Phytochemical and biological studies of *Helichrysum cymosum*

UNIVERSITY *of the*
WESTERN CAPE

By

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ABSTRACT

Diabetes mellitus (DM) is well known as a group of systemic metabolic disorders with a considerable mortality rate around the world. Hyperglycemia is the main consequence of DM, which results from the shortage in insulin production or degradation of produced insulin. Other internal and external factors including obesity, oxidative stress, and sedentary lifestyle have been also suggested as the causes of DM. Among the well-known existing types of DM, type 1 and 2 are the most common.

Treatment of type 1 necessitates insulin injection, while type II can be controlled by physical exercises, diet control in addition to different synthetic antidiabetic drugs. However, their effectiveness is restricted because of the high cost and unfriendly side effects. There is a significant need for producing alternative and more bioactive antidiabetic drugs from natural sources. Natural products are a well-known source for the discovery of new scaffold for drugs discovery, and South Africa is one of the most important megaflora with a high percentage of endemism.

South Africa is home to about 245 species of *Helichrysum* species, which are generally rich sources of secondary metabolites, especially flavonoids, which give them a great value for global acceptability and wide application in the pharmaceutical fields as antibacterial, anti-inflammatory, antioxidant, and antidiabetic agents.

The phytochemical investigation of the methanolic extract of *H. cymosum* resulted in the isolation and identification of seven pure compounds (**1**-**7**). Structural characterization of these isolated compounds was conducted using 1D NMR, in comparison with reported spectroscopic data.

The in vitro bio-evaluation of *H. cymosum* against alpha-glucosidase shown that **5** exhibited the highest alpha-glucosidase inhibitory activity with IC_{50} value of 13 µM, followed by **7** and **3** with IC_{50} values of 18.16 µM and 44.4 µM respectively. Additionally, strong total antioxidant

capacities were displayed by **6** and **2** as ORAC (122.86 ± 0.7 and 91.70 ± 0.4 µM TE/mL) respectively as well as **5** and **7** as FRAP (1006.34 ± 1.7; 977.79 ± 0.8) µM AAE/g.

This is the first scientific report to be carried out on alpha-glucosidase inhibitory activities and antioxidant capacities of *H. cymosum* constituents. The findings suggest that these compounds might become prominent natural candidates to inhibit alpha-glucosidase as well as oxidative stress related to diabetes with the prospect to be employed in the formulation of diabetes drugs upon further biological studies.

KEYWORDS

Alpha-glucosidase

Alpha-amylase

Asteraceae

Cape Flora

Diabetes mellitus

Flavonoids

Oxidative stress

Secondary metabolites

ABBREVIATIONS

AAE/g	Ascorbic acid per gram
AAPH	2,2-Azobis(2-methylpropionamidine) dihydrochloride, perchloric acid
ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammoniun salt
BHT	Butylated hydroxytoluene
$CDCl_3$	Deuterated chloroform
COSY	Correlation spectroscopy
d	Doublet
DIW	De-ionized water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EtOAc	Ethyl acetate
DPPH	2,2-diphenyl-1-picrylhydrazyl
Fig	Figure
g	Gram
H_2SO_4	Sulfuric acid
IC_{50}	Half maximal inhibitory concentration
KCl	Potassium chloride
L	Liter
mg	Milligram
mL	Milliliter

min	Minute
mM	Millimolar
M	Molar
MeOH	Methanol
NMR	Nuclear magnetic resonance
nm	Nanometer
ORAC	Oxygen radicals absorbance capacity
pNPG	*para*-nitrophenyl α-D-glucopyranoside
ROS	Reactive oxygen species
t	Triplet
TLC	Thin-layer chromatography
TPTZ	2,4,6-tri[2-pyridyl]-s-triazine
μL	Microliter
U	Unit
s	Singlet
SC_{50}	Concentration of sample required to scavenge 50% of DPPH radicals
V	Volume
^{1}H-NMR	Proton nuclear magnetic resonance
1D-NMR	One-dimensional nuclear magnetic resonance

DECLARATION

I, **Baraa Mohamed Ibrahim Soliman Jadalla** hereby declare that "Phytochemical and biological studies of *Helichrysum cymosum*" is my authentic work and has not previously been submitted for any degree or assessment purposes, at any other University. Similarly, all resources used have been indicated and acknowledged by means of comprehensive referencing.

Date…… Signed……Baraa……...

DEDICATION

I dedicate this dissertation to my family, particularly my father **Dr. Mohamed Jadalla**, my mother **Dr. Hanan Bedier** and my brother **Dr. Bassam Jadalla**, who gave me their unwavering support and have inspired me throughout my life. Similarly, to my wife, my son **Taha Jadalla**, my friends and to the whole academic communities.

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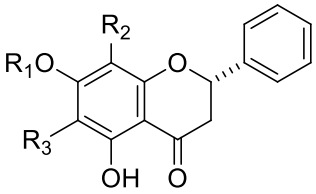
LIST OF ISOLATED COMPOUNDS



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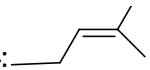
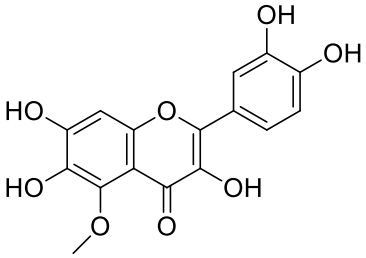
3 R_1: H; R_2: OH; R_3: H

4 R_1: H; R_2:  ; R_3: H



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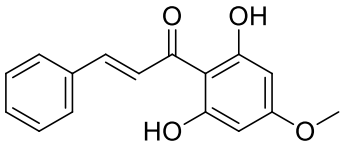

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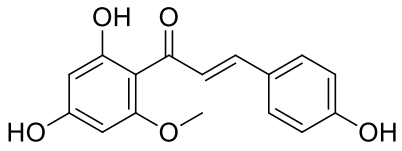

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CHAPTER ONE: GENERAL INTRODUCTION

1.1 Plants as source of new drugs

From the beginning of human existence, humans have familiarized themselves with plants and utilised them for food and medicine throughout the ages (Shakya, 2016). The relationship between man and plants has grown and numerous plants became useful as medicines. The knowledge for treating diseases continued to grow and a number of new plant-derived drugs increased likewise (Jamshidi-Kia, et al., 2018). The plant-based traditional medicine systems is still playing a key role in the health care systems, and the demand of health products, herbal based medicine, food supplements, pharmaceuticals, nutraceuticals, and cosmetics are increasing worldwide (Ekor, 2013). The usage of herbal remedies has been and continue to be a common practice in developing countries and an approximation of 80% of the population residing in Africa relies on folk medicine to treat different kinds of diseases (Mahomoodally, 2013). Herbal remedies play an essential part in South Africa, where it constitutes the backbone of the rural area with approximately 60% of the population consulting an estimation of 200000 traditional healers (Semenya, et al., 2013).

A plethora of medicinal plants contain very interesting bioactive secondary metabolites that serve as precursors to synthesized drugs (Atanasov, et al., 2015). Hence, the usage of herbal medicine is gaining valuable global recognition compared to synthetic drugs due to better cultural acceptability and minimal adverse side effects. In the 20st century, approximately 80% of cardiovascular, antimicrobial, anticancer, and immunosuppressive drugs were derived from plants and their sales surpassed US$ 65 billion in 2003 (Pan, et al., 2013). Additionally, natural products account for over 50% of all drugs in clinical trials and about 50% of the approved herbal drugs were obtained either directly or indirectly from natural sources (Pan, et al., 2013).

Furthermore, an estimation of 64% of newly-synthesized drugs for antihypertensive derived from natural product structures (Yuan, et al., 2016).

1.2 Overview of Diabetes

Diabetes mellitus (DM) is well known as a group of systemic metabolic disorders with considerable mortality around the world. Hyperglycemia is the main consequence of DM, which results from the shortage in insulin production or degradation of produced insulin (American, 2009; Gao, et al., 2016). The chronic hyperglycemia of diabetes is linked to long-term injury, disturbance, and failure of numerous organs including kidneys, eyes, nerves, heart, and blood vessels. Many internal and external factors including obesity, oxidative stress, and sedentary lifestyle are directly implicated in these cell alterations (Mamun-or-Rashid, et al., 2014; Ullah, et al., 2016). Regrettably, there is currently no cure available for diabetes, but it can be managed by controlling blood glucose levels through a healthy diet, physical exercise and medication, which can reduce/decrease the risk of long-term diabetes complications (Asif, 2014).

The International Diabetes Federation indicated that the number of people (adults) with diabetic was estimated to be 451 million in 2017 worldwide, which is projected to escalate to 693 million cases by 2045 (Cho, et al., 2018). Globally, an approximation of 50% of diabetes victims is undiagnosed, and most of them are predominantly found in poor- and average-income countries. Additionally, Africa accounts for about 69.2% of undiagnosed diabetes (Pheiffer, et al., 2018).

Diabetes mellitus is categorised into two main types, namely, type 1 DM commonly known as insulin-dependent diabetes mellitus (IDDM) and type 2 DM commonly referred to as non-insulin-dependent diabetes mellitus (NIDDM), which accounts for about 90-95% of diabetic patients (Mohammed, et al., 2015).

