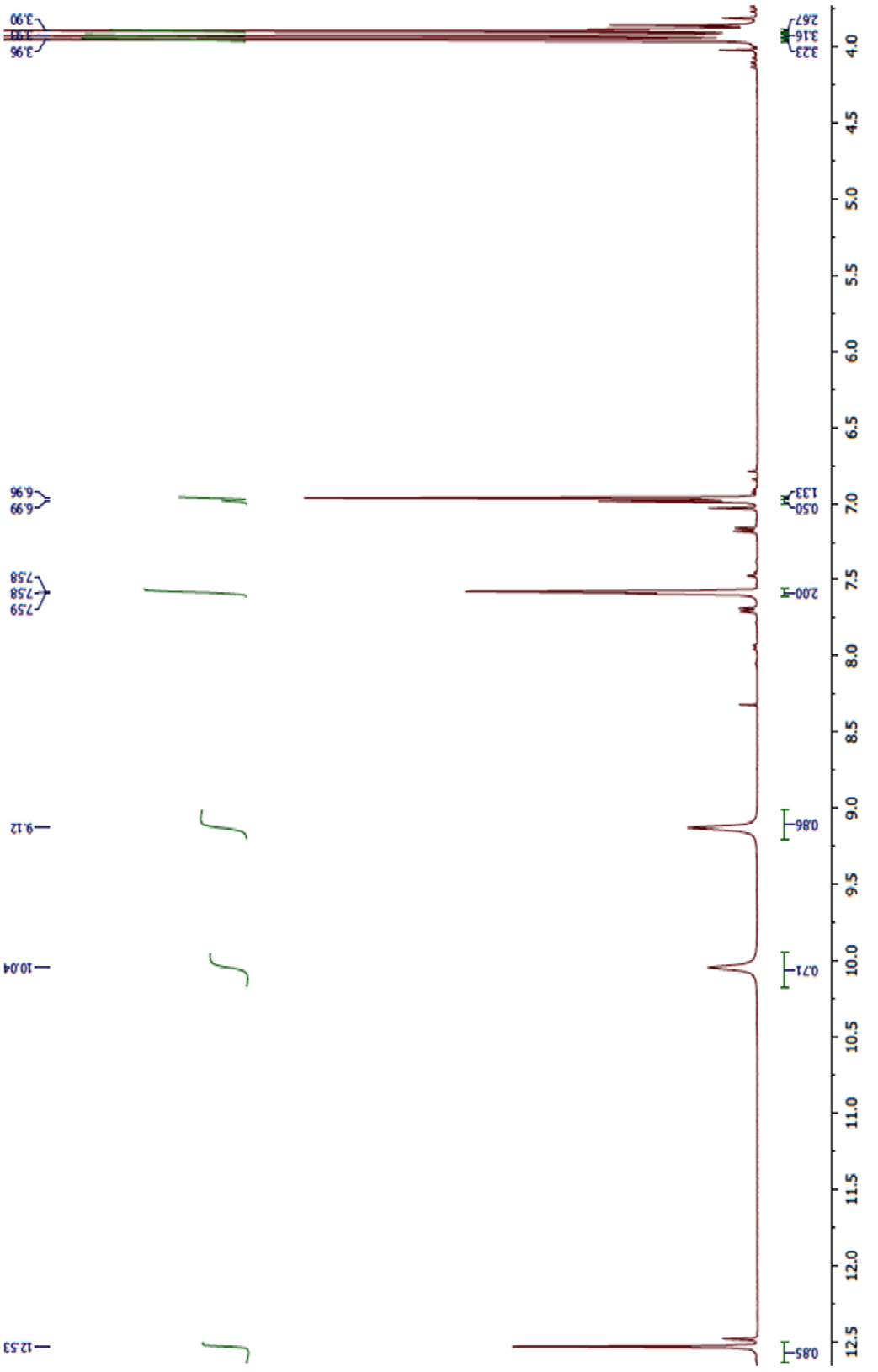



**Figure 22** $^{1}$HNMR spectrum of ML10 (5,6,3' trihydroxy-7,8,4'-trimethoxyflavones ) (400 MHz, DMSO)

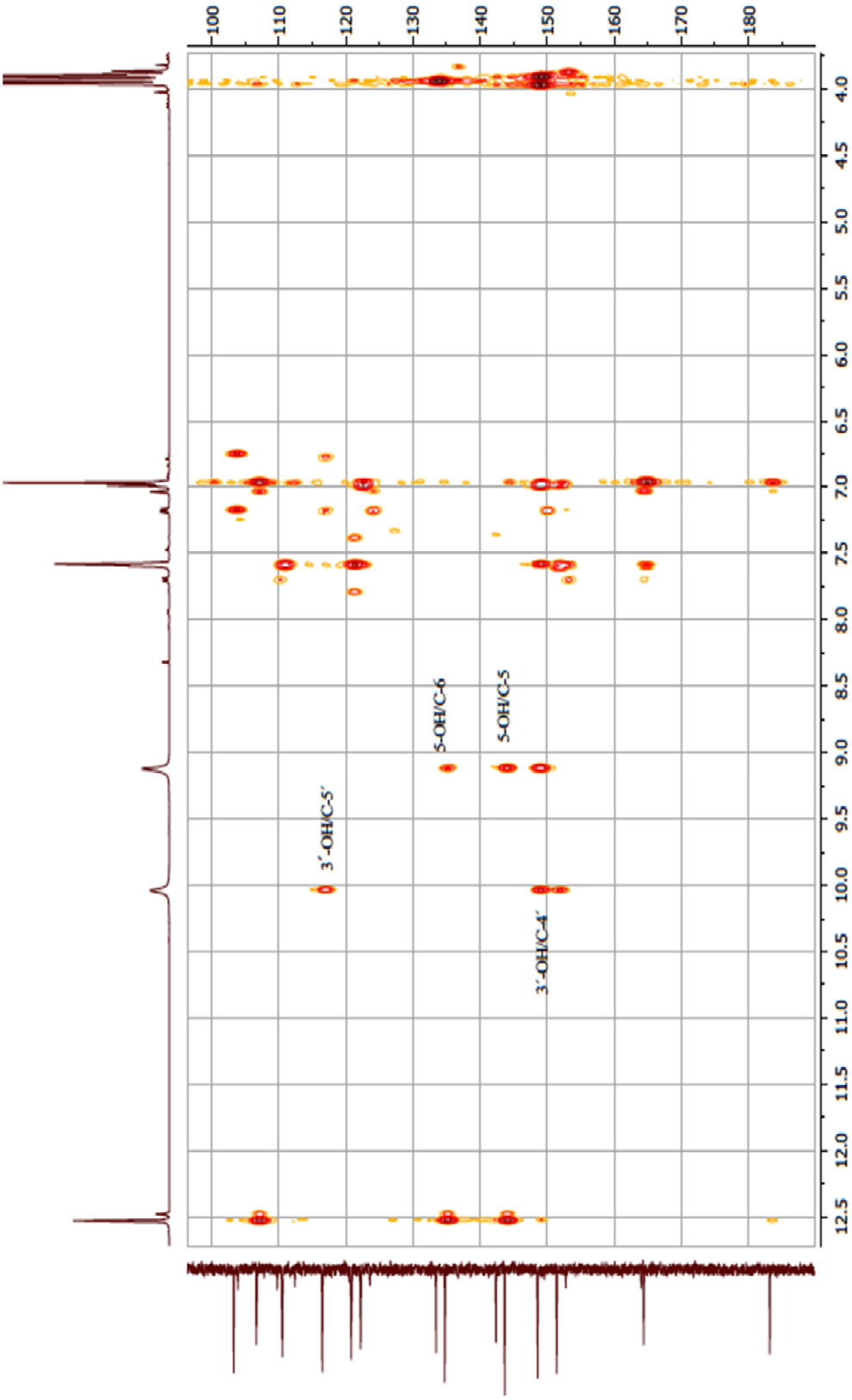



**Figure 23** HMBC spectrum of ML10 (5,6,3' trihydroxy-7,8,4'-trimethoxyflavones ) (400 MHz, DMSO)

### 3.4.2.3 Characterisation of ML16 as 4′,5,6-trihydroxy-7,8-dimethoxyflavone or sorbifolin

The high resolution positive mode HRESI-MS of **ML16** (Figure 24) gave an ion $[M+H]^+$ at 331.08091 suggesting a molecular formula of $C_{17}H_{14}O_7$.

From the $^1$HNMR and $^{13}$CNMR spectra of the three compounds ring A was the same. Therefore the only difference was in the B ring.

The $^1$HNMR spectrum for **ML16** (Figure 25) showed two *ortho, meta*, *para*-coupled AA′BB′ at $\delta_H$ 6.96 (2H, *m, J*= 8.8, 1.5Hz), 7.94(2H, *m*, *J*=8.8, 1.5Hz) suggesting 1,4-*para* disubstituted aromatic ring (Table 7).

In the HMBC spectrum (Figure 26) the protons at $\delta_H$ 7.94(H-2′/6′) and 6.965(H-5′/3′) showed $^3J$ and $^2J$ correlations to a phenolic carbon at 161.4 ppm and this established the presence of OH substitution at C-4′ on the B ring. Protons at $\delta_H$ 7.94(H-6′/2′) also showed $^3J$ correlations to another oxygen-bearing deshielded carbon at $\delta_c$ 164.5(C-2). The above spectral data for **ML16** were found to match with those of sorbifolin or 4′,5,6-trihydroxy-7,8-dimethoxyflavone (Ferreres *et al.*, 1985).

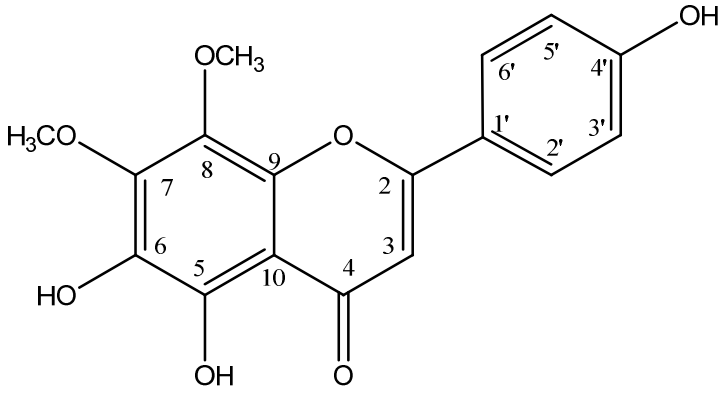



**Figure 24** structure of ML16 (sorbifolin)

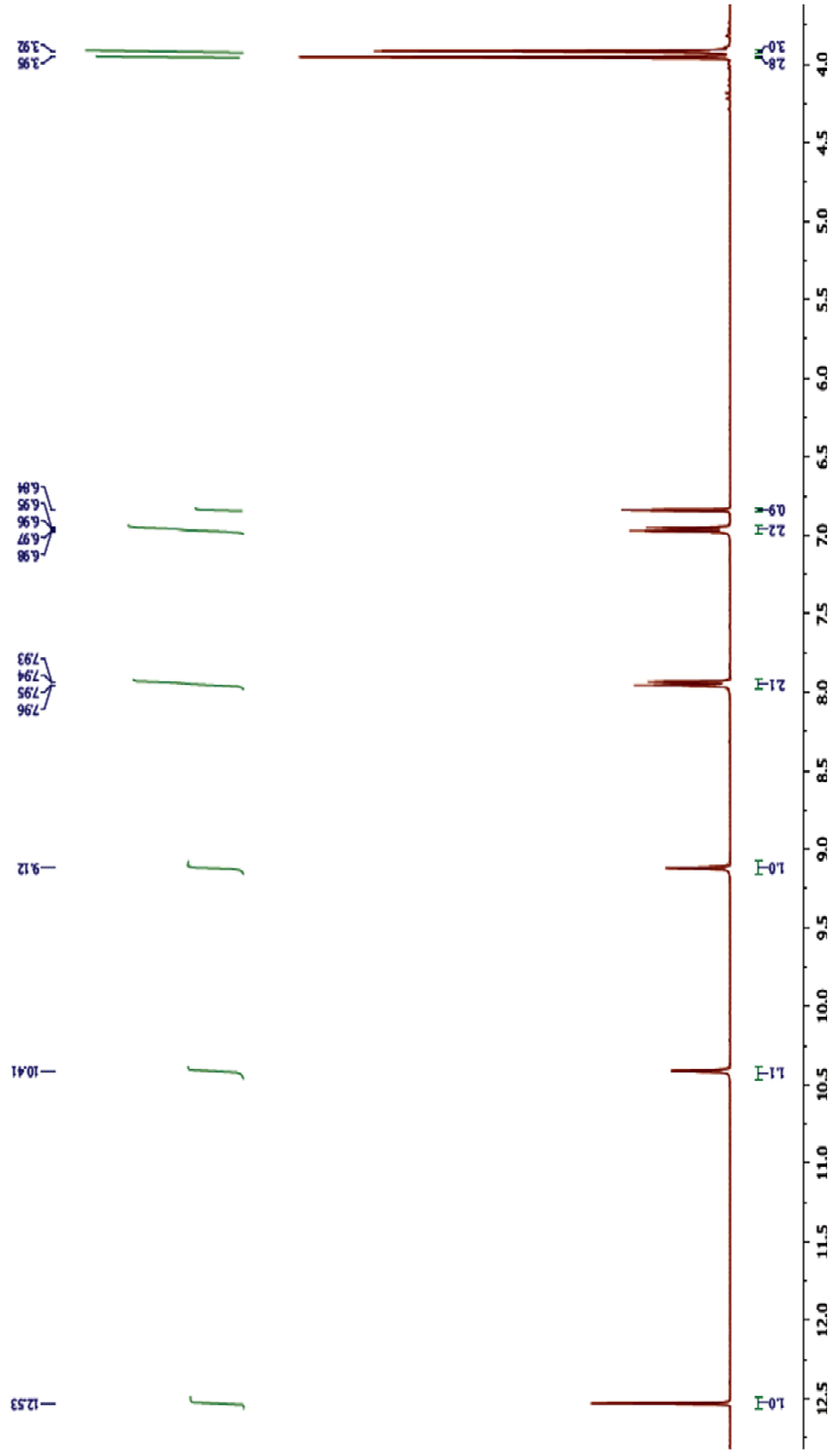


**Figure 25** $^{1}$HNMR spectrum of ML16 (sorbifolin) (400 MHz, DMSO)

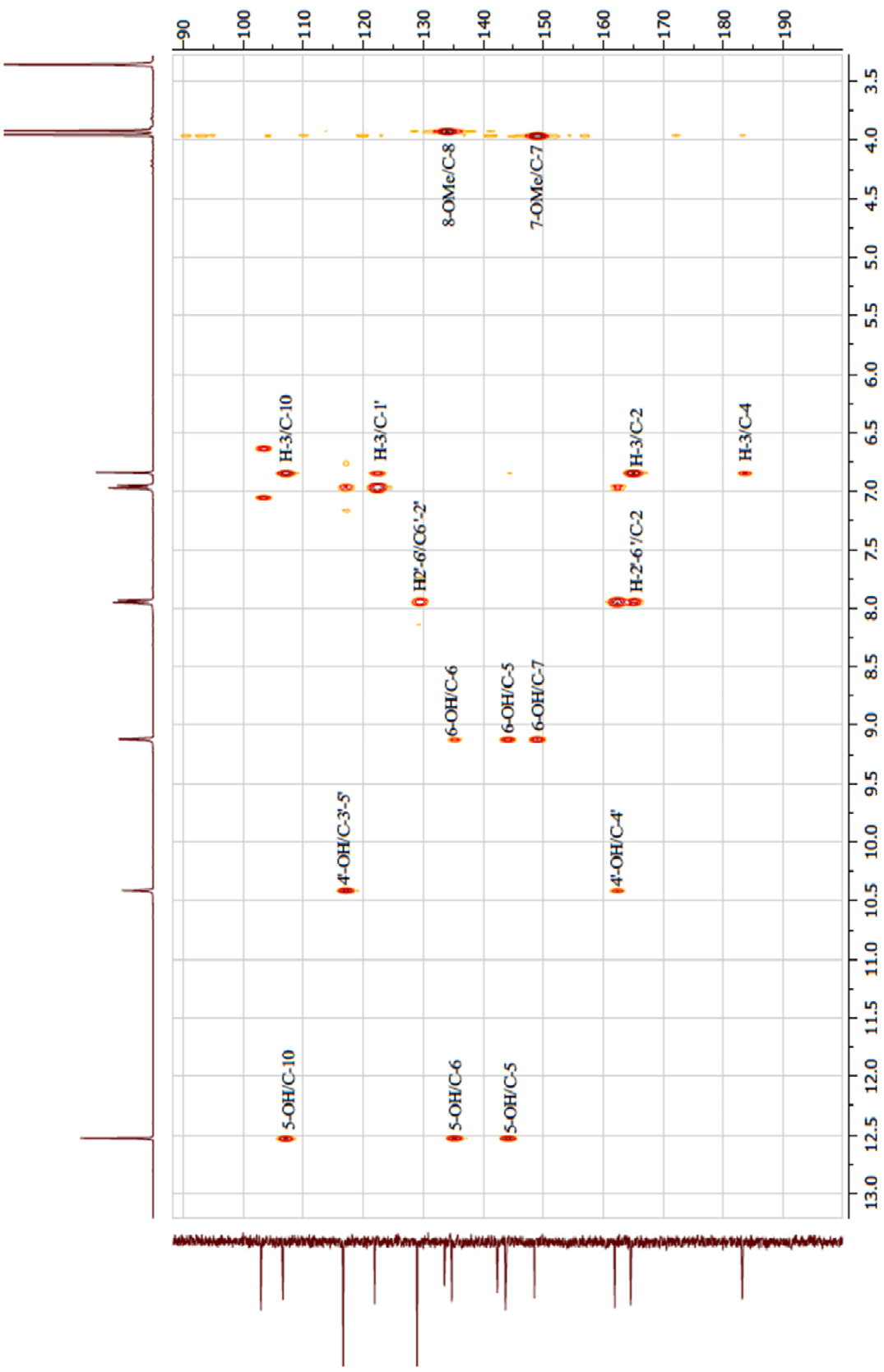



**Figure 26** HMBC spectrum of ML16 (sorbifolin) (400 MHz, DMSO)

**Table 7** $^{1}H$ (400 MHz) and $^{13}C$ (100 MHz) NMR data of ML7, ML10 and ML16 in DMSO

| Position | ML7 | | ML10 | | ML16 | |
|---|---|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | - | - | - | - | - | - |
| 2 | - | 164.0 | - | 164.3 | - | 164.5 |
| 3 | 6.95 (1H, s) | 103.8 | 6.96 (1H, s) | 103.2 | 6.84 (1H, s) | 102.9 |
| 4 | - | 183.2 | - | 183.2 | - | 183.4 |
| 5 | - | 143.5 | - | 143.6 | - | 143.6 |
| 6 | - | 134.7 | - | 134.7 | - | 134.7 |
| 7 | - | 148.5 | - | 148.6 | - | 148.5 |
| 8 | - | 133.4 | - | 133.4 | - | 133.5 |
| 9 | - | 142.4 | - | 142.3 | - | 142.3 |
| 10 | - | 106.6 | - | 106.6 | - | 106.6 |
| 1' | - | 123.5 | - | 122.5 | - | 121.8 |
| 2' | 7.53 (1H, *J*= 1.84 Hz) | 109.6 | 6.99 (1H,d, *J* =1.2Hz) | 120.8 | 7.94(2H, d, *J* = 8.8) | 128.9 |
| 3' | - | 149.5 | - | 148.5 | 6.96(2H, d, *J* = 1.5) | 116.6 |
| 4' | - | 152.7 | - | 151.4 | - | 161.9 |
| 5' | 7.16 (1H, *J*= 8.76) | 112.3 | 6.97(1H, d, *J* =1.7Hz) | 116.4 | 6.96(2H, d, *J* = 8.8) | 116.6 |
| 6' | 7.67 (1H, dd, *J*= 1.84, 8.76) | 120.1 | 7.58(1H, dd, *J* =1.7, 1.2Hz) | 110.5 | 7.94(2H, d, *J* = 1.5) | 128.9 |
| 5-OH | 12.42(1H, s) | 143.5 | 12.51(1H, s) | 143.5 | 12.53(1H, s) | 143.6 |
| 6-OH | 9.17(1H, s) | 134.7 | 9.1(1H, s) | 134.7 | 9.15(1H, s) | 134.7 |
| 7-OC $H_3$ | 3.94 (3H, *s*) | 61.5 | 3.95 (3H, *s*) | 61.5 | 3.95 (3H, s) | 61.5 |
| 8-OC $H_3$ | 3.91 (3H, *s*) | 62.3 | 3.93 (3H, *s*) | 62.3 | 3.95(3H, s) | 62.3 |
| 3'-OC $H_3$ | 3.86 (3H, *s*) | 56.29 | - | - | - | - |
| 4'-$OCH_3$ | 3.84 (3H, *s*) | 56.22 | 3.89 (3H, *s*) | 56.3 | - | - |
| 3'-OH | - | - | 10.0(1H, s) | - | - | - |
| 4'-OH | | | | | 10.41 (1H, s) | - |

### 3.4.3 Fractionation of Methanol extract of *Mentha longifolia*

The methanol extract for *Mentha longifolia* produced pale yellow crystals as precipitate in the flask after extraction. The crystals were filtered and weighed to yield 15g (**ML1**). The remaining extract was filtered and concentrated under vacuum pressure and partitioned with chloroform which produced a white precipitate (10g) in the chloroform phase. This was filtered and further purified on a C-18 flash column (**ML2**). The water phase was freeze dried. The $^{1}$HNMR spectrum of the fraction was similar to the one obtained for a commercial sample of sucrose and this fraction was not further investigated.

#### 3.4.3.1 Characterisation of ML1 as linarin

The positive mode HRESI-MS of **ML1** (Figure 27) gave an ion at $[M+H]^{+}$=593.1863, suggesting a molecular formula of $C_{28}H_{32}O_{14}$ .

The $^{1}$H NMR spectrum (Figure 28) indicated the presence of a 1,2,3,5-tetrasubstituted ring A of the flavonoid structure with the *meta* coupled protons at $\delta_H$ 6.78 (1H, *d,* 1.8Hz) and 6.45 (1H, *d*, 1.8 Hz). While *ortho* and *meta* coupled protons at $\delta_H$ 8.02 (2H, *dd*, 8.8Hz) and 7.12 (2H, *dd*, 8.8Hz) represented a 1,4-*para* disubstituted B ring. The 5- hydroxy proton appeared at $\delta_H$ 12.91(1H, s) and $\delta_H$ 6.91 (1H, s). The proton spectrum also indicated the presence of a methoxy group at $\delta_H$ 3.84 (3H, *s*), two sugar residues with their anomeric protons at $\delta_H$ 5.07 (1H, *d* 7.2 Hz) and 4.57 (1H, d, *J*= 1.1Hz) along with some oxymethines between $\delta_H$ 3.17 and 3.88 and a methyl doublet at 1.09 (3H, d, *J*= 6.1 Hz).

The $^{13}$C NMR spectra indicated the presence of 28 carbons including a carbonyl carbon at $\delta_C$ 197.0 (Table 8). Using $^{1}$H and COSY spectrum, the two sugar units were identified as β-D-glucose due to the large coupling constant (7.2 Hz) of its anomeric proton and α-L-rhamnose for the small coupling constant of the anomeric proton.

In the HMBC spectrum (Figure 29) the proton at $\delta_H$ 12.91 showed $^3J$ correlations to carbons at $\delta_C$ 162.2, 106.0 and 100.2. Proton at $\delta_H$ 6.91 showed $^3J$ correlations to $\delta_C$ 106.0, 123.2, 164.5 and 182.6 and $^2J$ correlations to $\delta_C$ 115.2. The proton at $\delta_H$ 6.78 showed $^3J$ correlations to the carbons at $\delta_C$ 106.0, 157.6 and 163.5 and $^2J$ correlations to $\delta_C$ 100.2. Protons at $\delta_H$ 6.45 showed $^3J$ to the carbons at $\delta_C$ 106.0, 161.7, 163.0 and $^2J$ correlation to the carbons at $\delta_C$ 95.3. Thus the protons at $\delta_H$ 6.78 and 6.45 were assigned as H-6/8, while $\delta_H$ 6.9 as H-3. The protons at $\delta_H$ 8.05 in ring B showed $^3J$ correlation to carbon $\delta_C$ 129.2 and to an oxygen bearing aromatic carbon at $\delta_C$ 163.5, these two protons were assigned as H-2' and H-6'. The other two protons at $\delta_H$ 7.12 showed $^3J$ correlations to carbon $\delta_C$ 115.2, 123.2 and 164.5 thus assigned as H-3'/5'.

Furthermore, the methoxy group at $\delta_H$ 3.83 (3H, *s*) showed $^3J$ correlation to the carbon at $\delta_C$ 163.5(C-4') proving its connectivity to ring B. The anomeric proton of α-L-rhamnose ($\delta_H$ 4.57) showed $^3J$ correlation to carbon at $\delta_C$ 66.1(C-6'') of glucose residue. While the anomeric proton of β-D-glucose at $\delta_H$ 5.07 showed $^3J$ correlation to carbon at $\delta_C$ 164.5 (C-7) proving its connectivity to ring A. Thus **ML1** was identified as acacetin-7-O-rutinoside or linarin. All the spectral data were in agreement with those published in the literature (Davydov *et al.*, 1985).

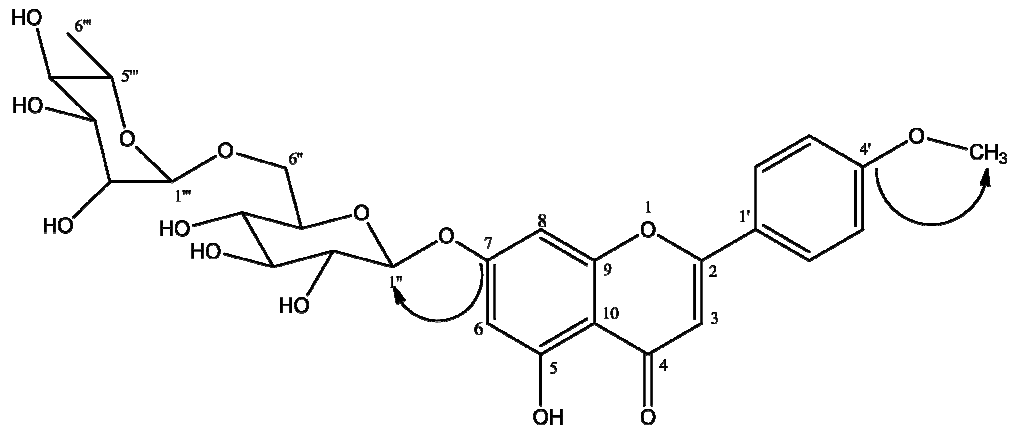



**Figure 27** structure of ML1 (Linarin)

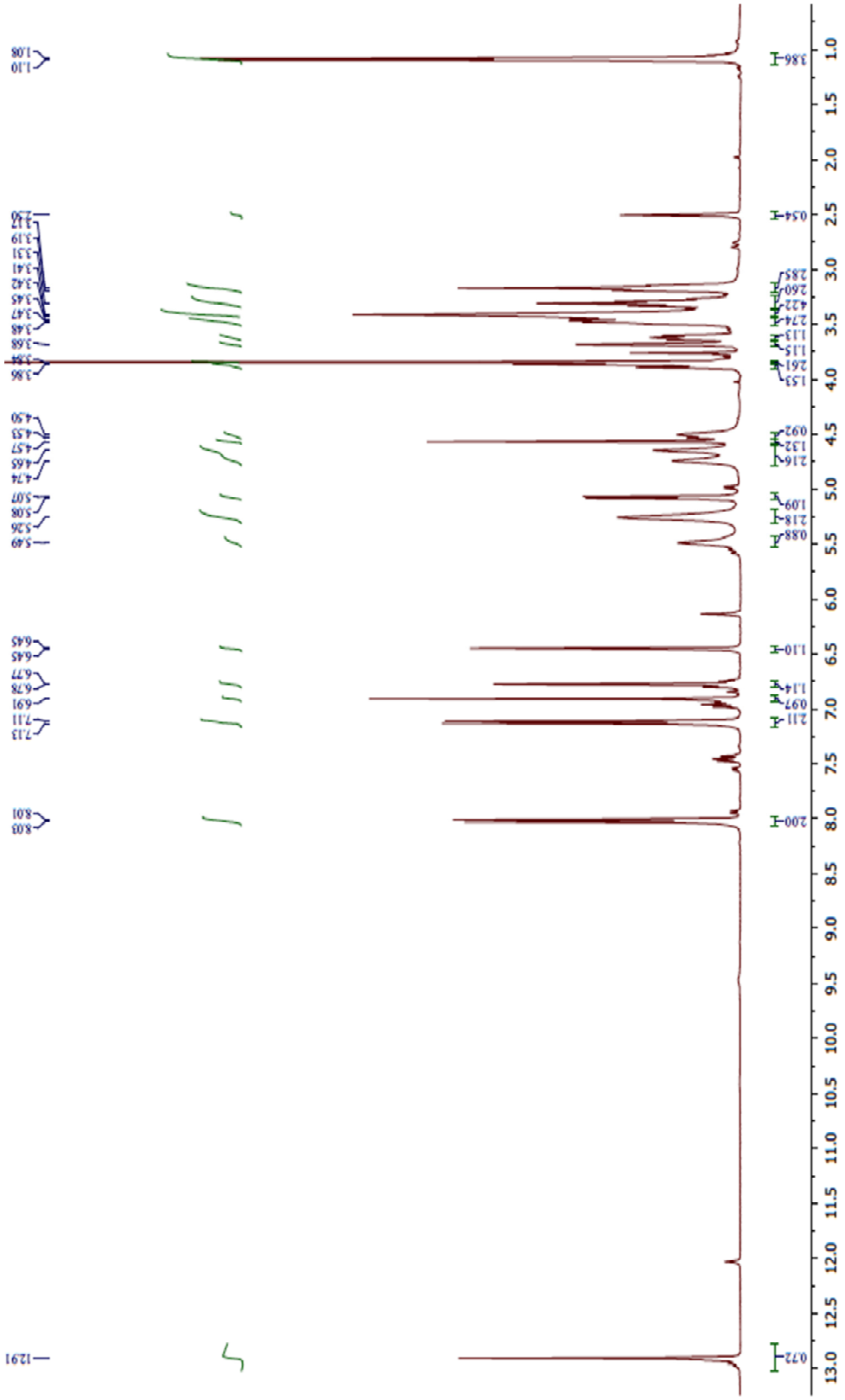


**Figure 28** $^{1}$H NMR spectrum of ML1 (linarin) (400 MHz, DMSO)

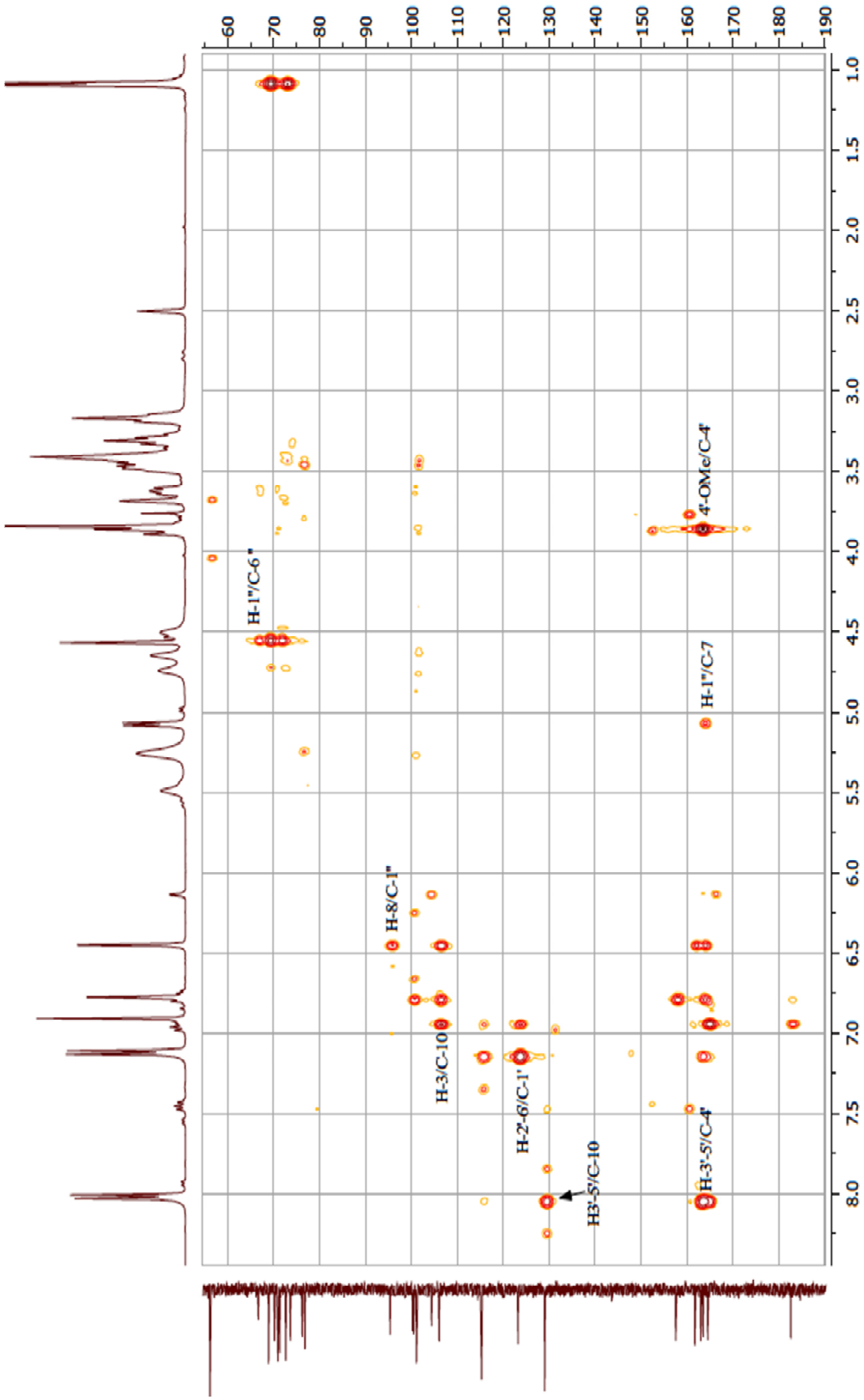



**Figure 29** Partial HMBC spectrum of ML1 (Linarin) (400 MHz, DMSO)

### 3.4.3.2 Characterisation of ML2 as didymin

The negative mode HRESI-MS data of **ML2** (Figure 30) gave an ion $[M-H]^-$ at *m/z* 593.1882, suggesting a molecular formula of $C_{28}H_{34}O_{14}$. This indicates two protons more than **ML1**.

The $^1H$ NMR spectrum (Figur 31) indicated the presence of a 1,2,3,5-tetrasubstituted ring A of the flavonoid structure with the *meta* coupled protons at $\delta_H$ 6.17 (1H, *d* Hz) and 6.19 (1H, *d* Hz). While *ortho*, *meta* coupled protons at $\delta_H$ 6.95 (2H, *dd* Hz) and 7.42 (2H, *dd* Hz) represented a 1,4-*para* disubstituted B ring. The compound was considered to be a flavanone due to the presence of methylene protons at $\delta_H$ 2.77 (1H, *dd* Hz) and 3.16 (1H, *dd* Hz) and a downfield oxymethine at $\delta_H$ 5.44 (2H, *dd* Hz). The proton spectrum also indicated the presence of a methoxy group at $\delta_H$ 3.80 (3H, *s*), two sugar residues with their anomeric protons at $\delta_H$ 4.68 (1H, *d* 1.3 Hz) and 4.93 (1H, *d* 7.5 Hz) along with some oxymethines between $\delta_H$ 3.43 and 3.98 and a methyl doublet at 1.18 (3H, *d* 6.2 Hz).

Using $^1H$ and COSY spectrum, the two sugar units were identified as β-D-glucose due to the large coupling constant (7.5 Hz) of its anomeric proton and α-L-rhamnose for the small coupling constant (1.3 Hz) of the anomeric proton. The $^{13}C$ NMR spectra indicated the presence of 28 carbons along with a carbonyl carbon at $\delta_C$ 197.0 (Table 7).

The HMBC spectrum (Figure 32) the proton at $\delta_H$ 6.17 in ring A showed $^2J$ correlations to carbons at $\delta_C$ 165.5 and 163.0. The proton at $\delta_H$ 6.19 showed $^2J$ correlations to highly deshielded oxygen bearing aromatic carbons at $\delta_C$ 165.5 and 163.7. These two protons also showed $^3J$ correlations to each other's carbons and to the carbon at $\delta_C$ 103.6. Thus these two protons were assigned as H-6 and H-8. The protons at $\delta_H$ 7.42 in ring B showed $^3J$ correlation to C-2 carbon ($\delta_C$ 79.1) and to an oxygen bearing aromatic carbon at $\delta_C$ 160.2 and these two protons were assigned as H-2' and H-6'. The other two protons at $\delta_H$ 6.95 showed correlations to C-2',6' and to the carbon at 160.2 and were assigned as H-3',5'. Further, the methoxy group at $\delta_H$ 3.80 (3H, *s*) showed $^3J$ correlation to the carbon C-4'. The anomeric proton of α-L-rhamnose showed $^3J$ correlation to C-5'' of glucose residue while the anomeric proton

of β-D-glucose showed $^{3}J$ correlation to carbon C-7 proving its connectivity to ring A. Determination of the optical rotation confirmed it to be *S* isomer. Thus the compound was identified as 5,7-Dihydroxy-4'-methoxyflavanone 7-O-[α-L-Rhamnopyranosyl-(1→6)-β-D-glucopyranoside] or didymin. All the spectral data were in agreement with those published by Pelter *et al.* (1976) and Shaiq Ali *et al.*(2002).