Type 1 DM is caused by the incapacity of the pancreas to produce insulin (Mohiuddin, et al., 2016). It results from an infectious or toxic environmental juncture in people that have immune systems which are genetically predisposed to evolve a robust autoimmune feedback against pancreatic B cell antigens (Filippi & Von Herrath, 2008). There are diverse factors that can damage pancreatic B cell such as viruses, chemical agents, destructive cytotoxins and antibodies liberated from sensitized immunocytes (Rekittke, et al., 2016). On the other hand, basal genetic defects in connection to pancreatic B cell replication or function might predispose a person to evolve pancreatic B cell failure after viral infections (Graham, et al., 2012). The pancreatic B cell injury appears to be minimised when immunosuppressive products such as cyclosporine or azathioprine are prescribed at the onset of type 1 DM to reinforce auto-aggression by the immune system as a main agent in the pathogenesis of this disease. Therefore, regular insulin injection is required for patients with type 1 DM (Li, et al., 2014).

Type 2 DM is mainly caused by degradation of produced insulin. It can be controlled by diet monitoring and consumption of synthetic anti-diabetic drugs (Mohiuddin, et al., 2016). Type 2 DM affects more people compared to all other types of diabetes, and patients with NIDDM do not depend on exogenous insulin for the prevention of ketonuria and ketosis (Preeti & Sushil, 2016). The pathogenesis in type 2 DM is characterized by the fact that the pancreas manufactures insulin, but it is not properly utilized by the body (Wu, et al., 2014). This is essentially by peripheral tissue insulin resistance where insulin-receptors or other intermediates in the insulin signalling pathways inside the cells are tactless to insulin. It results in the inability of glucose to go through the tissue, causing hyperglycaemia or elevated level of glucose concentrations in blood as shown in Figure 1.2 (Wilcox, 2005). Obesity is one of the major risk factors of type 2 DM, which generally results in impaired insulin action and most patients in this case are obese (Wu, et al., 2014). However, the use of multiple anti-diabetic drugs/agents is required to sustain glycaemic control (Krentz, 2005).

1.3 Diabetes mellitus in Africa

African societies are currently facing a huge increase in nutritional shortages, which is mainly related to poverty, socio-economic deprivation, malnutrition and war. Numerous rural areas are exposed to rural migration, which is one the main cause of the unavailability of food. These lifestyle changes developed against a background of raising the prevalence of diabetic complications in Africa, mainly in countries that have a low daily *per capita* dietary energy supply and poor grade of food (Azevedo, 2008).

The proportion of diabetes deaths in Africa accounts for approximately 77%, which occurs in respective person under the age of 60 years (Pheiffer, et al., 2018). In addition, as in different corners of the world, type 2 DM affect more than 90% of diabetes cases (Wu, et al., 2014).

An estimation of 45.8% of all diabetes cases, which is an approximation of 174.8 million people have undiagnosed diabetes mellitus (UDM) in 2013 globally (Beagley, et al., 2014). In 2013, DM caused the deaths of 74.9 thousand people, which was the seventh largest cause of death, and also led to 1.85 million of people with disability, which is the eighth leading source of disability worldwide (Asmelash & Asmelash, 2019). Additionally, health burden, diabetes-related health expenditure is experience high cost on individuals, medical care systems, and governments. The global health spending on DM was 376 billion USD in 2010. (Zhang, et al., 2010)

The prevalence of diabetes in Africa is almost 4.3% of adults, accounting for over 14 million people, and it caused approximately 40 thousand deaths in 2012 (Mohammed, et al., 2014). Diabetes in Africa mostly affects people in the age intervals between 40 and 60 years, putting down a burden/load on the economy, which consequently result to work desertion and reduced productivity (Pheiffer, et al., 2018; Awad, et al., 2019).

The prevalence of diagnostic Diabetes Mellitus (UDM) in Africa differs from one country to another because of differences in economy, social, and genetic variations. The prevalence of

UDM in North Africa varied from 18 to 75% of all diabetes victims. Additionally, the prevalence of UDM in different parts of Africa are illustrated as follows18.1% in South Africa, 9% in Tanzania, 7% in Nigeria, 6.3% in Cameroon, 4.77% in Mauritania, 4.64% in Senegal, 4.2% in Egypt, 3.19% in Guinea (Asmelash & Asmelash, 2019).

1.4 Diabetes mellitus in South Africa

In South Africa, there are about 6.5 million diagnosed diabetic patients and an estimated equal number that are currently undiagnosed (Pheiffer, et al., 2018). In 2009, about 2 million (9%) people aged from 30 years and above possessed diabetes (Pheiffer, et al., 2018). Numerous factors such as economic transition, ageing population, and urbanisation linked with nutrition transition and obesity are responsible of the raised of diabetes prevalence in Africa. In 2000, an estimation of 87% of diabetes victims in South Africa was ascribed to overweight (Frank & Hu, 2011). In 2013 an approximation of 38% of male (men) and ~69% of female (women) in South Africa were fell under the obese-prevalent category (Cois & Day, 2015). In 2015, the global burden of DM research study showed that obesity and hyperglycaemia were classified as the second and third main risk factors, respectively, after unprotected sex, for premature death and disability in South Africa (Pheiffer, et al., 2018).

South Africa is combating a quadruple burden of diseases because of high levels of infectious disorders, maternal and child mortality, non-communicable disease, and injury-related disorders, thus have restricted resources to encounter the raised health and economic costs of diabetes (Pheiffer, et al., 2018).

1.5 Diabetes complications

Diabetes complications are an outcome of long-term injury, dysfunction, and failure of some organs, mostly the kidneys (diabetic nephropathy), eyes (diabetic retinopathy), nerves (diabetic

neuropathy), blood vessels (atherosclerosis), and heart (myocardial infarction), which are associated to uncontrolled hyperglycemia (Cade, 2008). Other symptoms related to hyperglycemia enclose polyuria, polydipsia and weight loss. Impairment of growth and sensitivity to some infections might also go along with chronic hyperglycemia (American, 2009). Long-term complications related to diabetes including nephropathy can cause renal disorder, peripheral neuropathy with high risk of foot ulcers, autonomic neuropathy and amputations causing gastrointestinal, genitourinary, cardiovascular symptoms and erectile dysfunction. Additionally, other symptoms such as hypertension and dysfunction of lipoprotein metabolism are mostly present in people with diabetes (Ozder, 2014).

1.6 Alpha-glucosidase inhibitors

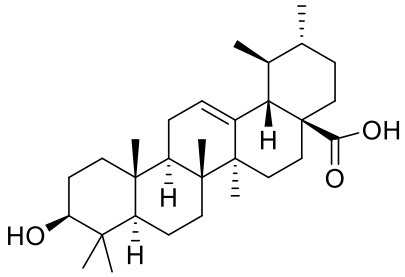
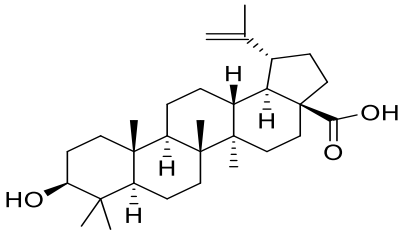
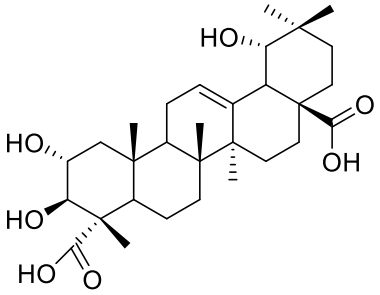
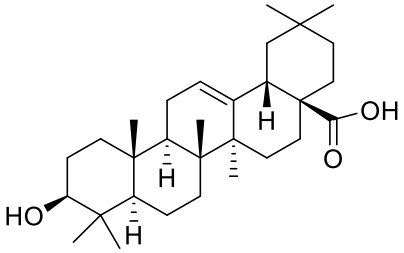
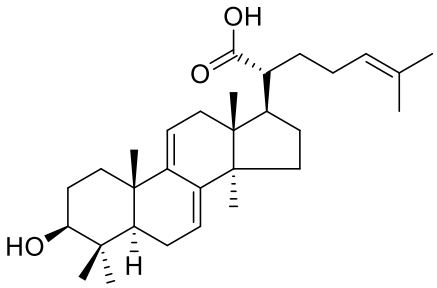
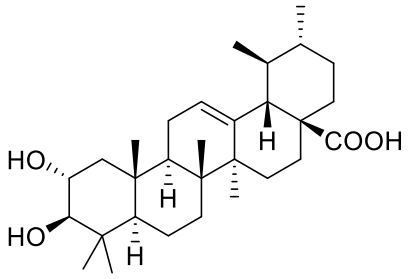
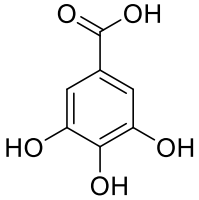
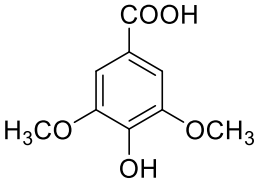
Alpha-glucosidase inhibitors are classes of oral anti-diabetic drugs employed for type 2 diabetes (Derosa & Maffioli, 2012). They delay the rate of complex carbohydrates (starches, oligosaccharides and disaccharides) digestion and delay the rate of glucose absorption (Lovegrove, et al., 2017). Acarbose and miglitol are the main two drugs available in this class (Dabhi, et al., 2013). Numerous classes of compounds isolated from natural sources such as terpenoids [ursolic acid , betulinic acid , bartogenic acid , oleanolic acid , dehydrotrametenolic acid , corosolic acid], gallic acid derivatives [gallic acid , syringic acid , vanillic acid], phenolic acids [chlorogenic acid], flavonoids [flavonolignans, feruloylglucosides], ellagic acid (Table 1) demonstrated strong alpha glucosidase inhibitory activities (Benalla, et al., 2010).