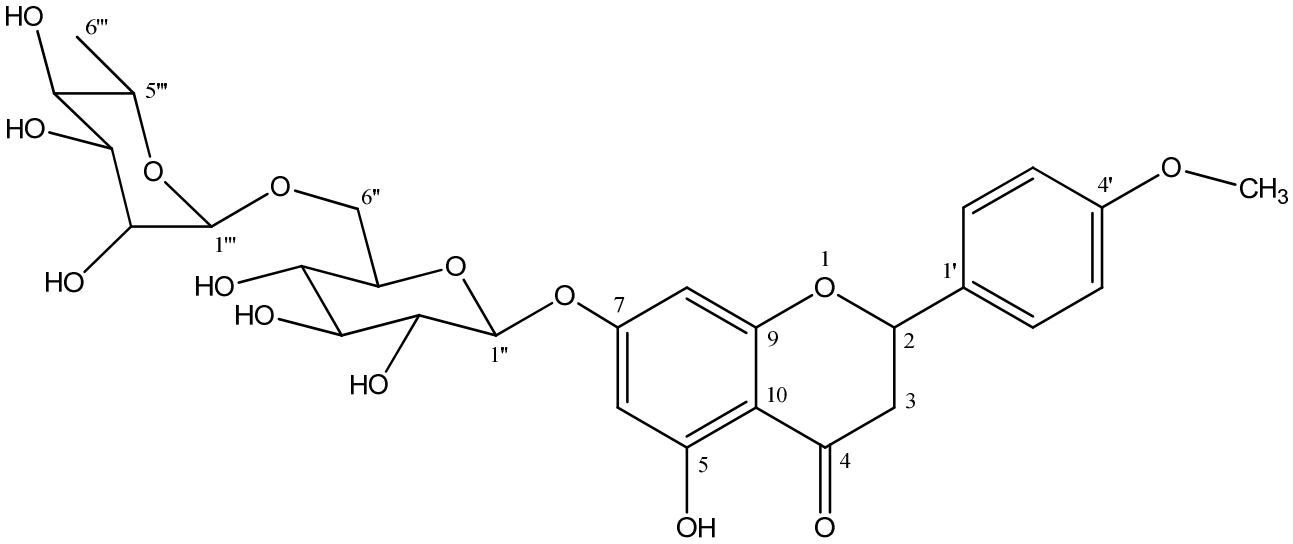



**Figure 30** structure of ML2 (didymin)

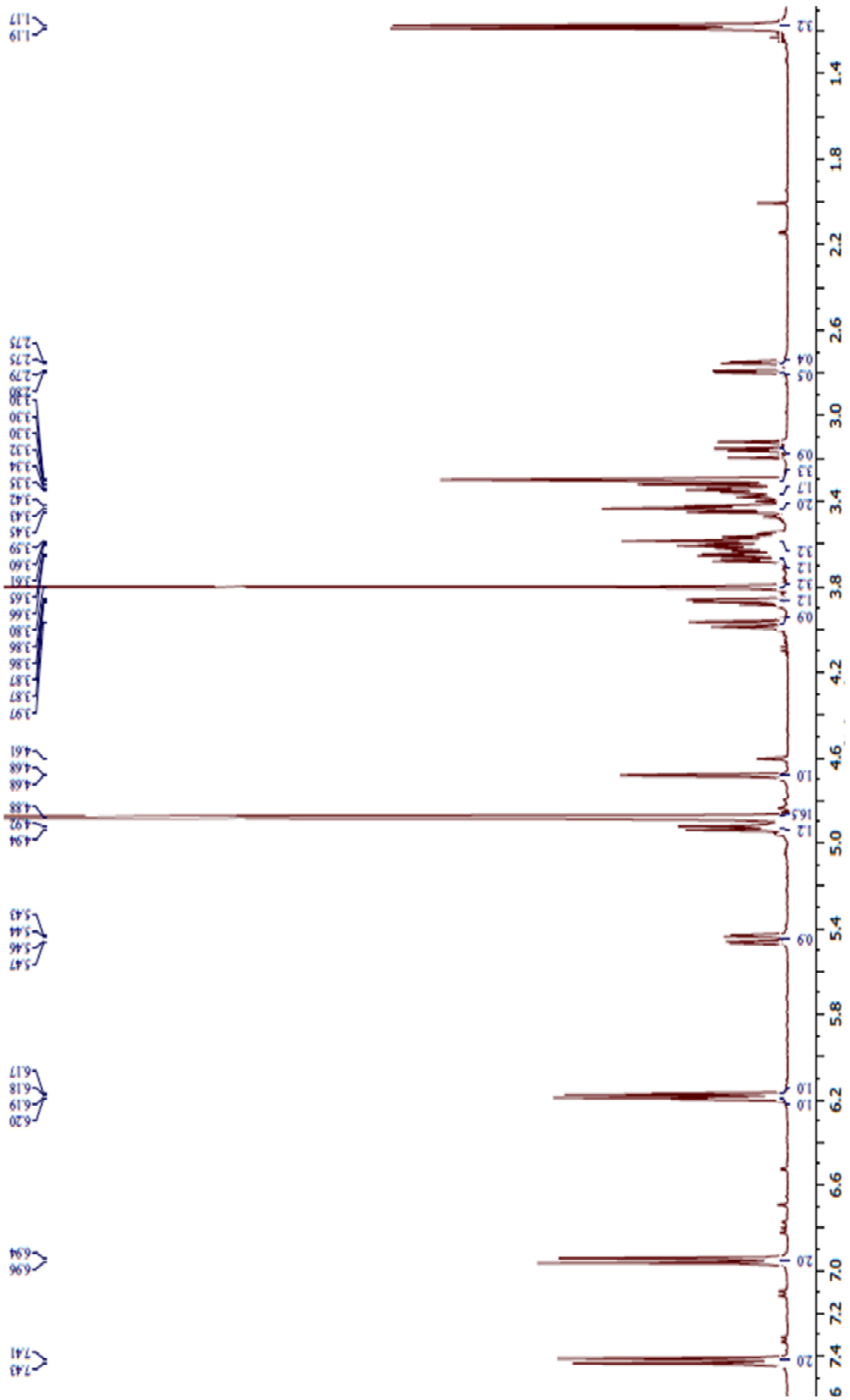


**Figure 31** $^{1}$HNMR spectrum of ML2 (didymin) (400 MHz, $CD_3OD$)

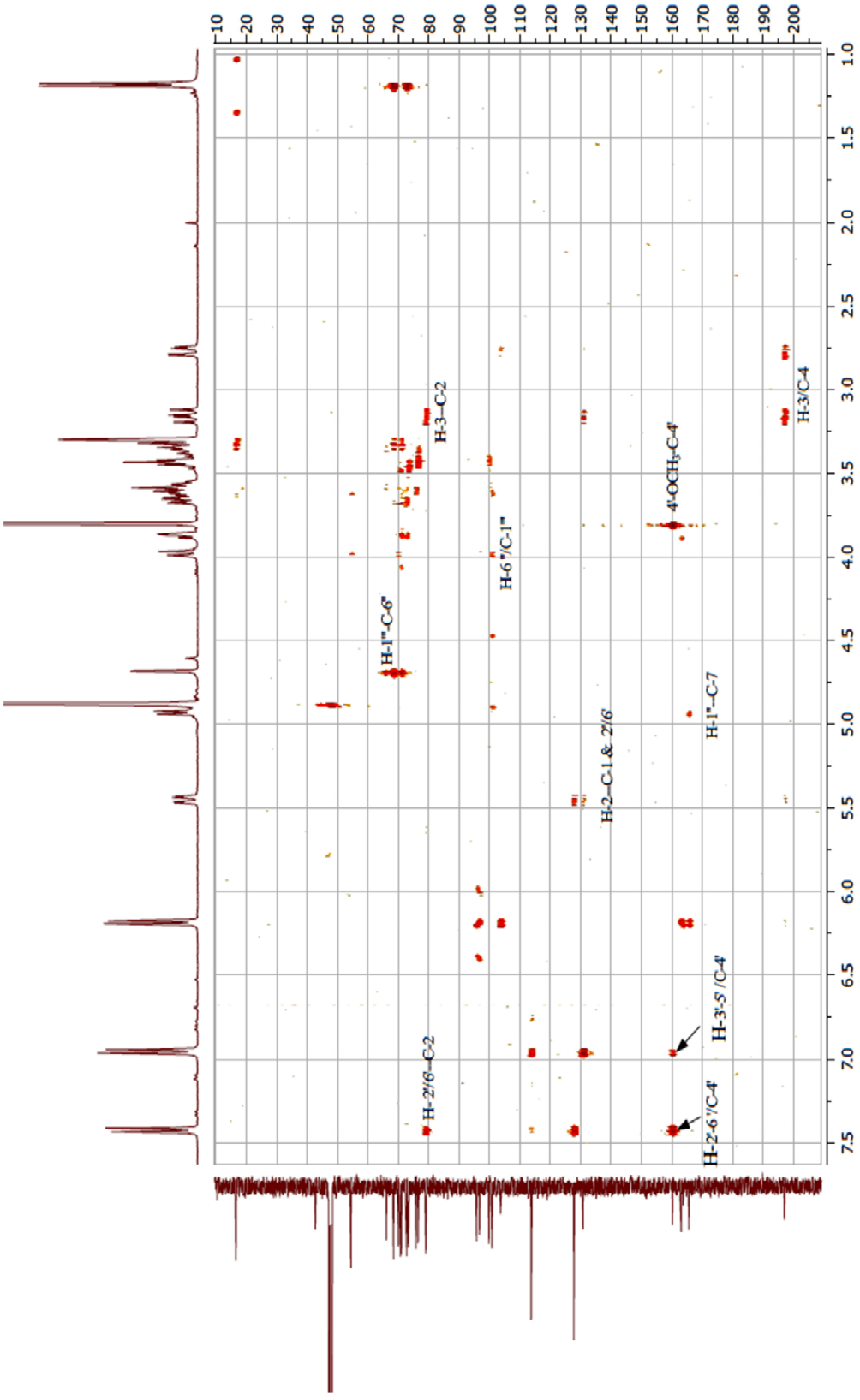



**Figure 32** HMBC spectrum of ML2 (didymin) (400 MHz, $CD_3OD$)

**Table 8** $^{1}H$ (400 MHz) and $^{13}C$ (100 MHz) NMR data of ML1 (linarin) and ML2 (didymin) in DMSO and $CD_3OD$.

| | ML1(linarin) | | ML2 (didymin) | |
|---|---|---|---|---|
| **Position** | **$\delta_H$** | **$\delta_C$** | **$\delta_H$** | **$\delta_C$** |
| 1 | - | - | - | - |
| 2 | - | 163.0 | 5.45 (1H, dd,*J*= 2.6, 12.8) | 79.1 |
| 3 | 6.91(1H, s) | 106.0 | 2.77 (1H, dd, *J*= 2.6, 17.2Hz), 3.16 (1H, dd, *J*= 12.8, 17.2 Hz) | 42.7 |
| 4 | - | 182.6 | - | 197.0 |
| 5 | - | 161.7 | - | 163.0 |
| 6 | 6.78 (1H, d, *J* = 1.8 Hz) | 95.3 | 6.17 (1H, d, *J*= 2.2Hz) | 95.7 |
| 7 | - | 164.5 | - | 165.5 |
| 8 | 6.45 (1H, d, *J* = 1.8 Hz) | 100.2 | 6.19 (1H, d, *J*= 2.2Hz) | 96.6 |
| 9 | - | 157.9 | - | 163.7 |
| 10 | - | 106.0 | - | 103.6 |
| 1' | - | 123.2 | - | 130.8 |
| 2' | 7.12 (2H, d, *J* = 8.9 Hz) | 129.0 | 7.42 (1H, d, *j*= 8.8Hz) | 127.8 |
| 3' | 8.02 (2H, d, *J* = 8.8 Hz) | 115.2 | 6.95 (1H, d, *j*= 8.8Hz) | 113.7 |
| 4' | - | 163.5 | - | 160.2 |
| 5' | 8.02 (2H, d, *J* = 8.8 Hz) | 115.2 | 6.95 (1H, d, *j*= 8.8Hz) | 127.8 |
| 6' | 7.12 (2H, d, *J* = 8.9 Hz) | 129.0 | 7.42 (1H, d, *j*= 8.8Hz) | 113.7 |
| 1'' | 5.07 (1H, d, *J* = 7.2 Hz) | 100.5 | 4.93 (1H, d, *j*= 7.5Hz) | 99.8 |
| 2'' | 3.43 (1H, *m*) | 73.3 | 3.43 (1H, *m*) | 73.3 |
| 3'' | 3.44 (1H, *m*) | 76.5 | 3.44 (1H, *m*) | 76.5 |
| 4'' | 3.58 (1H, *m*) | 75.8 | 3.58 (1H, *m*) | 75.8 |
| 5'' | 3.36 (1H, *m*) | 69.9 | 3.36 (1H, *m*) | 69.9 |
| 6'' | 3.60 (1H, m), 3.98 (1H, d, *J* =9.2Hz) | 66.1 | 3.60 (1H, *m*), 3.98 (1H, d, *J*=9.2Hz) | 66.1 |
| 1''' | 4.57 (*bro*, 1H) | 101.1 | 4.68 (1H, d, *J*= 1.3Hz) | 100.8 |
| 2''' | 3.88(1H, *dd* 1.8, 3.5) | 70.7 | 3.88(1H, *dd, J*= 1.8, 3.5) | 70.7 |
| 3''' | 3.65(1H, *dd* 3.5, 9.7) | 71.0 | 3.69(1H, *dd, J*= 3.5, 9.7) | 71.0 |
| 4''' | 3.32 (1H, *m*) | 72.8 | 3.32 (1H, *m*) | 72.8 |
| 5''' | 3.61 (1H, *m*) | 68.5 | 3.61 (1H, *m*) | 68.5 |
| 6''' | 1.09 (3H, d, *J* = 6.1 Hz) | 18.3 | 1.18 (3H, *d* ,*J*= 6.2) | 16.6 |
| 4'$OCH_3$ | 3.84 (3H, *s*) | 56.1 | 3.80 (3H, *s*) | 54.4 |

## 3.5 Isolation and structure elucidation of compounds isolated from *Acacia nilotica*

Three compounds were isolated from *Acacia nilotica*; all of them were hydrolysable tannins (derivatives of gallic acid). Seeds of *Acacia nilotica* plant were macerated with hot water then the extract filtered, concentrated and evaporated. The extract (28g) subjected to VLC. Fractions 1-8 were combined and subjected to silica gel column. This afforded two compounds **AN4** and **AN5**. Fractions 15-20 were subjected to Sephadex LH-20 affording **AN25** (Appendix III). The chemistry of *Acacia nilotica* has been previously investigated as outlined in the introductory section (3.1.2).

### 3.5.1 Characterisation of AN4 as methyl gallate

**AN4** was isolated from the water extract of *Acacia nilotica* as white needle crystals. On TLC it gave a dark spot under short UV and almost colorless after treatment with anisaldehyde reagent, with $R_f$ value of 0.57 in 80% ethyl acetate mobile phase.

The HREI-MS for **AN4** gave an ion at $[M-H]^-$=183.03 suggesting a molecular formula of $C_8H_8O_4$.

The $^1$HNMR spectrum consisted of aromatic protons at $\delta_H$ 6.94 (2H) and an esterified methyl group at $\delta_H$ 3.73 (3H, *s*). The $^{13}$CNMR spectrum exhibited the presence of 6 carbons including a carbonyl at $\delta_C$ 166.9, three oxygen-bearing aromatic quaternaries at $\delta_C$ 146.1 (×2) and $\delta_C$ 138.9. In the HMBC spectrum (Figure 33) the protons at $\delta_H$ 7.73 (H-2/6) showed their direct at $\delta_C$ 109.0 as well as 3*J*correlation to the same carbon indicating their relative *meta* positions. They also showed $^3J$ correlation to the carbonyl and one of the oxygen-bearing carbon ($\delta_C$ 138.9), and $^2J$ correlation to the other oxygen-bearing carbons ($\delta_C$ 146.1 and 119.8). The methyl group showed $^2J$ correlation to the carbonyl carbon. All the above information led to the conclusion that **AN4** was the methyl ester of gallic acid or methyl gallate. All spectral data were in agreement with previous reports (Wang *et al.*, 2007). This is the first report of methyl gallate from *Acacia nilotica*.

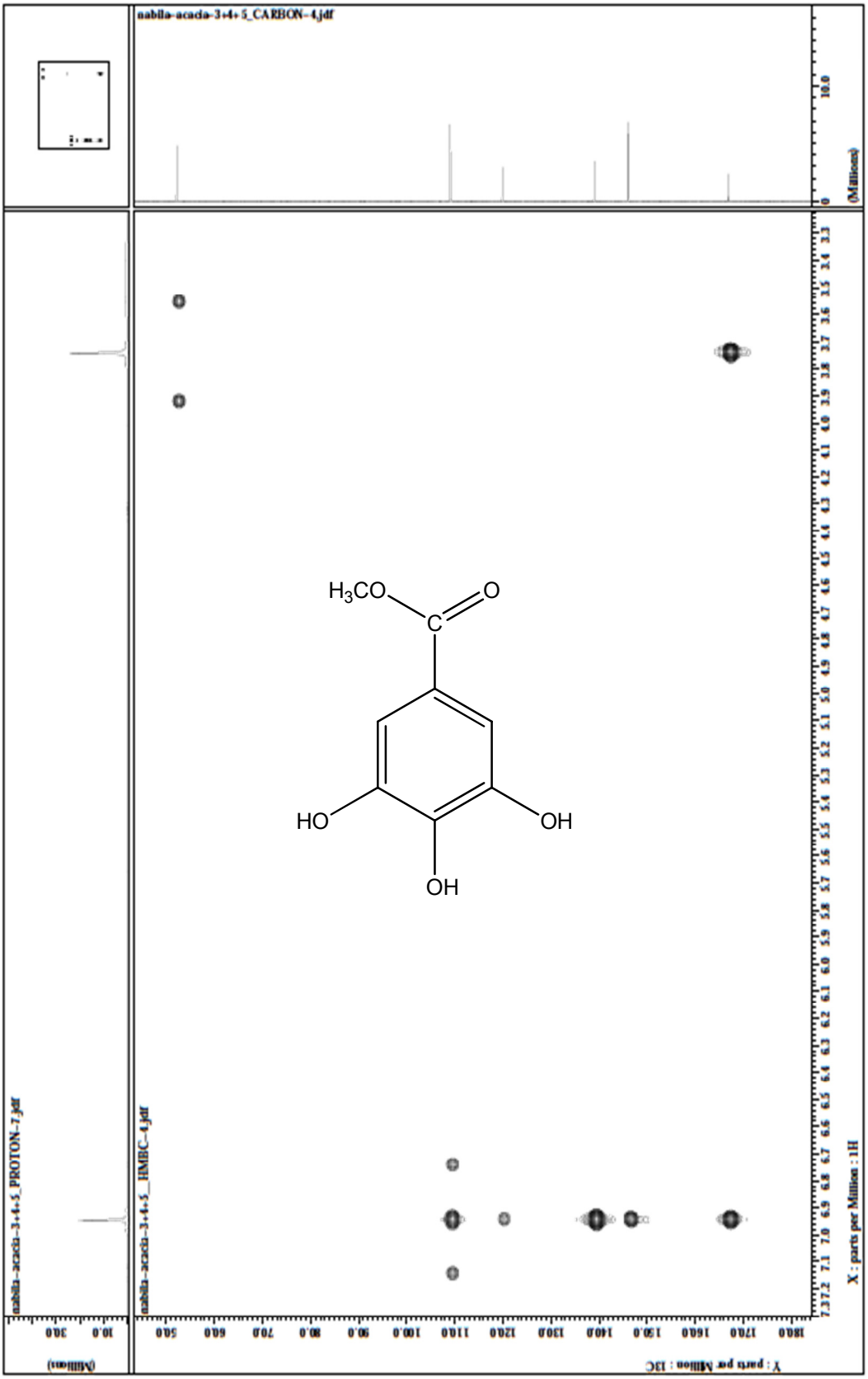



**Figure 33** HMBC spectrum and structure of AN4 (methyl gallate) (400 MHz, DMSO)

### 3.5.2 Characterisation of AN5 as gallic acid

**AN5** was isolated from the water extract of *Acacia nilotica* as white crystals. On TLC it gave a dark spot under UV and almost colourless after treatment with anisaldehyde reagent with a $R_f$ value 0.48 on 80% ethyl acetate.

HREI-MS showed an ion at $[M-H]^-$=169.0145 suggesting a molecular formula of $C_7H_6O_5$.

The $^1$HNMR spectrum had only one signal, a singlet at $\delta_H$ 7.02 (2H, *s*) accounted for H-2/6 protons, and two OH integrated to 3 OH. The $^{13}$C NMR spectrum showed 4 signals, this was including the carbonyl (169.5ppm, C-7) three oxygenated aromatic quaternary carbons signals (147.5ppm, double intensity,C-3/5, 140.9 ppm C-4) and two shielded aromatic methines 110.4 ppm double intensity, C2/6) and another quaternary carbon at 120.9ppm C-1.

The spectral data of **AN5** (Figure 34) were in agreement with those published for gallic acid (Wang et al., 2007).

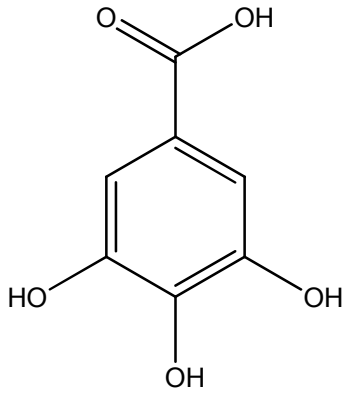



**Figure 34** structure of AN5 (gallic acid)

### 3.5.3 Characterisation of AN25 as 1-O-Galloyl-D-glucopyranose

**AN25** was isolated from water extract of *Acacia nilotica* as brown crystals. On TLC it gave a dark tail under short UV with $R_f$ 0.22 (5% methanol and ethyl acetate) and turned to orange after spray and heated with *p*-anisaldehyde.

The HREI-MS for **AN25** (Figure 35) gave an ion at $[M-H]^-$=331.0676 suggesting a molecular formula of $C_{13}H_{16}O_{10}$.

The $^1$H NMR spectrum (Figure 36) consisted of one aromatic protons at $\delta_H$ 7.13 (2H) and one sugar residues with its anomeric protons at $\delta_H$ 5.65 (1H, *d* 8 Hz) along with one oxymethylene between at $\delta_H$ 3.86, 3.68 and four oxymethines between 3.4 and 3.9.

The $^{13}$CNMR spectrum had 11 carbons including a carbonyl at $\delta_C$ 165.9, three oxygen-bearing aromatic quaternaries at $\delta_C$ 146.1 (×2), $\delta_C$ 140.3 and aromatic carbon at $\delta_C$ 107.1 (x2) which suggesting the presence of one benzene ring. Using $^1$H and COSY spectrum, the glucose moiety was assigned as as β-D-glucose due to the large coupling constant (8 Hz) of its anomeric proton (Table 9).

In the HMBC spectrum (Figure 37) the protons at $\delta_H$ 7.13 (H-2/6) showed their direct at $\delta_C$ 107.1 as well as $^3J$correlation to the same carbon indicating their relative *meta* positions. They also showed $^3J$ correlation to the carbonyl and two oxygen-bearing carbon ($\delta_C$ 146.1 and 140.3), $^2J$ correlation to carbons (122.2). The anomeric proton showed $^3J$ correlation to the carbonyl carbon, proving its connectivity to the carbonyl. All the above information led to the conclusion that **AN25** was 1-O-Galloyl-D-glucopyranose, which was the first report from *Acacia nilotica*. All spectral data were in agreement with that previously reported (Wang *et al.*, 2007).

**Table 9** $^{1}H$ (500 MHz) and $^{13}C$ (126 MHz) NMR data of AN25 (1-O-Galloyl-D-glucopyranose) in $CD_3OD$

| Position | $\delta_H$ | $\delta_C$ |
|---|---|---|
| 1 | - | 122.2 |
| 2/6 | 7.13 (s, 2H) | 107.1 |
| 3/5-OH | - | 140.3 |
| 4-OH | - | 146.1 |
| 7 | - | 165.9 |
| 1' | 5.65 (*d*, *J*=8 Hz,1H) | 95.8 |
| 2' | 3.48 (*d*, *J*=5 Hz, 1H) | 77.1 |
| 3' | 3.47 (*d*, *J*=3 Hz, 1H) | 73.1 |
| 4' | 3.41 (m, 1H) | 70.1 |
| 5' | 3.42 (m, 1H) | 78.1 |
| 6' | 3.85 (*dd,J*=1.5, 12 Hz, 1H) and 3.70 (*dd, J*=12, 5 Hz,1H) | 61.2 |

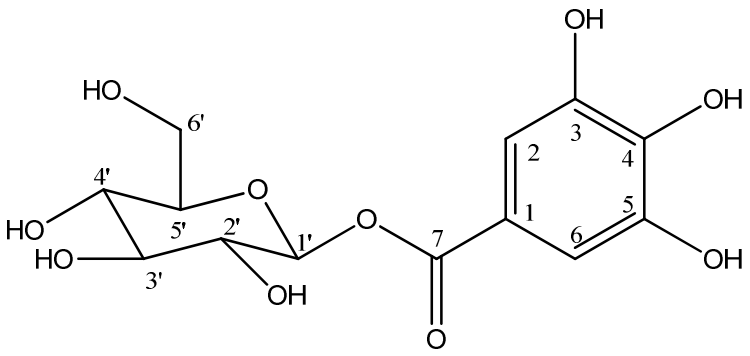



**Figure 35** structure of AN25 (1-O-Galloyl-D-glucopyranose)

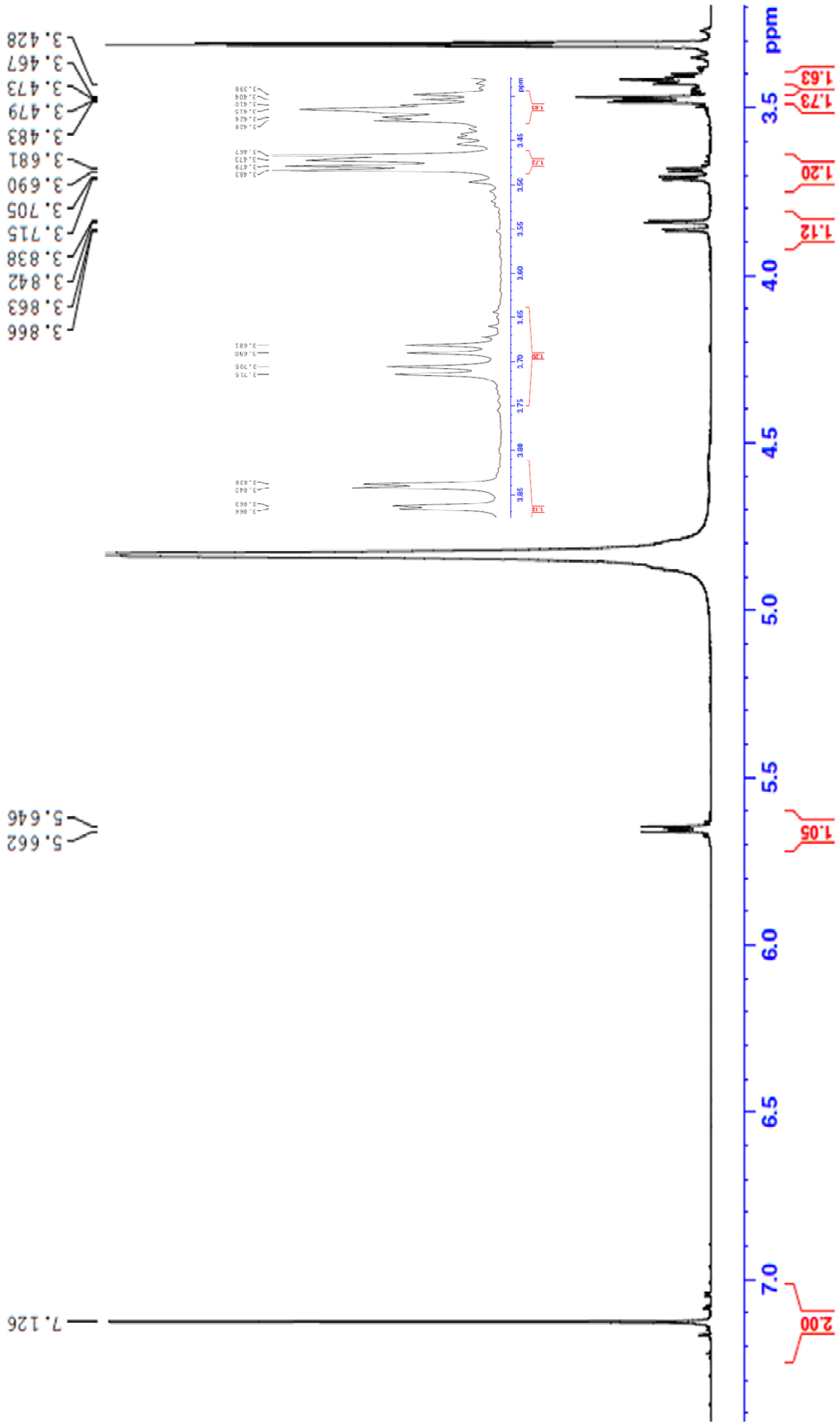



**Figure 36** $^{1}$HNMR spectrum of AN25 (1-O-Galloyl-D-glucopyranose) (400 MHz, $CD_3OD$)

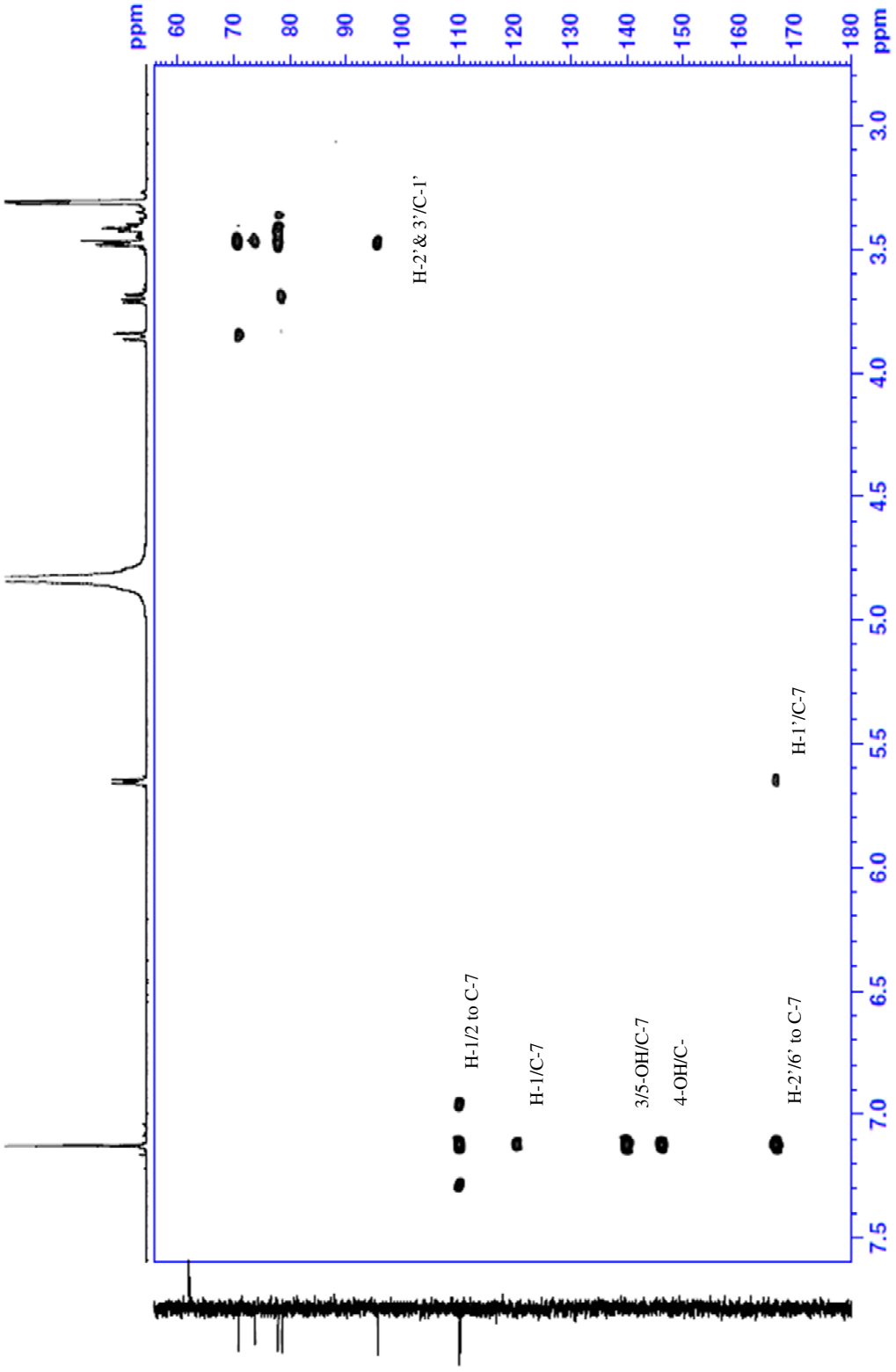



**Figure 37** MHBC spectrum of AN25 (1-O-Galloyl-D-glucopyranose) (400 MHz, $CD_3OD$)

## 3.6 Isolation and structure elucidation of compounds isolated from *Achillea fragrantissima*

The whole plant of *Achillea fragrantissima* (35g) extracted with methanol and partitioned by chloroform and *n*-butanol. The chloroform phase (12.5g) subjected to VLC eluting with hexane and increasing the polarity by 5% then purified over Sephadex LH-20 afforded **AF17** and **AF18**. The *n*-butanol (7.2g) subjected to Sephadex LH-20 afforded **AF25** and **AF36.**

### 3.6.1 Characterisation of AF17 as (3-acetoxy-1-oxo-13-hydroxy-4,7(11),10(14)-germacratrien-12,6-olide)

AF17 isolated as yellow oil (90mg) from chloroform phase of methanol extract from *Achillea fragrantissima.* On TLC it gave green colour, after spraying with *p*-anisaldehyde sulphuric acid reagent.

The positive mode HRESI-MS of **AF17** (Figure 39) gave a molecular ion $[M+H]^+$ at 321.1328, suggesting molecular formula of $C_{17}H_{20}O_6$ =320.

The proton spectrum (Figure 38) showed an exomethylene group at $\delta_H$5.73, 5.76 and two methyl groups at $\delta_H$ 1.65 and 1.98. Several $CH_2$ and CH protons were observed between 2.19 and 5.46 ppm. The $^{13}$CNMR showed 17 carbons typical of a sesquiterpene with acetyle group carbon side chain. The DEPT spectrum showed three methine (CH), five methylene ($CH_2$), two methyl ($CH_3$) and seven quaternary carbons including two carbonyl groups (Table 11).

In the NOE experiment, H-6 enhanced the signal of H-15; similar intensity enhancement was observed for H-5 on irradiation of H-3 and one of H-2. The structure was confirmed by correlation on the HMBC spectrum (Figure 40) and (Table 10) these data were in agreement with those published for the compound isolated by Abdel-Mogib *et al.*(1989).

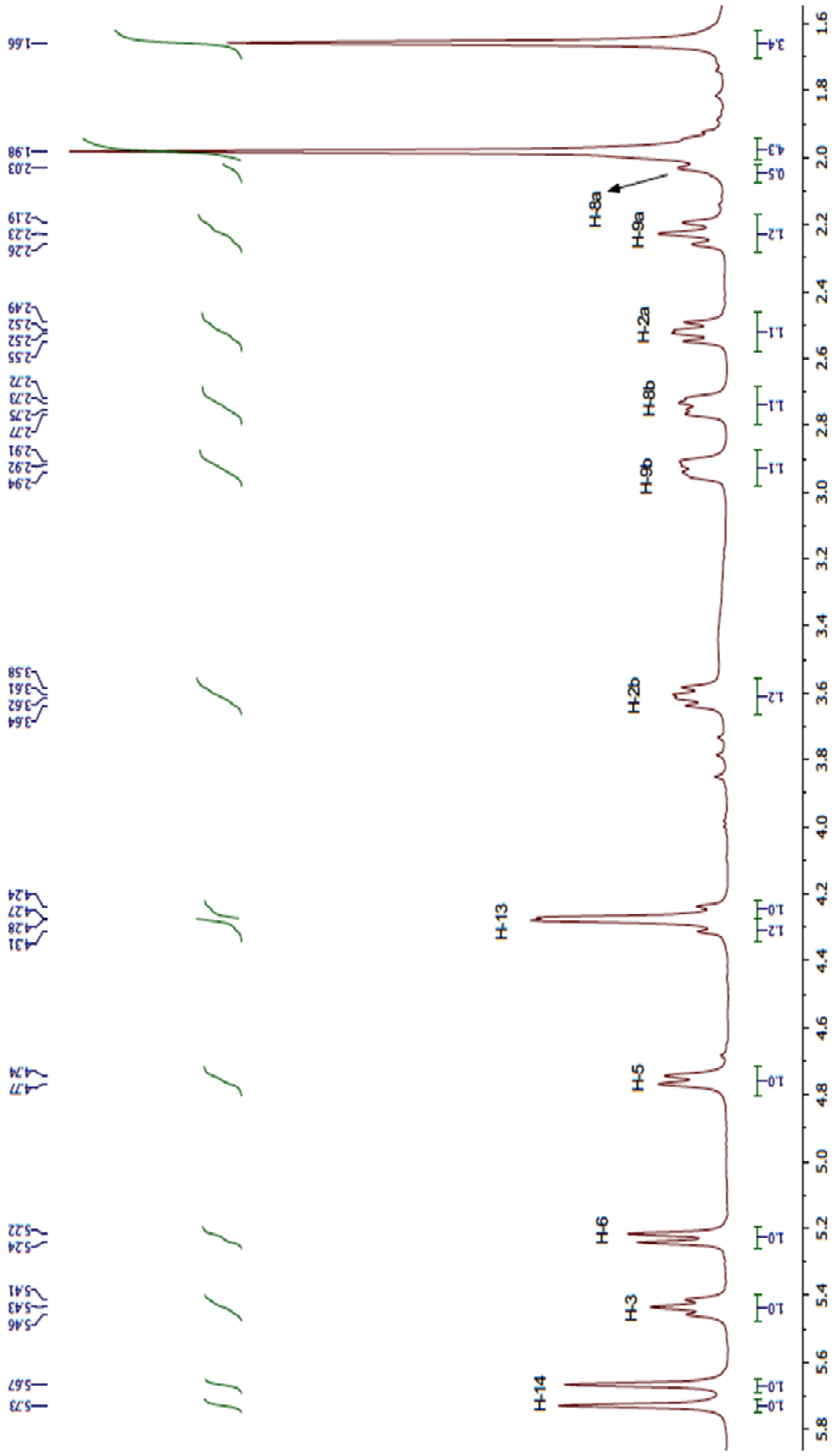



**Figure 38** $^{1}$HNMR spectrum of AF17 (3-acetoxy-1-oxo-13-hydroxy-4,7(11),10(14)-germacratrien-12,6-olide) (400 MHz, $CDCl_3$)

Table 10 Significant 2D NMR correlation for AF17

| | Multiple bond correlations ($^{13}C$-$^{1}H$ correlation in HMBC) | |
|---|---|---|
| **NO.** | **$^{13}C$** | **$^{1}H$** |
| 1 | 198.7 | 9 (2.23) (2.92), 2 (3.61), 14 (5.67) |
| 2 | 44.2 | 3 (5.44) |
| 3 | 75.5 | 15 (1.66), 17 (1.99), 2 (2.53)(3.61), 5 (4.76) |
| 4 | 136.3 | 15 (1.66), 2 (3.61), 6 (5.23), 3 (5.44) |
| 5 | 127.0 | 3(5.44), 15(1.66) |
| 6 | 79.5 | 15 (1.65), 8 (2.0)(2.75) |
| 7 | 165.9 | 6 (5.23), 13 (4.26)(4.29), 9 (2.92) (2.23), 8 (2.75) (2.0) |
| 8 | 23.6 | 6 (5.23), 9 (2.92)(2.23) |
| 9 | 33.4 | 14 (5.73) (5.67), 8 (2.75) (2.0) |
| 10 | 148.3 | 14 (5.67) (5.73) 9 (2.92) (2.23), 8 (2.75), 2 (2.0) |
| 11 | 127.7 | 8 (2.0)(2.75), 13 (4.26)(4.29), 6 (5.23) |
| 12 | 174.0 | 6 (5.23), 13 (4.26)(4.29) |
| 14 | 125.5 | 9 (2.92) (2.23) |
| 15 | 10.7 | 5 (4.76), 3 (5.44) |

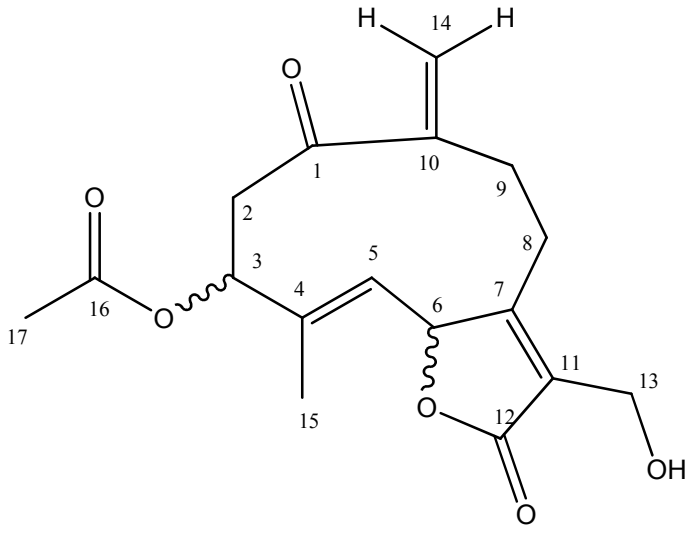



**Figure 39** Structure of AF17 (3-acetoxy-1-oxo-13-hydroxy-4,7(11),10(14)-germacratrien-12,6-olide)

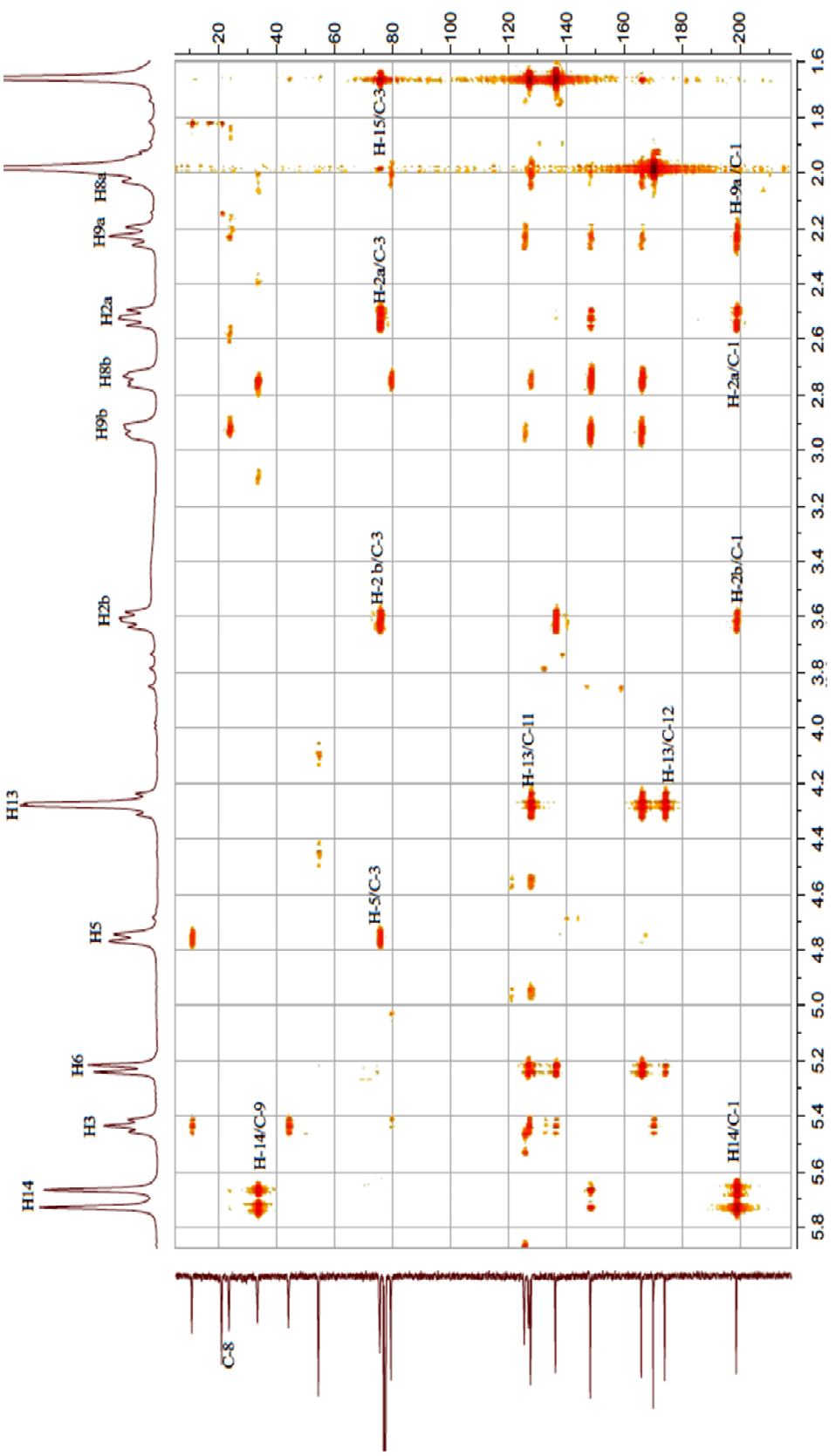



**Figure 40** HMBC spectrum of AF17 (3-acetoxy-1-oxo-13-hydroxy-4,7(11),10(14)-germacratrien-12,6-olide) (400 MHz, $CDCl_3$)

### 3.6.2 Characterisation of AF25 as 13-O-Desacetyl-1α-hydroxy-afraglaucolide

**AF25** was isolated from the butanol phase of the methanol extract of *Achillea fragrantissima* as white crystal. This compound was an analogue of **AF17**; it showed the absence of the ketone group ($\delta_C$ 198.7) at position one ($\delta_H$ 74.3) and this was replaced by CHOH (74.3). This was confirmed by mass spectrometry which differed by 2 mass units (Table 10). The positive mode HRESI-MS of **AF25** (Figure 41) gave aquasi-molecular ion peak at $[M+H]^+$= 323.1486 suggesting a molecular formula of $C_{17}H_{22}O_6$=322.

Further analysis of the spectral data using HMBC and NEOSY showed NOE between H-15 and H-6 (Figure 43) led to the identification of **AF25** as 13-O-Desacetyl-1-hydroxy-afraglaucolide. All the spectral data were in agreement with those found in the literature (Khafagy *et al.*, 1988).