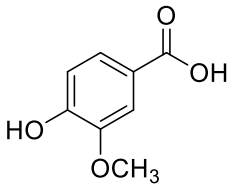
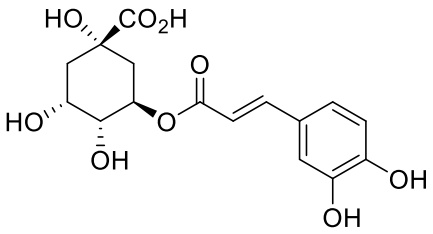
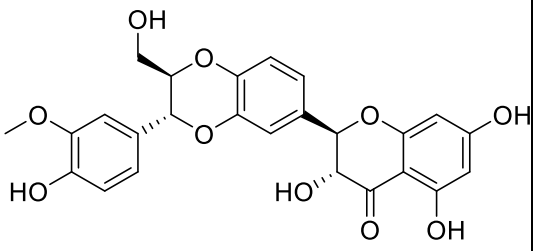
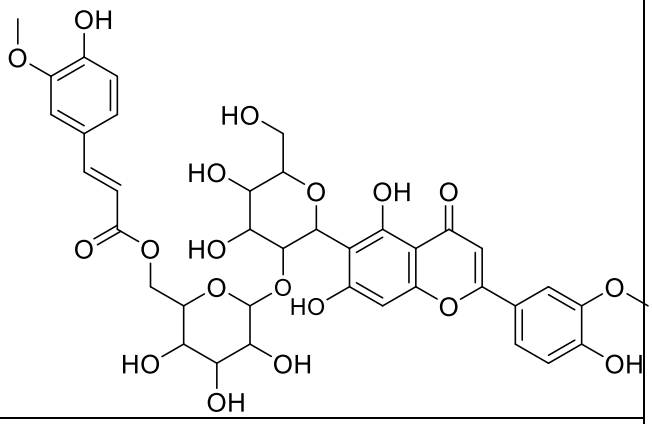
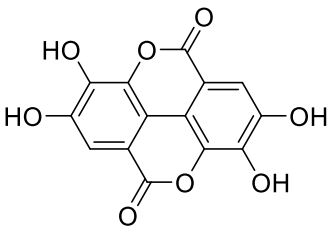
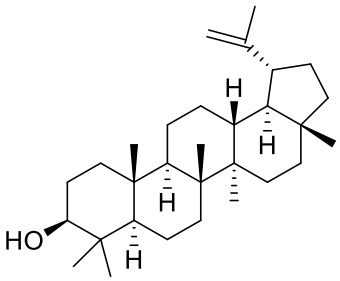
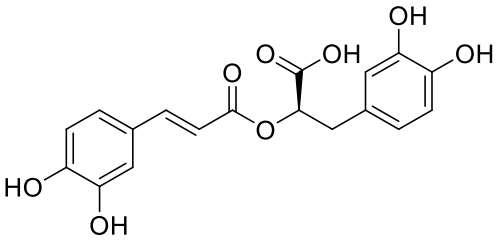
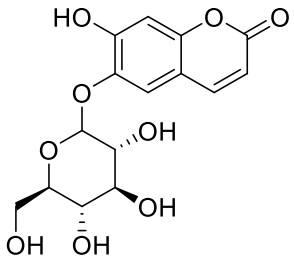
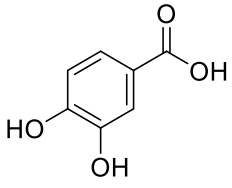
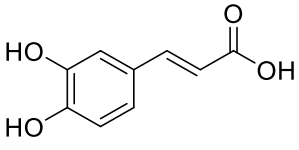
1.7 Alpha-amylase inhibitors

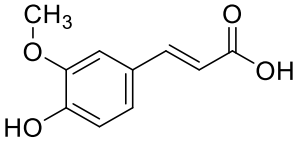
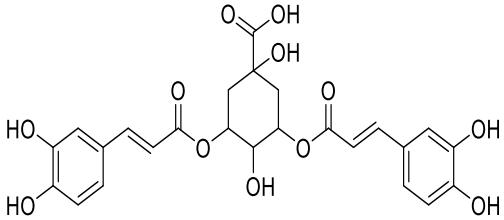
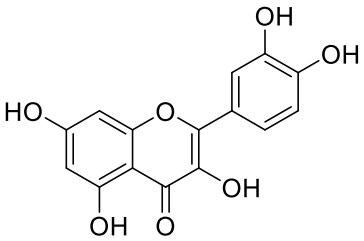
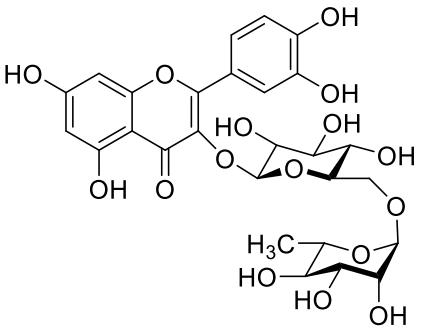
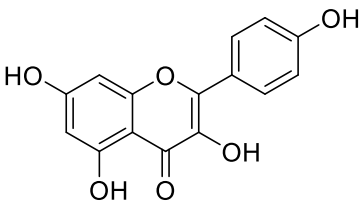
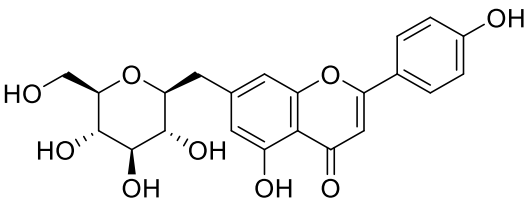
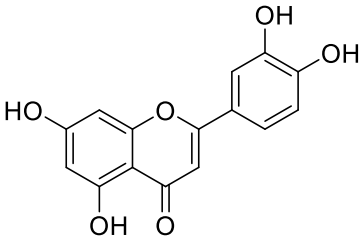
Alpha amylase inhibitors play a key role in the management of post-prandial hyperglycaemia. It is involved in the inhibition process of alpha-amylase, leading to depletion in starch hydrolysis to maltose and accordingly reduces post-prandial hyperglycaemia (Gautam, et al., 2013). Numerous classes of compounds isolated from natural sources such triterpenes [Ursolic

acid, Bartogenic acid, Oleanolic acid, lupeol], cinnamic acid [rosmarinic acid, esculin, Ellagic acid, protocatechuic acid, caffeic acid, ferulic acid], phenolic acids [Chlorogenic acid, isochlorogenic acid, flavonoids [quercetin, rutin, kaempferol, lupeol, apigetrin, luteolin] (Table 1) exhibit strong alpha-amylase inhibitory activities (Castellano, et al., 2013).

Table 1.1 List of alpha glucosidase and alpha amylase inhibitors

 **Ursolic acid**	 **Betulinic acid**
Ursolic acid	Betulinic acid
 Bartogenic acid	 **Oleanolic acid** Oleanolic acid
 **Dehydrotrametenolic acid**	 **Corosolic acid**
Dehydrotranetenolic acid	Corosolic acid
 **Gallic acid**	 **Syringic acid**
Garlic acid	Syringic acid

 **Vanillic acid**	 **Chlorogenic acid**
Vanillic acid	Chlorogenic acid
 **Flavonolignan**	
Flavonolignan	Feruloylglucoside
 **Ellagic acid**	 **Lupeol**
Ellagic acid	Lupeol
 **Rosmarinic acid**	
Rosmarinic acid	Esculin
	 **Caffeic acid**

Protocatechiuc acid	Caffeic acid
 **Ferulic acid**	 **Isochlorogenic acid**
Ferulic acid	Isochlorogenic acid
 **Quercetin**	 **Rutin**
Quercetin	Rutin
 **Kaempferol**	 **Apigetrin**
Kaempferol	Apigetrin
 **Luteolin**	
Luteolin	

1.8 Oxidative stress and diabetes complications

Diabetes mellitus is well known as one of the most dangerous metabolic disorders characterized by hyperglycaemia, which is mainly associated with oxidative stress, a phenomenon created by an imbalance between the free radicals or oxygen reactive species (ROS) generated and accumulated in cells and tissues as well as the capacity of a biological system to detoxify these reactive agents (Liguori, et al., 2018). However, oxidative stress happens when the fabrication of free radicals overwhelms the detoxification capability of cellular antioxidant system resulting in biological damage (Lobo, et al., 2010). It is involved in the development of diabetes complications such as cardiovascular and microvascular. The metabolic irregularities of diabetes result in mitochondrial superoxide overproduction, which is the principal activator of diabetes tissue injury, β-cell dysfunction, insulin resistance and impaired glucose tolerance (Giacco & Brownlee, 2010). Additionally, the excess nourishment linked with a sedentary lifestyle result in excess of glucose and fatty acid cumulation inside the muscle, pancreatic cells and adipose tissue, which lead to the overproduction of reactive oxygen species (ROS), mainly superoxide anion, via the mitochondrial electron-transport chain (Wright, et al., 2006). The principal origins of oxidative stress in diabetes encompass auto-oxidation of glucose, shifts in redox balances, reduced tissue concentrations of low molecular weight antioxidant agents, such as vitamin E and reduced glutathione (GSH), and impaired activities of antioxidant protection enzymes such as catalase (CAT) and superoxide dismutase (SOD) (Matough, et al., 2012).

1.9 Rationale of the study

The world is facing many challenges regarding nutritional deficiencies due to poverty, socio-economic deprivation, malnutrition, rural migration, war and consumption of fast food, which are the major factors for the development of various kinds of diseases including diabetes.

Diabetes mellitus (DM) is one of the most common endocrine ailments that affect about 6% of the worldwide population. It is projected to affect five times more people in the next ten (10) years as reported by the World Health Organization and American Diabetes Association.