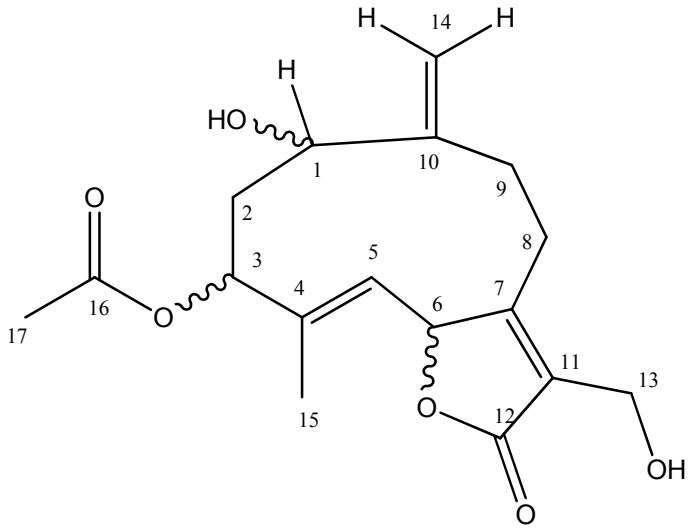



Figure 41 structure of 13-O-Desacetyl-1-hydroxy-afraglaucolide AF25

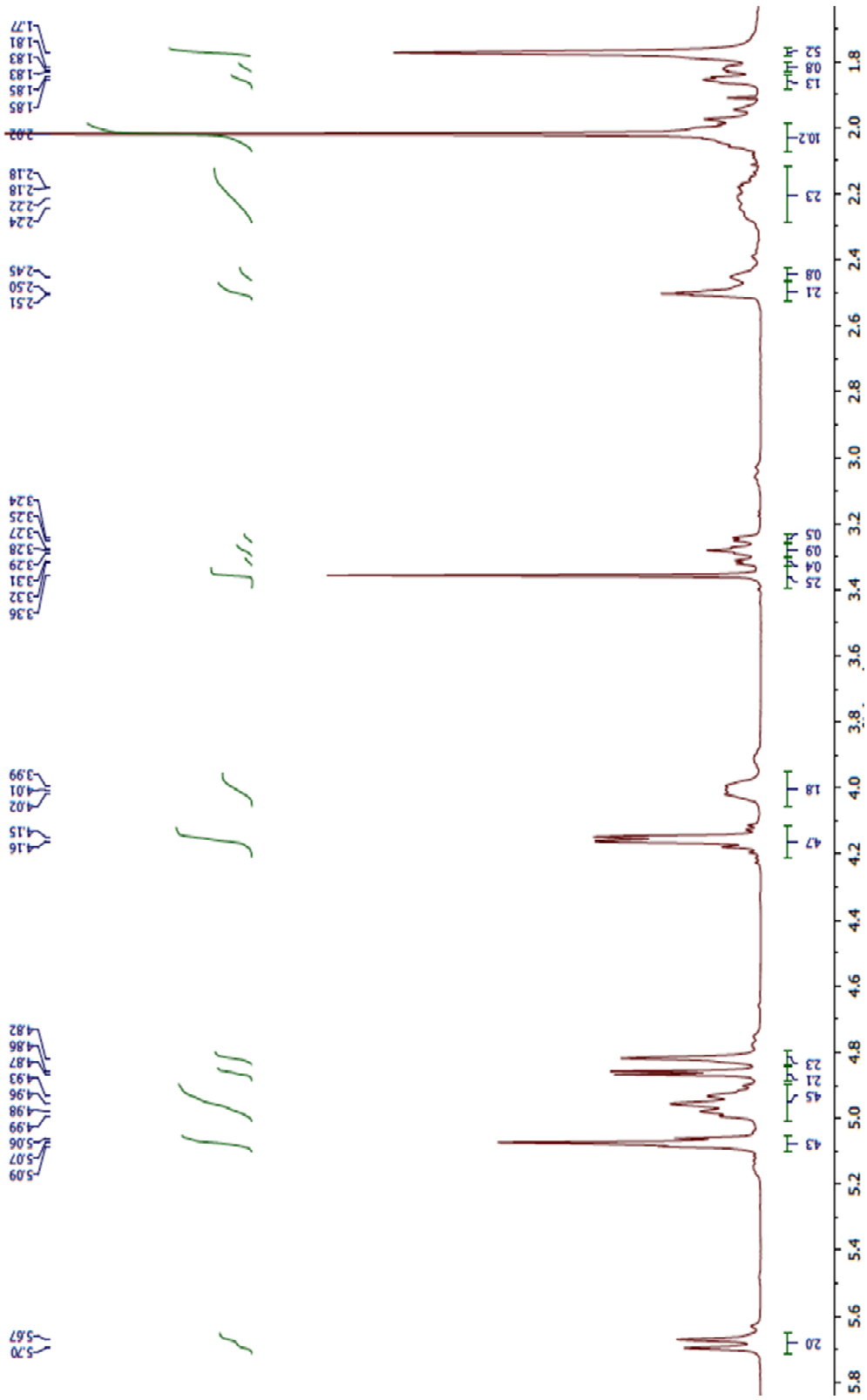


**Figure 42** $^{1}$HNMR spectrum of AF25 (13-O-Desacetyl-1-hydroxy-afraglaucolide) (400 MHz, DMSO)

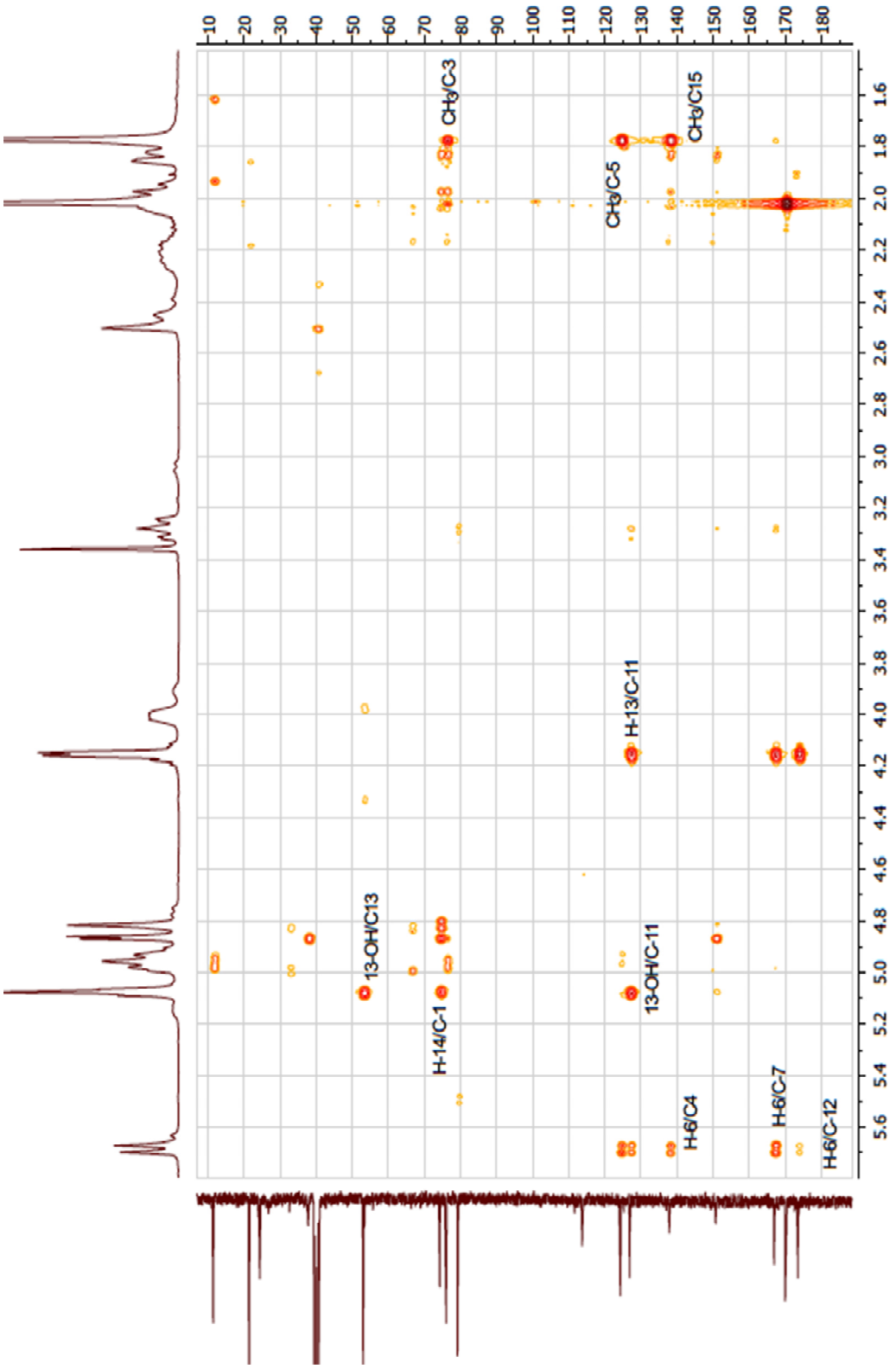



**Figure 43** HMBC spectrum of AF25 (13-O-Desacetyl-1-hydroxy-afraglaucolide) (400 MHz, DMSO)

**Table 11** $^{1}H$ (400 MHz) and $^{13}C$ (100 MHz) NMR data of AF17 in $CDCL_3$ and AF25 in DMSO

| | **AF17** | | **AF25** | |
|---|---|---|---|---|
| **Position** | **$\delta_H$** | **$\delta_C$** | $\delta_H$ | $\delta_C$ |
| 1 | - | 198.7 | 4.01 (1H, brs d) | 74.3 |
| 2 | 3.61(1H,dd, J= 12.9, 8.9Hz) / 2.53(1H, dd, J= 13.2, 8.8Hz) | 44.2 | 1.84 (1H, brs d) / 2.0 (1H, m) | 37.9 |
| 3 | 5.43(1H, t, J= 8.7Hz) | 75.5 | 4.95 (1H, m) | 76.0 |
| 4 | - | 136.3 | - | 137.9 |
| 5 | 4.76(1H, d, J= 10Hz) | 127.0 | 4.98 (1H, dd, J = 16.7, 7.3 Hz) | 124.3 |
| 6 | 5.23(1H, d, J=10.1Hz) | 79.5 | 5.68 (1H, d 10.2Hz) | 79.2 |
| 7 | - | 165.9 | - | 166.9 |
| 8 | 2.75(1H, J = 13.3, 5.4 Hz) / 2.0 (1H, brs) | 23.6 | 2.47 (1H, m)/ 3.28 (1H, t, J = 3.4 Hz) | 24.4 |
| 9 | 2.23 (1H, ,t, J=13.1Hz) / 2.92 (1H, *brs)* | 33.4 | 2.02 (1H, m)/ 2.21 (1H, m) | 26.8 |
| 10 | - | 148.3 | - | 150.7 |
| 11 | - | 127.7 | - | 126.9 |
| 12 | - | 174.0 | - | 173.5 |
| 13 | 4.30 (1H, d, J=13.5Hz) / 4.25 (1H, d, J=13.7Hz) | 54.5 | 4.14 (1H, brs d)/4.16 (1H, d, J = 5.3 Hz, 1H), | 53.0 |
| 14 | 5.67(1H, brs d) / 5.73(1H,brs d) | 125.5 | 4.82 (1H, *brs d*)/ 5.08 (1H *brs d*) | 113.7 |
| 15 | 1.66(3H, *bro s)* | 10.7 | 1.77 (3H, s) | 11.5 |
| 3-OAc | - | 170.1 | - | 170.0 |
| | 1.98(3H, s) | 21.1 | 2.02 (3H, s) | 21.4 |

### 3.6.3 Characterisation of AF18 as chrysosplenol D

**AF18** (120 mg) was isolated as yellow crystals. On TLC it gave a dark spot under short UV. After spraying with *p*-anisaldehyde sulphuric acid reagent it turned yellow.

The negative mode HRESI-MS of **AF18** (Figure 44) gave an ion $[M-H]^-$ at 359.07799 suggesting a molecular formula of $C_{18}H_{16}O_8$.

The $^1$HNMR spectrum (Figure 45) showed the presence of a hydroxyl signal at $\delta_H$ 12.35, and ABX aromatic spin system (at $\delta_H$ 7.54 dd, $J$=8.5, 2 Hz, $\delta_H$ 7.02, d, $J$=8.5Hz and $\delta_H$ 7.92d, $J$=2Hz) and one aromatic proton singlet at $\delta_H$ 6.51s. The $^{13}$CNMR spectrum had 18 signals including a carbonyl at $\delta_c$ (179.9ppm), ten quaternary carbons, four aromatic methine carbons as well as three methoxy groups (Table 12).

In the HMBC spectrum (Figure 46) the 5-OH at $\delta_H$ 12.35 showed $^3J$ correlations with carbons at $\delta_c$ 106.4 (C-10), $\delta_c$ 152.5 (C-5) and δc 132.3 (C-6), this indicates the presence of H-8 on the same ring of 5-OH. The proton at $\delta_H$ 6.51 (H-8) and the methoxy group at $\delta_H$ 3.94 showed $^2J$ and $^3J$ correlation with the carbon at $\delta_c$ 159.1 (C-7) respectively. The proton at $\delta_H$ 7.02 (H-5′) showed $^3J$ correlation to $\delta_c$ 143.7 (C-3′) and $\delta_c$ 122.4 (C-1′). The proton at $\delta_H$ 7.55(H-6′) showed $^3J$ correlation to $\delta_c$ 115.9 (C-2′) and $^2J$ correlation to $\delta_c$ 147.6 (C-4′), while the proton at $\delta_H$ 7.55 (H-2′) showed a $^3J$ correlation to $\delta_C$ 121.9 (C-6′) and at $\delta_c$ 147.6(C-4′). The methoxy groups at $\delta_H$ 3.91 and 3.79 showed $^3J$ correlation to $\delta_c$ at 159.1 and 138.6. On the basis of these results and by comparison with previously published data (Vieira *et al.*, 1997), **AF18** was identified as a 3',4',5-trihydroxy-3,6,7-trimethoxyflavone or chrysosplenol D.

**Table 12** $^{1}H$ (400 MHz) and $^{13}C$ (100 MHz) NMR data of AF18 (chrysosplenol D) in CDCL3 and AF36 (Jaceosidin) in DMSO.

| | **AF18** | | **AF36** | |
|---|---|---|---|---|
| **Position** | **$\delta_H$** | **$\delta_C$** | **$\delta_H$** | **$\delta_C$** |
| 2 | - | 156.9 | - | 164.7 |
| 3 | - | 138.6 | 6.72 (1H, s) | 103.1 |
| 4 | - | 179.9 | - | 182.5 |
| 5 | - | 152.4 | - | 153.0 |
| 6 | - | 132.3 | - | 132.3 |
| 7 | - | 159.1 | - | 159.0 |
| 8 | 6.51(1H,s) | 90.6 | 6.89 (1H, d, *J*=8.6Hz) | 91.9 |
| 9 | - | 152.5 | - | 152.5 |
| 10 | - | 106.4 | - | 105.5 |
| 1' | - | 122.4 | - | 121.7 |
| 2' | 7.92 (1H, *d*, *J*= 2 Hz) | 115.9 | 7.45(1H, dd, *J*=2.2, *br* Hz) | 119.5 |
| 3' | - | 143.7 | 6.89 (1H,s) | 116.4 |
| 4' | - | 147.6 | - | 150.3 |
| 5' | 7.02(1H, *d, J*= 8.5 Hz) | 115.2 | - | 146.2 |
| 6' | 7.54 (1H, *dd*, *J*= 8.5, 2 Hz) | 121.9 | 7.43 (1H*, d, br*) | 114.0 |
| 3-OMe | 3.79 (3H, s) | 60.1 | - | 103.1 |
| 6-OMe | 3.91 (3H, s) | 60.9 | 3.73 (3H, s) | 60.9 |
| 7-OMe | 3.94 (3H, s) | 56.3 | - | 159.0 |
| 5-OH | 12.35 (1H, s) | 152.5 | 12.35 (1H, s) | - |
| 3'-OMe | - | 143.7 | 3.92 (3H, s) | 57.4 |

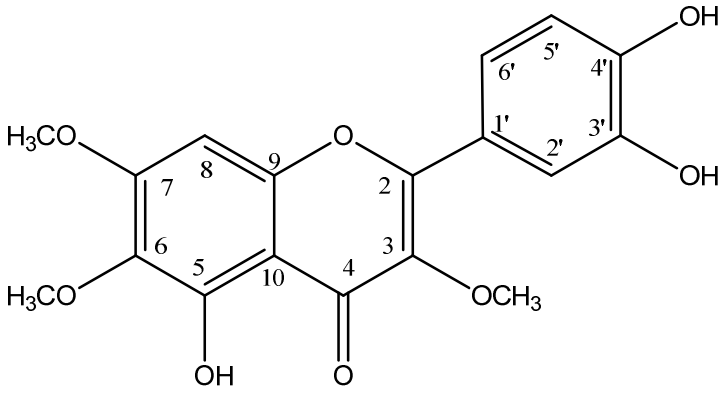



**Figure 44** Structure of AF18 (chrysosplenol D)

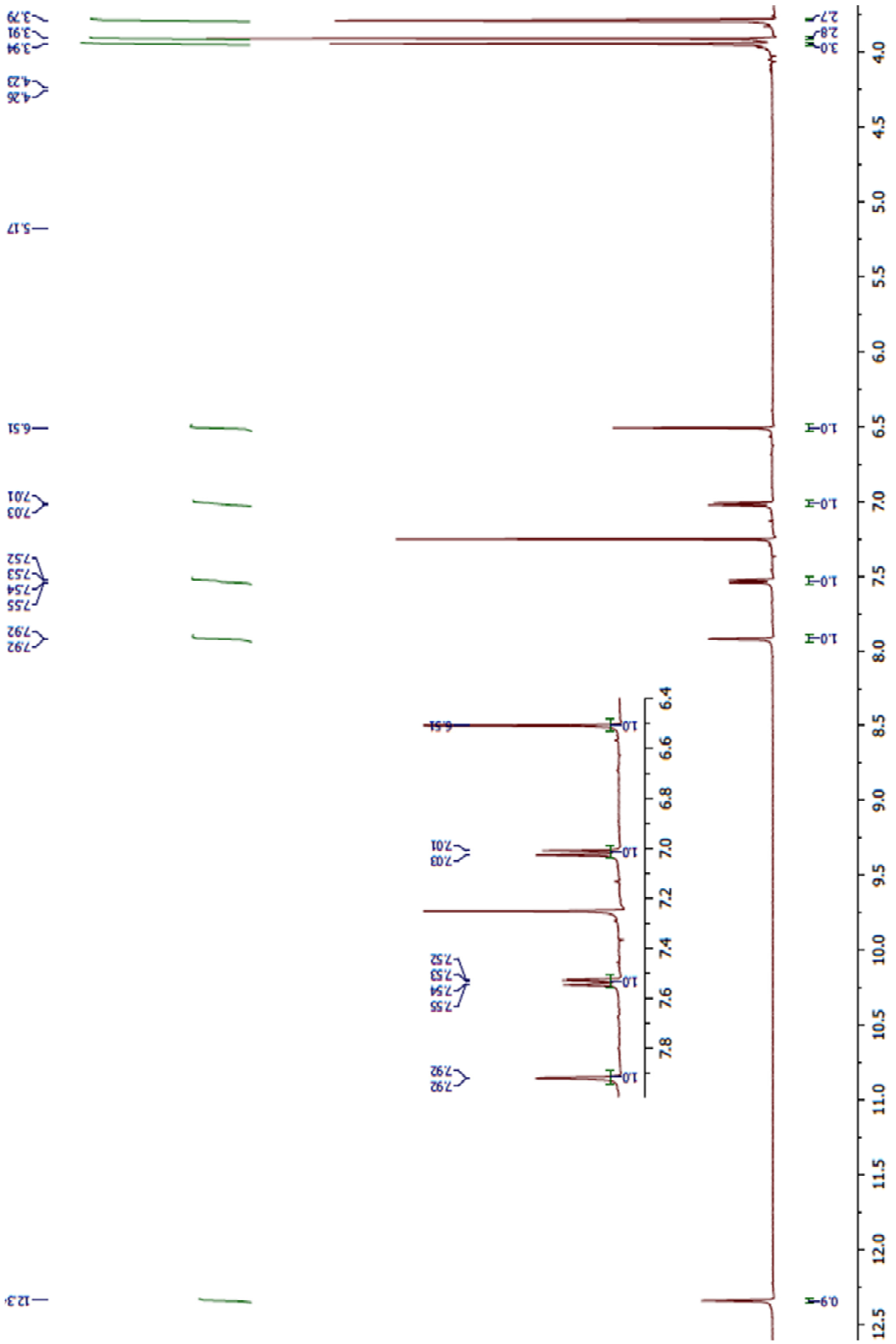


**Figure 45** $^{1}$HNMR spectrum of AF18 (chrysosplenol D) (400 MHz, $CDCl_3$)

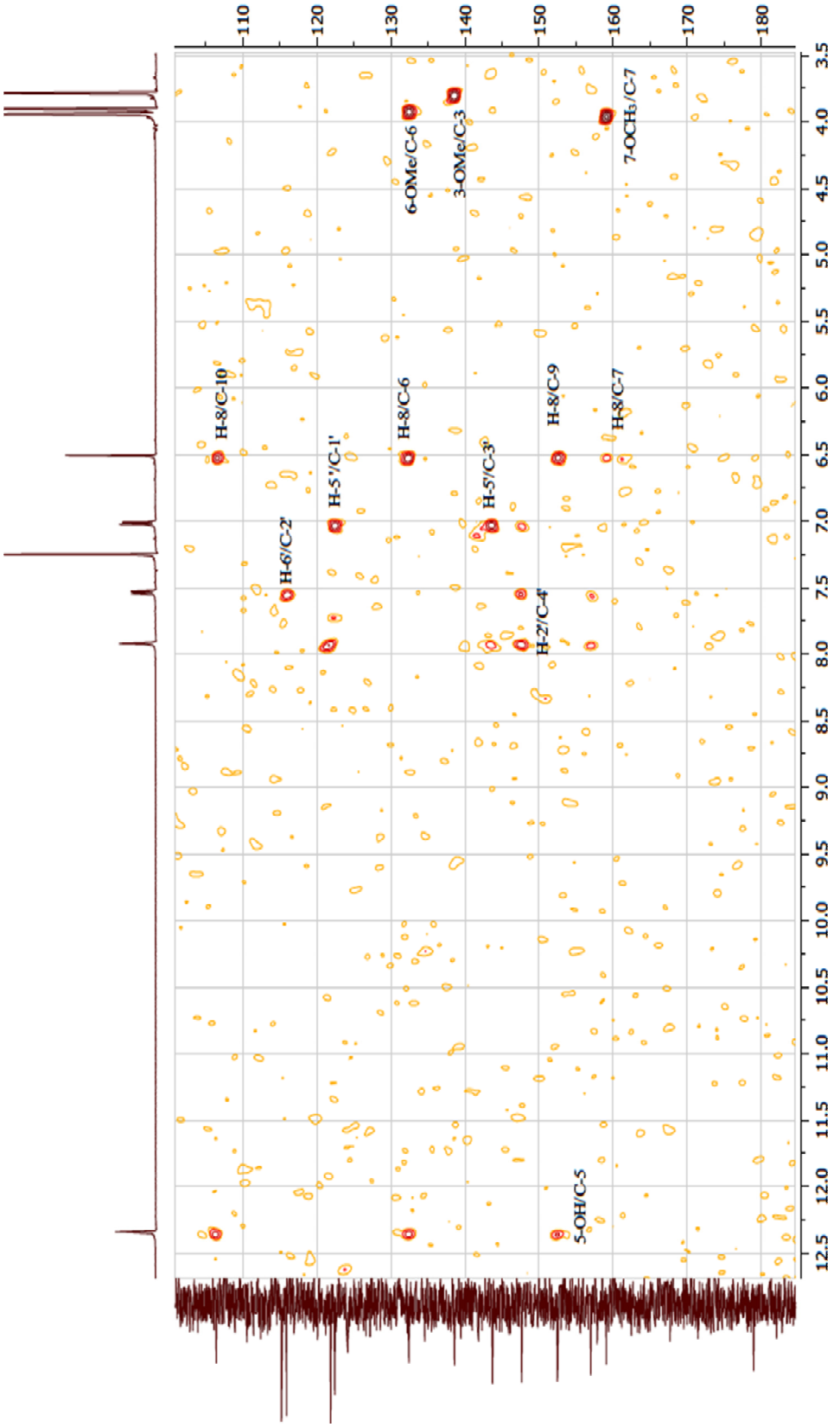



**Figure 46** HMBC spectrum of AF18 (chrysosplenol D) (400 MHz, $CDCl_3$)

### 3.6.4 Characterisation of AF36 as jaceosidin

**AF36** was isolated from the butanol phase of the methanol extract of *Achillea fragrantissima* as a yellow amorphous poweder. On TLC it gave a dark spot under UV 245nm and a yellow fluorescence under 365nm. After spraying with *p*-anisaldehyde sulphuric acid reagent it turned yellow.

Comparing the $^{1}$HNMR spectrum (Figure 48) of **AF36** to **AF18** was similar and therefore **AF36** could be analogues of **AF18** with different substitution pattern (Table 12). Results from the HRESI-MS of **AF36** (Figure 47) gave an ion $[M-H]^-$ at 329.06745 suggesting a molecular formula of $C_{17}H_{14}O_7$ =330, the difference in mass for **AF36** was 30 mass units compared to **AF18** corresponding to a methoxy group and this compound must have lost a methoxy group at position three. Using the HMBC (Figure 49) one methoxy moved from 7-OMe to 3'-OMe as well as added one in C-3 lost. Comparing to the previously published data at Schinella *et al.*(1998) **AF36** was identified as 5,7,4'-trihydroxy-3',6-dimethoxyflavone or Jaceosidin.

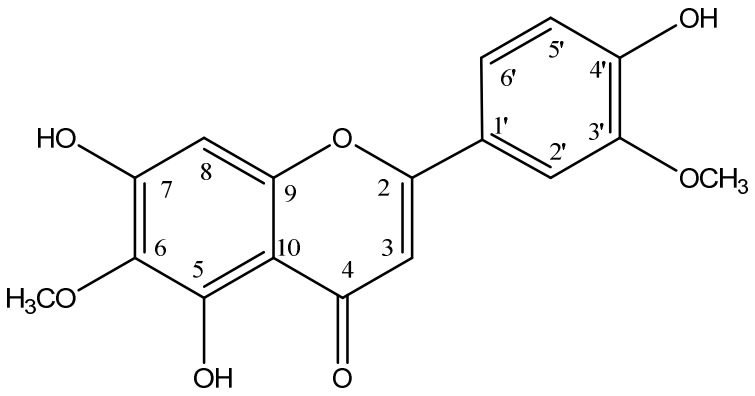



**Figure 47** structure of AF36

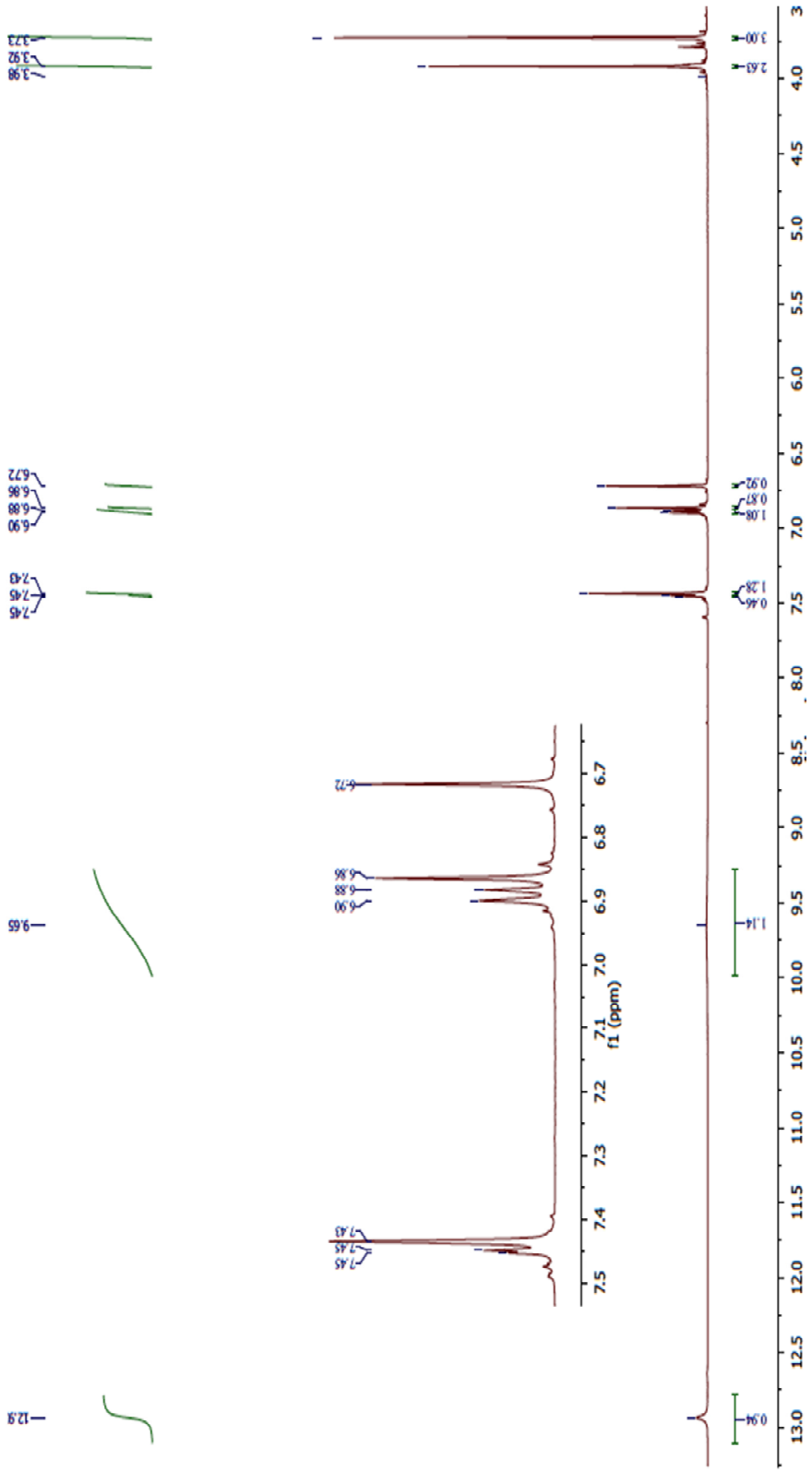



**Figure 48** HNMR spectrum of AF36 (Jaceosidin) (400 MHz, $CDCl_3$)

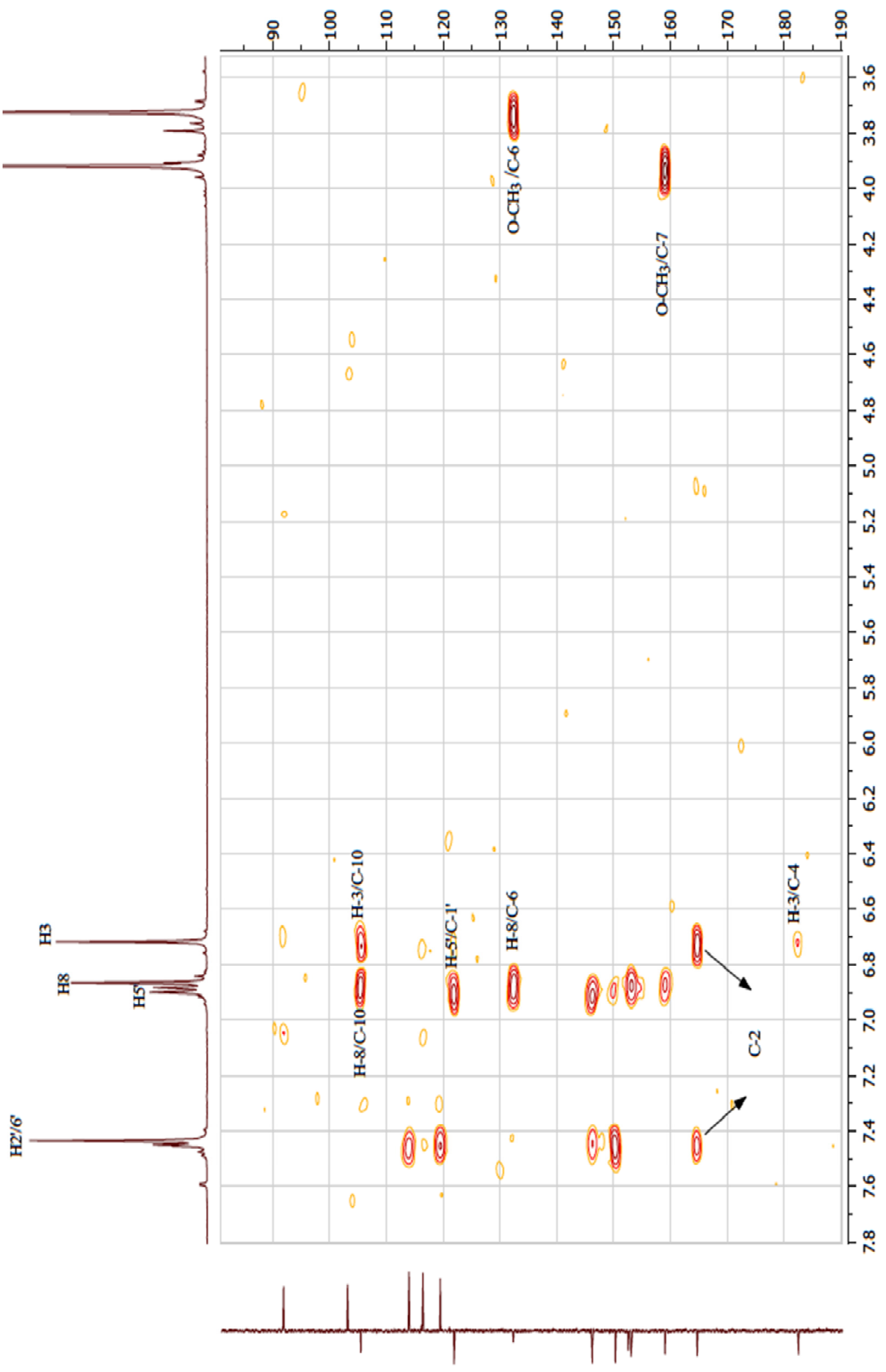



**Figure 49** $^{1}$HNMR spectrum of AF36 (Jaceosidin) (400 MHz, $CDCl_3$)

## 3.7 Isolation and structure elucidation of the compounds isolated from *Haloxylon salicornicum*

Plant material was extracted with methanol and water (1:1) mixture using a Soxhlet apparatus and the extract evaporated and freeze dried. The freeze dried solid (18 g) was re-dissolved in 100 ml water and was partitioned by adding 250 ml chloroform. The organic phase was dried over anhydrous sodium sulphate, filtered and evaporated to dryness on a rotary evaporator. The dried extract was dissolved in methanol and placed on a Sephadex LH 20 column and eluted with methanol to obtain 30 fractions 100 ml each. Fractions 1-15 identical on TLC, were combined to obtain sub-fraction **A.** Similarly fractions 16-30 were combined to obtain fractions **B**. Sub-fraction **B** was subjected to C-18 column chromatography and eluting with water/methanol to obtain 32 fratctions. Sub-fraction **C** made up of collected fractions 1-16. While the sub-fraction **D** made up of collected fraction 16-32 based on TLC analysis. Around 500 mg of dried sub-fraction **D** was chromatographed by MPLC utilising a gradient of EtOAc and MeOH from 100:0 to 90:10 at a flow rate of 20 mL/min for 35 min. This afforded 200 fractions of 5 mL each. The eluted compounds were pooled (TLC) to yield **HS1**, **HS2**, **HS3** and **HS4**. NMR studies (1D and 2D) and LC-MS were performed to elucidate the structures of the compounds.

### 3.7.1 Characterisation of HS1 as synephrine

The positive mode HRESI-MS for **HS1** (Figure 50) gave a quasi molecular ion peak at $[M=H]^+ = 168.1018$ suggesting a molecular formula of $C_9H_{12}O_2N$.

The $^1$HNMR spectrum (Figure 51) showed two multiplets signals at $\delta_H$ 6.79 and 7.23 with an AA'BB'-type coupling pattern and was assigned to a hydroxy benzene protons.

The $^{13}$CNMR spectrum showed seven distinct carbon signals. The signals at $\delta_c$ 157.5 and 131.5 were assigned to non-protonated aromatic carbons and the signals at $\delta_c$ 126.9 and 115.2 were assigned to protonated aromatic carbons. The two carbon signals at $\delta_c$ 68.5 and 55.4 were assigned to amino methylene and benzyl methine carbons, respectively (Table 13).

The HMBC spectra (Figure 52) showed that the carbon at $\delta_c$130 showed correlations to protons at 3.12, 4.82 and 6.79 the carbon at $\delta_c$ 126.9 showed correlations to protons at 4.83 and 7.23. The carbon at $\delta_c$ 68.4 showed correlation to 3.12 and 7.23. In COSY spectrum (Figure 53) the proton at $\delta_H$ 4.82 correlated with proton at $\delta_H$ 3.12. Comparing the data with the previously published data (Yokoo *et al.*, 1999) the compound was identified as synephrine.

**Table 13** $^{1}$H (400 MHz) and $^{13}$C (100 MHz) NMR data of **HS1 (**synephrine) in $CD_3OD$

| **Position** | **$\delta_H$** | **$\delta_C$** |
|---|---|---|
| 1-OH | - | 157.3 |
| 2/6 | 6.79 (2H, d, *J* = 8.5 Hz) | 115.2 |
| 3/5 | δ 7.24 (2H, d, *J* = 8.6 Hz) | 126.9 |
| 4 | - | 131.5 |
| 7 | 4.83 (1H, m) | 68.5 |
| 8 | 3.12 (1H, t), 2.74 (1H, *br t*) | 55.4 |
| 9-$NHCH_3$ | 2.72 (4H, s) | 32.2 |

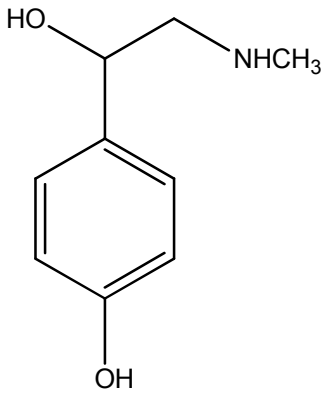



**Figure 50** structure of SH1 (synephrine)

### 3.7.2 Characterisation of HS2 as N-methyltyramine

This compound **HS2** was separated as a mixture with **SH1.** The positive mode HRESI-MS for **HS2** gave a molecular ion peak at $[M+H]^+$= 152.107 suggesting a molecular formula of $C_9H_{14}ON$. Compared to **HS1** it has one oxygen less and two protons extra. The $^1$HNMR spectrum (Figure 54) showed the same pattern for **HS1** two multiplets signals at δ6.88 and 7.19 with an AA'BB'-type coupling pattern was assigned to para-hydroxy aromatic protons. In the case of the $^{13}$CNMR spectrum, the same pattern was observed for **HS1** and **HS2** thus the compound was identified as N-methyltyramine (Yokoo et al., 1999).

### 3.7.3 Characterisation of HS3 as piperidine

The positive mode HRESI-MS for **HS3** gave a molecular ion peak at $[M+H]^+$=86.0963 suggesting a molecular formula of $C_5H_{12}N$.

In the $^1$HNMR spectrum (Figure 55) showed three peaks at $\delta_H$ 3.17-3.11 (m, 4H), 1.85-1.77 (m, 4H) and 1.68 (dt, *J* = 12.3, 3.5 Hz, 2H). In the $^{13}$CNMR showed only three carbons at $\delta_C$ 44.4, 22.4 and 21.0 (Table 14). The compound was identified as piperidine (Figure 55).

**Table 14** $^1$H (400 MHz) and $^{13}$C (100 MHz) NMR data of **HS3** (piperidine) in $CD_3OD$

| Position | $\delta_H$ | $\delta_C$ |
|---|---|---|
| 1-NH | | - |
| 2/6 | 3.17 – 3.11 (m, 4H) | 44.4 |
| 3/5 | 1.85 – 1.77 (m, 4H) | 22.4 |
| 4 | 1.68 (dt, *J* = 12.3, 3.5 Hz, 2H). | 21.8 |

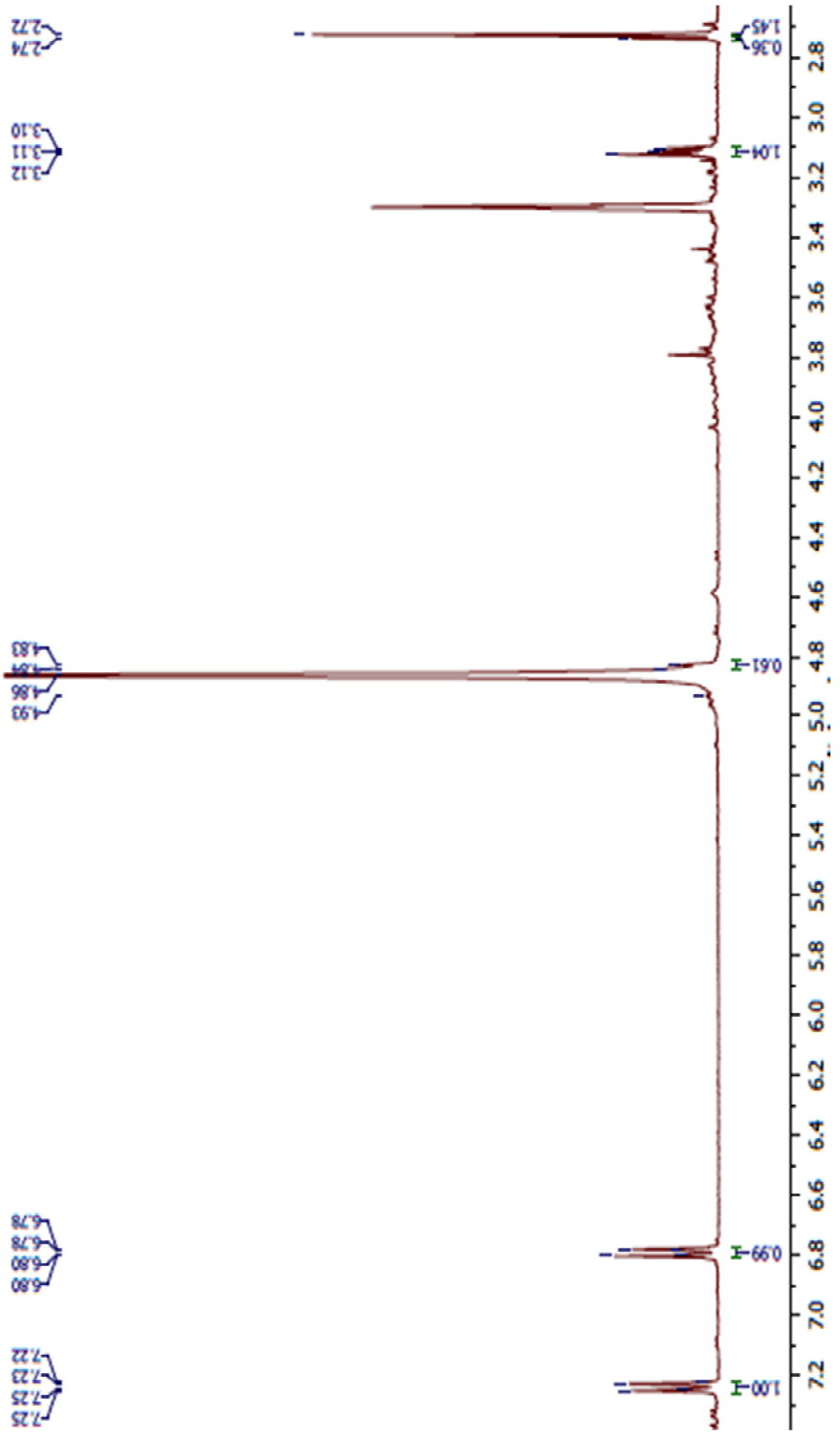



**Figure 51** $^{1}$HNMR spectrum of HS1 (synephrine) (400 MHz, $CD_3OD$)

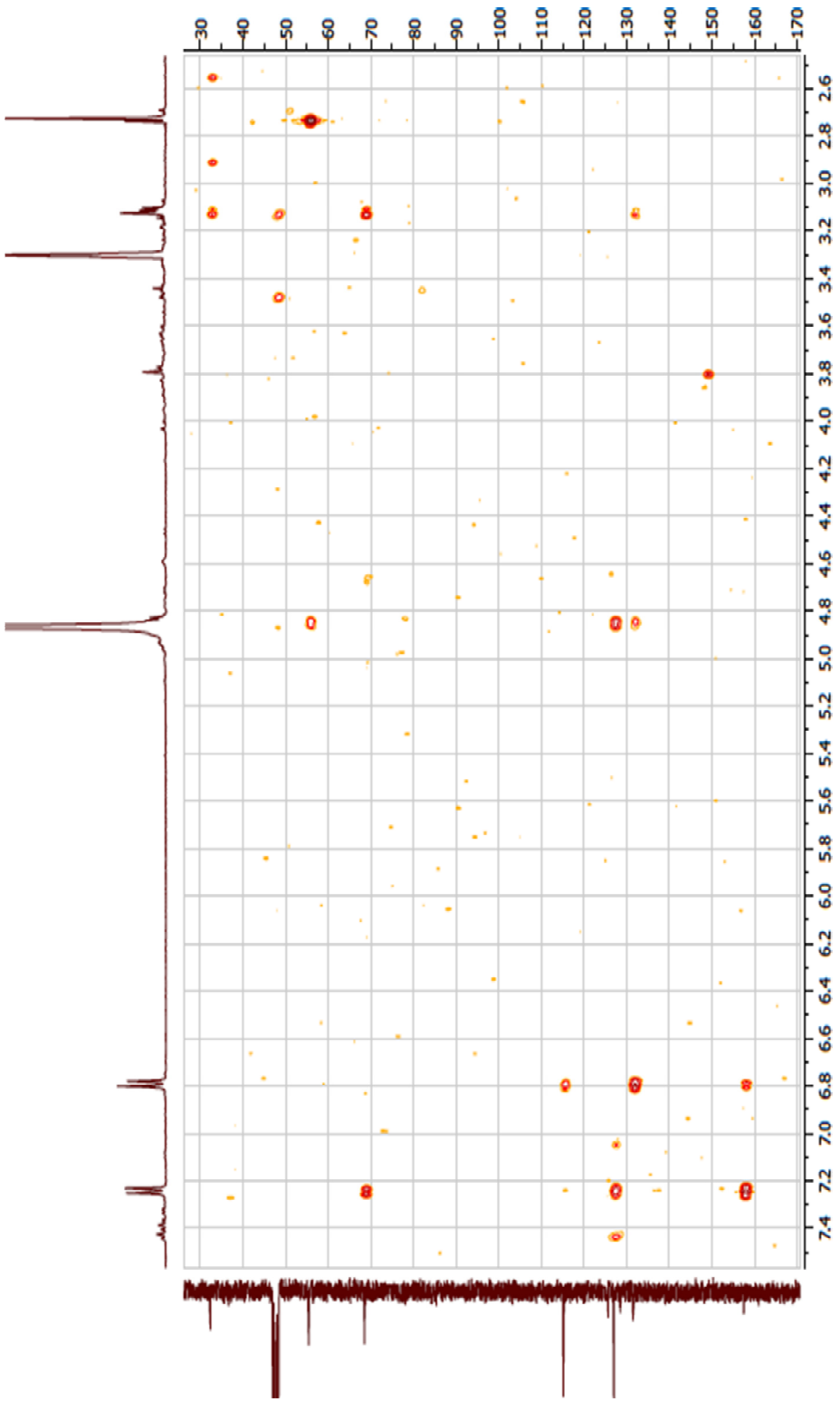



**Figure 52** HMBC spectrum of HS1 (synephrine) (400 MHz, $CD_3OD$)

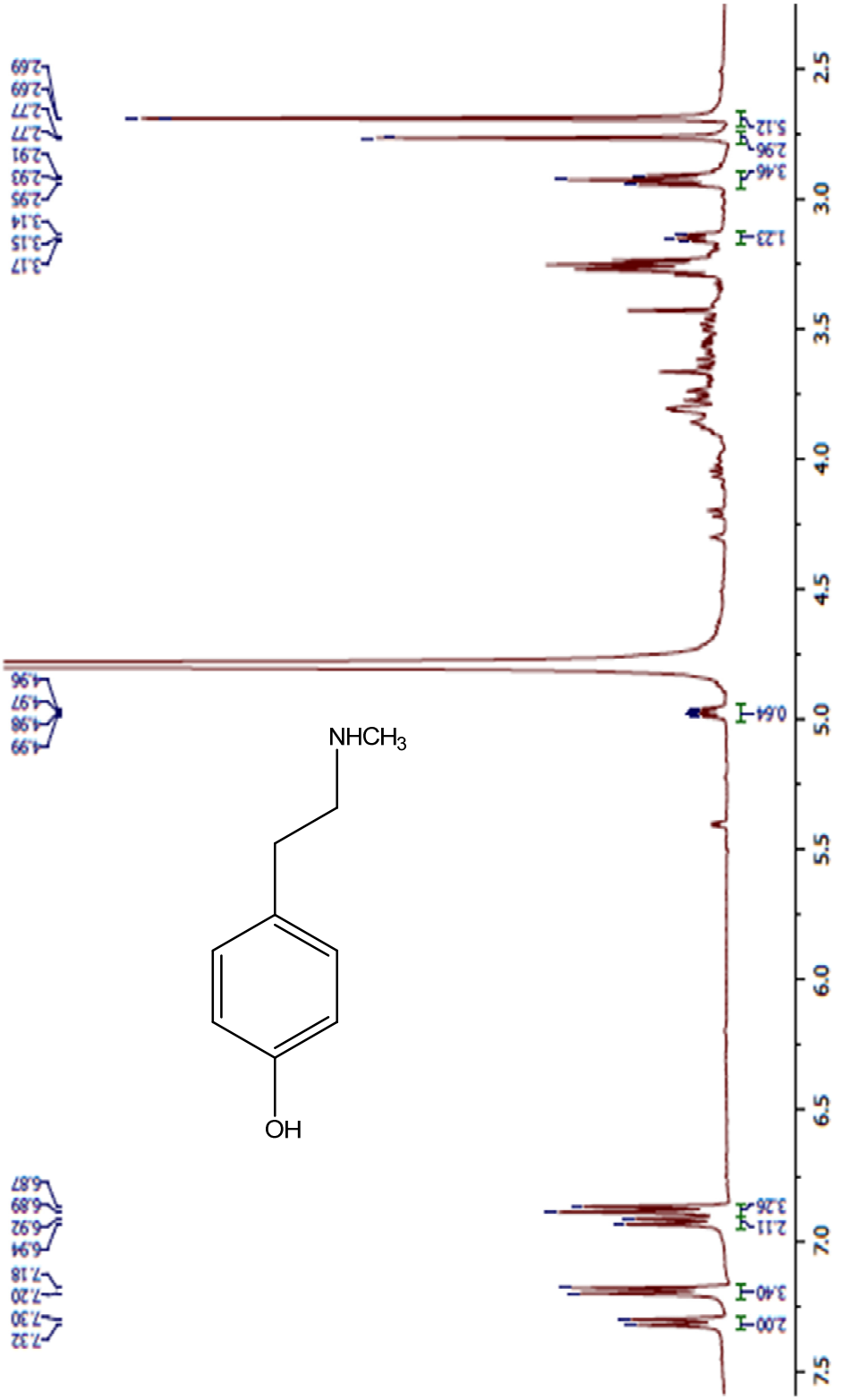



**Figure 53** $^{1}$HNMR spectrum mixture of HS1 and HS2 and structure of HS2 (N-methyltyramine) (400 MHz, $CD_3OD$)

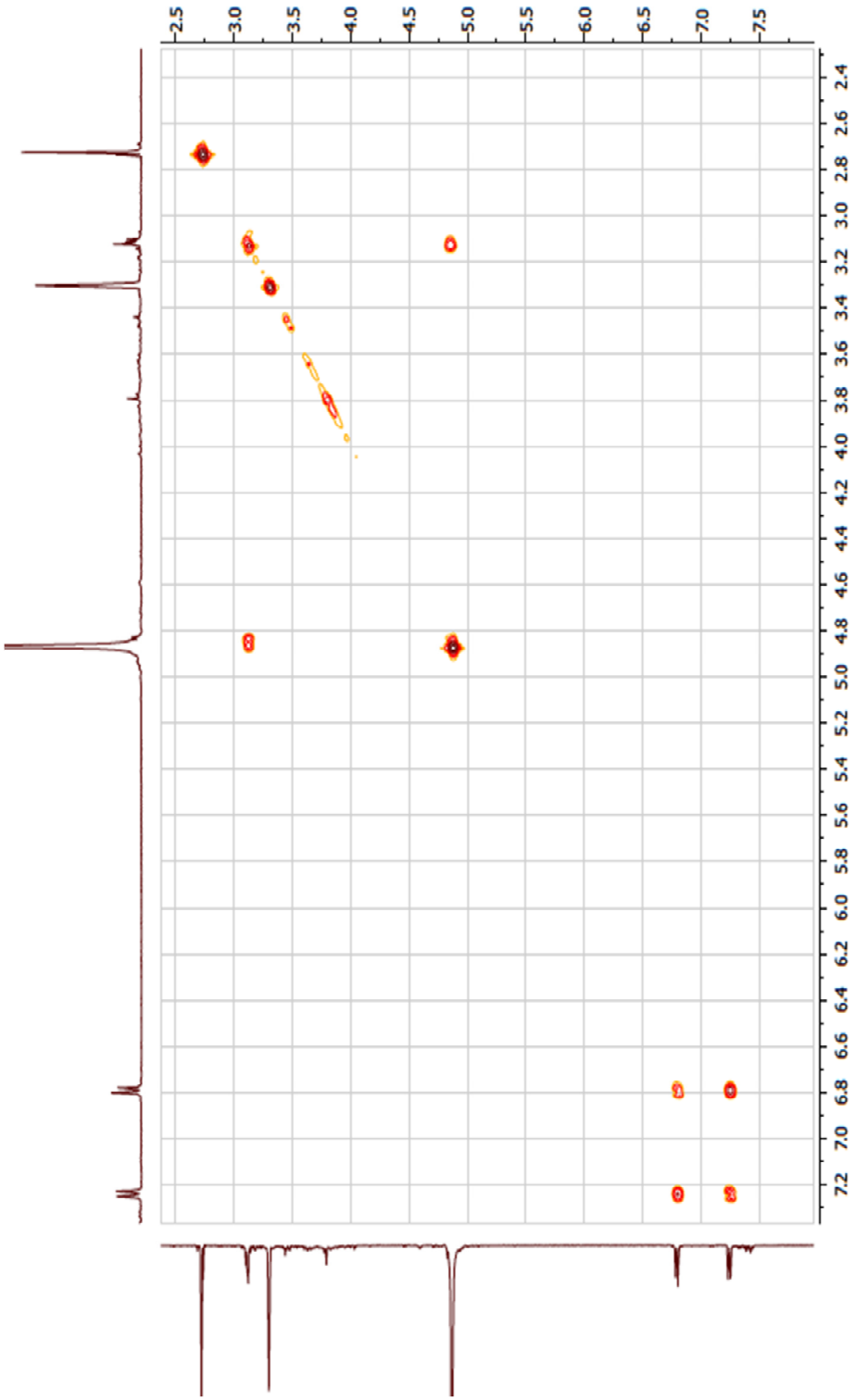



**Figure 54** COSY spectrum of HS1 (synephrine) (400 MHz, $CD_3OD$)

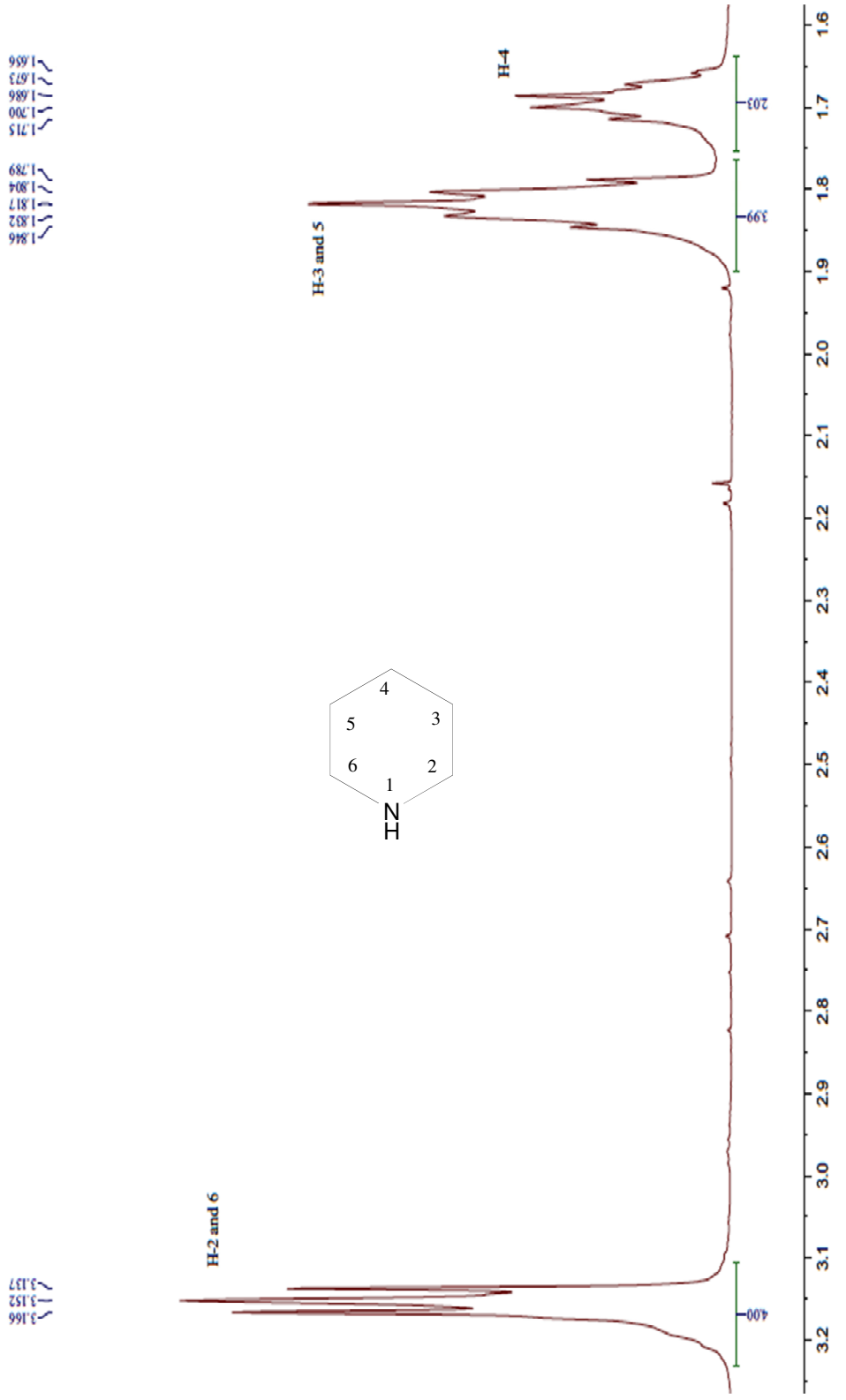



**Figure 55** $^{1}$HNMR spectrum and structure of HS3 (piperidine) (400 MHz, $CD_3OD$)

### 3.7.4 Characterisation of HS4 as Allantoin

**HS4** isolated as white needles obtained from MeOH fractions. The positive mode HRESI-MS for **HS4** gave a molecular ion peak at $[M+H]^+$=158.543 suggesting a molecular formula of $C_4H_6N_4O_3$.

The $^1$HNMR spectrum (Figure 56) of **HS4** indicated the presence of six protons in the region $\delta_H$ 5.20-10.54 ppm. The signals appeared as doublets at $\delta_H$ 6.99 ppm (J = 8.14Hz) and at $\delta_H$ 5.24 ppm (J = 8.13 Hz) assigned for 6-NH and H-4, respectively. The proton at $\delta_H$ 8.06 appeared as broad doublet. The remaining protons, appeared as singlet at δ 10.54and 5.83 ppm, were assigned to 1-NH and 8-$NH_2$, respectively (Table 15).

The HMBC spectrum (Figure 57) showed only four carbons present in this substance and indicated the presence of three carbonyl groups. The two carbonyl carbons C2 and C7 having similar environment appeared very close to each other at δ 157.3 and δ157.9 ppm, respectively, while C5 less shielded found at δ 174.1 ppm. The remaining carbon C4 appeared at δ 62.9 ppm. $^1$HNMR, and $^{13}$CNMR data were consistent with those previously reported by Rasheed *et al.* (2004).

**Table 15** $^1$H (400 MHz) and $^{13}$C (100 MHz) NMR data of **HS4** (allantion) in DMSO

| Position | $\delta_H$ | $\delta_C$ |
|---|---|---|
| 1-NH | 10.54 | - |
| 2 | - | 157.3 |
| 3-NH | 8.06 | - |
| 4 | δ 5.24 (J = 8.13 Hz) | 62.9 |
| 5 | - | 174.1 |
| 6-NH | 6.99 (J = 8.14Hz) | - |
| 7 | - | 157.9 |
| 8-$NH_2$ | 5.83 | - |

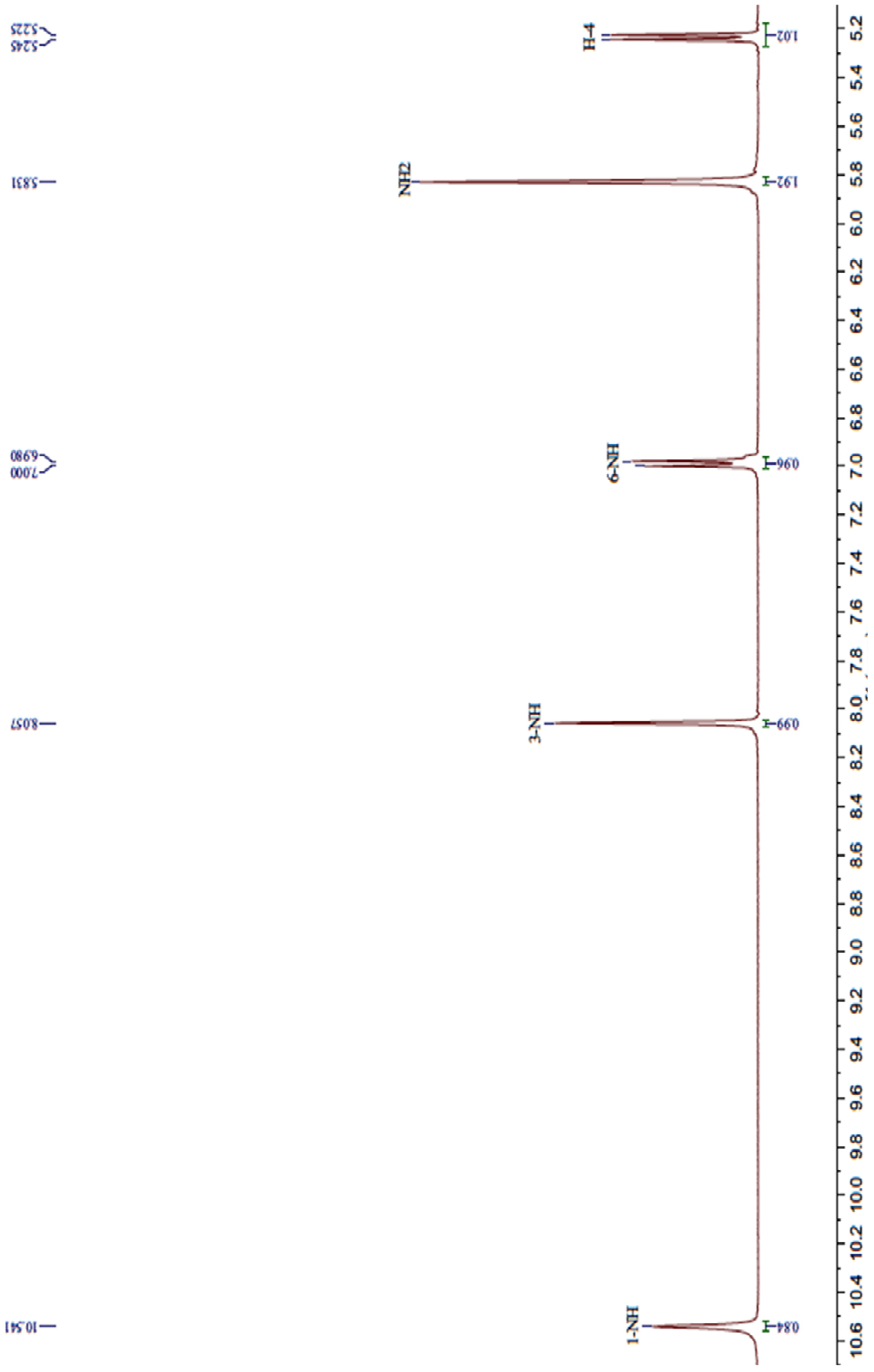



**Figure 56** $^{1}$HNMR spectrum of HS4 (allantion) (400 MHz, DMSO)

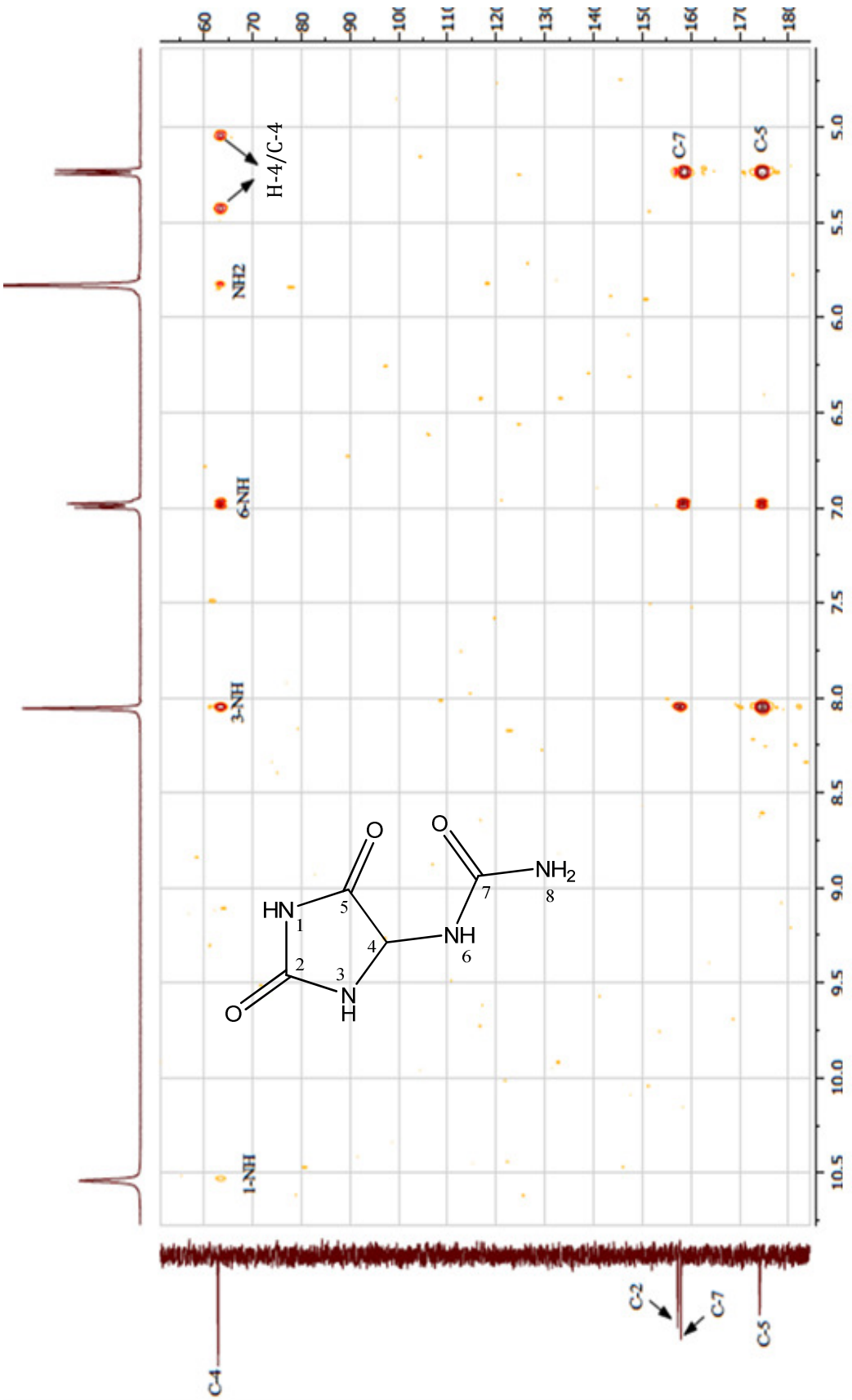



**Figure 57** HMBC spectrum of HS4 (allantion) (400 MHz, DMSO)

# CHAPTER FOUR

# PHARMACOLOGICAL EVALUATION

# 4. Pharmacological evaluation of the isolated compounds

## 4.1 Introduction

As outlined in chapter three, a total of nineteen compounds were isolated from four plants species under investigation. These compounds include monoterpenes (ML17 and ML 19), sesquiterpene lactones (AF 17 and AF 25), alkaloids (HS1 and HS2), tannins and flavonoids such as AF36 and AF35.

In this chapter, and based on the objectives of this study to identify anti-inflammatory, antimicrobial, antioxidant properties and uterine inhibitory effects of the isolated compounds, a brief, general introduction to infection and inflammation and the cellular events underlying these processes will be given.

### 4.1.1 Inflammation

The inflammatory process may be defined as a sequence of events that occurs in response to injury, pathogenic agents such as bacteria, fungal, viral or parasitic infections, various toxic agents as well as tumorgenesis. These responses are controlled by highly modulated interactions between mediators of inflammation and inflammatory cells (Sacca *et al.*, 1997). Furthermore, inflammatory responses can be triggered by the immune system's false recognition of tissues as infected or damaged, a condition known as an autoimmune response. As a result there are numerous pathophysiological conditions falling into the category of inflammatory diseases such as rheumatoid arthritis, Chrohn's disease and ulcerative colitis (inflammatory bowel disease), multiple sclerosis, chronic obstructive pulmonary disease (COPD), asthma, cardiovascular disease as well as other inflammation-related conditions such as cancer and wound healing (Calixto *et al.*, 2004).

### 4.1.2 Inflammatory mediators (cytokines)

Monocytes and macrophages are the key players in inflammatory responses and are also the major sources of pro-inflammatory cytokines and enzymes such as tumor necrosis factor (TNF-α), interleukins (ILs), cyclooxygenases (COX-1 and COX-2) and Nitric Oxide (NO). These pro-inflammatory mediators are strongly induced

during inflammation and are responsible for its initiation and persistence. TNF-α and IL-1β are the cytokines that act as signalling molecules for immune cells and coordinate the inflammatory responses. While mediation of inflammation against pathogen infection by TNF-α and IL-1β proteins can be beneficial to the host, over expression of these cytokines may cause serious disease (Chao *et al.*, 2005).

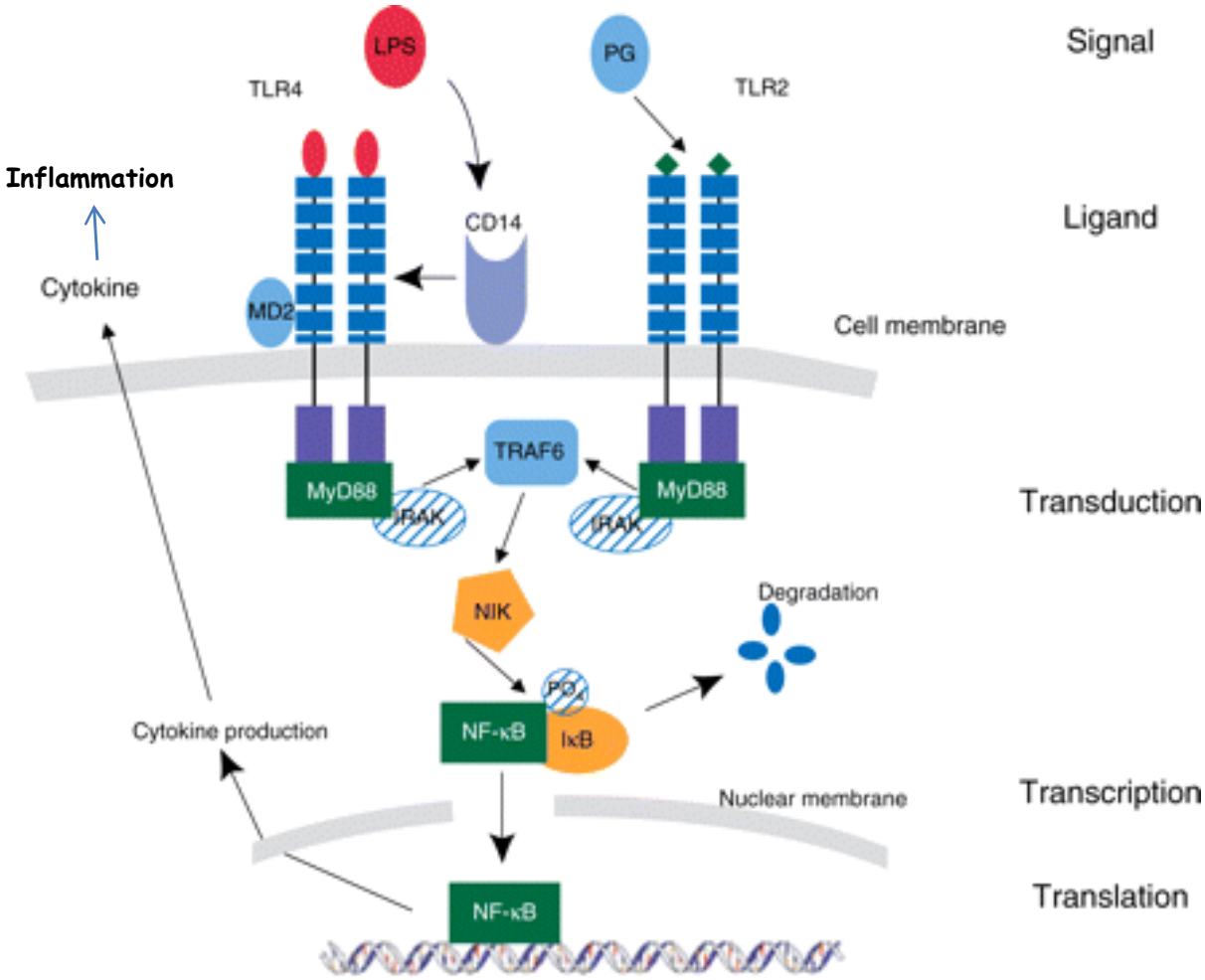



**Figure 58** Lipopolysaccharide-stimulated signal transduction pathways which increase cytokines (TNF-α) production in macrophages, adopted from (Webster, 2009)

The activation of the mitogen-activated protein kinases (MAPKs) and the transcriptional factor NFκB (Figure 58) are the two signalling pathways leading to the production of these cytokines (Philpott *et al.*, 2001). An inhibition of TNF-α released from macrophages *in vitro* is commonly used as an indicator for screening anti-inflammatory activity. For *in vivo* tests, inflammation can be induced by many substances in animals and carrageenan-induced rat paw oedema in rats is the most commonly used model to examine anti-inflammatory action (Shinde *et al.*, 1999).

### 4.1.3 Current anti-inflammatory agents

The current treatments available are classified as non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. NSAIDs typically relieve inflammation and associated pain by inhibiting COX enzymes (which act as nonselective inhibitors of the enzyme) involved in the production of prostaglandins (Chuan, 2000). However, many of the commonly used anti-inflammatory agents are becoming less acceptable due to serious adverse reactions such as gastric intolerance, bone marrow depression and water and salt retention, resulting from prolonged use (Maxwell *et al.*, 2005). In addition, high doses of corticosteroids such as cortisone and dexamethasone, which form one of the oldest and most effective classes of anti-inflammatory drugs, leads to reduced mineral bone density in human and animals (Hoegaerts *et al.*, 2005). This necessitates the continued search for potent anti-inflammatory agents with reduced or no side effects.

### 4.1.4 Natural products as anti-inflammatory agents

In this context, the identification of small, plant-derived compounds that are able to selectively interfere with the production and/or function of cytokines would constitute an important alternative for the treatment of many inflammatory diseases (Calixto *et al.*, 2004). A large number of plant derived anti-inflammatory substances have been reported in existing literature, which can be classified into several chemical groups (e.g., flavonoids, terpenoids, alkaloids, saponins, polyphenolic compounds, lignans and propanoids)(Molnar *et al.*, 2005). Studies based on the ethnobotanical use of plants have often proved to be a more efficient method of drug discovery than random plant screening (Khafagi, 2000). Natural plant compounds

which are able to suppress the production of inflammatory mediators from activated macrophages can act as potential anti-inflammatory agents. Therefore, this part of the study is aimed at exploring and evaluating the anti-inflammatory potential of isolated compounds from the plant extracts which are traditionally used as medicinal herbs by Bedouin women in Egypt.

In this chapter attempts were made to determine the effect of these compounds on the LPS-induced responses in human THP-1 monocytes. For the screening of such active components, pro-inflammatory TNF-α was chosen as the pro-inflammatory marker (Habeeb *et al.*, 2007).

### 4.1.5 Infection

Worldwide, infectious diseases are major causes of death, disability, and social and economic disruption for millions of people (WHO, 2008). Infectious diseases are caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi; the diseases can be spread, directly or indirectly, from one person to another. The major microbial infections in humans are infections of the upper respiratory organs, gastro-intestinal tract, skin, urinary tract, and sexually transmitted infections, they are a major cause of morbidity and infant death in developing countries, largely due to inadequate sewage disposal and contaminated water (Sleigh *et al.*, 1998).

Despite the existence of safe and effective interventions, many people lack access to much needed prevention methods and treatment. With an increase in antibiotic and multiple drug resistance, there is also a need for new therapeutics (Breman *et al.*, 2004). Antimicrobial resistance is a global concern and it is now recognized that resistant microorganisms are prevalent in both the inpatient and outpatient population.

### 4.1.6 Natural products as anti-microbial agents

Plants provide an extensive resource for anti-infection active components, and are rich in a wide variety of secondary metabolites, such as tannins, terpenoids,

alkaloids, and flavonoids, which have been found to have antimicrobial properties (Kong *et al.*, 2003). With the changing perception of TM there has been increased recognition and use of medicinal plants for primary health care by rural as well as urban populations (Cowan, 1999). Bedouin communities in Egypt have long used plants to prevent or cure infectious conditions.

### 4.1.7 Antioxidant

Antioxidants are widely believed to be an important line of defence against oxidative damage, which has been implicated in a range of diseases including, cancer, cardiovascular disease, arthritis and aging (Finkel *et al.*, 2000). Some antioxidants have anti-inflammatory properties. In addition to scavenging free radicals, there are antioxidants that actually block inflammation. The antioxidant effect (the blocking of certain oxidizing proteins) lowers the activation of inflammatory signals. Combinations of certain antioxidants have greater effect than single antioxidants on the inflammation (Wang *et al.*, 2004). The immune system produces cytokines and oxidant molecules, such as hydrogen peroxide and free radicals. The purpose of immune cell products is to destroy invading organisms and damaged tissue, bringing about recovery. However, oxidants and cytokines can also damage healthy tissue. Excessive or inappropriate production of these substances is associated with mortality and morbidity after infection and trauma, and in inflammatory diseases. Oxidants enhance IL-1 and TNF-α production in response to inflammatory stimuli by activating the NF-κB pathway. Sophisticated antioxidant defences directly and indirectly protect the host against the damaging influence of cytokines and oxidants. Indirect protection is afforded by antioxidants, which reduce activation of NF-κB , thereby preventing up-regulation of cytokine production by oxidants (Grimble, 1994) (Agbafor *et al.*, 2011).

### 4.1.8 Natural products as antioxidant agents

Medicinal plants possessing natural antioxidant polyphenolics such as flavonoids, which have received considerable attention in recent years due to their antioxidant activity. Natural antioxidants are considered useful agents for the prevention of

cardiovascular disease. Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury.

### 4.1.9 The effect of natural products on the myometrium

#### 4.1.9.1 Uterus

The uterus is a hollow organ with a well-differentiated lining layer (endometrium), a thick muscular coat (myometrium) and outer layer. Myometrial function may be of vital importance in physiological processes such as sperm and embryo transport and implantation, and in disorders such as dysmenorrhoea and endometriosis. Understanding of this physiology is essential to design and test interventions that can prevent or treat the important clinical problems (Aguilar *et al.*, 2010).

#### 4.1.9.2 Regulation of human myometrial contractility

Tocolytics are agents promoting uterine relaxation (used to suppress premature labour). The wide range of tocolytic agents in use is testament to the fact that we still do not have an ideal drug available (Tan *et al.*, 2006). Therefore, development of new safe and effective tocolytic agents is an important research topic.

#### 4.1.9.3 Physiological pathways and molecular mechanisms regulating uterine contractility

**i) Pathways to uterine contractility**

For significant interaction between myosin and actin in the uterus or any other smooth muscle, myosin must be phosphorylated (Wray *et al.*, 2003). Calcium ions binding to calmodulin activate it and therefore initiate the phosphorylation and subsequent cross-bridge cycling. There are two sources for the increase in activator $Ca^{2+}$: entry across the surface membrane through voltage gated L-type $Ca^{2+}$ channels and/or release from the sarcoplasmic reticulum (SR) (Wray, 2007). If $Ca^{2+}$ rises but myosin light-chain kinase (MLCK) is inhibited, uterine contractions also fail (Longbottom *et al.*, 2000). Thus, the $Ca^{2+}$ -calmodulin–MLCK pathway is vitally important for uterine mechanical activity (Figure 59). Release of $Ca^{2+}$ from uterine

sarcoplasmic reticulum (SR) has been demonstrated in human and animal myometrium preparations (Luckas *et al.*, 1999). Both inositol-triphosphate (IP3) and ryanodine (Ry) receptors have been identified on the SR. It now seems likely, however, that the $Ca^{2+}$ released from these receptors contributes little to the activation of contraction (Wray, 2007).

**ii) Pathways to uterine relaxation and Ca2+ sensitivity**

Relaxation of the myometrium follows a reversal of the $Ca^{2+}$–calmodulin–MLCK pathway. Thus, the myosin light chains (MLCK) are dephosphorylated by myosin phosphatase and $Ca^{2+}$ falls as L-type $Ca^{2+}$channels close and Ca2+ efflux mechanisms are stimulated. This causes a dissociation of $Ca^{2+}$ from calmodulin and inactivation of MLCK (Wray *et al.*, 2001). G-protein coupled receptor for inhibitory agonists, mainly regulating cyclic-adenosine monophosphate dependent protein (cAMP) formation and calcium channel function (Wray, 2003). Uterine agonists interact with a specific G-protein coupled receptor (GPCR) in the plasma membrane, which stimulate the phospholipase C (PLC). Activation of this enzyme stimulates production of inositol-triphosphate (IP3). IP3 interacts with a specific receptor at the level of the sarcoplasmic reticulum (SR) causing release of Ca2+ from its intracellular storage site and a subsequent rise in the internal level of Ca2+ and ultimately activation of MLCK through the intermediary activation of CaM.(Mitchell and Aguilar, 2010).

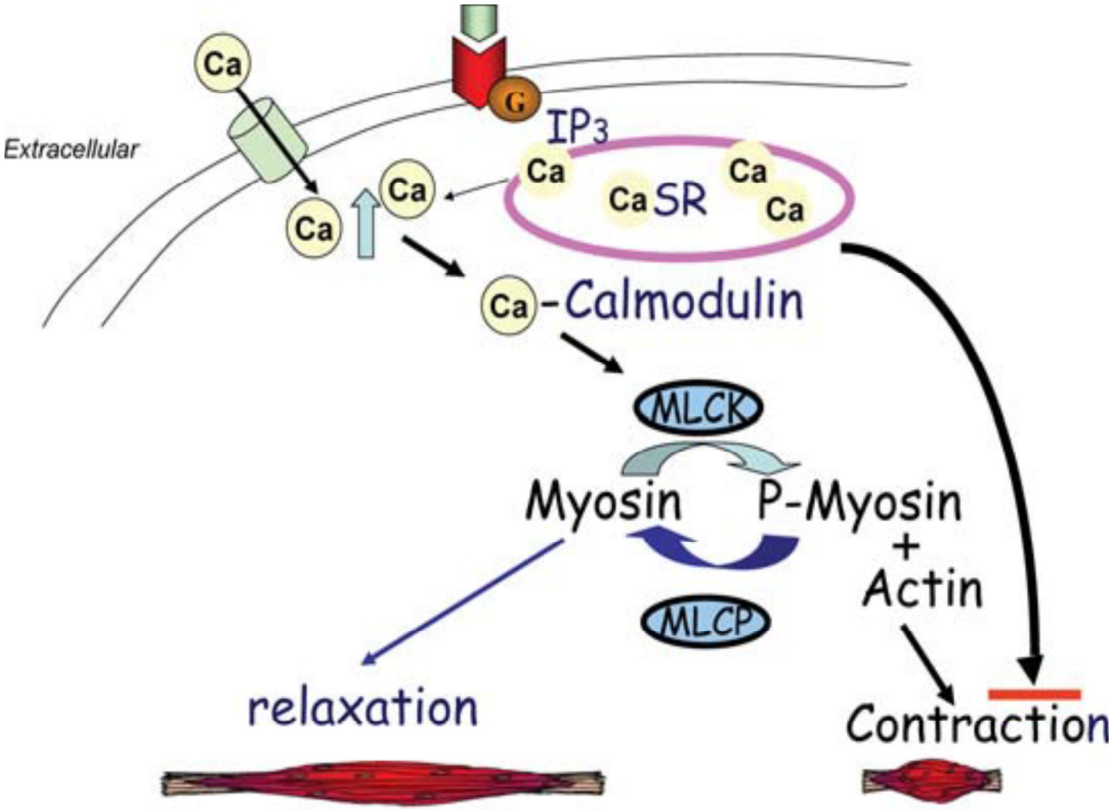



**Figure 59** Scheme to show how Ca2+ entry leads to smooth muscle contraction, adopted from (Wray *et al.*, 2001). MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; and SR, sarcoplasmic reticulum. The contribution of the SR to augmenting Ca2+ for contraction is not established for the uterus, but is indicated for completeness, and the red bar indicates its negative effect on contractility. Some Ca2+ entry through T-type channels may occur, but L-type Ca2+ entry predominates in the uterus.

#### 4.1.9.4 Perinatal problems

Perinatal and maternal related conditions caused a high mortality rates (AbouZahr, 2003). Dysfunctional uterine contractions can lead to premature delivery which is the predominant cause of prenatal mortality and morbidity (Shih *et al.*, 2009). Preterm labour (PTL) is not only a devastating problem for the family, but is also very costly in developing countries (Buxton *et al.*, 2000). PTL results in the deaths of thousands of premature infants each year (Gertrud Berkowitz, 1998). Dysmenorrhea may be another problem associated with uterine contractility (Aguilar *et al.*, 2010). There is

reasonable evidence to support the efficacy of the usual treatments including the contraceptive pill (Wong *et al.*, 2009) or non-steroidal anti-inflammatory drugs (Marjoribanks *et al.*, 2010). The mechanisms of these therapies appear to be directed towards limiting the contractile stimuli to the uterus (through steroid hormonal effects of suppression of inflammatory stimuli) rather than directly interfering with the contractions themselves. Hence, the duration of the effects (and side-effects) of treatment may actually be longer than the duration of the increased uterine contractility resulting in the painful contractions (Aguilar *et al.*, 2010).

Currently there is no effective therapeutic strategy for the treatment or prevention of preterm labour (Tribe, 2001). The regulation of the uterine smooth muscle that leads to birth is not fully understood. Knowledge, particularly on how best to induce relaxation of a uterus undergoing preterm labor, could revolutionize the treatment of this problem and potentially save thousands of lives and reduce morbidity greatly (Buxton, 2003). Uterine relaxation is responsible for maintaining uterine quiescence during pregnancy, and is required to facilitate complicated deliveries in some instances. Relaxation is important for uterus undergoing preterm labour.

For preventing or treating premature labor, $\beta_2$-adrenoceptor stimulants, such as ritodrine and terbutaline, that inhibit contractions of uterine smooth muscle by stimulating the production of cytosolic cAMP via $\beta_2$- adrenoceptor, have been used extensively. However, the $\beta_2$-adrenoceptor agonists used at present are not particularly selective for uterine smooth muscle, and side effects on the cardiovascular and metabolic systems are frequent (Kobayashi *et al.*, 2001).

### 4.1.10 Natural products as tocolytic agents

Tocolytic agents are drugs designed to inhibit the contractions of myometrial smooth muscle cells. Tocolytic agents are commonly used to treat preterm labour by inhibiting uterine contractions with the goal of postponing labor and improving neonatal outcomes (Tan, 2006). The aim of tocolysis is not only to stop uterine contractions and to prevent preterm delivery, but also to decrease perinatal morbidity

and mortality associated with preterm birth (Tsatsaris *et al.*, 2004). The main drugs used as tocolytics are indomethacin and other prostaglandins inhibitors (Ahmed A. *et al.*, 2010). Traditional medicine relies on the use of certain herbal and other remedies for beneficial effects during pregnancy, to induce labour, in the removal of retained placenta and management of post-partum bleeding and preterm births. Most often the biological effects elicited by these plants are due to the biomolecules that primarily act on the uterus. The nature of these actions may involve the modulation of uterine contractions at labour, resulting in either stimulation contraction or inhibition (tocolytic) of myometrial muscle contractions (Gruber *et al.*, 2011). Few studies have been carried out on the pharmacological properties of medicinal plant species with respect to reproductive health care in Egypt. The majority of plant species though widely used, have not been well researched. Thus, in this study the crude aqueous extracts of *Haloxylon salocornicum* and its pure compounds were screened by carrying out bioactivity tests in a mouse uterus model.

## 4.2 Material and methods

### 4.2.1 General reagents

- 0.22µm filter (Millipore, UK)
- 24-well plates (TTP, Switzerland)
- 96-well plates (TPP, Switzerland)
- 75 $cm^2$ cell culture flasks (Corning Incorporated, USA)
- 1,2-diphenyl-2-picrylhydrazyl (DPPH)
- DMSO (Sigma, UK)
- F16 Maxisorp Loose Nunc-immuno modules (Nunc, Denmark)
- Foetal calf serum (FCS) (Sigma, UK)
- Immuno™ 96-well MicroTitre™ Plates (Thermo Labsystems, USA)
- L-glutamine (Sigma, UK)
- Lipopolysaccharide (LPS) – *E. coli* (Sigma, UK)
- Neubauer-Improved Haemocytometer (Marienfeld, Germany)
- Penicillin (Sigma, UK)
- Resazurin (Sigma, UK)
- RPMI 1640 medium (Sigma, UK)
- Sodium chloride (BDH, UK)
- SpectraMax M5 Microplate Reader (Molecular Devices Corporation, California, USA)
- Streptomycin/ Penicillin (Cambrex, UK)
- THP-1 cells (European Collection Animal Cell Culture, UK)
- TNF-α ELISA Ready-Set-GO! Kit (eBioscience, UK)
- Tween 20 - Polyoxyethylenesorbitanmonolaurate (Sigma, UK)
- Mueller-Hinton Broth (MHB) (Becton Dickinson, UK)
- Mueller–Hinton Agar (MHA) (Becton Dickinson, UK)
- Sabroud Dextrose Agar (SDA) (Sigma-Aldrich, UK)
- Dextrouse Sabroud Broth (Sigma-Aldrich, UK)
- Gentamicine and Fluconazole (Sigma-Aldrich, UK)
- Iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, UK)

### 4.2.2 Anti-inflammatory assays

#### 4.2.2.1 Preparation of plant samples

Crude extract (20mg) and pure compounds (1mg) dissolved in DMSO or medium were filtered through a 0.22µm filter unit prior to use and further diluted in complete medium to obtain solution contining 0.1% (v/v) DMSO.

#### 4.2.2.2 Cell culture

THP-1 cells were maintained in complete RPMI-1640 medium prepared in a sterile flow hood. Complete medium consisted of 500ml RPMI 1640 medium, 50ml foetal calf serum, 5ml penicillin/streptomycin and 5ml L-glutamine. Cells were incubated at 37°C, 5% $CO_2$ and 100% humidity.