Synthetic anti-diabetic drugs such as miglitol, acarbose, metformin, sulfonylurea, thiazolidinedione are readily commercialized, but the usage of these products is limited because of high cost and high adverse side effects including hypoglycaemia, liver injury, diarrhoea, flatulence, dropsy, abdominal pain, drug- resistance, heart failure and weight gain.

In the search of natural antidiabetic agents, *Helichrysum* species were selected because numerous of them such as *Helichrysum graveolens*, *H. italicum, H. caespititium H. plicatum* etc are traditionally used in some geographic locations for the treatment of diabetes (Maroyi, 2019). Hence, *H. cymosum* was selected for this study based on the phytochemical profile as well as availability in the Cape Floristic Region. Phenolic compounds especially flavonoids are the main constituents of this family, and they have been reported to take part in the inhibition of alpha-glucosidase and alpha-amylase, which are enzymes implicated in diabetes complications as well as possessing high free radical scavenging ability.

Therefore, it is of great need to evaluate *H. cymosum* for the discovery of new bioactive compounds for their potential application in the prevention and management of diabetic complications with the potential of replacing synthetic drugs/products readily available in the market.

1.10 Aims of this study

The aim of this research project is directed towards the phytochemical study of *H. cymosum* as well as the *in vitro* bio-evaluation of the total extract and its isolated compounds against alpha-glucosidase, alpha-amylase enzymes in addition to their antioxidant properties.

1.11 Objectives of this study

The main objectives of the study are:

- Collection, documentation and identification of *H. cymosum*
- Extraction of the plant material with methanol.
- Perform chromatographic isolation of different phytochemical constituents present in *H. cymosum* extract using different chromatographic techniques.
- Structural characterization of the isolated compounds using different spectroscopic techniques such as 1D NMR.
- *In vitro* bio-evaluation of the crude extract and its isolates against alpha-glucosidase and alpha-amylase enzymes.
- Investigation of the antioxidant properties of the crude extract and its isolated compounds.

1.12 Scope of the study

The scope of this study is to systematically investigate the endogenous South African H.*Cymosum*, by using bioassay-guided fractionation using various chromatography techniques to isolate and purify H.*Cymosum* phytochemical compounds. In this study the pure plant material will be subjected to different spectroscopic techniques such as 1D NMR to elucidate the structures of compounds. The structure–activity relationship will be discussed in the course of the chemistry section of the thesis. The biological investigation of this study focuses on building a better understanding of the traditional use of the genus Helichrysum as treatment and management of the symptoms associated with a high blood-glucose level. Both alpha-glucosidase and alpha-amylase inhibition activity of each compound, in its pure form, will be assessed and compared to standard in different concentration. In addition, antioxidant activity will be assessed and reported for each isolated compound.

CHAPTER TWO: LITERATURE REVIEW

ANTIDIABETIC ACTIVITIES OF *HELICHRYSUM SPECIES*

2.1 Aim of this chapter

The aim of this chapter is to review the existing information from SciFinder on the traditional uses, phytochemical constituents, and antidiabetic activities of *Helichrysum* species traditionally used in some regions of the world. Furthermore, the antidiabetic activities of their notable chemical constituents are presented.

2.2 Asteraceae family

Asteraceae, also called Compositae, is a very big and widespread family of flowering plants worldwide. It comprises about 23 600 species assigned to 1620 genera of herbaceous plants, shrubs and trees. The family is divided into 13 subfamilies of which the Asteroideae contains more than 70% of the species currently recognised (Panero & Crozier, 2016). There are especially diverse in the tropical and subtropical regions of Southern Africa, Mediterranean region, Central Asia, South-western China, and Australia (Bremer, 1987; Heyman, 2009). Although the variability of the vegetation, this family is identifiable by capitulate and involucrate inflorescences in which many small flowers open first on the outside and only occasionally subtended by bracts. The anthers are combined and form a tube by which the style expands before the two stigmatic lobes separate and become recurved, while the single-seeded fruit commonly possess a plumose pappus (Fu, et al., 2016).

2.3 *Helichrysum* genus: Description, distribution, traditional uses and phytochemistry

The *Helichrysum* genus, commonly known as everlasting flowers and immortelles belongs to the Asteraceae family, tribe Inuleae and subtribe Gnaphaliinae. It comprises about 600 species

worldwide, which are originally from Africa and Madagascar, but it is also found in Australasia and Eurasia (Akaberi, et al., 2019). An approximation of 250 *Helichrysum* species are widespread in Southern Africa with wide morphological diversities (Abad, et al., 2013). *Helichrysum* species are generally considered as aromatic perennial herbs with dense hair or woolly leaves and persistent flower heads of different varieties (Van Wyk, et al., 2009). The flower heads are either compact or solitary or spreading inflorescences. The aerial parts are mostly hairy or woolly and the plant occurs as shrublets or herbs that are occasionally dwarfed and cushion forming (Lourens, et al., 2008). *Helichrysum* species have been used traditionally worldwide in the treatment of conditions associated with diabetes and numerous other diseases (Lourens, et al., 2008).

Helichrysum species are generally rich sources of secondary metabolites such as acylphloroglucinols, humulone derivatives, flavonoids, phenolic acids, chalcones, phthalides, sterols, pyrones, coumarins, diterpenes, sesquiterpenes, and polyacetylenes (Maroyi, 2019), which give them a great value for global acceptability and wide application in the pharmaceutical fields as anti-inflammatory, antibacterial and antioxidant, antidiabetic agents (Maroyi, 2019).

2.4 *Helichrysum* species used for the treatment diabetes

2.4.1 *Helichrysum graveolens*

Retrieved from: https://www.flickr.com/photos/inst_humboldt/19476914841

Figure 2.1: Morphology of *Helichrysum graveolens*

2.4.1.1 Traditional uses of *H. graveolens*

Helichrysum graveolens (Bieb.) Sweet (Asteraceae) is widely distributed in Turkey (Aslan, et al., 2007). Capitulums of *H. graveolens* are utilized in the treatment of different kind of diseases such as wound healing, jaundice, and as a diuretic agent in the rural zones of Anatolia. The decoction of *H. graveolens* is employed in the treatment of diabetes mellitus in different regions of Anatolia, Turkey, South Africa (Aslan, et al., 2007).

2.4.1.2 Phytochemical constituents of *H. graveolens*

The phytochemical investigation of the methanol extract of *H. graveolens* revealed the presence of chlorogenic acid, syringic acid, ferulic acid, caffeic acid, apigenin-7-glucoside, apigenin, and hesperidin, naringenin, luteolin, resveratrol and quercetin as the major phenolic compounds in the extract (Aslan, et al., 2007; Orhan, et al., 2014).

2.4.1.3 Antidiabetic activity of *H. graveolens*

The hydro ethanolic extract inhibited the alpha-glucosidase enzyme with IC_{50} value of 0.7129 mg/mL Additionally, the hydro ethanolic extract demonstrated 55.7 % ± 2.2 inhibition when tested against alpha-amylase enzyme at the concentration of 3 mg/mL (Orhan, et al., 2014). Additionally, the hydro-alcoholic extracts of *H. graveolens* has been reported to possess significant hypoglycaemic activity in normoglycaemic, glucose loaded and streptozotocin-induced diabetic rats (Orhan, et al., 2014).

2.4.2 *Helichrysum plicatum*

Retrieved from: https://species.wikimedia.org/wiki/Helichrysum_plicatum
Figure 2.2: Morphology of *Helichrysum plicatum*

2.4.2.1 Traditional uses of *H. plicatum*

H. plicatum is an herbaceous perennial plant, native to Iran, Balkan and Anatolian Peninsulas (Vujić, et al., 2020). The herbalist employed the decoction of the plant to treat the symptoms of diabetes mellitus in Turkey's folk medicine (Aslan, et al., 2007; Pereira, et al., 2017).

2.4.2.2 Phytochemical constituents of *H. plicatum*

Several flavonoids such as apigenin, isoastragalin, naringenin, isosalipurposide, helichrysin A and B were isolated as the main phytochemical constituents of *H. plicatum* (Aslan, et al., 2007).

2.4.2.3 Antidiabetic of *H. plicatum*

The bio-evaluation of hypoglycaemic and antioxidant activities of *H. plicatum* suggest that the plant could be useful for the protection and alleviation of diabetes complications (Ozkan, et al., 2016).

The hydro-alcoholic extract of *H. plicatum* has shown strong ABTS radical cation scavenging activity, which may be a good choice for complementary cure for type 2 diabetic patients (Orhan, et al., 2014). The antidiabetic activity demonstrated by *H. plicatum* might be related to

the high content of flavonoid (apigenin) present in the hydro-alcoholic extract (Aslan, et al., 2007).

2.4.3 *Helichrysum aureum*

Retrieved from: http://www.anniesannuals.com/signs/h/helichrysum_aureum.htm
Figure 2.3: Morphology of ***Helichrysum aureum***

2.4.3.1 Traditional uses *H. aureum*

H. aureum is a perennial herb, native to Mozambique and Cape Province (South Africa). ***H. aureum*** is traditionally used by Basotho people for the treatment of diabetes. Additionally, it is utilized for asthma, flu, cough, headache, bronchitis, epilepsy, and pain. The crude extract and the pure chemical derivatives are utilized in the treatment of migraine, malaria, epilepsy, nausea, glaucoma, acquired immune deficiency syndrome (AIDS), appetite activation for cancer patients (Balogun, et al., 2006).