#### 4.2.2.3 Cell viability assay

The cytotoxicity of crude extracts or pure compounds was determined using a resazurin reduction assay. Briefly, 200µl of extract was added to the first well of rows A, B, C and G of a 96 well tissue culture plate. Row G acted as a background control for coloured material as it only contained extract and medium. DMSO (200µl) was added to the first wells of rows D and E (solvent control), 100µl of medium was then added to all the remaining wells, and a 1:2 serial dilution carried out across the plate. Cells, 100µl ($1x10^6$ cells/well) suspended in complete medium containing 10% (v/v) resazurin was added to all the wells except row G (background control). To row F (cells only), which acted as a positive control, 100µl of medium plus 10% (v/v) resazurin was added. Next, medium in row H was carefully aspirated leaving behind the cells, and 200µl of NaCl solution (300mg/ml) plus 10% (v/v) resazurin was added which acted as a negative control (Figure 60). The plate was then placed in an incubator overnight at 37°C, 5% CO2 and 100% humidity.

The next day, 100µl supernatant from each well was carefully transferred to a new 96 well plate and read at OD 570nm and 600nm, to provide day 1 readings. Any remaining medium in the wells of the original plate was carefully aspirated and replaced with 100µl of fresh medium plus 10% (v/v) resazurin. The original plate

was then returned to the incubator overnight and read as before to give day 2 readings in the absence of test agent.

The % cell growth for each day was calculated using the following equation:

$$\%\ \text{Cell growth} = \frac{mean(OD570nm - OD600nm)test\ agent}{mean\ (OD570nm - OD600nm)\ positive\ control} \text{X100}$$

If the cell viability was over 50%, then the plant material was considered to be non-cytotoxic (Habeeb *et al.*, 2007).

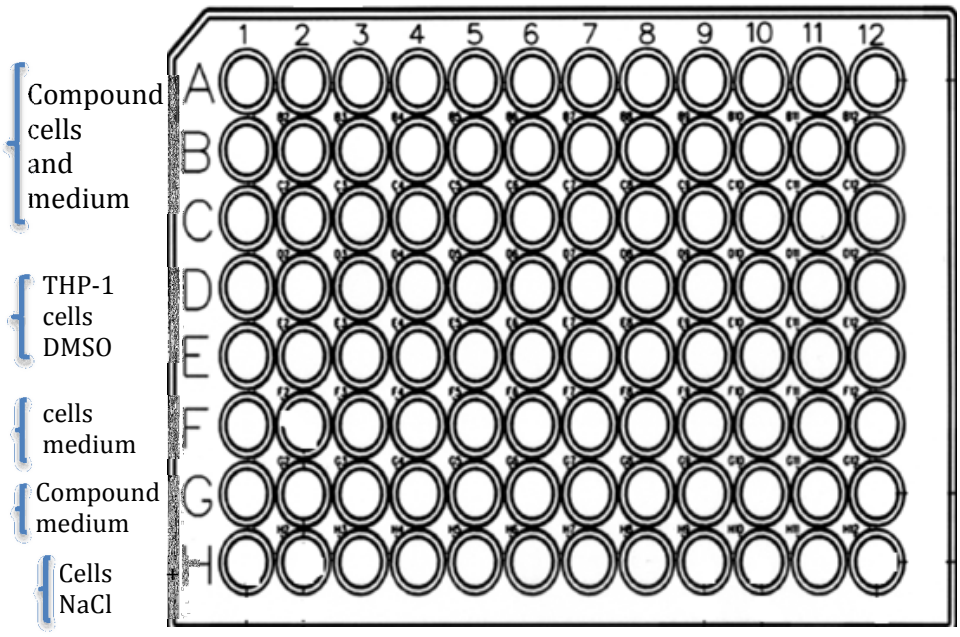



**Figure 60** Template of a cytoxicity plate layout

#### 4.2.2.4 LipoPolySaccharide (LPS) cell stimulation assay

One ml of THP-1 cells ($1x10^6$cells/well) was seeded in 24 well plates and treated with the compounds at different concentrations (the lowest concentration used was the one that showed at least 50% cell viability) for one hour (Kwon *et al.*, 2010) before adding the LPS (500pg/ml) the plate was incubated for 24 hours at 37°C, 5% $CO_2$ and 100% humidity. The contents of each well were centrifuged at 5000g for 5 minutes and the supernatants were collected and analyzed for the effect of the compounds on the levels of TNF-α produced by the cells. A kit was used according

to the manufacturer's instructions as described below. Supernatant from the cell stimulation was assayed by ELISA. Briefly, the wells were coated using 100µl capture antibody diluted 1:250 in coating buffer and left for 1 hour at 37°C. The wells were then washed three times in wash buffer, consisting of 2 litres of phosphate buffer saline (8g NaCl, 0.2g KCl, 1.15g Na2HPO4 and 0.02g KH2PO4 per litre of distilled water, pH 7.2) and 0.01% (v/v) Tween 20. The wells were then blocked using 200µl assay diluent (1:5 dilutions). This was then left for one hour at room temperature and washed as before. Supernatant (100µl) was added in triplicate. For the standards, a 1:2 serial dilution was carried out using a top concentration of 500pg/ml (run in triplicate). The plate was then incubated overnight at 4°C, and the following day washed five times. Detection antibody 100µl (1:250 dilution) was added and left for 1 hour at room temperature, followed by five washes. Avidin-HRP (100µl, 1:250 dilutions) was added for 30 mins, followed by 7 washes. Tetramethylbenzidine (TMB) solution 100µl was added and the reaction left for 15 minutes. The reaction was stopped by the addition of 50µl of 10% (v/v) sulphuric acid. The plate was then read at 450nm on a SpectraMax M5 microplate reader. From the standard curve the concentration of cytokine produced by the cells was quantified.

### 4.2.3 Antimicrobial assay

#### 4.2.3.1 Microorganisms used and inoculums preparation for antibacterial assay

Isolates of the following organisms: *Staphylococcus aureus* (ATCC 8095), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 25617), and *Candida albicans* (Mull 29903) were inoculated on Mueller–Hinton Agar (MHA). Prepared by adding 38 g of MHA to 1 L distilled water, autoclaved [121 C, 20 psi, 30 min] and allowed to cool to about 55° C, and then poured into petri dishes and incubated overnight at 37° C. The culture medium varied according to the microorganism being tested. In the case of the bacteria, four to five colonies were transferred to 3mL fresh sterile Mueller–Hinton Broth (MHB) (prepared by dissolving 29 g MHB in 1 L distilled water) and maintained at 37° C for

3–4 h until log phase growth was achieved. The inoculates were prepared by adjusting the turbidity of the medium to match a 0.5 McFarland standard which is equivalent to 1.5 x $10^8$ CFU/ml (spectrophotometrically achieved by an OD 625 nm of 0.008–0.10) (Galeano *et al.*, 2007). Bacterial suspensions were further diluted to 1.5X$10^4$ CFU in sterile 1% (w/v) NaCl solution.

#### 4.2.3.2 Extracts and compounds preparation

All the plant samples were dissolved in DMSO (1% v/v) and diluted with MHB at a concentration of 10 mg/ml for the crude extract and 1mg/ml for pure compounds. All solutions were filter sterilized through a 0.22µm filter unit.

#### 4.2.3.3 Colorimetric susceptibility assay

For the screening of antimicrobial activity, a microdilution assay according to the National Committee for Clinical Laboratory Standards with minor modifications was used (Salvat *et al.*, 2004). The test was performed in sterile 96-well microplates. Two hundred µl of plant extract was added to the wells of row A in triplicate, while the remaining wells in rows B to H received 100µl of MHB. Two-fold serial dilutions were prepared vertically in the plate. The last 100µl in row H was discarded (Figure 61). The inoculum (100µl) containing 1.5×$10^4$ CFU of microorganism was added to all the wells. Plates were incubated at 37º C and examined after 24 hours. As an indicator of bacterial growth, 50µl of 0.2 mg/ml INT was added to the wells and incubated at 37°C for 30 min. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured ormazan product by biologically active organisms (Eloff, 1998). Where bacterial growth was inhibited, the solution in the well remained clear after incubation with INT. A number of wells were also reserved in each plate for control of sterility (no inoculum added), inoculum viability (no extract added) DMSO and an antibiotic control consisting of gentamicin (50 µg/ml–0.19µg/ml). The minimum inhibitory concentration (MIC) was deemed to be the concentration in the first well in which no colour change was observed.

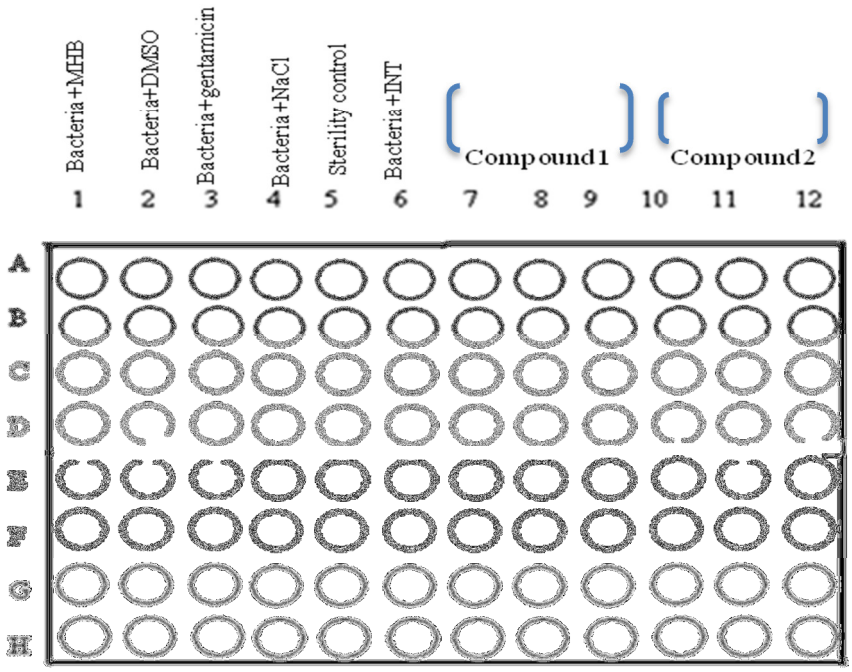



**Figure 61** Template of an antimicrobial plate layout

#### 4.2.3.4 Minimum bactericidal concentration determination

The minimum bactericidal concentration (MBC) was determined by spreading a 10µl volume on an agar plate from the wells showing no visible growth. The plates were incubated at 37°C for 24 h. The minimum concentration of compound that showed 99% reduction of the original inoculum was recorded as the MBC (Al-Bayati, 2009).

#### 4.2.3.5 Microorganisms used and inoculums preparation for antifungal activity

The antifungal activity of the extracts was evaluated against *Candida albicans* (Mull 29903) using the micro-dilution assay (Eloff, 1998) modified for fungi (Motsei *et al.*, 2003). C. *albicans* was aseptically inoculated on petri dishes containing autoclaved, cooled, and settled Sabroud Dextrous Agar (SDA). The petri dishes were incubated at 37°C for 48 h resulting in white round colonies. These were sub cultured on SDA slants. Inoculate were prepared by transferring several colonies to sterile Sabroud Dextrouse Broth (SDB) prepared by dissolving 29g SDB in 1L of distilled water and autoclaved.

The suspension was mixed to ensure homogeneity and subsequently diluted to match the turbidity of a 0.5 McFarland standard (i.e. OD = 0.12–0.15 at k = 530 nm, corresponding to 1–5X$10^6$ CFU/ml). Further dilutions in sterile distilled water were made to obtain the required working suspensions 1–5X$10^3$ CFU/ml. Colorimetric readings of the results employed resazurin as an indicator of cell growth. For this purpose, the working suspension (20 ml, 1–5X$10^3$ CFU/ml) was supplemented with 0.1ml sterilized solution of resazurin 20 mg/ml in water (Liu *et al.*, 2007). A colour change from blue to pink was taken as an indication of the growth. Fluconazole (1mg/ml) was used as a positive control. The plates were covered and incubated at 37° C overnight. The wells remained blue where there was inhibition. MIC values were recorded as the lowest concentrations that inhibited fungal growth after 48 h.

### 4.2.4 Antioxidant (DPPH) assay

An antioxidant assay was performed according to (Momtaz *et al.*, 2008) with slight modifications. For each compound, 50 µl/ well (concentration range between 100 to 0.012 µg/ml) was added to a 96 well plate and a dilution series (8 dilutions) prepared by adding methanol (100 µl as the dilution medium). Then 50ul of DPPH (0.5 µM) was added to each well [DPPH solution 20 mg/L (Backhouse *et al.*, 2008)]. The plates were covered and incubated at room temperature for 30 minutes and read on a SpectraMax M5 microplate reader. Ascorbic acid was used as a positive control and was tested at concentrations ranging from 50µg to 0.4µg/ml. The experiments were performed in triplicate and the average absorption was noted for each concentration. The absorbance of samples was plotted against the concentration and the $IC_{50}$ was calculated. Inhibition of radical scavenging was calculated as:

$(\%) = [(A_0 - A_1)/A_0 \times 100]$, with $A_0$ being the absorbance of the control and $A_1$ the absorbance of the test sample at 517 nm. $EC_{50}$ (effective concentration of 50%) was determined from the radical scavenging curve.

### 4.2.5 *Ex vivo* uterine contractile assay

#### 4.2.5.1 Animals

Adult virgin female mice (20-30g) were used in this study. The mice were humanely killed by cervical dislocation, and all experiments were performed in accordance with guidelines and principles for the care and use of laboratory animals at the University of Strathclyde.

#### 4.2.5.2 Tissue preparation and organ bath

The uterus was carefully removed and immediately immersed in cold physiological saline solution (PSS) containing (in mmol/L) 118.4 NaCl; 4.7 KCl; 1.2 $MgSO_4$; 11 glucose; 2.5 $CaCl_2$, 25 $NaHCO_3$ and 1.2 $KH_2PO_4$, pH 7.4. The uterus was then placed in a shallow dissecting dish containing PSS and the right and left uterine horns were carefully dissected under a microscope. The uterine horns were mounted vertically in 10 ml organ bath (Figure 62) and connected to a force displacement transducer (Grass FT03) using silk threads. A resting tension of 0.5 g was applied. The electrical signal from the transducer was amplified and converted to a digital signal and recorded on a personal computer using Chart software (AD Instruments Pty Ltd., Australia). The physiological saline solution was maintained at 37$^{o}$C and gassed with 95% $O_2$ and 5% $CO_2$. The uteri were allowed to contract spontaneously and an equilibrium period of 30-60 min was allowed before the application of any test agents.

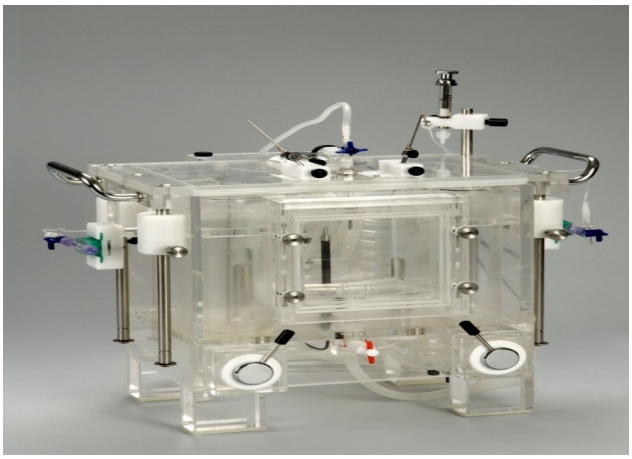


**Figure 62** Organ bath

#### 4.2.5.3 Effects of *Haloxylon salicornicum* extract and its compounds on spontaneous contractions of the mice

Following a 60-min equilibration period, the plant extracts and purified compounds (0.1, 0.5, 1 and 3 µg/ml) were added to the tissue in a cumulative manner at 10 minute intervals. In a parallel control experiment, the effect of the solvent vehicle (DMSO) on the contraction of the uterus was also tested.

#### 4.2.5.4 Effect of *Haloxylon salicornicum* extract and its compounds on $K^+$-induced contraction

To assess whether the inhibitory activity of the crude extract was through calcium channel blockade, $K^+$ was used to depolarize the preparations. KCl (60 mM/ml) was added to the tissue, producing sustained contraction of the uterus. Then the extract was added to the tissue in a cumulative manner and sometimes in a single concentration (3ug/ml).

#### 4.2.5.5 Effect of propranolol on the inhibition of produced by *Haloxylon salicornicum* extract and its compounds

Propranolol (20 µM/ml) was added to the bath solution, and 20 min later the effect of the extract on the contraction was determined.

### 4.2.6 Statistics

All graphs were produced using Microsoft Excel Version 9. Minitab Statistical Software, Version 15 was used. Statistical differences between the experimental groups were examined by analysis of variance and the statistical significant was determined at $P \leq 0.05$. The data presented are means ± SD of three determinations.
The results for uterus are expressed as the mean ± SEM of several preparations (n) from different animals, statistical evaluation was performed with Prism, and values of $p < 0.05$ were considered statistically significant.

## 4.3 Results

### 4.3.1 Evaluation of anti-inflammatory activity

#### 4.3.1.1 Cell viability assay

Prior to assessment of the anti-inflammatory responses, a cytotoxicity assay was performed on the plant materials. The cytotoxicity assay is important to determine the highest concentration that can be used in the cell stimulation assay without killing the cells or which does not induce cytokine production as the cells become non-viable. Toxicity of crude extract and pure isolated compounds in THP-1 cells was assessed using a resazurin reduction assay. The cytotoxicity of the compounds is expressed as $IC_{50}$, which is the drug concentration that causes 50% growth inhibition. Only concentrations of compounds with toxicity less than 20% after day 2 were progressed to anti-inflammatory evaluation.

### 4.3.1.2 Cytotoxicity assay for *Achillea fragrantissima* crude methanol extract

The methanol crude extract of *A. fragrantissima* was not cytotoxic to THP-1 cells after 24 hours incubation. All concentrations caused a significant increase ($P<0.05$) in growth with a range from 80% to 100% (Figure 63). On the second day in the absent of the test agent *A. fragrantissima* was not toxic to the cells.

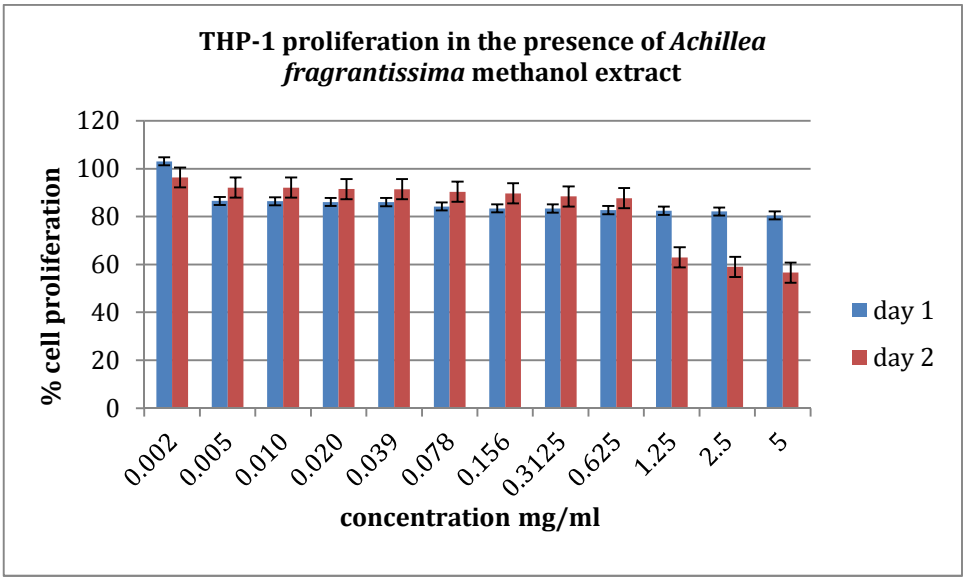



**Figure 63** % cell growth of THP-1 cells incubated with *Achillea fragrantissma* methanol extract, at different concentrations. Cell viability was measured by reduction of resazurin, at 570nm and 600nm and calculated as a % of untreated controls. Each bar is representative of the mean ±SD of triplicate readings.

### 4.3.1.3 Cytotoxicity assay for AF17, AF18 and AF36

The results show that AF17 is moderately toxic and produces a decrease in the percentage of cell growth for all concentrations (Figure 64). The highest growth was 95% for the 0.002mg/ml concentration and the lowest percentage was 45% at 5mg/ml, the compound had a similar cytotoxic effect on the second day in the absence of test agent.

AF18 showed $IC_{50}$ toxicity at 1.25mg/ml and above on day 1 (Figure 65); however, on day 2 in the absence of test agent, the toxicity was observed at 0.3125mg/ml and above.

In the case of AF36, toxicity was observed at 0.3125mg/ml and above on day 1 and 2.5mg/ml for day 2 (Figure 66). Therefore, 0.030mg/ml of AF17, AF18 and AF36 is the highest concentration that was used for anti-inflammatory assessment.

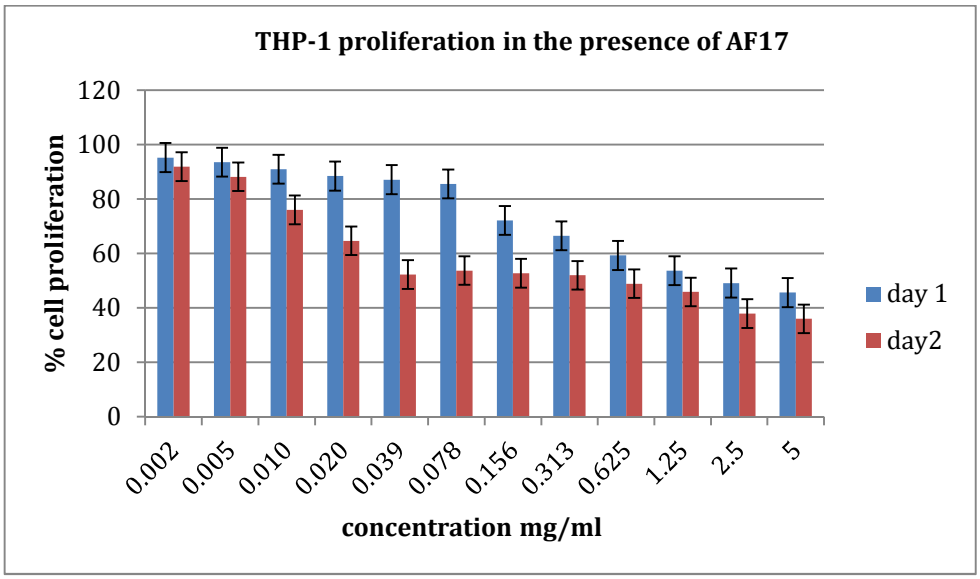



**Figure 64** % cell growth of THP-1 cells incubated with AF17 at different concentrations. Cell viability was measured by reduction of resazurin, at 570nm and 600nm and calculated as a % of untreated controls. Each bar is representative of the mean ±SD of triplicate reading

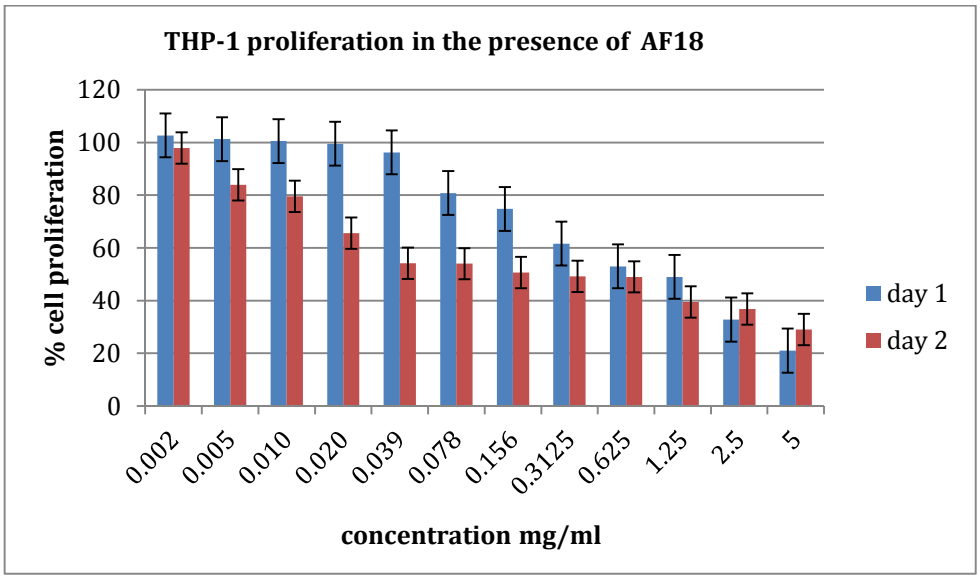



**Figure 65** % cell growth of THP-1 cells incubated with AF35 at different concentrations. Cell viability was measured by reduction of resazurin, at 570nm and 600nm and calculated as a % of untreated controls. Each bar is representative of the mean ±SD of triplicate reading.

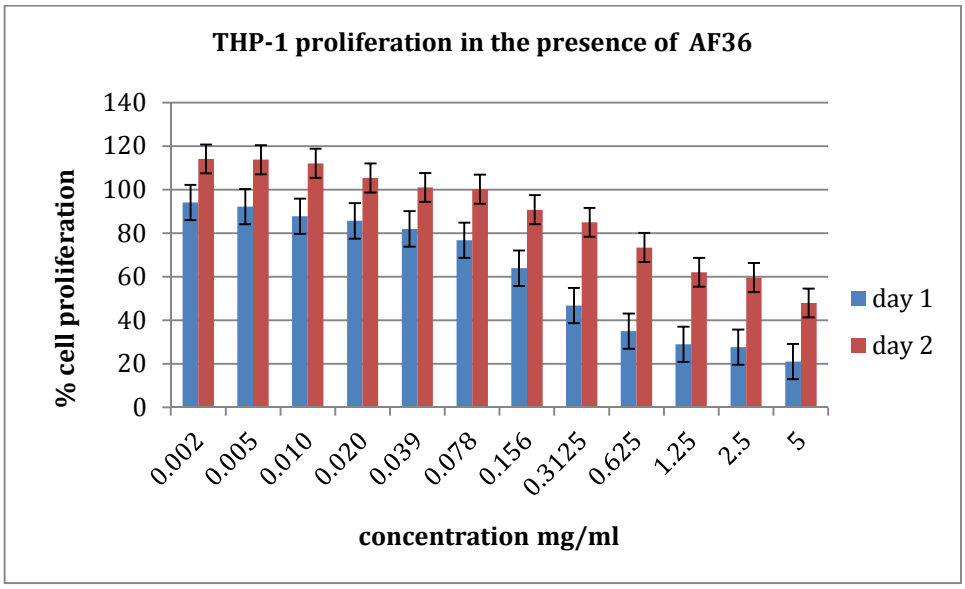



**Figure 66** % cell growth of THP-1 cells incubated with AF36 at different concentrations. Cell viability was measured by reduction of resazurin, at 570nm and 600nm and calculated as a % of untreated controls. Each bar is representative of the mean ±SD of triplicate reading.

#### 4.3.1.4 Cytotoxicity assay for the compounds isolated from *Mentha longifolia* ML7, ML10, ML16, ML1 and ML2

ML7 showed toxicity at 0.625mg/ml on the first day, while the toxicity increased to 0.156mg/ml on day 2 (Figure 67). ML15 showed toxicity at 1.25mg/ml for day 1 and then decreased to 0.625mg/ml for day 2 (Figure 68). ML10 showed a high percentage of growth for all concentrations on day 1 while it decreased to 0.625mg/ml for day 2 (Figure 69). The flavonoids ML1and ML2 were non-toxic to the THP-1 cells on both days (Figures 70 and 71).

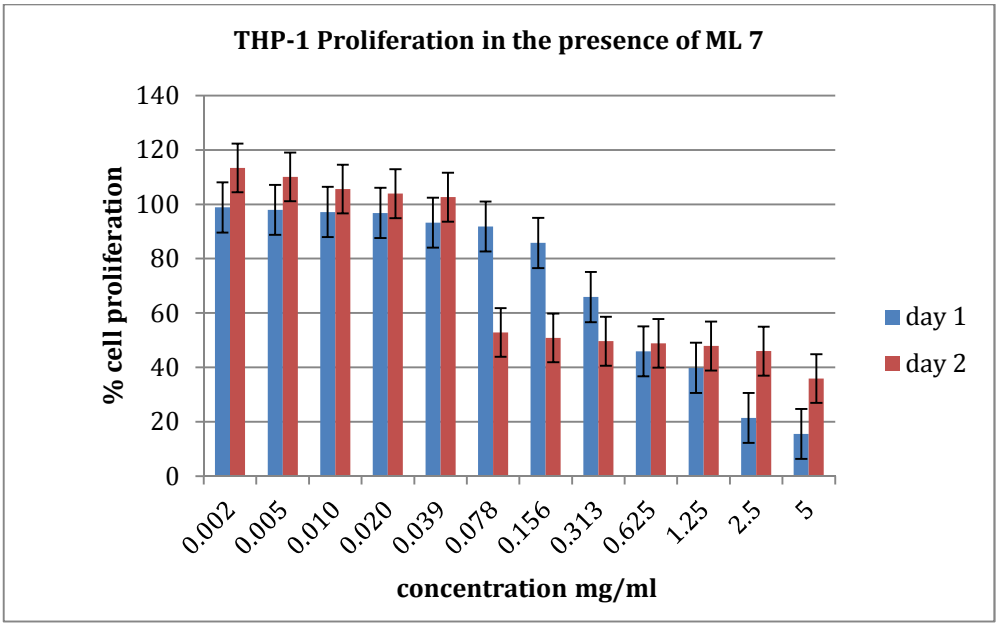



**Figure 67** the Effect of ML7 on the viability of THP-1 cells. Cell viability was assayed by resazurin at 570nm and 600nm. Each bar is representative of the % mean ± SD of 3 sets of data.

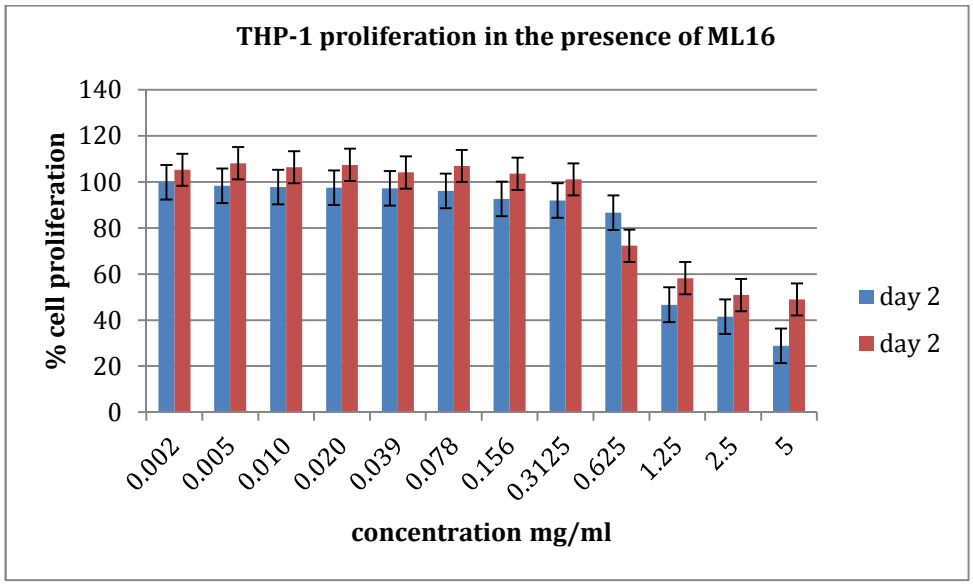



**Figure 68** the Effect of ML15 on the viability of THP-1 cells. Cell viability was assayed by resazurin at 570nm and 600nm. Each bar is representative of the % mean ± SD of 3 sets of data.

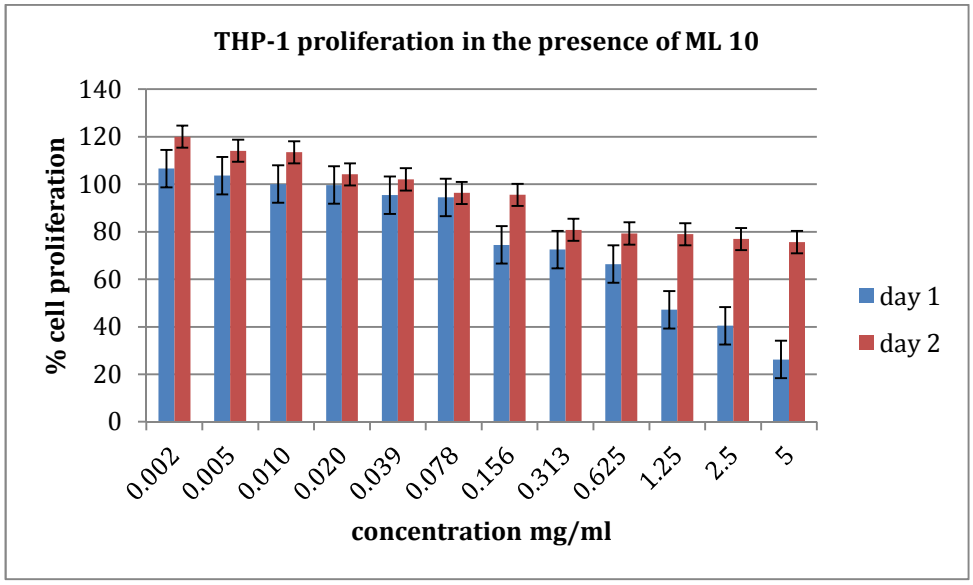



**Figure 69** the Effect of ML10 on the viability of THP-1 cells. Cell viability was assayed by resazurin at 570nm and 600nm. Each bar is representative of the % mean ± SD of 3 sets of data.

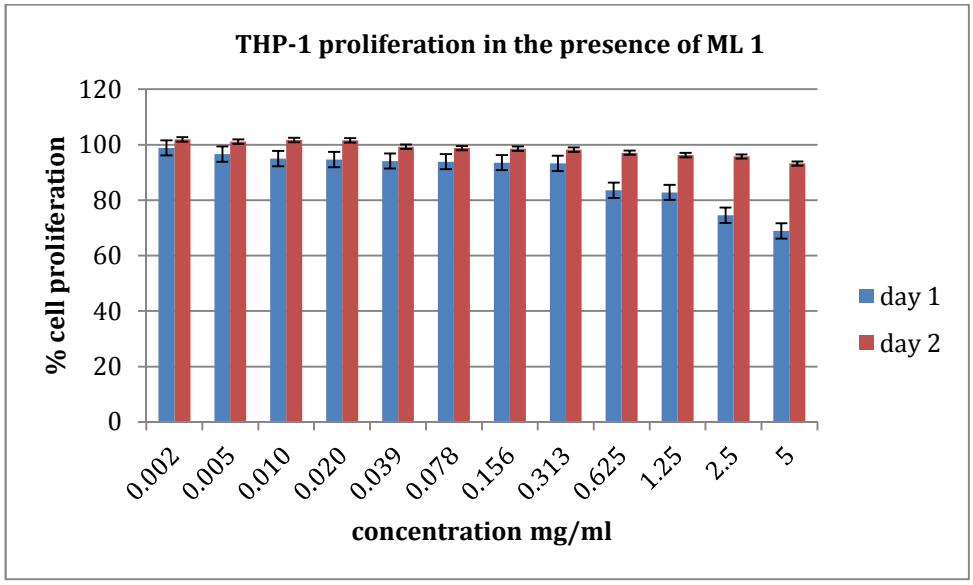



**Figure 70** the Effect of ML1 on the viability of THP-1 cells. Cell viability was assayed by resazurin at 570nm and 600nm. Each bar is representative of the % mean ± SD of 3 sets of data.

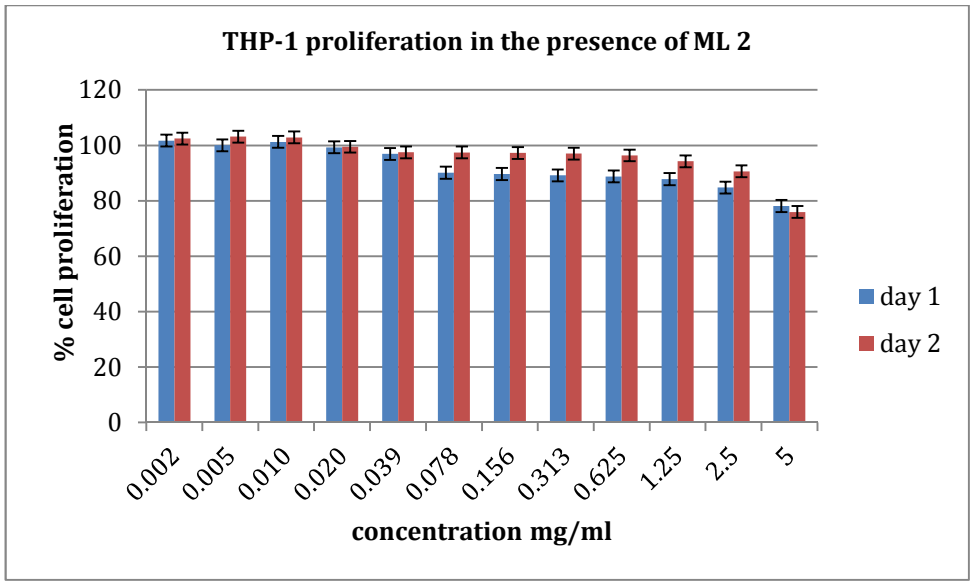



**Figure 71** the Effect of ML2 on the viability of THP-1 cells. Cell viability was assayed by resazurin at 570nm and 600nm. Each bar is representative of the % mean ± SD of 3 sets of data.

### 4.3.2 Effect of isolated compounds on LPS-induced TNF-α release in THP-1 cells

The response of cells to activation agents such as LPS are well documented and lead to the secretion of several inflammatory cytokines such as TNF-α and the inflammatory mediators NO and COX-2 (Eliopoulos *et al.*, 2002). LPS evokes a time concentration-dependant increase in TNF-α production in THP-1 cells assessed by ELISA, TNF-α level is hardly detectable in un-stimulated cells. Cells were treated with the compounds for one hour prior to stimulation with LPS (500pg/ml) for at least 20 hours. The compound concentration was chosen based on the average toxicity profile that compounds exhibited on the THP-1 cell cytotoxicity assay. The levels of TNF-α were then quantified by ELISA. A typical standard curve is shown in Figure 72. This assay demonstrated whether the plant extracts were exhibiting any anti-inflammatory properties by comparing the LPS stimulated cells with the cells stimulated in the presence and absence of the plant extracts. If there was a reduction in the levels of TNF-α produced by the LPS stimulated cells in the presence of the plant extract, this meant that the extract at that concentration had an anti-inflammatory effect. The concentrations of plant extract shown in the figures are the final concentrations which were present in the well.

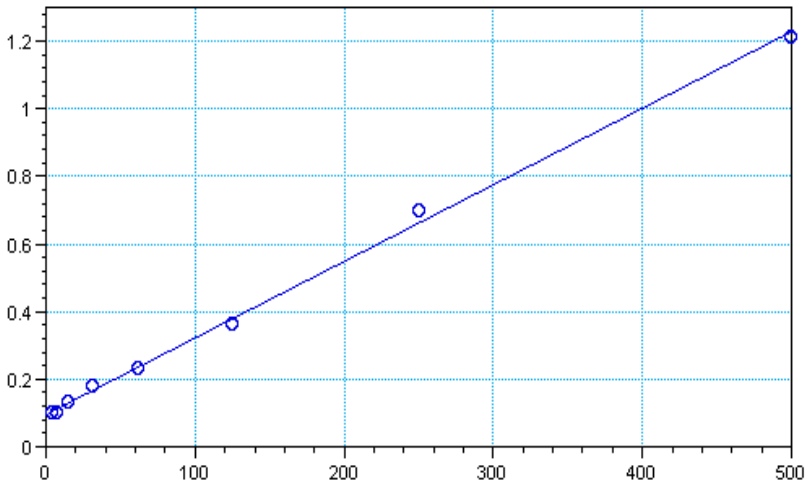



**Figure 72** A typical example of a standard curve that was used to determine the concentration of TNF-α produced by the cell stimulation assay. A new standard curve was created each time the ELISA was performed. The $R^2$ value for this curve was 0.998.

#### 4.3.2.1 Effect of compounds isolated from *Achillea fragrantissima* on TNF-α release

##### 4.3.2.1.1 Effect of AF17 on TNF-α release

The data showed that AF17 strongly inhibited LPS-induced TNF-α release from THP-1 cells in a concentration-dependent manner. The results are expressed as a mean of triplicate readings ± SD, n=3. Cells were treated with decreasing concentrations of AF17 (30, 15 and 7µg/ml) for one hour prior to stimulation with LPS (500pg/ml) and the presence of TNF-α in the supernatants was analysed. Stimulation with LPS resulted in a 300 fold increase over the control. Stimulated cells showed dramatically reduced levels of TNF-α in a dose dependant manner from 304±11 pg/ml to zero ($p \leq 0.001$), 141±15 and 176±17 pg/ml by 30, 15 and 7 µg/ml of AF17, respectively (Figure 73).

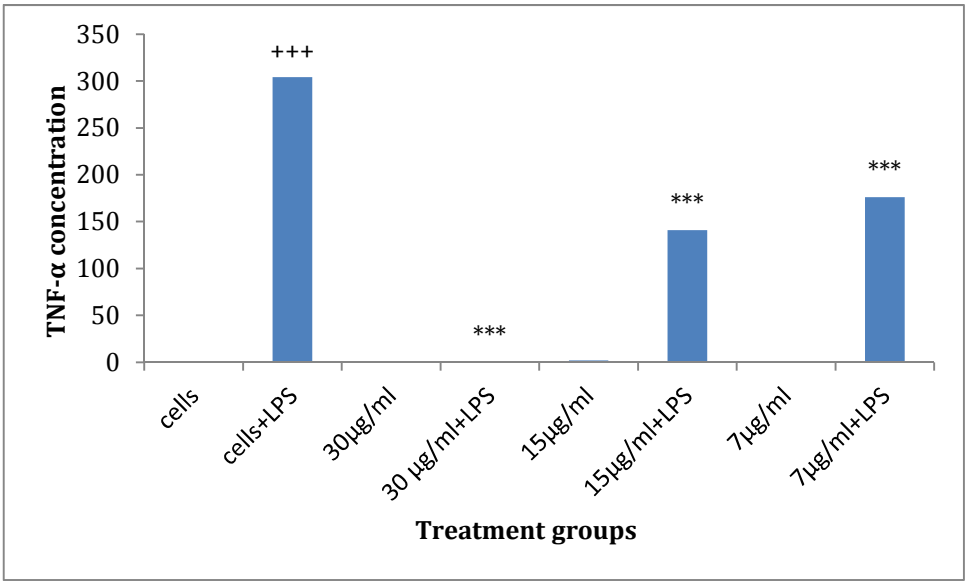



**Figure 73** Effect of AF17 on TNF-α released from THP-1 cells stimulated with LPS (500pg/ml). Three concentrations were used 30, 15 and 7µg/ml. Each bar represents the mean of nine sets of data ± SD (was always below 20pg/ml).

***indicates significantly ($p \leq 0.05$) lower values compared with the cells+LPS.

+++ indicates significantly ($p \leq 0.05$) higher values compared with cells only

### 4.3.2.2 Effect of AF18 (Chrysoplenol D) and AF36 (jaceosidin) on TNF-α release

Two flavonoids AF18 (chrysoplenol D) and AF36 (jaceosidin) were isolated from the methanol extract of *A. fragrantissima*. AF18 was examined for its potential inhibitory effects on LPS stimulated cells, which reduced the levels of TNF-α from 101±8 pg/ml to 31±10, 28±11 and 50±10pg/ml at concentration of 30, 15 and 7 µg/ml respectively. Expression of TNF-α induced a 100-fold increase in stimulated cells with respect to non-stimulated cells, the result was still significant ($p$=0.001) (Figure 74). While the addition of 30µg/ml AF36 (jaceosidin) almost abolished the TNF- α released and gave a significant ($p<0.05$) decrease in TNF-α production from 1101±20 pg/ml to 360±18, 464±11 pg/ml using 15 and 7 µg/ml from AF36, respectively (Figure 75).