2.4.3.2 Phytochemical constituents *H. aureum*

The aerial part (flowers and leaves) of ***H. aureum*** are the rich source of phenolic compounds including flavonoids (isosalipurposide, naringenin, naringenin-5-*O*-glucoside, helichrysin A, helichrysin B, quercetin, luteolin, apigenin), phenolic acids (chlorogenic acid, caffeic acid, ferulic acid, 3,4-methylendioxycinnamic acid , sinapic acid, syringic acid, protocatechuic, gentisic acid), phthalides (5,7-dihydroxyphthalide, 5-methoxy-7-

hydroxyphthalide, helichrysumphtalide, everlastoside H), coumarins (umbelliferone, scopoletin), and pyrones (homoarenol, . (Pljevljakušić, et al., 2018).

2.4.3.3 Antidiabetic activity of *H. aureum*

To date, the scientific validation of the antidiabetic activity has not been reported (Balogun, et al., 2006).

2.4.4 Helichrysum caespititium

Retrieved from:
https://keys.lucidcentral.org/keys/v3/helichrysum/key/Helichrysum/Media/Html/Helichrysum_caespititium.htm

Figure 2.4: Morphology of ***Helichrysum*** *caespititium*

2.4.4.1 Traditional uses of *H. caespititium*

H. caespititium is widely distributed in South and Central Africa (Maroyi, 2019). The whole plant of *H. caespititium* is traditionally used in South African folk medicine to alleviate diabetes mellitus (Makhafola, et al., 2019). *H. caespititium* is employed for treating many medical conditions such as respiratory infections, tuberculosis, bronco-pneumonia, and intestinal ulceration, sexually transmitted infections, wounds, headache, nausea, ulceration, skin infection diseases, respiratory problems, gastro-intestinal tracts, and diarrhoea, headache, chest colds as well as for the treatment of internal wounds such as intestinal ulceration (Maroyi, 2019).

2.4.4.2 Phytochemical constituents of *H. caespititium*

Several compounds were isolated from whole plant of *H. caespititium* such as phloroglucinol, caespitin (A) and caespitate (B) (Maroyi, 2019).

2.4.4.3 Anti-diabetic activity of *H. caespititium*

The scientific validation of the antidiabetic activity has not been reported.

2.4.5 Helichrysum italicum

Retrieved from: http://plantinfo.co.za/plant/helichrysum-italicum/

Figure 2.5: Morphology of ***Helichrysum*** *italicum*

2.4.5.1 Traditional uses of *Helichrysum italicum*

H. italicum is a perennial plant native to Mediterranean. The most commonly reported traditional uses of *H. italicum* are associated to digestive, respiratory, wound healing, skin inflammatory conditions, and diabetes complications (Pereira, et al., 2017).

2.4.5.2 Phytochemical constituents of *H. italicum*

Several compounds were isolated from *H. italicum* including acetophenones (acetophenones 4-hydroxy-3-(3-methyl-2-butenyl), acetophenone,4-hydroxy-3-(2-hydroxy-3-isopentenyl) acetophe-none), flavonoids and pholoroglucinol derivatives (tiliroside, apigenin, gnaphaliin,

quercetin, luteolin and the prenylated α-pyrone-pholorglucinol etherodimer arzanol) (Pereira, et al., 2017).

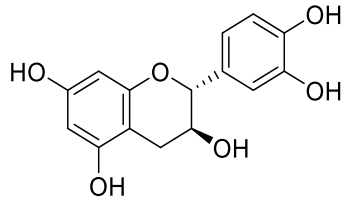
2.4.5.3 Antidiabetic activity of *H. italicum*

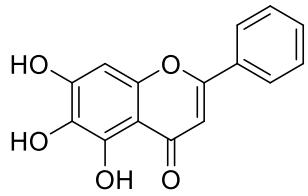
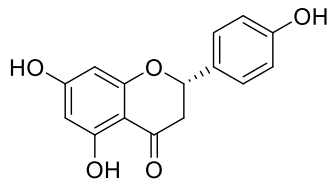
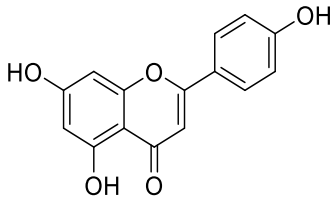
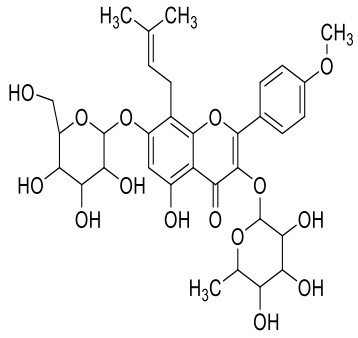
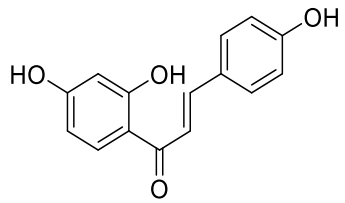
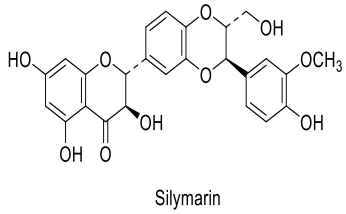
H. italicum has been reported to ameliorate hyperglycemia by suppressing digestive enzyme capacities and SGLT1-mediated glucose uptake. Additionally, *H. italicum* extracts showed inhibitory activity against alpha-glucosidase and alpha-amylase with the IC_{50} values of 0.19 and 0.83 mg/mL, respectively (Pereira, et al., 2017).

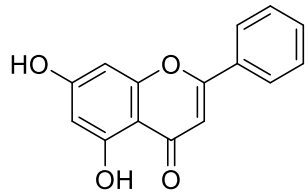
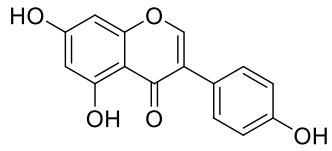
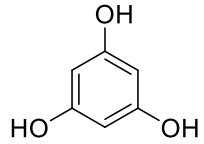
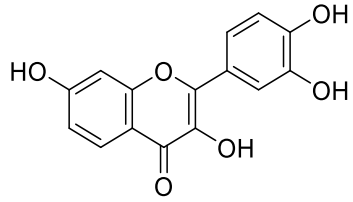
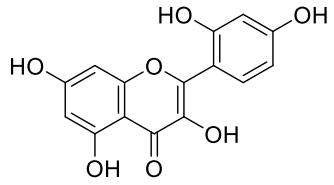
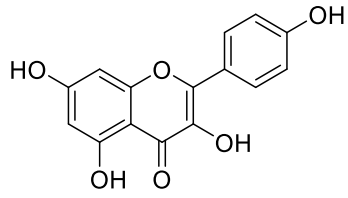
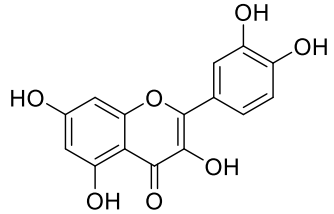
2.5 Antidiabetic activity of some notable chemical constituents isolated from *Helichrysum* species

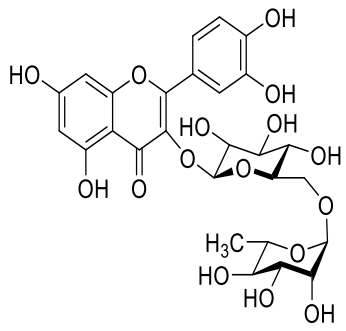
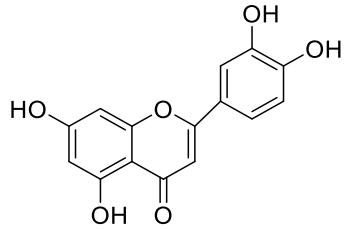
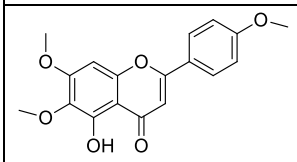
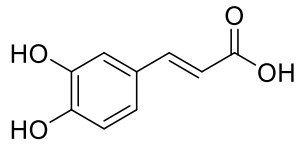
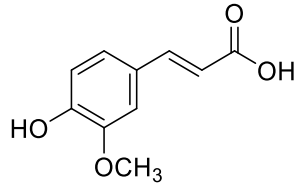
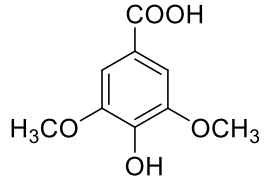
Numerous classes of compounds have been reported to be the bioactive constituents of countless plant species of *Helichrysum* species used in folk medicine for their potential applications in the management of diabetes related complications. Among these bioactive compounds, flavonoids demonstrated significant antidiabetic activity due to the OH group allocated at C-3 on the C-ring and additional OH groups attached to the A and B-ring, which play a vital role in the inhibition process of alpha-glucosidase and alpha amylase enzymes as well as hypoglycaemia, insulin activation, glucose uptake activation (Wang, et al., 2010). Hence, the mode of action of such compounds is illustrated in Table 2.1.