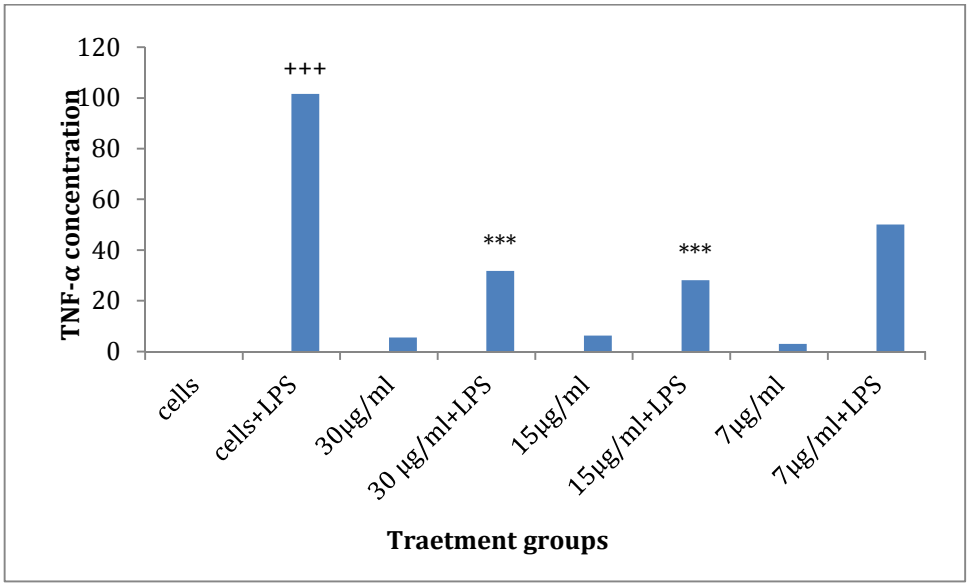



**Figure 74** the inhibitory effect of AF18 on TNF-α release from THP-1 cells stimulated with LPS (500pg/ml). Three concentrations were used 30, 15 and 7ug/ml. Each bar represents the mean of nine sets of data ± SD (was always below 20pg/ml).

***indicates significantly ($p\leq0.05$) lower values compared with the cells+LPS.

+++ indicates significantly ($p\leq0.05$) higher values compared with cells only

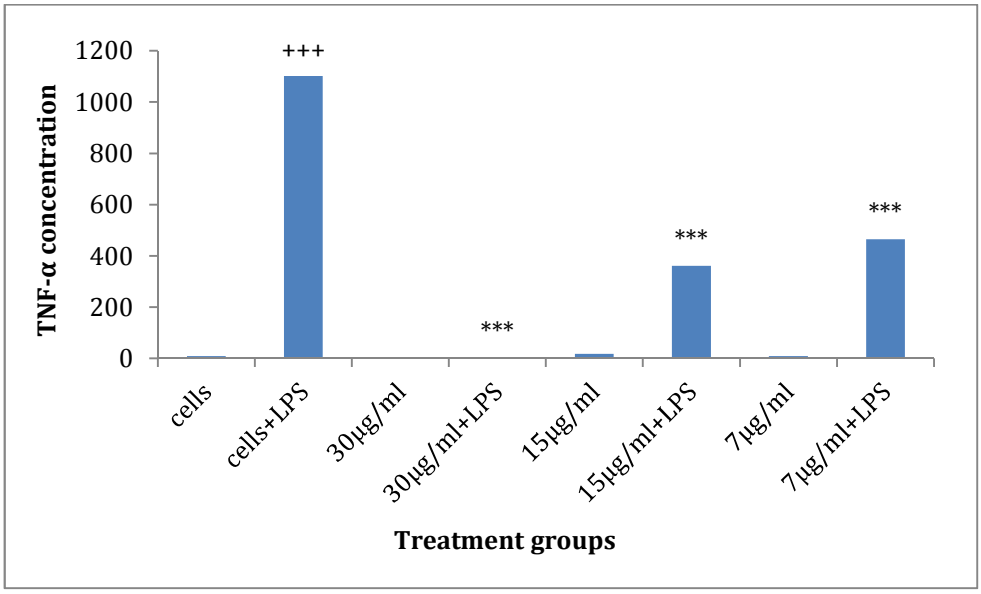



**Figure 75** the inhibitory effect of AF36 on TNF-α release from THP-1 cells stimulated with LPS (500pg/ml). Three concentrations were used 30, 15 and 7ug/ml. Each bar represents the mean of nine sets of data ± SD (was always below 20pg/ml).

***indicates significantly ($p \leq 0.05$) lower values compared with the cells+LPS.

+++ indicates significantly ($p \leq 0.05$) higher values compared with cells only

#### 4.3.2.3 Effect of compounds isolated from *Mentha longifolia* on TNF-α release

##### 4.3.2.3.1 Effect of ML7 on TNF-α release

The inhibitory effect of ML7 isolated from *M. longifolia* ethyl acetate extract was evaluated for its ability to inhibit TNF-α production in THP-1 cells stimulated with LPS (500pg/ml). ML7 (7, 15 and 30 µg/ml) markedly inhibited TNF-α production in a concentration dependant manner from 901±16 pg/ml to 832±15, 138±13 and 0 pg/ml, respectively. No TNF-α was detected in the un-stimulated cells (control) (Figure 76).

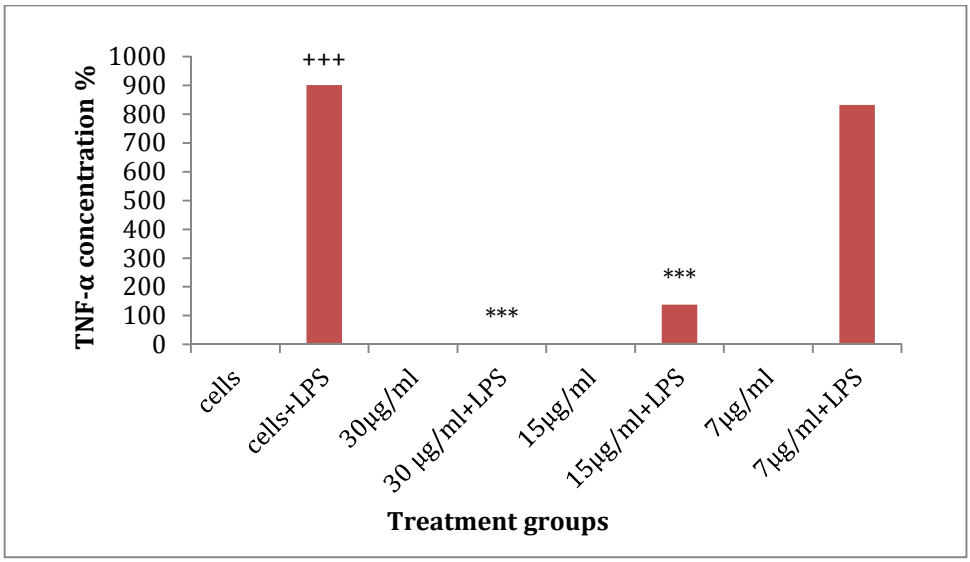



**Figure 76** the inhibitory effect of ML7 on TNF-α release from THP-1 cells stimulated with LPS (500pg/ml). Three concentrations were used 30, 15 and 7ug/ml. Each bar represents the mean of nine sets of data ± SD (was always below 20pg/ml).

***indicates significantly ($p \leq 0.05$) lower values compared with the cells+LPS.

+++ indicates significantly ($p \leq 0.05$) higher values compared with cells only

#### 4.3.2.3.2 Effect of ML10 and ML16 on TNF-α release

ML16 had an anti-inflammatory effect; however, it was not as potent as ML7. The 30, 15 and 7µg/ml concentrations reduced TNF-α from 584±19pg/ml to 227±14, 371±11 and 573±12pg/ml, respectively. In the case of ML10, reduction was from 756±17 pg/ml to 392±10, 562±15 and 766±11pg/ml for 30, 15 and 7 µg/ml, respectively. Both of the compounds showed TNF-α inhibition in a concentration-dependent manner (Figures 77 and 78).

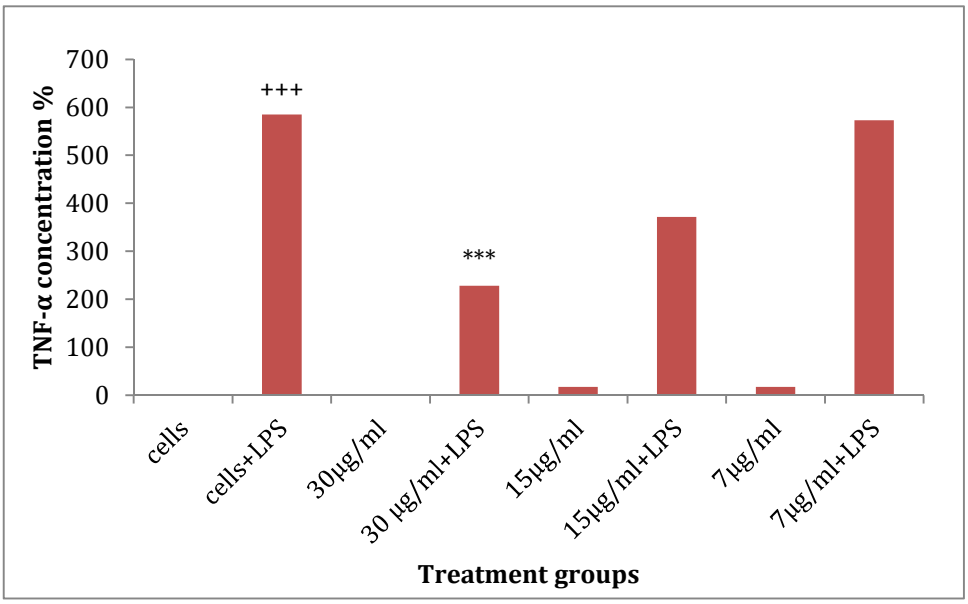



**Figure 77** the inhibitory effect of ML15 on TNF-α release from THP-1 cells stimulated with LPS (500pg/ml). Three concentrations were used 30, 15 and 7ug/ml. Each bar represents the mean of nine sets of data ± SD (was always below 20pg/ml).

***indicates significantly ($p \leq 0.05$) lower values compared with the cells+LPS.

+++ indicates significantly ($p \leq 0.05$) higher values compared with cells only

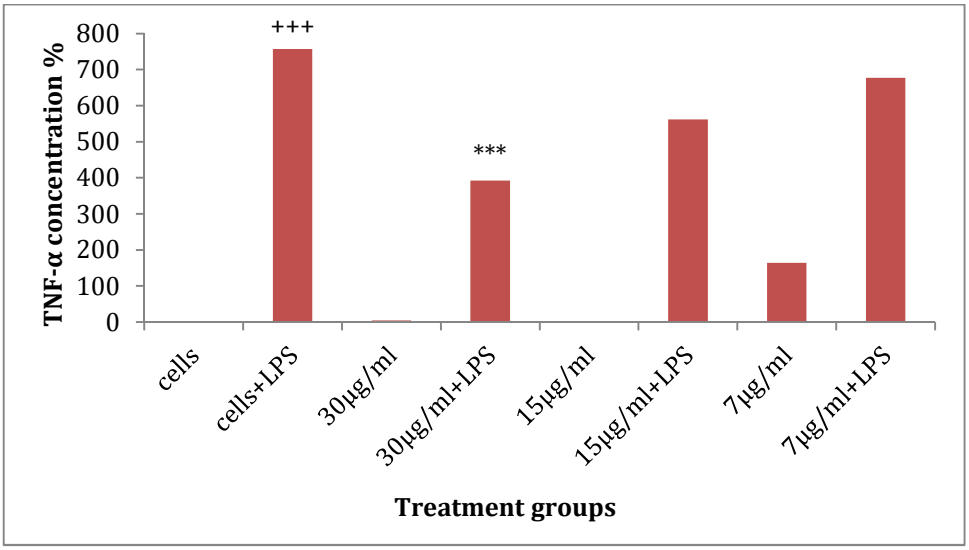



**Figure 78** the inhibitory effect of ML10 on TNF-α release from THP-1 cells stimulated with LPS (500pg/ml). Three concentrations were used 30, 15 and 7ug/ml. Each bar represents the mean of nine sets of data ± SD (was always below 20pg/ml).

***indicates significantly ($p \leq 0.05$) lower values compared with the cells+LPS.

+++ indicates significantly ($p \leq 0.05$) higher values compared with cells only

#### 4.3.2.3.3 Effect of ML1 and ML2 on TNF-α release

LPS-induced cells treated with linarin (ML1) and didymin (ML2) showed low to no TNF-α inhibition. Only the higher concentration of 30 µg/ml gave low inhibition from 1618.7±5.4 to 1012.5±9.3 pg/ml in the case of linarin and 1249±4.6 to 864±6.1 pg/ml in the case of didymin (Figure 79 and 80).

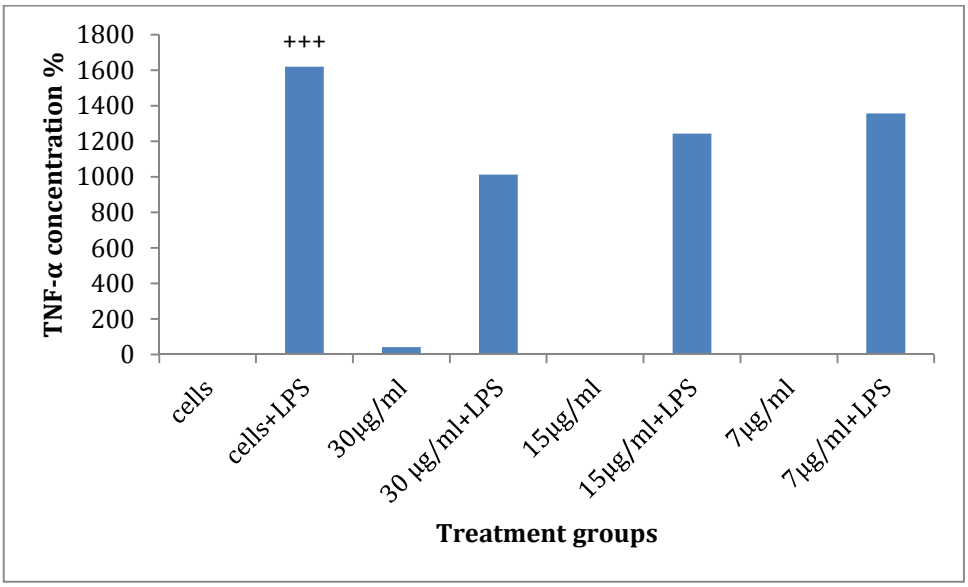



**Figure 79** the inhibitory effect of ML1 on TNF-α release from THP-1 cells stimulated with LPS (500pg/ml). Three concentrations were used 30, 15 and 7ug/ml. Each bar represents the mean of nine sets of data ± SD (was always below 20pg/ml).

+++ indicates significantly ($p \leq 0.05$) higher values compared with cells only

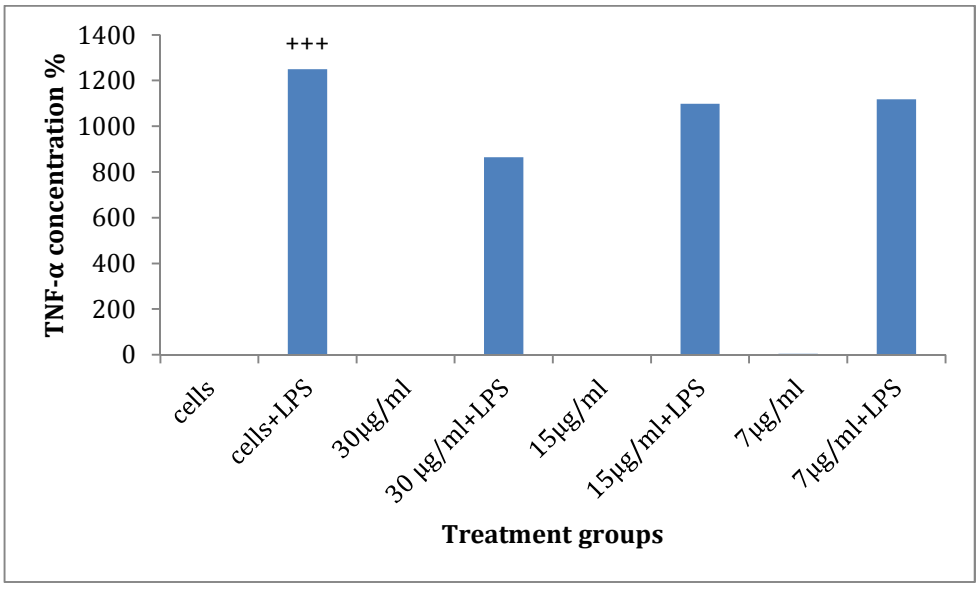



**Figure 80** the inhibitory effect of ML2 on TNF-α release from THP-1 cells stimulated with LPS (500pg/ml). Three concentrations were used 30, 15 and 7ug/ml. Each bar represents the mean of nine sets of data ± SD (was always below 20pg/ml).

+++ indicates significantly ($p \leq 0.05$) higher values compared with cells only

### 4.3.3 Evaluation of antibacterial activity in crude extract

The results of the evaluation of the antimicrobial activity of *Achillea fragrantissima*, *Mentha longifolia* and *Acacia nilotica* extracts and their isolated compounds against *B. subtilis*, *S. aureus*, *P.aeruginosa* and *E. coli* are reported in Tables 16 and 17. MICs of active extracts and compounds are in yellow and bold. The initial screening of antibacterial activity of the different plants extracts was assayed by a broth microdilution assay using serial concentrations in triplicate. All the extracts tested started at concentrations of 10mg/ml, the highest concentration of DMSO used was 1% (v/v).

All extracts tested showed some antibacterial activity against at least one bacterial strain. MIC of the extracts was substantially smaller than that caused by the antibiotic control. The most active plant extract was water extract of *A. nilotica* seeds with a MIC of 0.63 mg/ml against *E. coli,* 0.31 mg/ml against *S. aureus,* 0.625 mg/ml against *P.aeruginosa* and 0.63 mg/ml against *B. subtilis*. Followed by the chloroform extract of *A. fragrantissima* (MIC 1.25 mg/ml for *B. subtilis* and *S. aureus*) and ethyl acetate extract of *M. longifolia* (MIC 1.25 mg/ml *E. coli*, *B. subtilis* and *S. aureus*). The antibiotic gentamicin was active against all reference bacteria with a MIC in the range 50 µg/ml to 0.19 µg/ml. Based on these observations, no conclusion can be drawn regarding the correlation between activity and extract polarity. Generally, aqueous extracts showed poor or moderate antibacterial activity in most of the plant species, however, the aqueous extract of *Acacia nilotica* showed exceptional activity with MIC of 0.31 mg/ml against Gram-positive followed by 0.63 mg/ml for Gram negative bacteria.

A literature search on the traditional uses of *A. nilotica, A. fragrantissima* and *M. longifolia* identified them as species reputed to be effective in the treatment of infection, inflammation and wound healing (Boulos, 2000; Duke, 2002; Etuk *et al.*, 2009; Ndamba *et al.*, 1994). The antimicrobial activity of these plants has already been reported by various researchers (Banso, 2009); (Solomon-Wisdom *et al.*, 2010);

(Barel *et al.*, 1991); (Maswadeh *et al.*, 2006) (Gulluce *et al.*, 2007) & (Sarac *et al.*, 2009).

**Table 16** Antimicrobial activities of the plant crude extracts

| **MIC of the plant crude extracts (mg/ml)** | | | | | |
|---|---|---|---|---|---|
| **Plant extract** | ***Escherichia coli*** | ***Staphylococcus aureus*** | ***Bacillus subtilis*** | ***Pseudomonas aeruginosa*** | ***Candida albicans*** |
| **Acacia-water** | **0.63** | **0.31** | **0.63** | **0.63** | **0.31** |
| **Achillea-chloroform** | **2.50** | **1.25** | **1.25** | **2.5** | **1.25** |
| Achillea-butanol | 5.00 | 2.50 | 2.50 | 5 | 2.50 |
| Achillea-water | 10.00 | 5.00 | 10.00 | 10.00 | 5.00 |
| Mentha-hexane | 2.50 | 2.50 | 2.50 | 2.50 | 2.50 |
| **Mentha-ethyl acetate** | **1.25** | **2.50** | **1.25** | **1.25** | **1.25** |
| Mentha-methanol | 2.50 | 2.50 | 2.50 | 2.5 | 2.50 |
| Gentamicin | 0.78 | 0.39 | 0.39 | 3.125 | 0.78 |

*A. fragrantissima* is recognized as a powerful medicinal plant in the Bedouin community of Sinai, widely used as an eye lotion, for colic, indigestion, stomach pains and for female vaginal infections (used as decoction or infusion). The genus Achillea is also widely distributed and has been used medicinally by a number of other cultures for thousands of years (Frey *et al.*, 2010). In this study it showed antibacterial activity against *S. aureus, B. subtilis* and *C. albicans* with MIC of 1.25 mg/ml. A few numbers of studies have investigated the antibacterial properties of this species although attention was only given to the activity of its essential oils (Barel *et al.*, 1991) (Al-Gaby *et al.*, 2000). Furthermore, many studies have shown antibacterial properties of Yarrow (Stojanovic *et al.*, 2005) which is from the same genus and the results were similar to the ones presented here. To the best of my knowledge this is the first report of the effect of a polar extract for *A. fragrantissima.*

*A. nilotica* is used by the Bedouin community in Wadi Allaqi as an antiseptic, for treatment of ulcer, diarrhoea and genital infections (used as a decoction or infusion). Results show that water extracts of *A. nilotica* inhibited some of the species of bacteria used in this study with MIC of 0.625 to 0.3125 mg/ml. There are a few other

investigations of *A. nilotica* in the literature showing antibacterial, antifungal and antitrypanosomal properties (Banso, 2009) (Solomon-Wisdom *et al.*, 2010) (Satish *et al.*, 2009) (Mann *et al.*, 2011). In addition, there are studies demonstrating antimicrobial activity against urinary tract infections (Sharma *et al.*, 2009). Tannins are antimicrobial agents and soluble in water (Mlambo *et al.*, 2009). In this study, tannins were the main compounds found in *A. nilotica* extract mainly hydrolysable tannins, which were most likely to be responsible for the activity of the extract.

The ethyl acetate extract showed the best antibacterial activity for all the *M. longifolia* extracts and presented MICs in the range 2.5-1.25 mg/ml against *S. aureus, B. subtilis* and *E. coli*. *M. longifolia* is a highly valued plant within Bedouin communities in Sinai and Siwa. It has been used as an antispasmodic and for treatment of colds, stomach pain, for menstruation pain and sometimes as an antiseptic for genital infections. Most of the literature on *M. longifolia* described it for its anti-inflammatory, antioxidant and antispasmodic properties (Conforti *et al.*, 2008) (Mkaddem *et al.*, 2009) & (Shah *et al.*, 2010). Many studies have also been carried out on the antimicrobial activity of the essential oils (menthol, piperitenone oxide and piperitone) (Al-Bayati, 2009) (Koliopoulos *et al.*, 2010) (Shahverdi *et al.*, 2004) from *M. longifolia*.

### 4.3.4 Antifungal activity of the crude extract

*C. albicans* is an opportunistic pathogen that causes severe inflammation and plays a role in sexual transmitted diseases (Mulaudzi *et al.*, 2011). The results obtained showed that *C. albicans* is resistant to most of the plant extracts (Buwa *et al.*, 2006). Only the water extract of *A. nilotica* seeds showed antifungal activity with MIC value of 0.3125 mg/ml, followed by the *A. fragrantissima* chloroform phase extract (1.25 mg/ml) and ethyl acetate extract for *M. longifolia*. Regarding the other extracts, the activity was between 2.5 and 5mg/ml which are not considered high.

### 4.3.5 Antimicrobial activity for the isolated compounds

Results of the evaluation of the activity of the isolated compounds against *S. aureus, E. coli, B. subtilis*, *P. aeruginosa* and *C. albicans* are presented in Table 17. Most of

the compounds isolated exhibited activity against at least one organism tested. The concentrations tested ranged from 500 to 3.9 µg/ml; while the positive control gentamicin for bacteria and fluconazole for Candida ranged from 50 µg/ml to 0.19 µg/ml. Compounds with MIC less than MIC ≤100 µg/ml are presented in these results, while all the isolated compounds with their MIC are displayed in Table 17.

**Table 17** Antimicrobial activities of the isolated compounds from different plant extracts.

| MIC of the isolated compounds (µg/ml) | | | | | |
|---|---|---|---|---|---|
| **Isolated compounds** | ***Escherichia coli*** | ***Staphylococcus aureus*** | ***Bacillus subtilis*** | ***Pseudomonas aeruginosa*** | ***Candida albicans*** |
| **AF 17** | 250 | 250 | 250 | 500 | 500 |
| **AF 25** | 500 | 500 | 500 | 250 | 500 |
| **AF 18** | **125** | **62.5** | **62.5** | **62.5** | **31.25** |
| **AF 36** | **125** | **62.5** | **62.5** | **62.5** | **31.25** |
| **ML38** | 500 | 500 | 500 | 500 | 500 |
| **ML17** | 250 | 500 | 500 | 500 | **62.5** |
| **ML19** | 500 | 500 | 500 | 500 | **125** |
| **ML 7** | **62.5** | **125** | **62.5** | **125** | **7.8** |
| **ML10** | **125** | **125** | **125** | **125** | **125** |
| **ML16** | **7.8** | **31.25** | **62.5** | **31.25** | **125** |
| **ML 1** | 500 | 500 | 500 | 500 | 500 |
| **ML 2** | 500 | 500 | 500 | 500 | 500 |
| **AN 4** | **31.25** | **62.5** | **7.8** | **62.5** | **15.62** |
| **AN 5** | **62.5** | **62.5** | **62.5** | **62.5** | **62.5** |
| **AN 25** | 125 | 125 | 125 | 125 | 125 |
| **Gentamicin** | 0.78 | 0.39 | 0.39 | 3.125 | 0.78 |
| **Fluconazole** | - | - | - | - | 3.123 |

Gram-positive *B. subtilis* was the most susceptible bacterium; seven compounds showed good activity against the bacterium, with a MIC of 7.8 µg/ml for compound AN4 and 62.5 µg/ml for AN5, ML16, ML7, AF18 and AF36. The gentamicin positive control was 0.39 µg/ml. Six compounds (AF18, AF36, ML15, AN4, AN5 and ML7) with MIC ranging between 62.5 and 31.2 µg/ml, showed good activity against *S. aureus*. While the MIC for *S. aureus* was 31.2 µg/ml with ML15 and 62.5

µg/ml with AN4, AN5, AF18 and AF36. The best MIC was 7.8 µg/ml for ML15, while ML7, AN4 and AN5 showed good inhibition for *E. coli* with MIC of 62.5, 31.2 and 62.5 µg/ml, respectively. The activity against *P. aeruginosa* with a MIC value of 31.2 µg/ml was for ML16, while it was 62.5 µg/ml for AN5, AN4, AF18 and AF36. The best fungicidal activity was shown by ML7 with a MIC of 7.8 µg/ml with *C. albicans*, while the MIC were 62.5 µg/ml for AN5 and ML17, 15.6 µg/ml for AN4 and 31.2 µg/ml for both AF18 and AF36.

### 4.4 Evaluation of the antioxidant activity for the isolated compounds

The DPPH test provides information on the reactivity of compounds with a stable free radical DPPH that gives a strong absorption band at 517 nm in visible region. When the odd electron becomes paired off in the presence of a free radical scavenger the absorption reduces and the DPPH solution is decolorized as the colour changes from deep violet to light yellow. The degree of reduction in absorbance is reflective of the radical scavenging (antioxidant) power of the compounds (Agbafor *et al.*, 2011). The antioxidant activities of isolated compounds with DPPH are summarized in Table 18, compared with ascorbic acid as the positive control. Most compounds exhibited a concentration-dependent increase in their DPPH radical scavenging activity. The concentration range of the compounds tested was between 0.012 and 100µg/mL.

The radical scavenging activity displayed by compounds ML7 and ML16 (Figure 81) isolated from *M. longifolia* ethyl acetate extract with $IC_{50}$ of 5.7 and 1.9µg/ml, respectively, while the ascorbic acid showed an $IC_{50}$ of 3.7µg/ml. The maximum inhibition for the two compounds was 88% at 12.5µg/ml. The difference between the tested compounds and control was statistically significant with $p$ value $< 0.01$. The results are in agreement with (Fernandes *et al.*, 2008). For ML10 (Figure 82) scavenging activity had the same $IC_{50}$ value (3.7µg/ml) as ascorbic acid. The results are in agreement with the conclusion that the ability of flavonoids to inhibit oxidation processes is controlled by the presence of the double bond between C-2 and C-3 and a free hydroxyl in C-3 (Perez *et al.*, 2004).

Compounds ML1 and ML2 (Figure 83) showed moderate antioxidant activity with an $IC_{50}$ value of 39.3 and 42.8µg/ml, respectively. Compounds AF18 and AF36 (Figure 84) showed strong inhibitory activities with percentage inhibitions of 89 % and 86 % at a concentration of 100 µg/ml, while the $IC_{50}$ was 2.8 and 15.6 µg/ml, respectively.

Compounds AN4, AN5 and AN25 (Figure 85) isolated from the water extract of *Acacia nilotica* displayed very obvious antioxidant activity in the DPPH assay, up to 90% inhibition at 25 µg/ml for AN4, 86% inhibition at 25 µg/ml for AN5 and 75% inhibition at 25 µg/ml for AN25. The $IC_{50}$ was very high 0.4, 3.3 and 4µg/ml respectively) in comparison with ascorbic acid as standard 3.6µg/ml. Given the $IC_{50}$ for ascorbic acid it is not showed that scavenging activity for AN5, AN25 are very different from ascorbic acid, however, AN4, ML16, and AF18 appear to be much stronger scavenging activity.

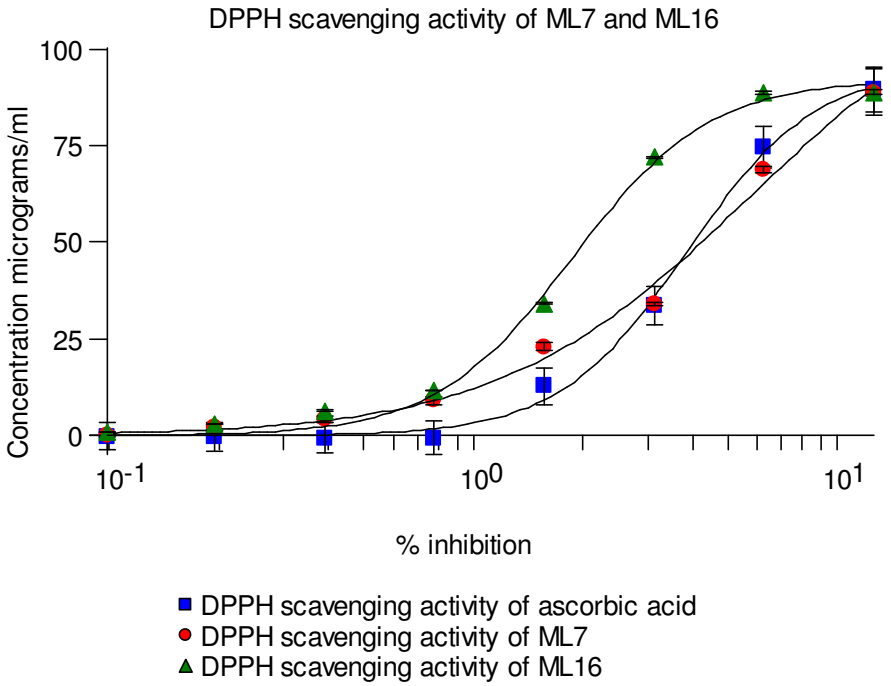



**Figure 81** DPPH antioxidant activity of ML7, ML16 and ascorbic acid (Asc.) as a positive control. Each points represents mean ± SD of triplicate reading. Data fitted to the Hill equation using Biograph Software (John Dempster, University of Strathclyde).

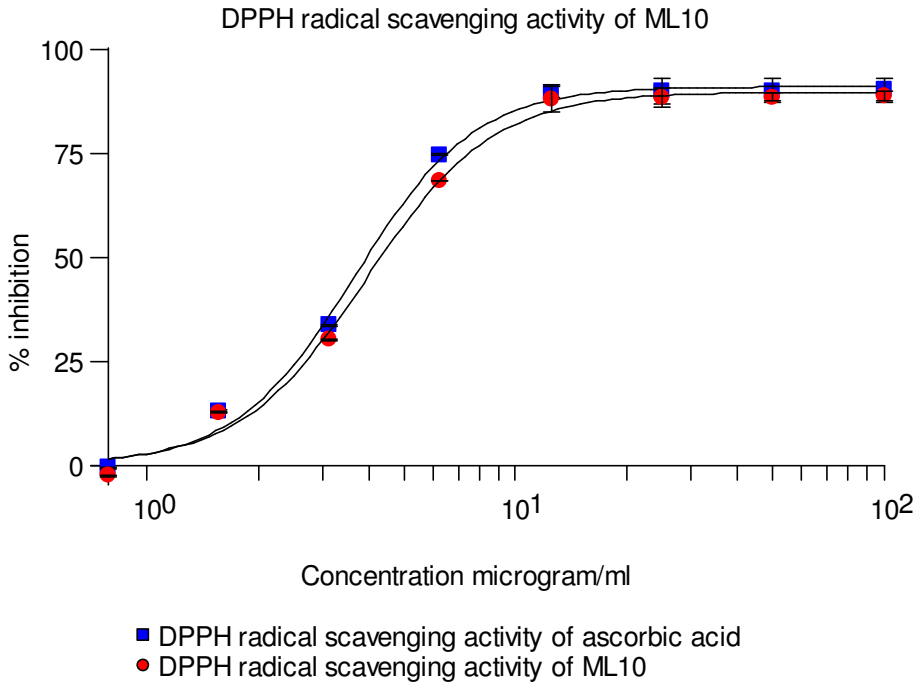



**Figure 82** DPPH antioxidant activity of ML10 and ascorbic acid (Asc.) as a positive control. Each points represents mean ± SD of triplicate reading. Data fitted to the Hill equation using Biograph Software (John Dempster, University of Strathclyde).

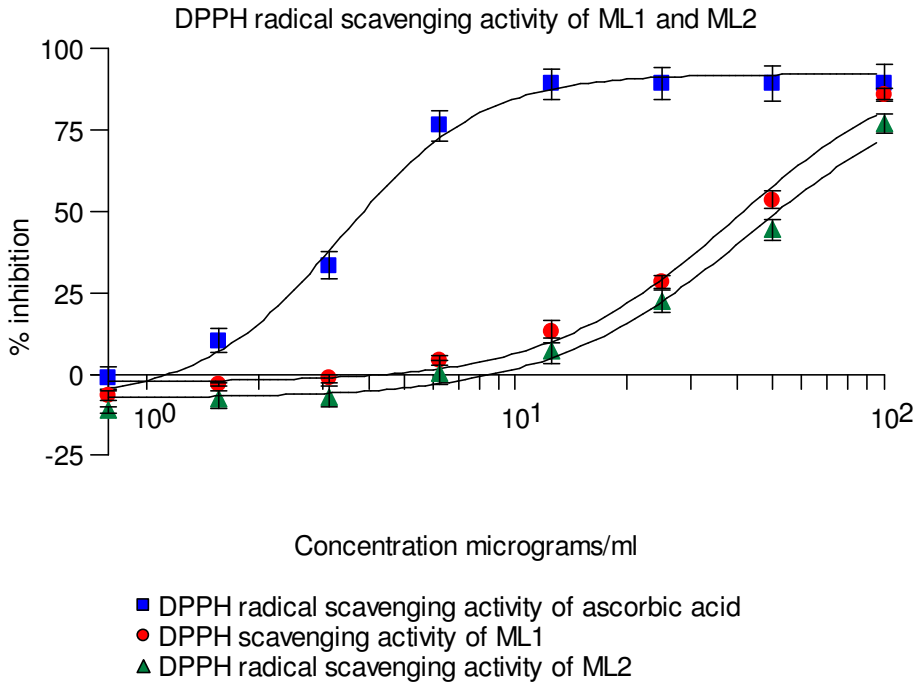



**Figure 83** DPPH antioxidant activity of ML1, ML2 and ascorbic acid (Asc.) as a positive control. Each points represents mean ± SD of triplicate reading. Data fitted to the Hill equation (with Rmin and Rmax fixed at 100%) using Biograph Software (John Dempster, University of Strathclyde).

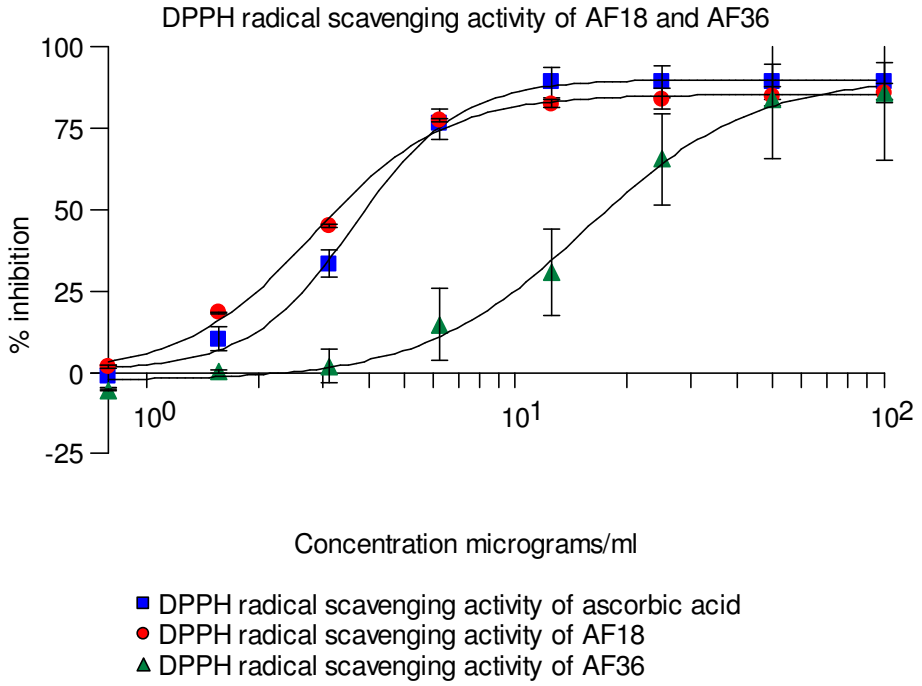



**Figure 84** DPPH antioxidant activity of AF18, AF36 and ascorbic acid (Asc.) as a positive control. Each points represents mean ± SD of triplicate reading. . Data fitted to the Hill equation (with Rmin and Rmax fixed at 100%) using Biograph Software (John Dempster, University of Strathclyde).

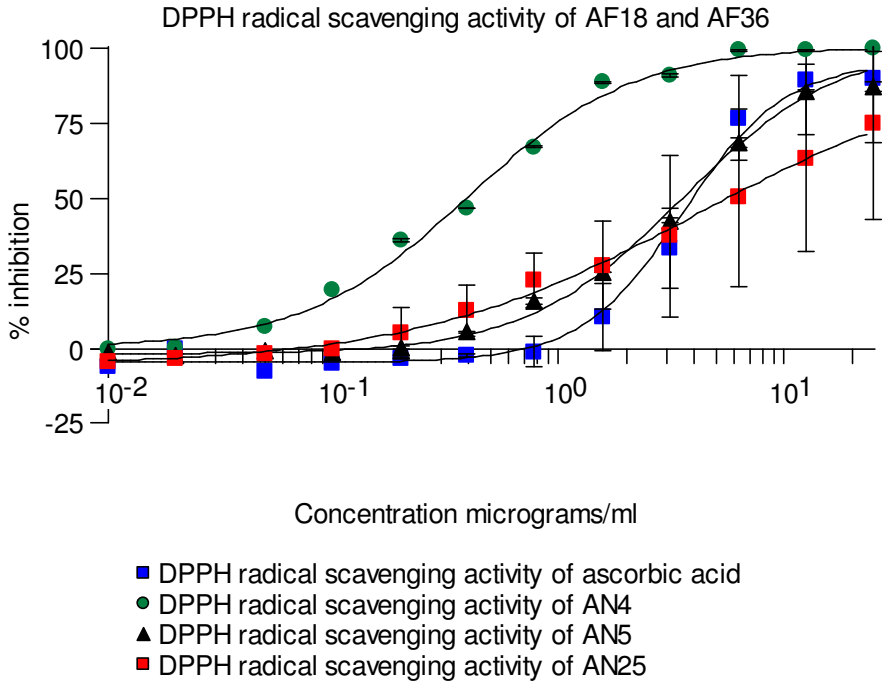



**Figure 85** DPPH antioxidant activity of An4, AN5, AN25 and ascorbic acid (Asc.) as a positive control. Each points represents mean ± SD of triplicate reading. Data fitted to the Hill equation (with Rmin and Rmax fixed at 100%, weighted to standard error for last eight points) using Biograph Software (John Dempster, University of Strathclyde).

**Table 18** Shows the DPPH radicals scavenging activity expressed in the $IC_{50}$ (µg) that is able to quench 50% of in a 200uM solution. Data represent means ± SD of three independent experiments performed in triplicate.

| **Compounds** | **$IC_{50}$ (µg)** |
|---|---|
| ML17 | - |
| ML19 | - |
| ML38 | - |
| ML7 | 5.7 |
| ML10 | 3.9 |
| ML16 | 1.9 |
| ML1 | 39.3 |
| ML2 | 42.8 |
| AF18 | 2.8 |
| AF36 | 15.6 |
| AF17 | - |
| AF25 | - |
| AN4 | 0.4 |
| AN5 | 3.3 |
| AN25 | 4 |
| Ascorbic acid | 3.7 |

## 4.5 Effects of *Haloxylon salicornicum* crude extract of on spontaneous contractions of the mouse uterus

In this part, the effect of the crude extract for *Haloxylon salicornicum* and its isolated compounds will be presented. Under control conditions, spontaneous contractions could be recorded, allowing the effects of the different concentrations of the extract to be examined. The crude extract, decreased uterine spontaneous contractility in a concentration-dependent manner (n = 5). At the concentration examined (3 µg/ ml), the extract almost abolished the contractions. The stimulatory effects of the crude extract could be seen within one minute of application and were maintained as long as the extract was present in the bath; the effect was reversed upon washout, an example of this is shown in Figure 86, whereas the addition of vehicle alone (0.1% DMSO) had no effect.

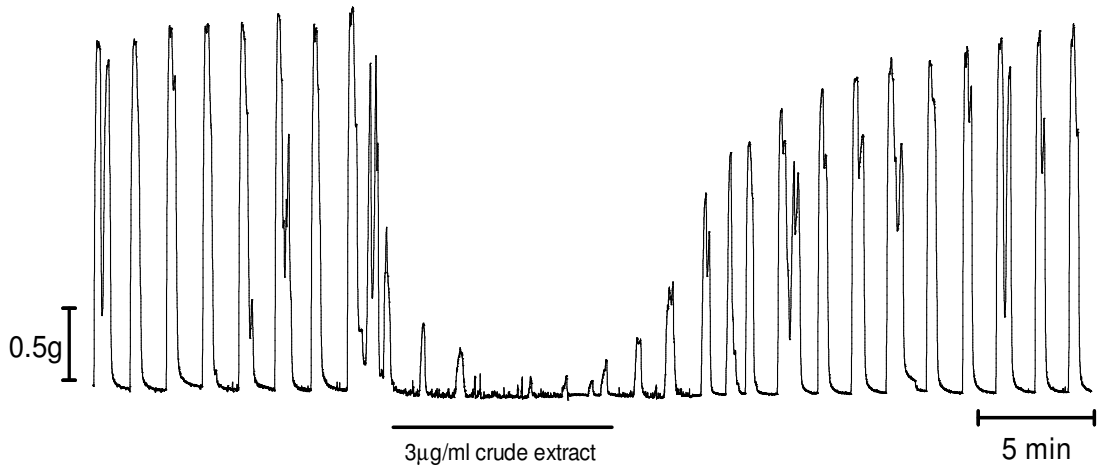



**Figure 86** the effect of 3µg/ml of the *Haloxylon salicornicum* crude extract on the spontaneous activity in nonpregnant uterine strips from mouse (35 min recording).