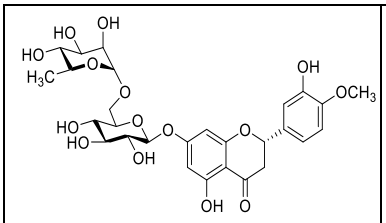
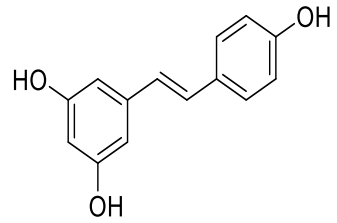
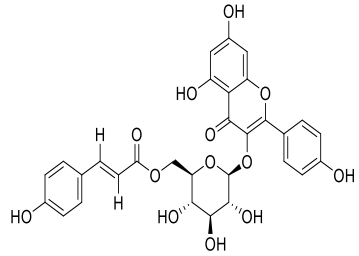
Table 2.1: Antidiabetic activity of different constituents of *Helichrysum* species

Compounds	Plant source	Biological activity	References
 Catechin	*Helichrysum* species	Potential antidiabetic agent with strong alpha glucosidase activity IC_{50} = 87.55 ± 2.23 µg/mL	(Mrabti, et al., 2018)

 Baicalein	*H. graveolens*	Good antidiabetic, hypolipidemic, and hypoglycemic capacities in streptozotocin (STZ)-induced diabetic rats	(Bai, et al., 2019)
 Naringenin	*H. graveolens*	Prevent obesity-related diseases and to activate PPARγ	(Yoshida H1, et al., 2017)
 Apigenin	*H. graveolens*	Antihyperglycemic effect Protective ability on pancreatic β-cell destruction in streptozotocin-induced diabetes Reduces blood glucose	(Huang, et al., 2018) (Süntar, et al., 2013) (Osigwe, et al., 2017)
 Icariin	*H.* arenarium *H. odoratissimum*	Useful candidate in the treatment of diabetic retinopathy	(Xin, et al., 2012) (Zai-Biao, et al., 2015) (De Canha, et al., 2020)
 Isoliquiritigenin	*H. maracandicum*	Potential candidate for controlling blood glucose level/diabetes	(Gaur, et al., 2014)
 Silymarin	*Helichrysum* species	Good activity in the restoration of liver function Good efficacy in managing blood glucose level in diabetes patients with liver problems	(Abraham & Narmadha, 2011)

 Chrysin	*H. italicum*	Induces antidyslipidemic and antidiabetic, anti-inflammatory effects in athymic nude diabetic mice	(Ramírez-Espinosa, et al., 2018)
 Genistein	*H. italicum*	Anti-diabetic effects on β-cell proliferation, glucose stimulated insulin secretion and protection against apoptosis	(Gilberta & Liub, 2013)
 phloroglucinol	*H. caespititium*	Oral antihyperglycemic candidate in the management of diabetes mellitus	(Murugan, 2018)
 Fisetin	*Helichrysum.* species	Inhibits high glucose induced cytokine production in monocytes Improve the development of diabetic cardiomyopathy in STZ- induced DM rats	(AL-Ishaq, et al., 2019)
 Morin	*Helichrysum.* species	Protective effect against diabetic induced osteopenia	(Abuohashish , et al., 2013)
 Kaempferol	*Helichrysum.* species	Improves streptozotocin-induced diabetes Suppress Hepatic Glucose Production	(Alkhalidy, et al., 2018)
 Quercetin	*H. italicum*	Stimulate β-cells to liberate more insulin	(Jadhav & Puchchakayala, 2012) (Kladar, et al., 2015)

 Rutin	*H. leucocephalum*	Stimulates β-cells to produce more insulin Anti-hyperglycemic capacity in streptozotocin-induced diabetic rats	(Jadhav & Puchchakayala, 2012) (Niture, et al., 2014)
 Luteolin	*H. italiensis*.	Positive effects of anti-diabetes on KK-A(y) mice	(Zang, et al., 2016)
 Salvigenin	*Helichrysum* species	Improves diabetes by decreasing lipid profile, blood glucose level, HbA1c. Increases insulin secretion	(Sadeghi, et al., 2016)
 Caffeic acid	*H. graveolens*	Potential antidiabetic agent Attenuates hepatic glucose output Enhances adipocyte glucose uptake, insulin secretion, and antioxidant capacity.	(Jung, et al., 2006)
 Ferulic acid	*H. graveolens*	Therapeutic and protective effects on diabetic nephropathy by reducing oxidative stress and inflammation	(Choi, et al., 2011)
 Syringic acid	*H. graveolens*	Ameliorates glycoprotein components abnormalities	(Muthukumaran, et al., 2013)

 Hesperidin	*H. graveolens*	Hypolipidemic and hypoglycemic effects in streptozotocin-induced marginal diabetic rats	(Akiyama, et al., 2010)
 Resveratrol	*H. graveolens*	Activator in a genetic model for type-2 Diabetes	(Sharma, et al., 2011)
 Tiliroside	*H. italicum*	Significant glucose consumption-enhancing effects in IR-HepG2 cells	(Zhu, et al., 2010)

2.6 Conclusion

This chapter describes the traditional uses, phytochemical constituents, and antidiabetic activities of *Helichrysum* species used traditionally in some geographic locations for the treatment of diabetes. Additionally, it highlighted the biological importance of some notable chemical constituents isolated from *Helichrysum* species for their potential application in the prevention and management of type 2 diabetes complications using different mechanism of action.

HELICHRYSUM. CYMOSUM: BACKGROUND AND LITERATURE REVIEW

2.7 Background information on *Helichrysum cymosum*

2.7.1 Botanical description of *H. cymosum*

Helichrysum cymosum, a member of the Asteraceae family, it is commonly referred to as daisy, aster, or sunflower family. In South Africa, *H. cymosum* is well known as gold carpet or yellow-tipped strawflower in English, goute tapyt in Afrikaans, and impepho in isiXhosa or Zulu. There are two subspecies of *H. cymosum* that exist in nature, which are *H. cymosum* subsp. cymosum and *H. cymosum* subsp. calvum Hilliard. The leaves possess variable shape and size, becoming smaller and more distant upward. The leaves are elliptic-oblong or linear-oblong in shape, the apex is acute, sometimes acuminate, mucronate, slightly narrowed, and clasping at the base (Hayman, 2013). The leaf margins are flat or sub-revolute, upper surface covered in thin silvery grey and paper-like hairs, while the lower surface has white-woolly hairs. The flower heads are disciform sometimes discoid, crowded in terminal corymbs, cylindric, glossy, and bright canary-yellow in color as shown in Figure 2.6 (Maroyi, 2019)

Figure 2.6: *Helichrysum cymosum* description

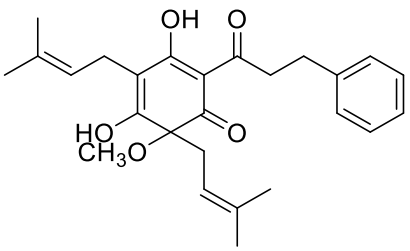
2.7.2 Traditional uses of *H. cymosum*

The aerial parts (flowers, leaves, roots, twigs) and whole plant parts of *H. cymosum* are used in traditional medicine for various applications. *H. cymosum* is mostly used as herbal medicine

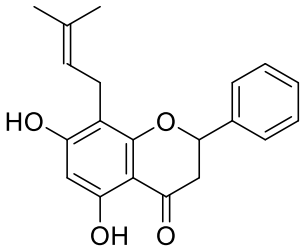
for colds, cough, fever, headache, and wounds. Other minor medicinal applications include blocked nose, boost immunity, cardiovascular problems, diarrhoea, dizziness, eye problems, flatulence, improve appetite, influenza, insect repellent, insomnia, kidney problems, laxative, menstrual pain, pertussis, pulmonary problems, skin infections, urinary problems, varicose veins, vomiting, and weak bones (Hayman, 2013). The leaves, stems, and twigs of *H. cymosum* are sold as herbal medicines in the informal herbal remedies' markets in the Gauteng and the Western Cape provinces in South Africa (Maroyi, 2019).

2.7.3 Phytochemical constituents of *H. cymosum*

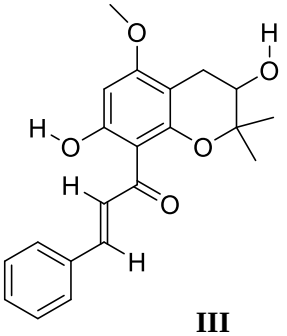
Numerous compounds have been isolated from the alcoholic extract of the leaves and roots of *H. cymosum* including helihumulone (**I**), helichromanochalcone (**II**), 5-hydroxy-8-methoxy7-prenyloxyflavanone (**III**), 1-Benzopyran-4-one,2,3-dihydro-5-hydroxy-8-methoxy-7-[(3-methyl-2-butenyl)oxo] (**IV**), pinocembrin chalcone (**V**), (2E)-3-Phenyl-1-[2,4,6-trihydroxy-3-(3-methyl-2-buten-1-yl)phenyl]-2-propen-1-one (**VI**), 2,3-Dihydro-6,8-dihydroxy-5-methoxy-2-phenyl-4H-1-benzopyran-4-one (**VIII**), 2,3-Dihydro-5,8-dihydroxy-6-methoxy-2-phenyl-4H-1-benzopyran-4-one (**IX**), 1-Benzopyran-4-one, 2,3-dihydro-5-hydroxy-2-phenyl (**X**), 2,3-Dihydro-5,8-dihydroxy-6-prenyl-2-phenyl-4H-1-benzopyran-4-one (**XI**), gnaphaliin (**XII**), 8-Hydroxykaempferol 5,6-dimethyl ether (**XIII**), 2*H*-1-Benzopyran-5-carboxylic acid, 7-methoxy-2,2-dimethyl (**XIV**) (Fig. 2.) (Jakupovic, et al., 1988; Van Vuuren, et al., 2006; Popoola, et al., 2015). Helihumulone is a known phloroglucinol derivative with the antimicrobial activity (MIC) ranges, from 0.016 to 0.125 (mg/mL) (Van Vuuren, et al., 2006).



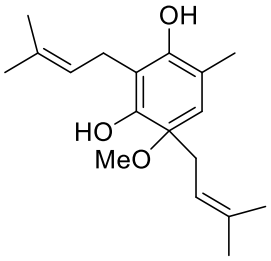
I



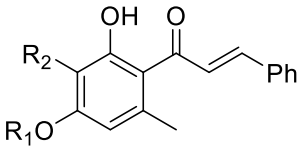
II



III

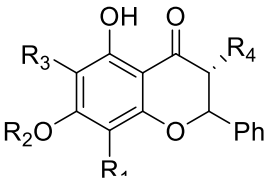


IV

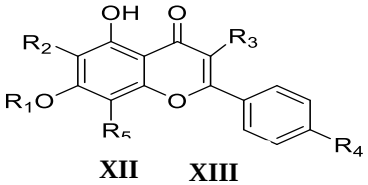


V **VI**

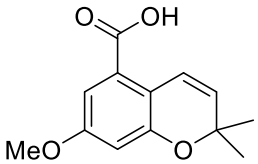
R1: H H
R2: H Prenyl



	VII	VIII	IX	X	XI
R1:	OMe	OMe	OH	H	Prenyl
R2:	Prenyl	H	Me	H	H
R3:	H	H	H	H	H
R4:	H	H	H	H	H



	XII	XIII
R1:	H	Me
R2:	H	H
R3:	OMe	OH
R4:	H	OH
R5	OMe	OMe



XIV

Figure 2.7: List of isolated compounds from *H. cymosum*

2.7.4 Medicinal studies of *H. cymosum*

The following biological properties have been reported from the extract of *H. cymosum* as well as its isolated compounds such as antimicrobial, antioxidant, antifungal, antiviral, anti-HIV, anti-inflammatory, antimalarial, and cytotoxicity activities (Lourens, et al., 2008; Maroyi, 2019; Van Vuuren, et al., 2006).

2.7.4.1 Antibacterial activities

Stafford et al. 2005 reported on the antibacterial activities of the ethanol extract of whole plant of *H. cymosum* against *S. aureus, Bacillus subtilis*, *E. coli*, and *K. pneumonia* using micro plate method. The extract demonstrated activities with MIC values ranging from 0.8 to 1.6 mg/mL (Stafford, et al., 2005). Additionally, the extract displayed activities against *E. faecalis, B. subtilis, B. cereus,* and *S. aureus* with zone of inhibition ranging from 3.7 to 8.0 mm, which was in a comparative manner to 3.0 mm to 11 mm shown by the positive control (Van Vuuren, et al., 2006).

2.7.4.2 Antioxidant activities

The extract of *H. cymosum* exhibited radical scavenging activities with SC50 value of 6.3 g/L, which was less than the value of the positive control (7.0 mg/L) (Franccedil, et al., 2010).

2.7.4.3 Antifungal activities

The extract of *H. cymosum*, essential oil and helihumulone displayed activities with MIC values ranging from 0.03 to 4.0 mg/mL (Van Vuuren, 2007). Additionally, the extract showed activities with 54.7% inhibition starting from 2.5 to 5 mg/mL (Franccedil, et al., 2010).

2.7.4.4 Anti-HIV activities

The methanol:water of *H. cymosum* extract demonstrated slight toxicity with Cytopathic effect (CPE) of 400. µg/mL in comparison to 0.8 µg/ml displayed by the positive control (Heyman, 2009). The evaluation of the anti-HIV activities of dichloromethane and methanol/water extracts of the aerial parts of *H. cymosum* using the DeCIPhR method displayed activities with median lethal concentration ranging from 10.0 to 21.0 µg/mL (Heyman, et al., 2015)

2.7.4.5 Anti-inflammatory activities

Stafford *et al.* investigated the anti-inflammatory activities of aqueous and ethanol extracts of whole plant parts of *H. cymosum* using the cyclooxygenase (COX-1) inhibition assay. The COX-1 inhibitory activities displayed by aqueous and ethanol extract was 52.0% and 100.0%, respectively (Stafford, et al., 2005).

2.7.4.6 Antimalarial activities

The extract of *H. cymosum* was evaluated for the antimalarial activities and the result showed activity with IC_{50} value of 60.8 µg/mL, compared to the positive controls, which displayed IC_{50} values ranging from 0.09 to 0.1 µg/mL (Van Vuuren, et al., 2006).

2.7.4.7 Cytotoxicity activities

The chloroform and methanol:water extracts of *H. cymosum* exhibited IC_{50} values of 36.5 µg/mL and 59.7 µg/mL, respectively, which were higher than 1.3 µg/mL displayed by the positive control (Heyman, 2009).

CHAPTER THREE: METHODOLOGY

3.1 General experiment procedure

3.1.1 Reagents and solvents

Organic solvents such as methanol and deuterated chloroform were purchased from Merck (Cape Town, South Africa). Sulfuric acid, ethyl acetate, hexane, ethanol, dichloromethane, were purchased from Kimix (Cape Town, South Africa).

3.1.2 Chromatography

3.1.2.1 Thin-layer chromatography (TLC)

Pre-coated TLC plates of silica gel 60 F_{254} (Merck, Germany) was utilised for TLC analysis. Visualization of TLC plates was carried out by observing the spots after development under UV lamp (CAMAG, Switzerland) at 254 and 366 nm wavelengths, followed by vanillin-sulfuric acid spray reagent and then heated for the identification of secondary metabolites. The solvent systems commonly utilized for the TLC development of *H. cymosum* fractions are shown in Table 3.1.

Table 3.1: TLC solvent systems

Solvent system	Ratio	Assigned code
Hex - EtOAc	9:1	A
DCM - MeOH	8:2	B
Hex - EtOAc	7:3	C
DCM - MeOH	9.7:0.3	D
DCM - MeOH	9.5:0.5	E

Hex: hexane; EtOAc: ethyl acetate; DCM: dichloromethane; MeOH: methanol

3.1.2.2 Column chromatography

Column chromatography (different diameters) was carried out using silica gel 60 H (0.040-0.063 mm particle size, Merck, South Africa) and Sephadex LH-20 (Sigma-Aldrich, South Africa) as stationary phases.

3.1.3 Spectroscopy

3.1.3.1 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were conducted at 20 ^{o}C, using deuterated chloroform on a Bruker Avance 400 MHz NMR spectrometer (Germany), Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were recorded relative to tetramethylsilane (TMS) as internal reference.

3.2 General experimental procedure for biological assays

3.2.1 Reagents

EGCG (Epigallocatechin gallate), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and other reagents including potassium peroxodisulfate, ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), AAPH (2,2-Azobis(2-methylpropionamidine) dihydrochloride, fluorescein sodium salt, perchloric acid, copper sulfate, iron (III), hydrogen peroxide, alpha-amylase (procaine pancreas), alpha-glucosidase, 4-nitrophenyl α-D-glucopyranoside, 3,5, dinitrosalicylic acid (DNS), sodium carbonate (Na_2CO_3), disodium hydrogen phosphate, sodium dihydrogen phosphate, were supplied by Sigma-Aldrich, South Africa.

3.2.2 Bioassay of Alpha-glucosidase

The alpha-glucosidase assay of the tested compounds (**1-7**) was conducted according to the standard method with slight modification (Telagari & Hullatti, 2015). Inside the 96-well plate, 50 µL of PBS (pH = 6.8, 100 mM), 10 µL of alpha-glucosidase enzyme (1 U/mL), 20 µL of samples and standard (acarbose) of different concentration were incubated for 15 min at 37 °C. Briefly, 20 µL of 5 mM substrate (4-Nitrophenyl β-D-glucopyranoside) was added to each well and left for incubation for 20 min at 37 °C. The reacting mixture was stopped after incubation by adding 0.1 M sodium carbonate (50 µL). The released of *p*-nitrophenol of the reacting mixture relate to the activity of the enzyme was read at wavelength of 405 nm utilizing multiplate reader. The results represent mean of three independent experiments and expressed as percentage inhibition calculated as stated below.

Inhibitory activity (%) = $(1 - A/B) \times 100$

Where, A is the absorbance in the presence of test substance and B is the absorbance of control.

3.2.3 Bioassay of Alpha-amylase

The alpha-amylase assay of the tested compounds (**1**–**5**) was conducted according to the standard method with slight modification (Telagari & Hullatti, 2015). In a 96-well plate, 50 µL of PBS (pH = 6.8, 100 mM,), 10 µL of alpha-amylase enzyme (2 U/mL), 20 µL of varying concentrations of sample (100–31.2 µg/mL), and standard. The mixture was allowed to incubate for 20 min at 37 °C before the addition of 1% soluble starch (10 µL) and further incubated for another 30 min. A color reagent DNS (100 µL) was added and boiled at 95 °C for 15 min. The change in color was read at a wavelength of 450 nm using a multiplate reader. The results are the mean of three independent studies and calculated as percentage inhibition as stated below:

Inhibitory activity (%) = (1 − A/B) ×100

Where, A is the absorbance in the presence of test substance and B is the absorbance of control.

3.2.4 Antioxidant Assays

3.2.4.1 Automated oxygen radical absorbance capacity (ORAC) assay

The method of Cao and Prior (1998) was employed to measure Oxygen Radical absorbance Capacity (ORAC) and as reported in our previous study (Cao & Prior, 1998). The method measures the scavenging potential of compounds against decomposition of peroxyl radical of 2,2-azobis(2-amino-propane) dihydrochloride (AAPH) as peroxyradical (ORAC ROO˙). The antioxidant capacity of compound is measure of fading fluorescence of probe (fluorescein) using area under the curve (AUC) plot in relation to control blank. The florescence of the probe was programmed to be measured at every two minutes for 2 h after adding AAPH at the excitation wavelength and emission set at 485 nm and 530 nm respectively. The ORAC results were estimated following a regression equation ($Y = a + bX + Cx^2$) between Trolox concentration (Y in μM) and the net area under the fluorescence decay curve (X). ORAC values were expressed as μMTE/mg of test sample.