### 4.5.1 Effects of HS1 on spontaneous contractions of the mouse uterus

HS1 (0.1-3µg/ml) inhibited the spontaneous contractions in a concentration-dependent manner (Figure 87). Treatment with 1µg/ml from HS1 decreased the contractile response by 27.2±4.7 % when compared with control response (area under the curve or integral) (n=5) ($p<0.05$). This was due to HS1 (1µg/ml) decreasing the frequency from 0.017±0.001 to 0.006±0.002 Hz and decreasing the amplitude from 1.25±0.03 to 0.29±0.09g. The highest concentration of HS1 (3µg/ml) used in this study completely abolished the contraction in four out of five tissue examined (Figure 88). This inhibitory effect was reversed when the tissue was washed.

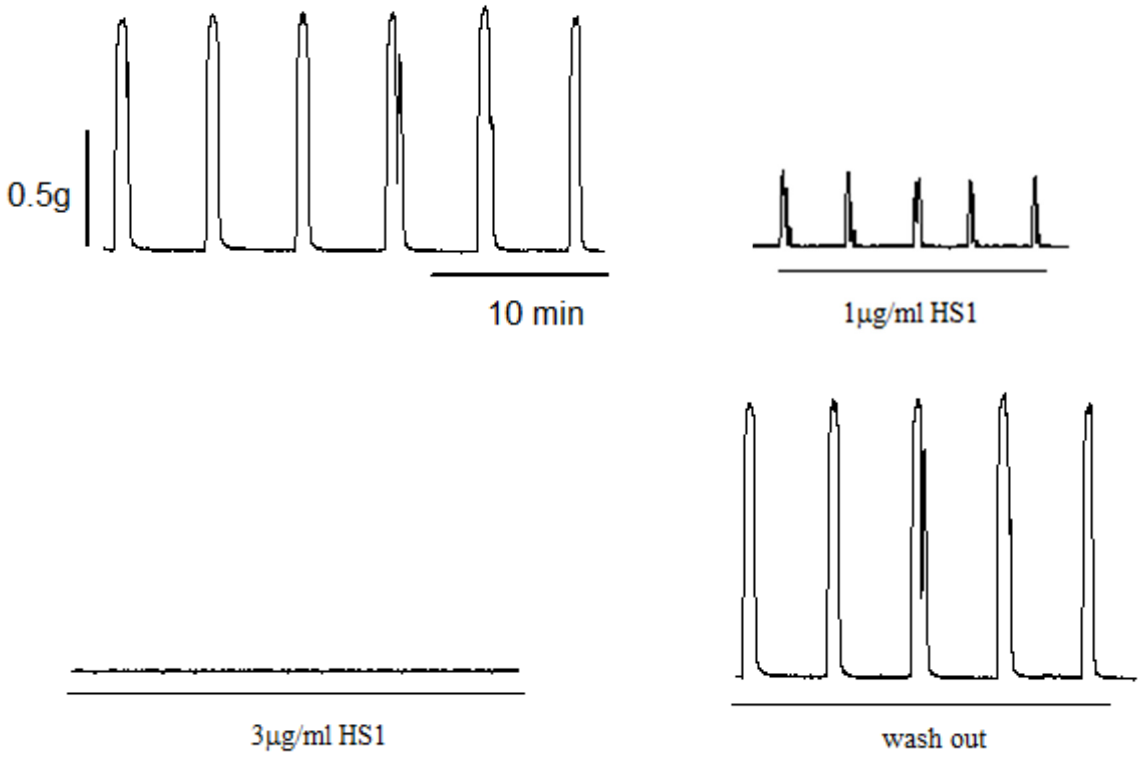
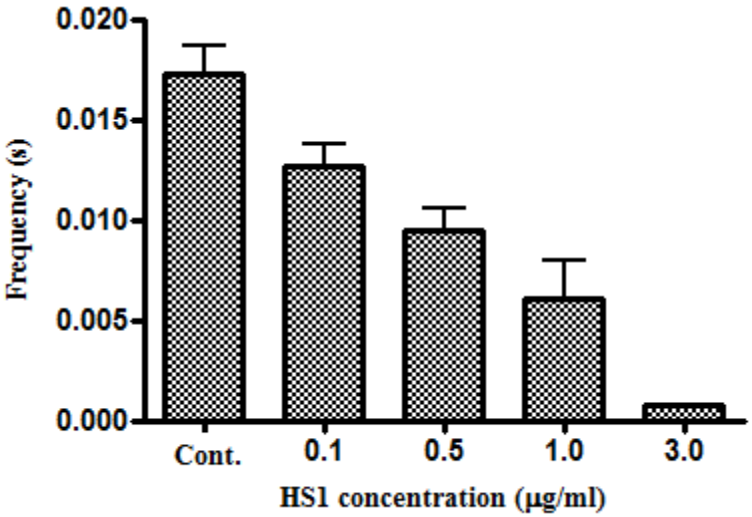
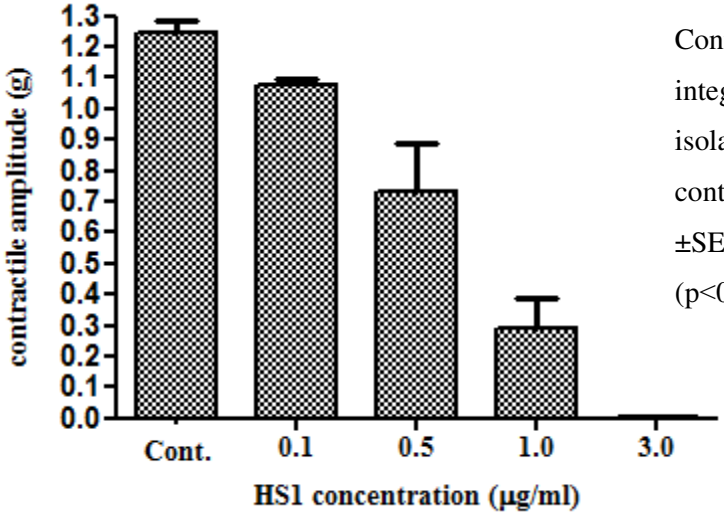



**Figure 87** Effect of HS1 on spontaneous contraction of the mouse uterus, traces showed control, the effect when adding 1µg/ml from HS1, the effect when adding 3µg/ml from HS1 and the recovery of the tissue after HS washed out.



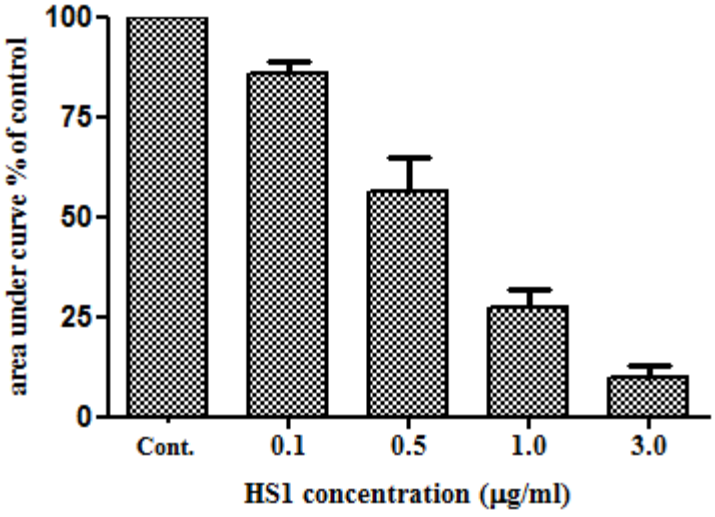




**Figure 88** Effect of HS1 on the spontaneous contractile activity of the mouse uterus. Contractile amplitude, frequency and integral of spontaneous contractions of isolated mice uterus (n =5). Primary contraction frequency and amplitude (mean ±SEM) indicate significant difference ($p<0.05$) compared to control.

### 4.5.2 Effects of synephrine on spontaneous contractions of the mouse uterus

Synephrine (1µg/ml) significantly ($p<0.05$) inhibited uterus spontaneous contraction (area under the curve) to 12.2±4.2 % when compared with control; while the contractions were completely abolished by the highest concentration of synephrine used (3µg/ml). Synephrine decreased the amplitude from 1.8±0.2 to 0.44 g, and decreased the frequency from 0.008±0.01 to 0.001 Hz (Figure 89).

### 4.5.3 Effects of HS2 on spontaneous contractions of the mouse uterus

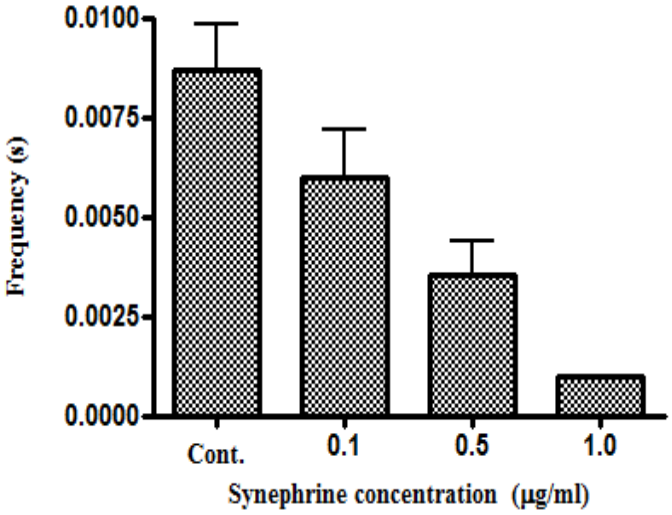
Application of HS2 (0.1-3µg/ml) also inhibited spontaneous contractions of mice uterus in a concentration-dependent manner (Figure 90). HS2 (1µg/ml) significantly ($p<0.05$) decreased spontaneous uterine contractions (area under the curve) to 34.17±6.7 % compared to the untreated contol. HS2 decreased the amplitude of spontaneous contractions from 1.76 ± 0.33 to 0.94 ± 0.05g, while the frequency was decreased from 0.014±0.004 to 0.002 ± 0.001 Hz.

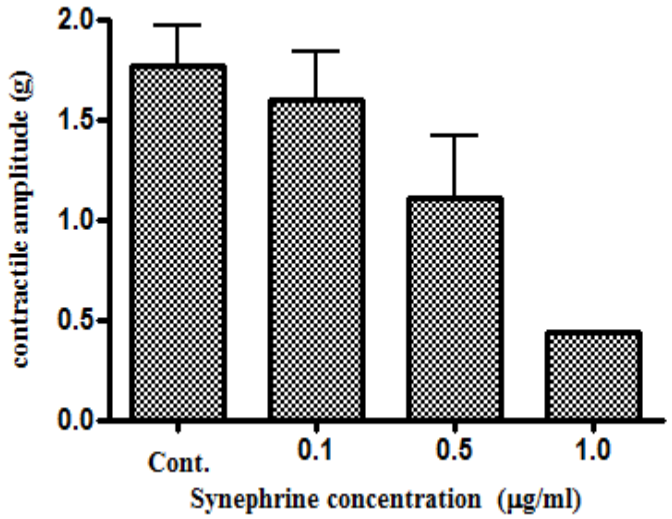
### 4.5.4 Effect of propranolol on the inhibition of produced by HS1, synephrine and HS2

The β-adrenoceptor blocker propranolol was added to the uterus and had no effect on the spontaneous contraction. The inhibition decreased to 98.4±8.0 when compared with control. However, in the presence of propranolol the inhibition effect of HS1 was blocked (Figure 91). The spontaneous contraction showed non-significant inhibition 89.2±6.9 % when compared with the control. Addition of HS2 did not show inhibition and the area under the curve decreased to 93.1±3.4 % and 85.4±3.4 in the case of synephrine.

### 4.5.5 Effect of HS1 on uterine contractions induced by $K^+$ depolarization

Adding KCl caused an initial rapid, phasic contraction; followed by a sustained tonic contraction. Adding HS1 (0.1-3 µg/ml) cumulatively inhibited of the contractions (Figure 92) and was completely blocked by the highest concentration of HS1 (3µg/ml).



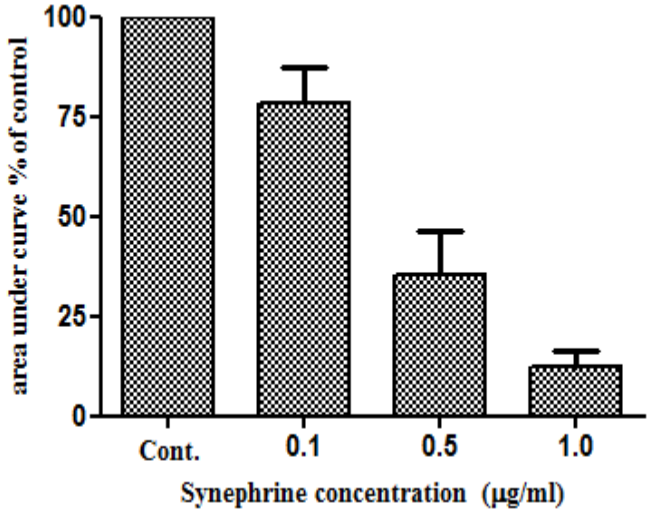
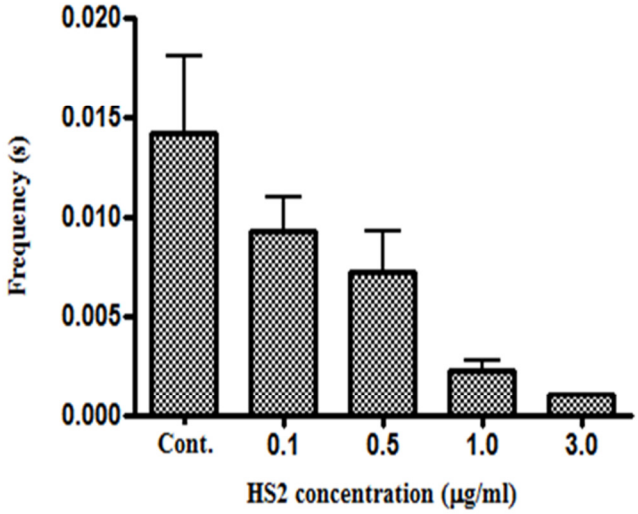
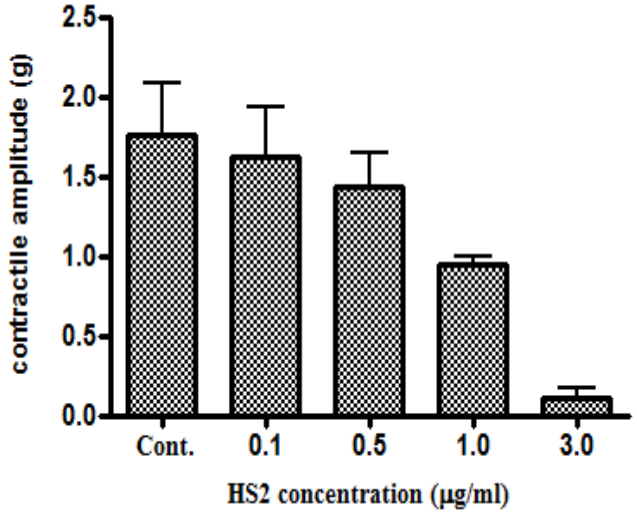




**Figure 89** Concentration–response bars for synephrine on the uterus. Contractile amplitude, frequency and integral of spontaneous contractions of isolated mice uterus (n =4). Primary contraction frequency and amplitude (mean±SEM) indicate significant difference ($p<0.05$) compared to control.



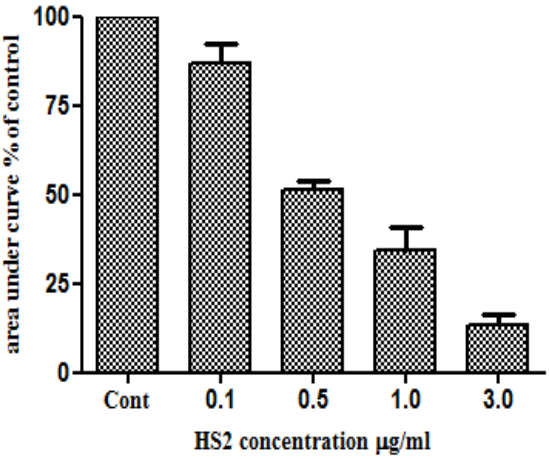




**Figure 90** Effect of HS2 on the spontaneous contractions of isolated mice uterus, amplitude, frequency and integral (n =4). Primary contraction frequency and amplitude (mean ± SEM) indicate significant ($p<0.05$) difference compared to control.

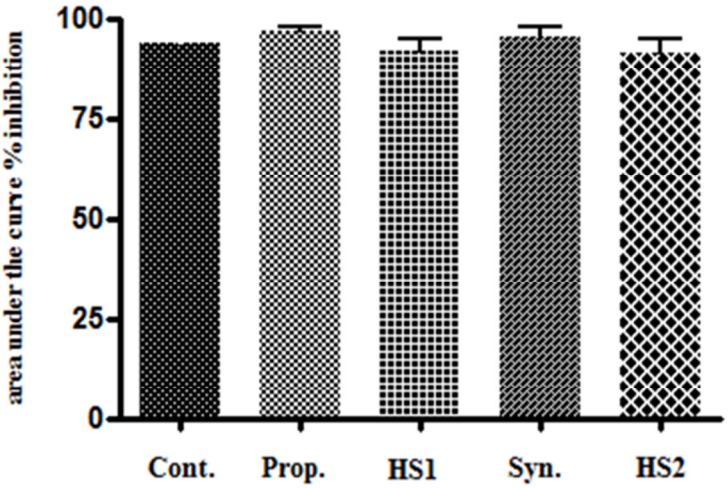



**Figure 91** Propranolol (20 µM/ml) had no significant ($p>0.05$) effect on either the curve or its parameters (amplitude and frequency) on non-pregnant mouse uterus. Treatment with propranolol antagonized the inhibitory response to HS1, synephrine and HS2 in the uterine muscle.

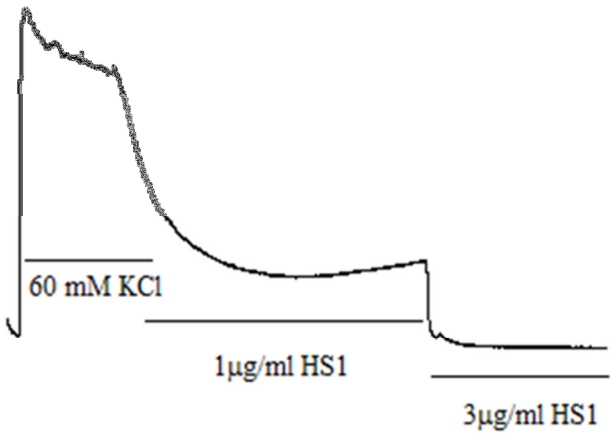



**Figure 92** shows a representative tracing of a KCl (60 mM)-induced contraction of the isolated mouse uterus. The inhibitory effect of HS1 on a high concentration of potassium (KCl 60 mM)-induced uterine contractions.

# CHAPTER FIVE

# DISCUSSION

# 5. Discussion

## 5.1 Ethnopharmacological information

Ethnopharmacological studies differ from natural products pharmacology in several ways, including that results should be meaningful for traditional users (Etkin, 2001). Bedouin communities rely on herbal remedies because of difficulty to access western medicine for culture, and socio-economic reasons. In this study, an ethnopharmacological survey was performed within Bedouin community in Egypt to identify the traditional uses of plants used by Bedouin women for illnesses related to the female reproductive health. Data collected showed that Bedouin use more than 40 plant species for treating their illnesses. The results also revealed that men and women are different in terms of their traditional knowledge about medicinal plants for primary health care (Singhal, 2005). For example, although *Haloxylon Salicornicum*, *Achillea fragrantissima*, *Artemisia judaica*, *Cleome droserifolia*, *Mentha longifolia* and *Solenostemma argel* have been recorded for their medicinal uses this is the first record of using them to treat female health and related symptoms. The fact that several traditional healers from different areas use these plants is a possible sign of their efficacy and safety. However, to produce phytoremedies from these plants that will be accepted as conventional medicine and sold as regulated drug, the existing and claimed biological activities need to be confirmed by scientific studies.

Although traditional medicine is well established in Egypt and has been a part of cultures and traditions for centuries, not much information has been documented in the scientific literature about Egyptian medicinal plants used by Bedouin women for their illnesses. Information on traditional medicine in these areas has been dominated by oral tradition. Since elderly people hold this information, the need for documentation is urgent. The development of drugs from traditional medicine by the scientific community would help to increase availability of drugs to a large majority of the impoverished communities and would maintain the biodiversity through a cultivation of plants to face their extended use.

## 5.2 Phytochemical investigation

From the ethnopharmacological survey, four plants species were chosen for phytochemical investigations, namely *Achillea fragrantissima*, *Acacia nilotica*, *Mentha longifolia* and *Haloxylon Salicornicum*. The isolation strategy used in this study was not based on bioassay-guided fractionation, although the latter was initially considered for this study; this approach was found inappropriate when based on the ELISA assay for TNF-α detection. This was due to two steps requirement (testing of compounds on THP-1 monocytes and detection of TNF-α using an ELISA assay) of this experiment that proved time consuming and yielded little information on the isolation of the active compounds. Instead, isolation was carried out independently of the activity of the isolated compounds in case of anti-inflammatory and anti-oxidant activity while the bioassay-guided was used for the antimicrobial activity and myometrium studies. The phytochemical investigation of these species led to the isolation of nineteen compounds (Chapter 3). These compounds included monoterpenes (ML17 and ML 19), sesquiterpene lactones (AF 17 and AF 25), alkaloids (HS1 and HS2), tannins and flavonoids (ML7, ML10, ML16, AF36 and AF18).

## 5.3 The anti-inflammatory effect of the isolated compounds

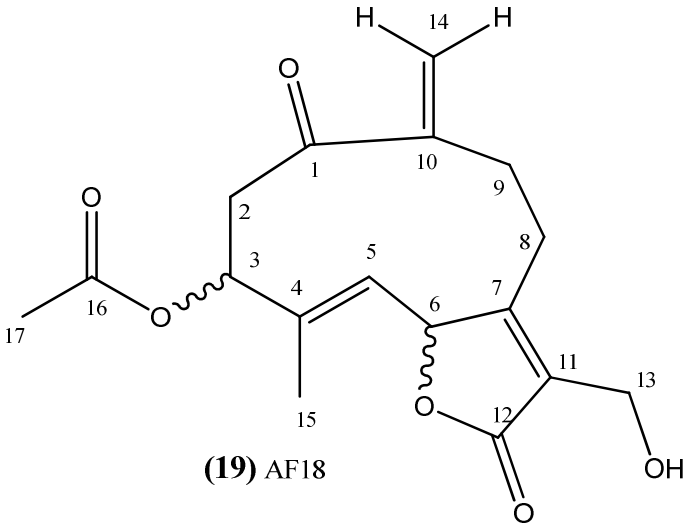
Cytokines and mediators contribute to the regulation of inflammation (Figure 58). TNF-α is a critical cytokine involved in inflammation and plays a complex and central role in responses to infection (Chao *et al.*, 2005). Inhibition of this factor is an important treatment strategy for inflammation-related diseases (Kwon *et al.*, 2010). In this study, eight compounds were isolated from *M. longifolia* and *A. fragrantissima* extracts suppressed production of TNF-α in THP-1 cells induced by LPS. Moreover, these compounds inhibited the cytokine generation in a dose-dependent manner. They proved highly efficacious in modifying the inflammatory responses induced by LPS in monocytes. THP-1 cells provide a good model for this study as they produce TNF-α and IL-1 pro-inflammatory cytokines when stimulated with LPS (Lee *et al*., 2008). Determination of the cytotoxicities of the crude extract and the isolated compounds revealed that they did not exhibit cytotoxicity on THP-1 cells at different concentration over the two days. This is an important assay and was

performed to calculate the highest concentration that could be used in the cell stimulation assay without killing the cells. TNF-α ELISA was carried out for the compounds and the pro-inflammatory cytokine (TNF-α) was analysed. Most of the compounds (AF17, AF18, AF36, ML7, ML10 and ML16) showed significant ($p \leq 0.05$) anti-inflammatory activity. However, the level of cytokine (TNF-α) produced was variable, this may have been due to different reasons as the ELISA kit used (sensitivity of the antibodies and the standard) or the possible mycoplasm contamination.

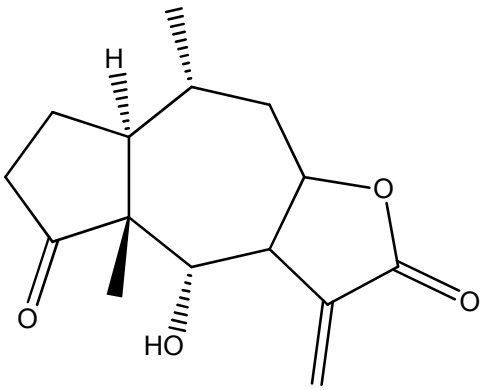
### 5.3.1 Sesquiterpene Lactones (SLs)

AF17 and AF25 (Sesquiterpene Lactones, SLs) were purified from the chloroform phase of *A. fragrantissima* methanol extract. AF17 (**19**) inhibited the release of TNF-α in stimulated cells in a concentration dependant manner. The suppression of LPS induced TNF-α and NF-κB for SLs has already been reported (Cho *et al.*, 2000; Feltenstein *et al.*, 2004). SLs are terpenoid compounds characteristic of the Asteraceae (Picman, 1986), which constitute a large and diverse group of plant constituents. Their wide variety of chemical structures is matched by a diversity of biological activities, including anti-tumor, anti-ulcer, anti-inflammatory, antimicrobial and cardiotonic activities (Robles *et al.*, 1995) and hepato-protective activity (Malarz *et al.*, 2002). They are also known to exhibit an inhibitory effect on TNF-α levels in LPS-activated systems *in vitro* and *in vivo* (Lee *et al.*, 1999).

Structural studies identified the exomethylene group in conjugation with the lactone group in SLs are the reason for the inhibitory activity (Hall *et al.*, 1979). Lacking the lactone or exomethylen group in the α-position to the lactone was shown to have no inhibitory effect on the pathway leading to NF-κB activation (Dirsch *et al.*, 2000). Recently, it has been confirmed that the types of biological activities displayed by SLs is due to the presence of the α-methylene-γ-lactones and α,β-unsaturated cyclopentenone ring (Chaturvedi, 2011). These structural elements react with nucleophiles, especially sulfhydryl groups by Michael type addition, thus rendering exposed thiol groups such as cysteines in protein molecules as their primary targets (Lyss *et al.*, 1997). In an oedema-induced carrageenan inflammation screen, the 6-hydroxy group of helenalin (**20**) was also required for potency (Itoh *et al.*, 2009).

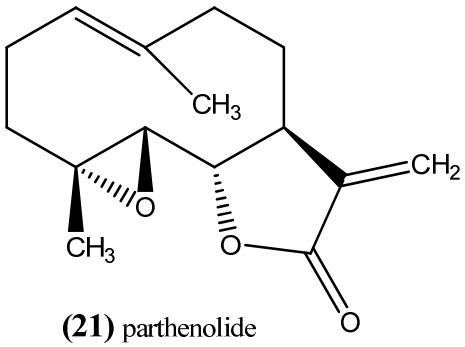



**(19)** AF18




**(20)** Helenalin

Since NF-κB plays a central role in most disease processes, and since it can regulate the expression of many key genes involved in inflammatory as well as in a variety of human cancers (Smahi *et al.*, 2002), NF-κB represents a relevant and promising target for the development of new chemo-preventive and chemotherapeutic agents (Chaturvedi, 2011). SLs have displayed potential anti-inflammatory activity through the NF-κB pathway (Castro *et al.*, 2000). Some of the SLs have displayed anti-inflammatory activity in a dose dependently inhibited LPS-induced NF-κB activation (Li-Weber *et al.*, 2002). Helenalin (**20**) and parthenolide (**21**) inhibit NF-κB activation in response to four different stimuli in T-cells, B-cells and epithelial cells (Hehne *et al.*, 1999).




**(21)** parthenolide

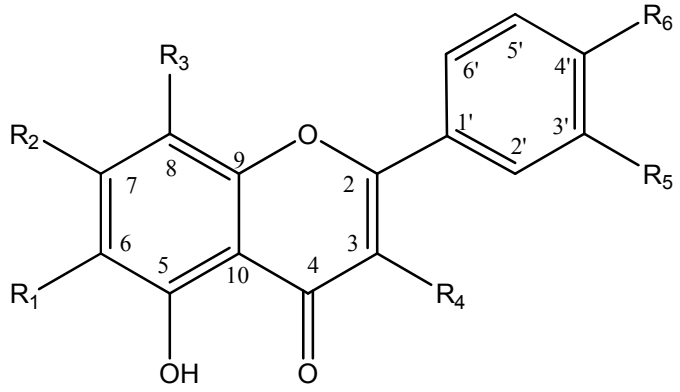
These results in this study entirely fit into the notion that SLs could serve as lead compounds for the development of potent anti-inflammatory drugs for the treatment of inflammatory disorders such as rheumatoid arthritis (Castro *et al.*, 2000) and justified Bedouin women use for these plants as analgesic, reducing or relieving pain as well as inflammation.

### 5.3.2 Flavonoids

Flavonoids are polyphenolic compounds that are reported to have promising anti-inflammatory properties (Middleton *et al*., 2000(Havsteen, 2002) besides being anti-oxidant and anti-microbial agents (Ueda *et al.*, 2004). Some known representatives of them are quercetin, myricetin, rutin and apigenin which are more efficient than traditional vitamins (Rao *et al.*, 2005). Many types of flavonoids including trifolirhizin, luteolin, chrysin and anthocyanidin inhibited LPS-induced expression of pro-inflammatory cytokines including TNF-α and interleukin-6 (IL-6) (Zhou *et al.*, 2009). The activity of some flavonoids related to their ability to interfere with NF-κB which is responsible for the expression of these pro-inflammatory cytokines and a down-regulation of its activity is a possible explanation for the observed reduction in TNF-α levels (Xagorari *et al.*, 2001). The ability of flavonoids to inhibit the arachidonate metabolism has been suggested as the reason for their anti-inflammatory action. Therefore, the protection of membrane lipids is important in inducing both anti-inflammatory and antioxidant activities (Middleton *et al.*, 2000).

*In vivo* and *in vitro* anti-inflammatory effects have been reported for several flavonoids. Thus, some 6-methoxylated flavones have been found to be active in different experimental models (Cottiglia *et al.*, 2005) and this was the situation in case of AF18 and AF36 which has been isolated for the first time from *A. fragrantissima*. The results have proved the potent *in vitro* inhibitory effects of AF18 and AF36 on TNF-α release, the results for AF36 (jaceosidin) section 4.3.2.2 were in agreement with (Min *et al.*, 2009) who described that jaceosidin markedly reduced TNF-α and IL-1β levels and inhibited COX-2 expression and NF-κB activation. In the literature, AF36 (Jaceosidin) inhibits IgE induced hypersensitivity (Lee *et al.*, 2007) suppresse E6 and E7 oncoproteins of HPV 16 cells (Min *et al.*,

2009) and induced apoptosis in ras-transformed human breast epithelial cells (Kim *et al.*, 2007). Orally administered AF36 (jaceosidin) blocked paw oedema induced by carrageenan (Lee *et al.*, 2007). A number of studies show that jaceosidin (AF36) has a significant effect on inflammation which it can reduce by inhibition of NF-κB (Nagao *et al.*, 2002); (Min *et al.*, 2009); (Clavin *et al.*, 2007; Lee *et al.*, 2007). AF36 also inhibits phorbol-ester-induced up-regulation of COX-2 and matrix metalloproteinase-9 as well as ear oedema in mouse (Jeong *et al.*, 2007). AF36 suppresses inducible nitric oxide synthase expression in LPS induced RAW264.7 macrophages (Moscatelli *et al.*, 2006). AF18 was found to have anti-malaria (Stermitz *et al.*, 2002) and anticancer activity which may be contributed by an induction of apoptosis involving cytochrome *c* release from mitochondria to cytosol (Lv *et al.*, 2008). However, this is the first report for the anti-inflammatroty activity for it.




| | R1 | R2 | R3 | R4 | R5 | R6 |
|---|---|---|---|---|---|---|
| ML7 = | OH | OCH3 | OCH3 | H | OCH3 | OCH3 |
| ML10= | OH | OCH3 | OCH3 | H | OH | OCH3 |
| ML16= | OH | OCH3 | OCH3 | H | H | OH |
| AF18 = | OCH3 | OCH3 | H | OCH3 | OH | OH |
| AF36 = | OCH3 | OH | H | H | OCH3 | OH |

The anti-inflammatory activity of the isolated flavonoids ML7, ML10 and ML16 from *M. longifolia* was also evaluated on LPS induced THP-1 cells. These flavonoids were found to have anti-inflammatory activity by down regulating TNF-α level (pages 147-149). B-ring substituted flavones are capable of inhibiting carrageenan and ear oedema in rats (Cottiglia *et al.*, 2005). Nevertheless, the influence of the substitution pattern on the inhibition of inflammation is not yet completely understood (Clavin *et al.*, 2007), this could explain the anti-inflammatory activity (which is recorded for the first time) of these flavones dispite their different substitution patterns.

The effect of ML1 and ML 2 on LPS-induced cytokines production showed low inhibition for TNF-α. This may be a result for using very low concentrations (starting from 30µg/ml) hence using higher concentrations may be more effective especially as these two compounds did not show any toxicity on the THP-1 cells with concentrations up to 5 mg/ml. *In vivo* experiments may imply other physiological effects not found in *in vitro* experiments (Ueda *et al.*, 2004). The acid hydrolysis and absorption of flavonoids in the stomach may also be an important factor in judging their many alleged health effects. This could be the case with ML1 (linarin) and ML2 (didymin) isolated in this study because they are flavonoids glycoside. Didymin has selective anticancer effects on human non-small-cell lung cancer (Hung *et al.*, 2010). Also the antioxidant activity of flavone glycosides including didymin have been highlighted (Ramful *et al.*, 2010). While the anti-inflammatory activity of linarin has been reported (Martinez-vazquez *et al.*, 1998) as well as the analgesic and antipyretic activities (Martinez-vazquez *et al.*, 1996) sedative and sleep-enhancing properties (Fernández *et al.*, 2004) and selective inhibitory effect on acetylcholinesterase (Oinonen *et al.*, 2006).

Recently, linarin was found to be a potent therapeutic compound against Alzheimer's disease acting through both acetylcholinesterase inhibition and neuroprotection (Lou *et al.*, 2011) and can protect osteoblasts against hydrogen peroxide-induced osteoblastic dysfunction and may exert anti-resorptive actions (Kim *et al.*, 2011). For

exact confirmation of the anti-inflammatory effects of ML1 and ML2 *in vivo* investigation is needed.

## 5.4 Antimicrobial effect of isolated compounds

The results presented in this study demonstrate that *M. Longifolia*, *A. fragrantissima* and *A. nilotica* used by the Bedouin women to treat infectious diseases have good antimicrobial activity.

Only one study has documented the antimicrobial activity of the phenolic compounds in *M. longifolia* (Gursoy *et al.*, 2009). This study produced the first report for antimicrobial activity of these flavones ML7, ML10 and ML16 (from ethyl acetate extract) and the potent antioxidant activity (section 4.4). Flavones, flavonoids, and flavonols have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Cushnie *et al.*, 2005). According to the literature, the activity of a number of structurally different flavonoids including flavones have shown that 5-hydroxyflavanones and 5-hydroxyisoflavanones with one, two or three additional hydroxyl groups at the 7, 2' and 4' positions inhibited the growth of *Streptococcus mutans* and *Streptococcus sobrinus* (Cushnie and Lamb, 2005). This could be applied in the case of ML16, which has additional one OH group on 4' positions. Flavones with different hydroxy and methoxy substitution patterns, showed a good range of activity on tested microorganisms ((Wang *et al.*, 1989). Results in this study correlate well with those of the literature as ML7, ML10 and ML16 showed a wide range of activity from 7.8 to 125 µg/ml against *B. subtilis*, *S. aureus*, *P.aeruginosa*, *E. coli* and *C. albicans*. While M17, a terpenoid from *M. longifolia* hexane extract, showed a MIC of 62.5ug/ml for *Candida albicans,* this result was consistent with (Manenzhe *et al.*, 2004).

The results showed the antimicrobial activity of AF18 (chrysosplenol D) and AF36 (jaceosidin) isolated from *A. fragrantissima*, which is used by Bedouin women of Sinai for the treatment of infections related to the genital tract. AF18 is a methylated flavonoid isolated from the chloroform phase of the methanol extract of *A.*

*fragrantissima*. It was initially isolated from the Chinese medicinal plant *Artemisia annua* (Kraus *et al.*, 2008). Since then was been characterized from many different species including some of the *Achillea* genus (Valant-Vetschera *et al.*, 1994). In this study, this is the first report of chrysoplenol D from *A. fragrantissima*. The importance of AF18 comes from its ability to potentiate the anti-malarial activity of artemisinin and from its ability to potentiate the activity of norfloxacin, and inhibition of multidrug resistance (MDR) efflux pumps in *Plasmodium falciparum* and in *S. aureus* (Stermitz *et al.*, 2002). Jaceosidin isolated from the herb of the genus *Artemisia* (Lv *et al.*, 2008) also has an inhibitory effect on COX-2 and MMP-9 in human mammary epithelial cells, suppressing E6 and E7 oncoproteins of HPV 16 (Min *et al.*, 2009), inhibits IgE induced hypersensitivity (Lee *et al.*, 2007) and inducing apoptosis in ras-transformed human breast epithelial cells (Kim *et al.*, 2007). This is the first isolation for jaceosidin from *A. fragrantissima*. No previous reports on the antibacterial activity of the jaceosidin could be found in literature.

The antimicrobial activity for AF17, isolated from *A. fragrantissima*, was found to be higher than that AF25 has, this could be due to an extra keton group of AF17. SLs are known for their anti-protozoa, anti-bacterial and anti-fungal properties (Karamenderes *et al.*, 2006). Most of the literature describes antiprotozoal activity of SLs against *Plasmodium falciparum* (Maas *et al.*, 2011), antitrypanosomal activity (Julianti *et al.*, 2011) and antibacterial activity against methicillin resistant *S. aureus* ((Bach *et al.*, 2011). The structure relationship activity for SLs was significantly correlated with cytotoxicity and the major determinants for activity are α,β-unsaturated structural elements (Schmidt *et al.*, 2009). Despite this cytotoxicity, SLs are candidates for evaluation as antibiotics in topical preparations against skin-associated pathogens (Bach *et al.*, 2011). The antimicrobial results in this study could explain the use for *A. fragrantissima* as an antiseptic by Bedouin women.

The present results raise some interesting expectations, regarding the potential use of *A. nilotica* extract as a source of some sort of antimicrobial agents. Phytochemical analysis for the seeds of *A. nilotica* showed a high percentage of tannins containing up to 25% of gallic acid and methyl gallat (AN4 and AN5) respectively. Numerous

studies have reported the antibacterial activity of such compounds against various microbial pathogens (Adesina *et al.*, 2000) (Panizzi *et al.*, 2002). Several tannins with different structures have also inhibited microbial growth *in vitro* (Chung *et al.*, 1998; Salvat *et al.*, 2001).

In general, there were only four compounds that showed activity against both gram-negative (*P. aeruginosa* and *E. coli*) and the gram positive (*B. subtilis S. Aureus*) bacteria. Gram-negative bacteria are more resistant to drugs than their gram-positive. Gram-negative bacteria have an outer membrane acting as a barrier to environmental substances including antibiotics, the observed results agreed with those of (Nasar-Abbas *et al.*, 2004). According to a number of references ((Gibbons, 2004) (Ríos *et al.*, 2005) (van Vuuren, 2008) natural products with MIC values below 1.0 mg/ml are considered noteworthy. In this study, most of the plant extracts failed to exhibit this level of activity (less than 1 mg/ml) against the tested microorganisms. However, negative results do not mean that the plant extracts are inactive or that there is an absence of bioactive compounds. Active compounds may be present in insufficient quantities in the crude extracts for them to show activity with the concentrations assayed. For example, solubility issues became quite challenging in preliminary screening; most of the hexane extracts showed very weak activity, or no activity which could have been due to the poor solubility of non-polar ingredients in a water-based medium. However, further testing was carried out on the purified compounds from hexane extract.

The Microtitre plate assay is an effective method used to assess antimicrobial activity and MIC. However, in some cases the process proved difficult. When using fluorescence as a viability indicator, the indicator could interfere with the compounds and lead to misinterpretation of results (Clarke *et al.*, 2001). The first attempt was to use Alamar blue or MTT as an indicator, but the interference was very high so p-iodonitrotetrazolium violet ( *p*-INT) was used instead, which has been shown to be the most stable indicator when determining the MIC of plant extracts (Eloff, 1998) (Figure 93). To avoid this problem small concentration from the extract was used and the more traditional method, such as plating out a serial dilution (Othman *et al.*,

2011) used in conjunction with the tetrazolium assay. Moreover, it is possible that the antimicrobial effect of these plants is mediated through immune-stimulation rather than direct inhibition of bacterial or fungal growth. Also we have no evidence that the bacteria used in this study (e.g *B. subtilis*) were indeed the causal agents underlying the symptoms associated with the use of these plants (e.g., decoctions of *A. nilotica* plants to treat vaginal infection).

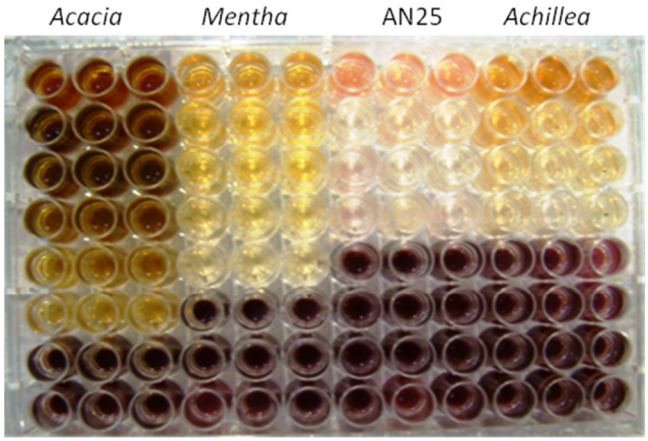



**Figure 93** Example of the colour interference of the test extract with *p*-INT. Results obtained for *Acacia nilotica* water extract, *M. longifolia* ethyl acetate *A. fragrantissima* chloroform extract and AN25 from *A. nilotica* against *E. coli*.

## 5.5 Antioxidant effect of the isolated compounds

The flavonoids isolated from *M. longifolia* and *A. fragrantissima* showed high antioxidant activity. The free radical-scavenging activity of flavonoids is dependent on the presence of free OH groups. Flavonoids with a 3-OH and 3', 4'-cathecol are reported to be 10-fold more potent than the corresponding catechol and 3-OH free flavonoids (Sharififar *et al.*, 2009), which was in agreement with the result from AF18, AF36, ML7, ML10 and ML16. It is known that the antioxidant activity of the flavonoids is closely associated with its structure, such as substitutions on the aromatic ring and side chain structure (Majo *et al.*, 2005). Antioxidant activity decreases with glycosylation and is enhanced with hydroxylation and the presence of C2–C3 double bond in conjugation with a 4-oxo function (Rice-Evans *et al.*, 1996).

This could explain the low activity for ML1 and ML2 that are substituted on C-8 by two sugars.

Gallic acid (AN5) and methyl gallate (AN4) isolated from *A. nilotica* are well-known natural antioxidants found widely in plants. Methyl gallate was reported to have protective effects against hydrogen peroxide-induced oxidative stress and DNA damage in MDCK cells (Hsieh *et al.*, 2004). The hydrolysable tannins act as tyrosinase and hyaluronidase inhibitors, suggesting they are useful additives not only for antioxidation in food, but also for anti-melanogenesis, antiallergy and anti-inflammation in food and cosmetics (Sugimoto *et al.*, 2009). They have shown cytoprotective properties (Gonzales *et al.*, 2000) and have been associated into antiulcerogenic activity (Ramirez *et al.*, 2003) and prevent ulcer development due to their protein precipitating and vasoconstrictions effects (Berenguer *et al.*, 2006). This result justifying Bedouin women used for the water extract from *Acacia nilotica* as antiseptic and for vaginal infection.

### 5.6 Myometrium inhibition by *Haloxylon salicornicum*

Bedouin women in Egypt used *Haloxylon salicornicum* to treat dysmenorrhoea or painful periods and to cleanse uterus. The present study characterized the inhibitory response of *Haloxylon salicornicum* aqueous extract on spontaneously contracting muscle preparations from mouse uterus. Phytochemical analysis showed that HS1 (synephrine) and HS2 (N-methyltyramin [NMT]) isolated from this plant caused inhibition of muscle contractility in a dose dependent manner.

Synephrine (commercially available ) was first reported as a synthetic compound by Legerlotz in Germany in 1927 and the first isolation from natural source was by Stewart et al. (1964) while, NMT isolated from acacia seeds and barley (Adams *et al*., 1966; Hirofumi Koda *et al*., 1999). Phenolic amines are a class of secondary metabolites found in plants that have potential health promoting (Bartley *et al*., 2010; Mercader *et al*., 2011). Synephrine is used as a sympathomimetic agent (Mercader, 2011) and as an antihistamine in the treatment of common colds (Stewart, 1964). Recently, synephrine has received increasingly more attention as results from several

animal studies and human clinical trials (Haaz *et al*., 2006) suggest that it has promise as an aid to weight management and obesity reduction (Mercader 2011). It is also a vasoconstrictor and bronchiectatic agent (Harumi *et al*., 1999). NMT is reported to increase blood pressure in anesthetized rats, to relax guina pig ileum and to increase both the intensity and rate of contraction of guinea pig right atrium by inducing the release of norepineprine (Evans *et al*., 1979).

The results demonstrate that HS1 (synephrine), one of the major components of *Haloxylon salicornicum*, exerted significant relaxation effects on spontaneous uterine contractions in a dose dependent manner. The relaxation activity of HS1 and HS2 was attenuated in KCl contracted uteri and blocked by the addition of propranolol (non-selective β-adrenceptor antagonist). This could explain that these compounds effect as β-adrenoceptors agonist.

β-adrenoceptors are responsible for smooth muscle relaxation in many organs including uterine muscle (Emilien *et al*., 1998). $\beta_2$-Adrenoceptor agonists are of therapeutic potential due to their use for asthma (D'Urzo *et al*., 2010) and to inhibit pre-term labour (Giles *et al*., 2007), which is still a medical challenge (Clouse et al., 2007). A few $\beta_2$-adrenoceptor drugs are of a natural origin (Ahmed *et al*., 2011), this encouraged attempts to isolate and test the pharmacological features of isolated compounds on the $\beta_2$-adrenergic system in this study. $\beta_2$-adrenoceptor agonists are effective in delaying delivery for 48 h and have no effect on perinatal mortality or morbidity but have significant maternal side effects (Giles *et al*., 2007). The mechanism of $\beta_2$-adrenoceptor inhibition of smooth muscles is via cAMP-dependent protein kinase-mediated inhibitory phosphorylation of myosin light chain kinase. In obstetric use, the $\beta_2$-agonists are the preferred treatment for premature labor (Emilien *et al*., 1998; Weis *et al*., 2008). Previous studies of myometrial β-adrenoceptor binding sites and of responsiveness to β-adrenoceptor agonists in the uterus of several species (rat, human and guinea-pig) indicated that $\beta_2$ is a dominant subtype and is involved in the inhibition of myometrial contractility by β-adrenoceptor agonists (Kitazawa *et al*., 2001). The most widely reported adverse effects of therapeutic doses of $\beta_2$-adrenoceptor agonists mediated via β2-adrenoceptors are

skeletal muscle tremor (Emilien *et al*., 1998) and cardiac effects (Fernandes *et al*., 2004).

Although the literature has revealed the isolation and identification of synephrine (HS1) and N-methyle tyramine (HS2) before and the pharmacological effects of HS2 as β-adrenoceptor agonists which increases blood pressure. This side effect was not observed or reported between Bedouin women who using this plant for dysmenorrhoea and for birth pain. This may be due to that the crude extract has different fractions with different effect. In this study, all the fractions had relaxation effect on the uterus and vasoconstriction effects (results not shown) on the artery except fraction **C** that had a relaxation effect, which may compensate and cause balance in the effect.

These results provide scientific evidence to support the traditional use of *Haloxylon salicornicum* and demonstrate that the inhibitory effect might be attributable to the activation of β-adrenoceptors. HS1, HS2 and synephrine caused inhibition of muscle contractility. Propranolol was employed to assess the involvement of β-adrenoceptor in the effect of HS1, HS2 and synephrine on the uterus. It appeared that their inhibitory effect was through the activation of β-adrenoceptor agonists. Since *Haloxylon salicornicum,* exerts β-adrenoceptor agonistic activity it can be considered a natural source of potential significance for the treatment of premature labour and relaxations of the lung smooth muscle, and thus use for asthma treatment. Studying medicinal plants used by Bedouin women in Egypt can serve as a starting point in future drug development aimed at the production of a new safe, effective and bio-accessible therapeutic agent.

Some of this work justifies the use of this plant in the Bedouin communities for the treatment of perinatal problems and provide a scientific correlation between traditional medicinal plant use among the Bedouin and the pharmacological basis for their administration.

## 6. Conclusion

The results presented in this study validate the use of most of these plants as antimicrobial and anti-inflammatory agents by the Bedouin women in Egypt. Although the non-aqueous extracts were evaluated in this study, they are not accessible to traditional healers; water is the most commonly used solvent by many traditional healers. The activity of aqueous extracts from medicinal plants could be said to be dose dependent. Weak antibacterial activity observed in aqueous extracts could suggest that most of the active compounds present in the evaluated medicinal plants are less polar therefore; they are extracted in small quantities because of their weak affinity to water. However, good antibacterial activity could be achieved at higher concentrations. Practically, more cups of tea or decoction prepared from the medicinal plants would be prescribed for effective treatments to be achieved in traditional medicine.

The isolated compounds from *M. longifolia* and *A. fragrantissima* extracts were shown to possess some potent *in vitro* inhibitory effects on TNF-α. However, to the best of my knowledge the anti-inflammatory activity of these plant components are not well defined yet. TNF-α is known as the main proinflammatory cytokine secreted during the early phase of acute and chronic inflammatory diseases, such as asthma, rheumatoid arthritis, septic shock (Hasko *et al.*, 1999). Therefore, the crude extracts from traditionally used medicinal plants could be valuable sources of some new potential anti-inflammatory compounds that have not yet been explored. These extracts may have therapeutic potential for the modulation and regulation of macrophage activation, and may provide safe and effective treatment options for a variety of inflammation-mediated diseases. The mounting evidence shows that altered immune function plays a crucial role, modulating the inflammation could be an alternative approach (Lu *et al*., 2010). Plants used by Bedouin women for the treatment of menstrual pains were screened for TNF-α inhibition, which was giving a potent inhibitory effect. Hence, Inhibition of TNF-α may lead to relief of menstrual pain.

In fact, this type of research is extremely valuable because as discussed with the Bedouin, their customs have started to change and now western medicine is becoming more available to them. They may use this traditional knowledge less and it is in danger of being lost. This study was in response to the lack of in-depth literature on medicinal plants use by Bedouin women in Egypt.

**Future work**

Many questions have arisen from this study: Regarding to the immunomodulatory effects of these compounds and considering the attempt to study their mechanism of action further study could contribute to the complete understanding of action of these compounds. Finally, it should be noted that the present study is limited in terms of its *in vitro* experimental design and that extrapolations to *in vivo* conditions should be carried out with caution. Future studies should aim to define the *in vivo* actions of these compounds using animal models. As any other derived compounds will also require rigorous toxicological and pharmacokinetic testing as well as clinical verification of the findings obtained *in vitro*.

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# Appendix I

# Questionnaire

**Questionnaire**

Date:

Location:

Name: Age:

Tribe: Sex:

1- How do you deal with diseases?
2- What are the most common diseases in your area?
3- What are the most common diseases in women?
4- If you have a disease, do you use plants by yourself or go to herbalist in your tribe?
5- Is he male or female?
6- When do you go to see doctor?
7- How do you deal with the following diseases:

   Abdominal

   Respiratory

   Abscess

   Eye and mouth inflammation

   Reproductive system infections

   Menstruation pains

   During pregnancy

   After delivery

**Data about plant uses and practice specifications**

1-Plant (local name)

2-Type (tree, herb, shrub, climber)

3-Uses

4-Plant part(s) used

5-Availability (easily available, available with difficulty, hardly available, unavailable)

6-Ethnomedicinal preparation

7-Collection

8-Storage

9-Preparation

10-Frequency of treatment

11-Duration of treatment

12-Accompanied side effect(s)

13-Conservation needs and efforts

14-Other information

15-Other treatments from different environmental sources

16-Source(s) of knowledge of herbal treatment

**Arabic translation of the questionnaire**

معلومات عامة :

تاريخ :

الموقع :

الاسم : العمر :

القبيلة : الجنس :

1 -- كيف تتعامل مع الأمراض؟

2 -- ما هي الأمراض الأكثر شيوعا في منطقتك؟

3 -- ما هي الأمراض الأكثر شيوعا بين النساء؟

4 -- إذا كان لديك مرض، لا يمكنك استخدام النباتات من قبل نفسك أو انتقل إلى الأعشاب في قبيلتك؟

5 -- هل هو ذكر أم أنثى؟

6 -- عندما تذهب إلى الطبيب؟

7 -- كيف تتعامل مع الأمراض التالية :

البطن

تنفسي

خراج

التهاب العين والفم

التهابات الجهاز التناسلي

آلام الحيض

خلال فترة الحمل

بعد الولادة

بيانات حول النباتات ويستخدم مواصفات الممارسة

1 - النباتية (الاسم المحلي)

2 - نوع (الشجرة، عشبة، شجيرة، متسلق)

3 الأغراض

4 - النبات جزء (ق) المستخدمة

5 الإتاحة (سهل المتاحة، وهي متاحة بصعوبة ، بالكاد المتاحة، غير متوفر)

6 إعداد

7 كوكتيل

8 التخزين

# Appendix II

# List of Bedouin interviewed

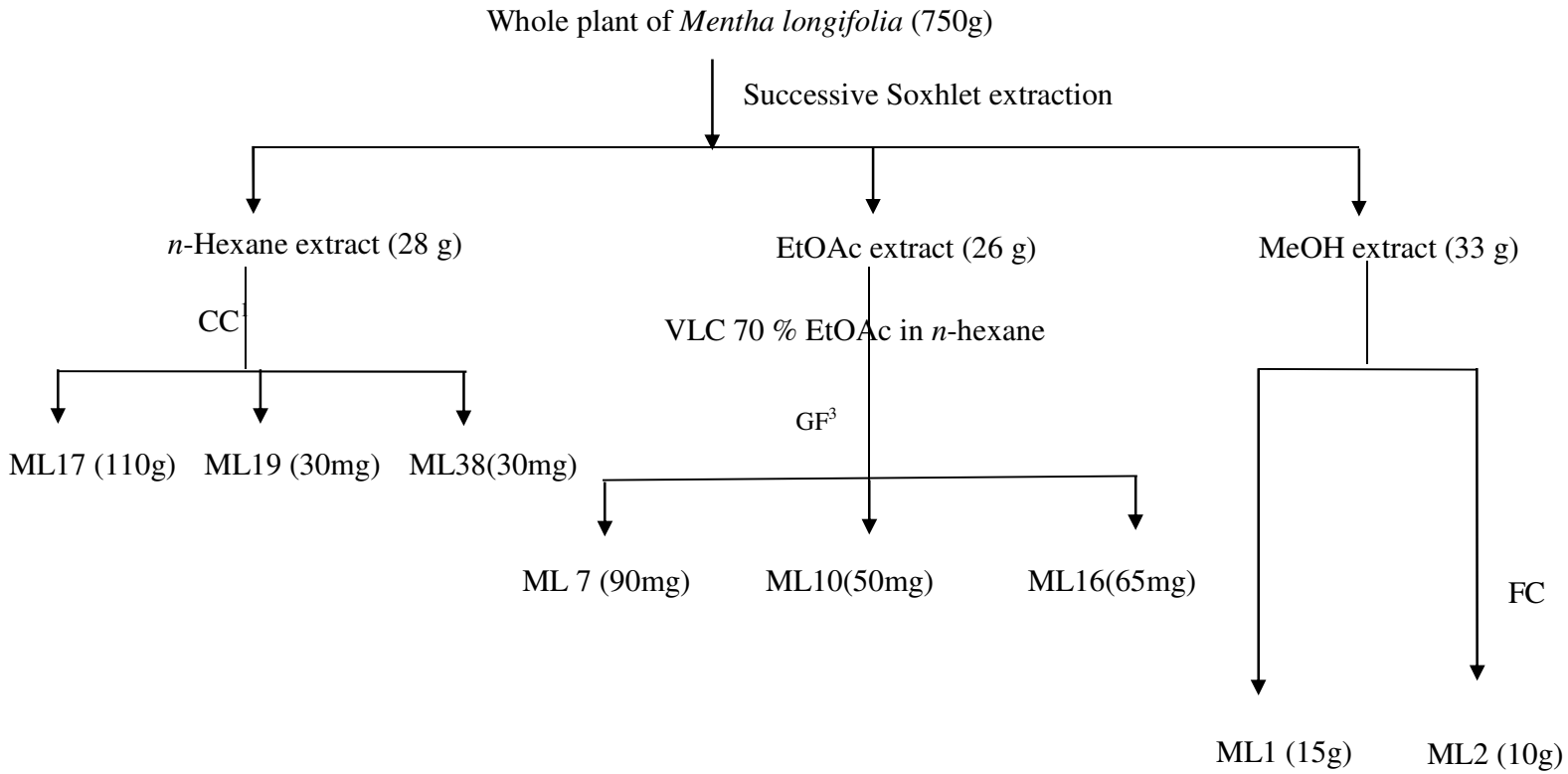
**List of Bedouin interviewed**

| clan | No. | English name | Sex | Age | area |
|---|---|---|---|---|---|
| **Geibalyia Bedouin from St. Cathrine** | | | | | |
| | 1 | Salam | Female | 35 | Wadi Abu Sila |
| | 2 | Um Mahmoud | Female | | Wadi Abu Sila |
| | 3 | Naeema | Female | | Wadi Abu Sila |
| | 4 | Um salma | Female | | Wadi Abu Sila |
| | 5 | Frehaa | Female | | Asquf-Ala |
| | 6 | Sebeha | Female | | Asquf-Ala |
| | 7 | Fatma | Female | | Asquf-Ala |
| | 8 | Subhya | Female | | Asquf-Sufla |
| | 9 | Sabha | Female | | Asquf-Sufla |
| | 10 | Handya | Female | | Asquf-Sufla |
| | 11 | Fedya | Female | | Asquf-Sufla |
| | 12 | Um Musa | Male | 55 | Sbaeya |
| | 13 | Hamda | Female | 53 | Sbaeya |
| | 14 | Hamada | Female | 23 | Sbaeya |
| | 15 | Helala | Female | 60 | Wadi Nusub |
| | 16 | wedad | Female | 42 | Wadi Nusub |
| | 17 | Subhya | Female | 54 | Darba |
| | 18 | Fatma | Female | 34 | Darba |
| | 19 | Salaha | Female | 38 | Darba |
| | 20 | Nadia | Female | 24 | Darba |
| | 21 | Fathya | Female | 59 | Darba |
| | 22 | Mabsota | Female | 41 | Mariya |
| | 23 | Awad | Male | 57 | Nusub Ala |
| | 24 | Awad wife | Female | 36 | Nusub Ala |
| | 25 | Second wife | Female | 40 | Nusub Ala |
| | 26 | Hameda | Female | 46 | Sbaeya Olya |
| | 27 | Mohamed Salamah | Male | 50 | Sbaeya Olya |
| | 28 | Ayda | Female | 19 | Sbaeya Olya |
| | 29 | Um Musa | Male | 60 | Sbaeya Olya |
| | 30 | Haja Sultanah | Female | 70 | Mulqah |
| | 31 | Um Husien | Male | 60 | Mulqah |
| | 32 | Haja Subheya | Female | 65 | Mulqah |
| | 33 | Um Nasser | Female | 47 | Talah |
| | 34 | Farhanah | Female | 43 | Talah |
| | 35 | Hameda | Female | 40 | Talah |
| | 36 | Hajar | Female | 20 | Talah |
| | 37 | Salma | Female | 23 | Mulqah |
| | 38 | Um Ramdan | Female | 65 | Mulqah |
| | 39 | Um Esra | Female | 26 | Mulqah |
| | 40 | Um Rajab | Female | 54 | Mulqah |

| | | | | | |
|---|---|---|---|---|---|
| | 41 | Alyan Atwah | Male | 57 | Wadi Feran |
| | 42 | Alyan wife | Female | 39 | Wadi Feran |
| | 43 | Feraha salem | Female | 28 | Wadi Feran |
| | 44 | Gemea | Female | 31 | Wadi Feran |
| | 45 | Siekh salem wife | Female | 46 | Joqa |
| | 46 | Um Jamel | Female | 35 | Joqa |
| | 47 | Um fathi | Female | 31 | Joqa |
| | 48 | Sheikh Mansur | Female | 30 | Joqa |
| | 49 | Um Husien | Female | 26 | |
| | 50 | Um Atyia | Female | 28 | |
| | 51 | Mahmoud Saleh Oda | Male | 56 | |
| | 52 | Ahmed Saleh | Male | 60 | |
| **Siwa Bedouin** | | | | | |
| | 53 | Agha | Female | 70 | Garah |
| | 54 | Shiekh husine | Male | 60 | Garah |
| | 55 | Um Mohamed | Female | 53 | Bahy Eldien |
| | 56 | Um Rajab | Female | 48 | Bahy Eldien |
| | 57 | Hajah Khamela | Female | 63 | Bahy Eldien |
| | 58 | Haj Mohamed | Male | 54 | Maragy |
| | 59 | Sunosy | Male | 51 | Maragy |
| | 60 | Dief Hassan | Male | 64 | Maragy |
| | 61 | Dief wife | Female | 46 | Maragy |
| **Wadi Allqi Bedouin** | | | | | |
| | 62 | Neffesah | Female | 45 | Sadenab |
| | 63 | Amriya | Female | 36 | |
| | 64 | Redina | Female | 49 | |
| | 65 | Aminah | Female | 24 | |
| | 66 | Auhod | Female | 16 | |
| | 67 | Eda | Female | 32 | |

# Appendix III

# Isolation schemes

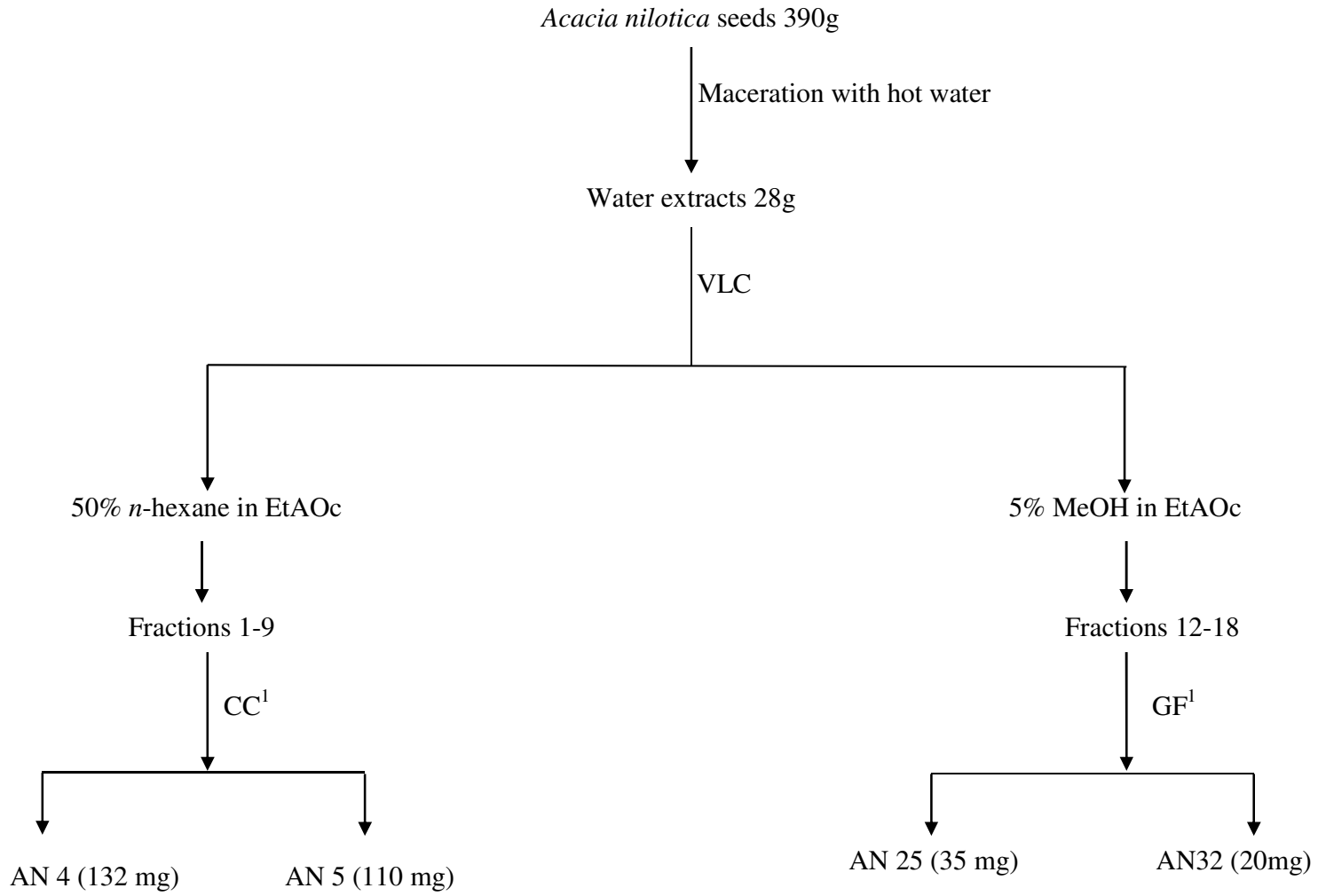



CC[1]: Gradient Si gel column started with 5% *n*-hexane in EtOAc, with the gradual increase of EtOAc;

FC: Flash chromatography reverse phase C-18. Eluted with 100% H2O with gradual increase of MeOH

GF[3]: Gel filtration eluted with 100% MeOH.

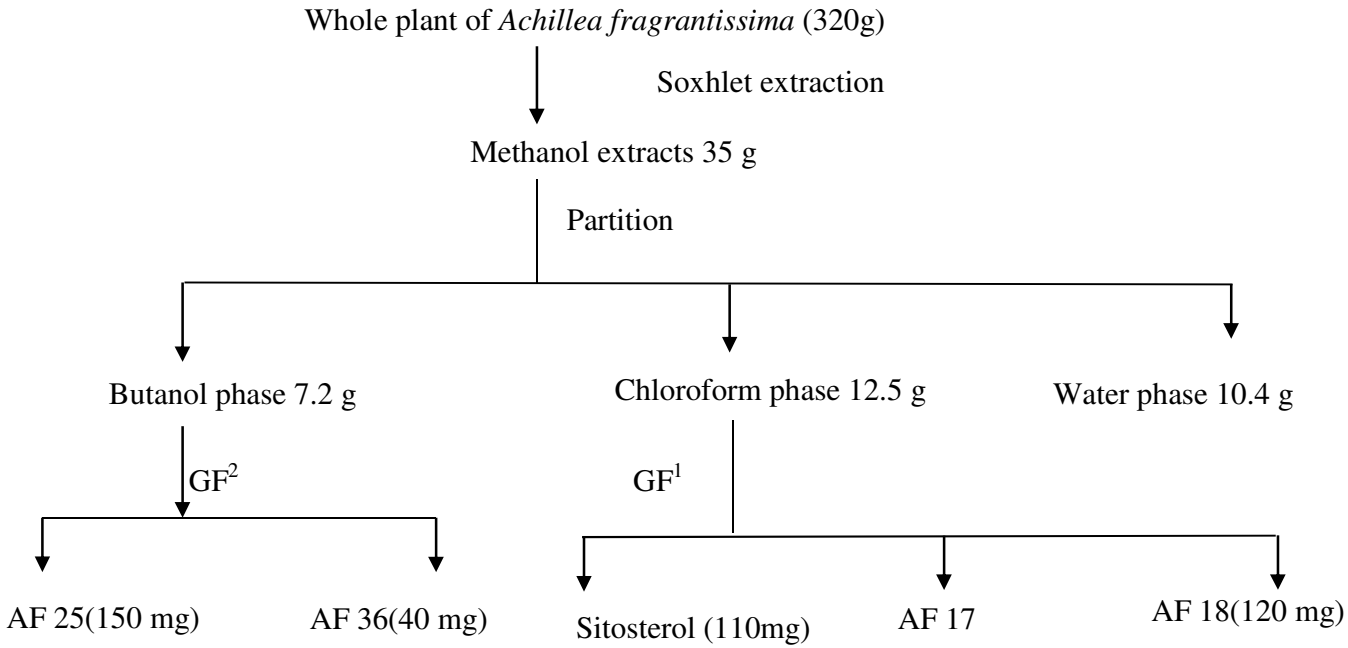
**Scheme 1: Isolation of compounds from the *Mentha longifolia* extracts**




GF[1]: Gel filtration eluted with MeOH

CC[1]: Gradient column eluted with 70% n-hexane, increasing polarity by addition of EtOAc.

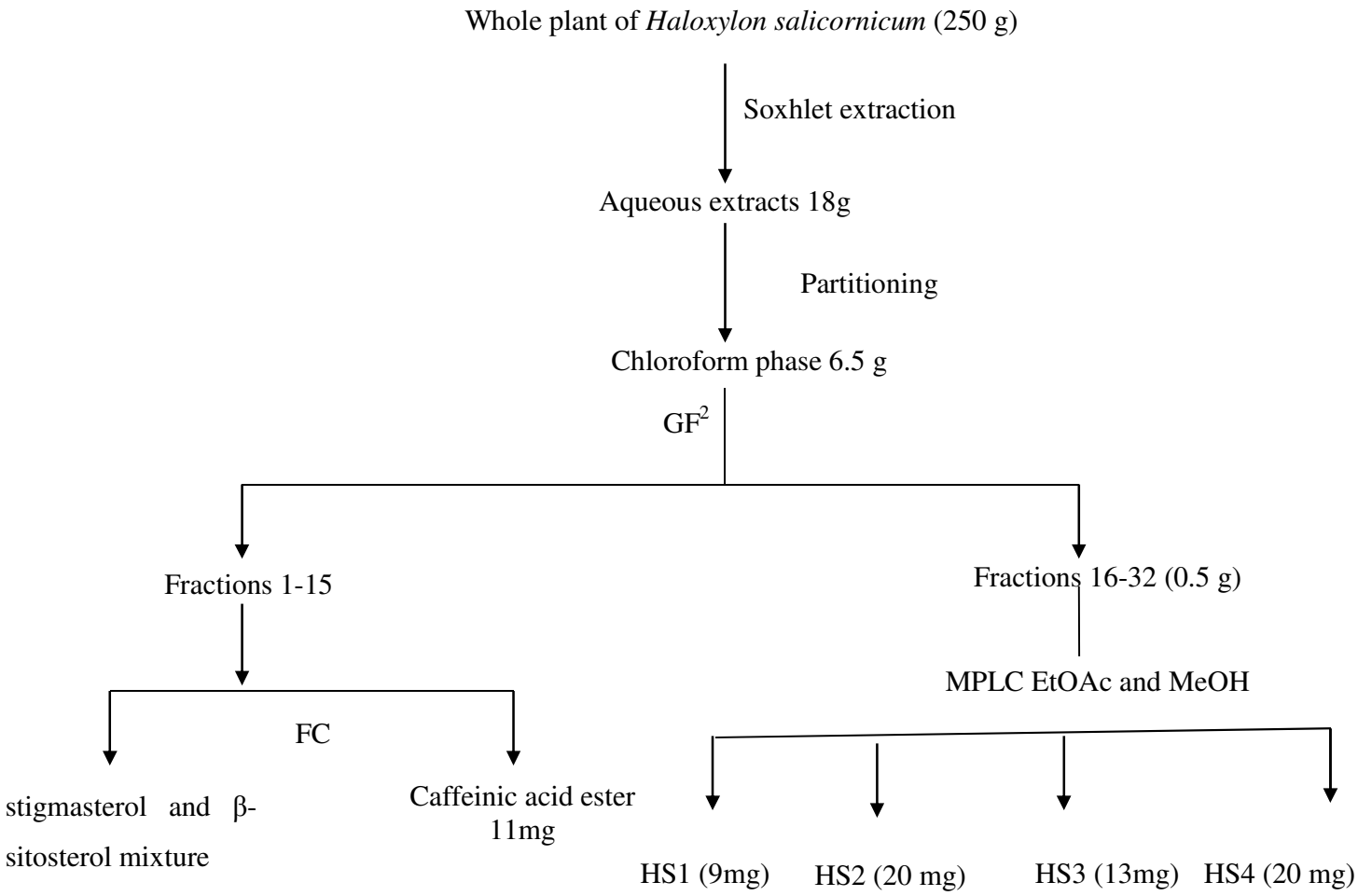
**Scheme 2: Isolation of pure compounds from the water extract of *Acacia nilotica***




GF[1]: Gel filtration eluted with 5% *n*-hexane in $CHCl_3$, with the gradual increase of $CHCl_3$, followed by the addition of MeOH

GF[2]: Gel filtration eluted with MeOH

**Scheme3: Isolation of compounds from the Methanol: water extract of *Achillea fragrantissima***




FC: Flash chromatography using Si. gel column. Eluted with 100% $CHCl_3$ with gradual addition of MeOH

$GF^3$: Gel filtration eluted with 100% MeOH.

**Scheme 4: Isolation of pure compounds from the MeOH extract of *Haloxylon salicornicum***

# Appendix IV

# Compounds properties

## Compound properties

**ML17**

Light orange oil, $^{1}$HNMR (400 MHz, chloroform-D) δ 5.81 (dd, J = 2.6, 1.3 Hz, 1H), 2.59 (t, J = 6.1 Hz, 3H), 2.23 (t, J = 6.2 Hz, 4H), 2.02 (s, 3H), 1.86 (s, 3H), 1.79 (s, 3H). $^{13}$CNMR (101 MHz, chloroform-D) δ 191.76 (s), 159.77 (s), 142.54 (s), 128.86 (s), 31.94 (s), 28.01 (s), 23.88 (s), 22.97 (s), 22.58 (s).

**ML19**

White powder, $^{1}$HNMR (400 MHz, chloroform-D) δ 8.82 (s, 1H), 6.95 (d, J = 7.9 Hz, 1H), 6.70 (d, J = 0.4 Hz, 1H), 6.63 (dd, J = 7.9, 0.4 Hz, 1H), 2.27 (s, 3H), 1.65 (s, 6H). $^{13}$CNMR (101 MHz, CHLOROFORM-D) δ 155.61 (s), 139.17 (s), 128.37 (s), 125.23 (s), 120.39 (s), 118.20 (s), 30.49 (2Xs), 29.79 (s).

**ML38**

Yellow needles, $^{1}$HNMR (400 MHz, chloroform-D) δ 12.75 (s, 1H), 7.52 (dd, J = 8.4, 2.1 Hz, 1H), 7.33 (d, J = 2.1 Hz, 1H), 6.98 (d, J = 8.5 Hz, 1H), 6.59 (s, 1H), 6.55 (s, 1H), 3.99 (s, 3H), 3.97 (s, 3H), 3.96 (s, 3H), 3.92 (s, 3H), 1.58 (s, 1H). $^{13}$CNMR (101 MHz, CHLOROFORM-D) δ 182.72 (s), 164.07 (s), 158.84 (s), 153.32 (s), 153.18 (s), 152.38 (s), 149.43 (s), 132.99 (s), 123.89 (s), 120.17 (s), 111.26 (s), 108.86 (s), 106.46 (s), 104.59 (s), 94.67 (s), 90.68 (s), 60.97 (s), 56.44 (s), 56.22 (s).

**ML7**

Yellow amorphous powder; $^{1}$HNMR (400 MHz, DMSO-D6) δ 12.42 (s, 1H), 9.17 (s, 1H), 7.65 (dd, J = 8.5, 1.9 Hz, 1H), 7.53 (d, J = 1.9 Hz, 1H), 7.14 (d, J = 8.6 Hz, 1H), 6.95 (s, 3H), 3.95 (s, 3H), 3.92 (s, 3H), 3.87 (s, 1H), 3.84 (s, 3H) $^{13}$CNMR (101 MHz, DMSO-$D_6$) δ 183.21 (s), 164.05 (s), 152.76 (s), 149.54 (s), 148.57 (s), 143.56 (s), 142.42 (s), 134.72 (s), 133.43 (s), 123.51 (s), 120.52 (s), 112.39 (s), 109.63 (s), 106.66 (s), 103.81 (s), 79.61 (s), 62.39 (s), 61.57 (s), 56.27 (s).

**ML10**

Yellow amorphous powder, $^{1}$HNMR (400 MHz, DMSO-$D_6$) δ 12.52 (s, 0H), 10.03 (s, 1H), 9.11 (s, 1H), 7.58 (dd, J = 5.4, 1.8 Hz, 1H), 6.99 (d, J = 1.2 Hz, 1H), 6.97 (d, J = 1.7 Hz, 1H), 6.96 (s, 1H), 3.96 (s, 3H), 3.93 (s, 3H), 3.90 (s, 3H). $^{13}$CNMR (101 MHz, DMSO-$D_6$) δ 183.20 (s), 164.38 (s), 151.42 (s), 148.61 (s), 148.53 (s), 143.67 (s), 142.38 (s), 134.74 (s), 133.46 (s), 122.21 (s), 120.82 (s), 116.49 (s), 110.54 (s), 106.66 (s), 103.29 (s), 62.39 (s), 61.53 (s), 56.37 (s).

**ML16**

Yellow amorphous powder; $^{1}$HNMR (400 MHz, DMSO-D6) δ 12.53 (s, 1H), 10.40 (s, 1H), 9.16 (s, 1H), 7.94 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 8.8 Hz, 2H), 6.84 (s, 1H), 3.95 (s, 3H), 3.92 (s, 3H). $^{13}$CNMR (101 MHz, DMSO-$D_6$) δ 183.15 (s), 164.55 (s), 161.91 (s), 148.53 (s), 143.69 (s), 142.36 (s), 134.77 (s), 133.52 (s), 128.98 (s), 121.89 (s), 116.68 (s), 106.65 (s), 102.96 (s), 62.45 (s), 61.51 (s).

**ML1**

Yellow powder; $^{1}$HNMR (400 MHz, DMSO-$D_6$) δ 12.91 (s, 1H), 8.02 (d, J = 8.8 Hz, 2H), 7.12 (d, J = 8.9 Hz, 2H), 6.91 (s, 1H), 6.78 (d, J = 1.8 Hz, 1H), 6.45 (d, J = 1.8 Hz, 1H), 5.49 (s, 1H), 5.26 (s, 2H), 5.07 (d, J = 7.2 Hz, 1H), 4.69 (d, J = 38.1 Hz, 2H), 4.57 ( d, J= 1.1Hz, 1H), 4.52 (d, J = 11.8 Hz, 1H), 3.88 (d, J = 11.0 Hz, 2H), 3.84 (s, 3H), 3.68 (s, 1H), 3.65 – 3.59 (m, 1H), 3.52 – 3.44 (m, 3H), 3.42 (d, J = 5.3 Hz, 4H), 3.30 (dd, J = 16.7, 8.9 Hz, 3H), 3.17 (t, J = 8.9 Hz, 3H), 1.09 (d, J = 6.1 Hz, 4H). $^{13}$CNMR (101 MHz, DMSO-$D_6$) δ 182.61 (s), 164.52 (s), 163.53 (s), 163.01 (s), 161.72 (s), 157.55 (s), 129.04 (s), 123.24 (s), 115.29 (s), 106.04 (s), 104.38 (s), 101.11 (s), 100.51 (s), 100.23 (s), 95.36 (s), 76.83 (s), 76.24 (s), 73.65 (s), 72.64 (s), 71.32 (s), 70.93 (s), 70.17 (s), 68.91 (s), 66.67 (s), 56.15 (s), 18.39 (s).

**ML2**

Bright yellow amorphous powder, $^{1}$HNMR (400 MHz, methanol-D3) δ 7.42 (d, J = 8.7 Hz, 2H), 7.42 (d, J = 8.7 Hz, 2H), 6.95 (d, J = 8.7 Hz, 2H), 6.19 (d, J = 2.2 Hz, 1H), 6.17 (d, J = 2.2 Hz, 1H), 5.45 (dd, J = 12.7, 2.9 Hz, 1H), 4.93 (d, J = 7.3 Hz, 1H), 4.68 (d, J = 1.4 Hz, 1H), 3.98 (d, J = 9.3 Hz, 1H), 3.89 – 3.84 (m, 1H), 3.80 (s,

3H), 3.66 (dd, J = 9.5, 3.4 Hz, 1H), 3.64 – 3.55 (m, 3H), 3.44 (dd, J = 7.0, 4.5 Hz, 2H), 3.41 – 3.33 (m, 2H), 3.31 (dd, J = 5.7, 4.1 Hz, 3H), 3.16 (dd, J = 17.2, 12.7 Hz, 1H), 2.79 (d, J = 3.1 Hz, 1H), 2.75 (d, J = 3.0 Hz, 1H), 1.18 (d, J = 6.2 Hz, 3H). $^{13}$CNMR (101 MHz, methanol-D3) δ 197.03 (s), 165.63 (s), 163.66 (s), 162.97 (s), 160.16 (s), 130.55 (s), 127.85 (s), 113.58 (s), 103.41 (s), 100.92 (s), 99.81 (s), 96.77 (s), 95.74 (s), 78.98 (s), 76.52 (s), 75.78 (s), 73.32 (s), 72.77 (s), 71.02 (s), 70.73 (s), 69.97 (s), 68.45 (s), 66.05 (s), 54.44 (s), 42.73 (s), 16.51 (s).

**AN4**

White powder, $^{1}$HNMR (400 MHz, DMSO-$D_6$) δ 9.26 (s, 1H), 6.95 (s, 1H), 4.23 (m, 1H). $^{13}$CNMR (101 MHz, DMSO-D6) δ 166.90 (s), 146.13 (s), 138.97 (s), 119.86 (s), 109.06 (s), 52.16 (s).

**AN25**

Brown crystal, $^{1}$HNMR (500 MHz, MeOD) δ 7.12 (s, 2H), 5.65 (d, J = 8.0 Hz, 1H), 3.86 (dd, J =1.5, 2 Hz, 1H), 3.71 (dd, J = 5.0, 4.5 Hz, 1H), 3.485 (d, J = 2.0Hz, 1H), 3.475(d, J= 3.0 Hz, 2H), 3.428-3.398 (m, 2H). $^{13}$CNMR (126 MHz, MeOD) δ166(s), 146(2xs), 140.05(s), 122(s), 110 (2xs), 96 (s), 78.05(s), 77.05(s), 73.05(s), 70.05(s), 61.09(s)

**AF17**

Yellow oil; $^{1}$HNMR (400 MHz, chloroform-D) δ 5.73 (brs, 1H), 5.67 (brs, 1H), 5.43 (t, J = 8.7 Hz, 1H), 5.23 (d, J = 10.1 Hz, 1H), 4.76 (d, J = 10.0 Hz, 1H), 4.30 (d, J = 13.5 Hz, 1H), 4.25 (d, J = 13.7 Hz, 1H), 3.61 (dd, J = 12.9, 8.9 Hz, 1H), 2.92 (m, 1H), 2.75 (dd, J = 13.3, 5.4 Hz, 1H), 2.53 (dd, J= 13.2, 8.8Hz 1H), 2.23 (t, J = 13.1 Hz, 1H), 2.03 (brs, 1H), 1.98 (s, 3H), 1.66 (s, 3H). $^{13}$CNMR (101 MHz, chloroform-D) δ 198.71 (s), 174.05 (s), 170.05 (s), 165.94 (s), 148.33 (s), 136.32 (s), 127.69 (s), 127.04 (d, J = 16.7 Hz), 125.54 (t, J = 11.1 Hz), 79.47 (d, J = 16.1 Hz), 75.64 (d, J = 14.6 Hz), 54.45 (t, J = 9.9 Hz), 44.18 (d, J = 11.2 Hz), 33.48 (d, J = 9.4 Hz), 23.6 (m), 21.1 (q, J = 9.8 Hz), 10.7 (m).

**AF 18**

Yellow crystal; $^{1}$HNMR (500 MHz, CDCl3) δ 12.34 (s, 1H), 7.92 (d, J = 2.0 Hz, 1H), 7.54 (dd, J = 8.5, 2.1 Hz, 1H), 7.02 (d, J = 8.5 Hz, 1H), 6.51 (s, 1H), 3.94 (s, 3H), 3.91 (s, 3H), 3.79 (s, 3H). $^{13}$CNMR (126 MHz, CDCl3) δ 178.93 (s), 159.13 (s), 156.97 (s), 152.39 (s), 147.62 (s), 143.65 (s), 138.61 (s), 132.20 (s), 123.92 (s), 122.34 (s), 121.86 (s), 115.90 (s), 115.21 (s), 106.36 (s), 90.60 (s), 60.87 (s), 60.15 (s), 56.38 (s).

**AF 36**

Jaceosidin; yellow amorphous powder; $^{1}$HNMR (500 MHz, DMSO) δ 12.93 (s, 1H), 7.45 (dd, J = 2.2, brs Hz, 1H), 7.43 (brs d,1H), 6.89 (d, J = 8.6 Hz, 1H), 6.86 (s, 1H), 6.72 (s, 1H), 3.92 (s, 3H), 3.73 (s, 3H). $^{13}$CNMR (126 MHz, DMSO) δ 182.57 (s), 164.72 (s), 159.06 (s), 153.07 (s), 152.57 (s), 150.32 (s), 146.27 (s), 132.35 (s), 121.92 (s), 119.52 (s), 116.43 (s), 114.00 (s), 105.53 (s), 103.18 (s), 91.92 (s), 60.49 (s), 56.90 (s).

**AF25**

White crystal; $^{1}$HNMR (400 MHz, DMSO-D6) δ 5.68 (d, J = 10.2 Hz, 1H), 5.08 (t, J = 5.1 Hz, 1H), 4.98 (dd, J = 16.7, 7.3 Hz, 1H), 4.95(m, 1H), 4.86 (d, J = 4.0 Hz, 2H), 4.82 (brs d, 1H), 4.16 (d, J = 5.3 Hz, 1H), 4.14 (brs d, 1H), 3.36 (s, 2H), 3.28 (t, J = 3.4 Hz, 1H), 2.47 (m, 1H), 2.21 (m, 1H), 2.02 (m, 1H), 1.84 (brs d, H), 1.77 (s, 3H). $^{13}$CNMR (101 MHz, DMSO-D6) δ 173.52 (s), 169.97 (s), 166.89 (s), 150.63 (s), 137.80 (s), 126.90 (s), 124.31 (s), 113.73 (s), 79.19 (s), 76.02 (s), 74.26 (s), 53.04 (s), 24.38 (s), 21.43 (s), 11.47 (s).

**HS1**

$^{1}$HNMR (400 MHz, METHANOL-D3) δ 7.24 (d, J = 8.6 Hz, 2H), 6.79 (d, J = 8.5 Hz, 2H), 4.83 (m, 1H), 3.12 (t, 1H), 2.74 (bro, 1H), 2.72 (s, 4H). $^{13}$CNMR (101 MHz, METHANOL-D3) δ 157.37 (s), 131.35 (s), 126.94 (2xs), 115.24 (2xs), 68.57 (s), 55.46 (s), 32.29 (s).

**HS2**

Amorphous powder; $^{1}$HNMR (400 MHz, D2O) δ 7.31 (d, J = 8.6 Hz, 1H), 7.19 (d, J = 8.3 Hz, 2H), 6.93 (d, J = 8.4 Hz, 1H), 6.88 (d, J = 8.3 Hz, 2H), 4.97 (dd, J = 8.1, 4.8 Hz, 1H), 3.18 – 3.13 (m, 1H), 2.93 (t, J = 7.3 Hz, 2H), 2.77 (d, J = 0.9 Hz, 2H), 2.69 (d, J = 0.9 Hz, 3H).

**HS3**

$^{1}$HNMR (400 MHz, METHANOL-D3) δ 3.17 – 3.11 (m, 4H), 1.85 – 1.77 (m, 4H), 1.68 (dt, J = 12.3, 3.5 Hz, 2H). $^{13}$CNMR (101 MHz, methanol-D3) δ 44.41 (2xs), 22.40 (2xs), 21.82 (s).

**HS4**

White needles; $^{1}$HNMR (400 MHz, DMSO-D6) δ 10.54 (s, 1H), 8.06 (s, 1H), 6.99 (d, J = 8.1 Hz, 1H), 5.83 (s, 2H), 5.24 (d, J = 8.1 Hz, 1H). $^{13}$CNMR (101 MHz, DMSO-D6) δ 174.12 (s), 157.97 (s), 157.31 (s), 62.95 (s).