3.2.4.2 Ferric-ion reducing antioxidant power (FRAP) assay

The FRAP assay was assessed according to the method of Benzie and Strain (1996). FRAP reagent containing the mixture of acetate buffer (300 mM, pH 3.6), tripyridyl triazine (TPTZ) (10 mM in 40 mM HCl) and 20 mM $FeCl_3.6H_2O$ in ratio 10:1:1 (v/v/v). Extract, compounds or standard (10 μL) was added to 300 μL FRAP reagent, incubated for 30 min in dark at room temperature. The reacting mixture was read at a wavelength of 593 nm in Multiplate Reader. Ascorbic acid was used as standard at varying concentration of 0 to 1000 μM. The result was

presented as a mean of independent triplicate experiments and presented as µM ascorbic acid equivalents per milligram dry weight (µM AAE/g) of the test samples.

3.3 Collection and identification of the plant material

The aerial part of *Helichrysum cymosum* species was collected in January 2018, from University of Western Cape campus, Bellville. A voucher specimen (No. Ahmed-UWC-25-2018) was identified by Prof Christopher Cupido.

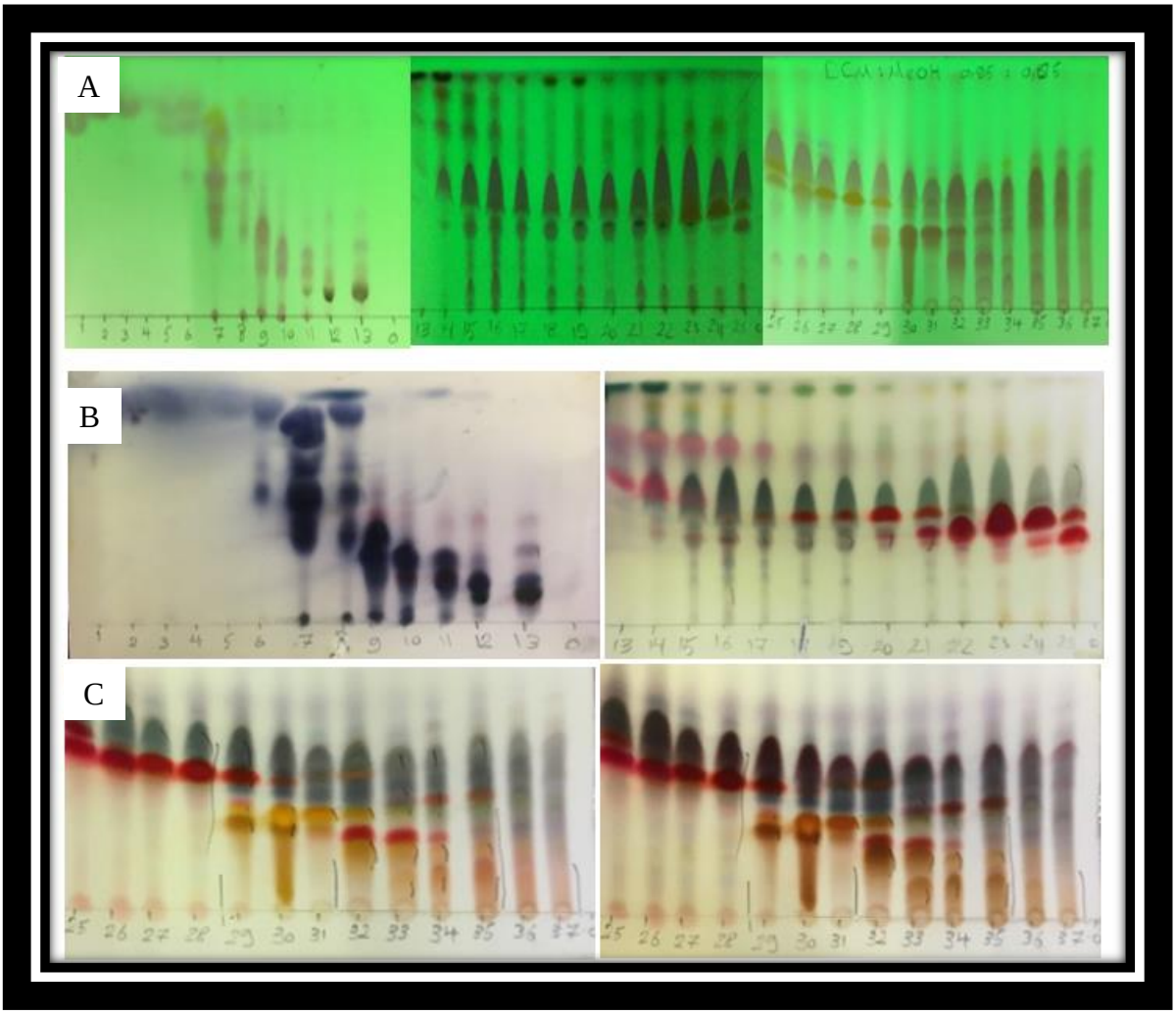
3.4 Extraction and fractionation of the total extract

The dried aerial parts of *Helichrysum cymosum* (330 g) were grounded and extracted with methanol (2.5 L) at ambient temperature (25 ^{o} C) for 24 h. The methanol extract was filtered and then evaporated to dryness using a rotary evaporator under low pressure at 40 ^{o}C to produce 90.2 g (27.3%). The total extract (90 g) was subjected to a silica gel column (30 x 18 cm) and eluted using gradient of hexane (Hex) and ethyl acetate (EtOAc) in order of raising polarity. Forty-one fractions (500 mL each) were collected and combined according to their TLC similarities (Fig. 3.1) to yield twelve main fractions labelled I-XII as shown in Table 3.2.

Table 3.2: Fractionation of the extract of *H. cymosum*

Solvent system			**Fraction collected**
Hexane	Ethyl acetate	MeOH	
100%	0%		1-4
90%	10%		5-8
85%	15%		9-12
80%	20%		13-16
75%	25%		17-20
70%	30%		21-2
60%	40%		24-27
50%	50%		28-30
30%	70%		31-33

10%	90%		34-35
	100%		3637
	90%	10%	38-39
		100%	40-41



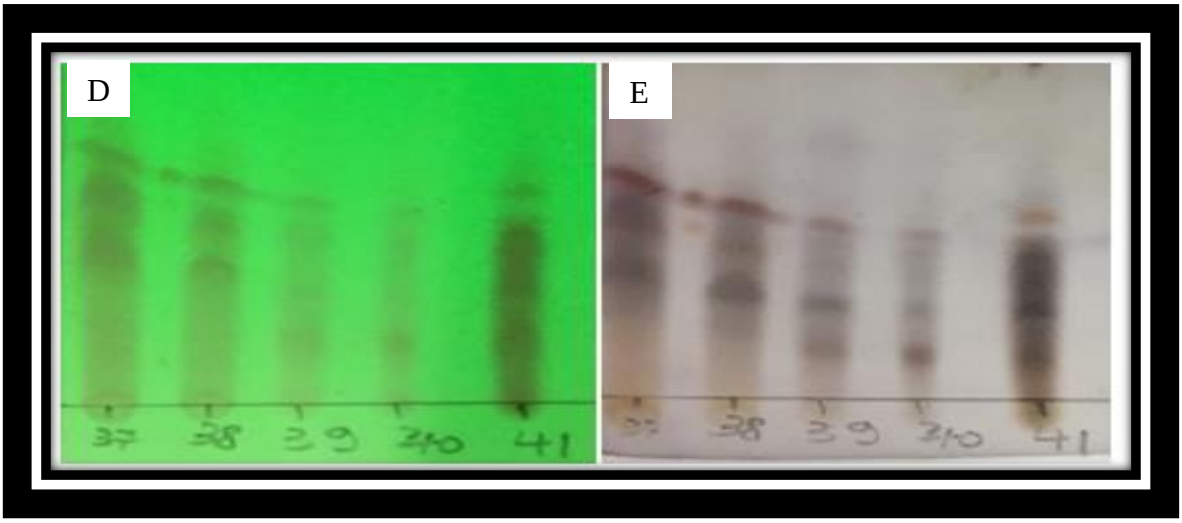


Figure 3.1: TLC plate (silica gel) of collected fractions under UV (254 nm; A&D), and after spraying with H_2SO_4/vanillin and then heated (B-C&E). TLC was developed using solvent system (A, D-E)

The collected fractions (1-41) were evaporated and combined according to their TLC profiles using solvent system E [DCM - MeOH (9.5:0.5)] to produce twelve main fractions (Fig. 3.2). The roman numbers (I-XII) were used as to code the obtained fractions and the results are summarized in Table 3.3

Table 3.3: Fractions obtained upon fractionation of total extract of *H cymosum*

Combined fraction	Designated number	Code	Weight of fractions (mg)
1-5	I	Br-I	235.5
6-8	II	Br-II	352.1
9-11	III	Br-III	452.3
12-13	IV	Br-IV	1025.8
14-17	V	Br-V	852.9
18-21	VI	Br-VI	325.5
22-24	VII	Br-VII	628.8
25-28	VIII	Br-VIII	1435.2
29-31	IX	Br-IX	2701.3
32-35	X	Br-X	1325.4
36-38	XI	Br-XI	1582.2
39-41	XII	Br-XII	2974.6

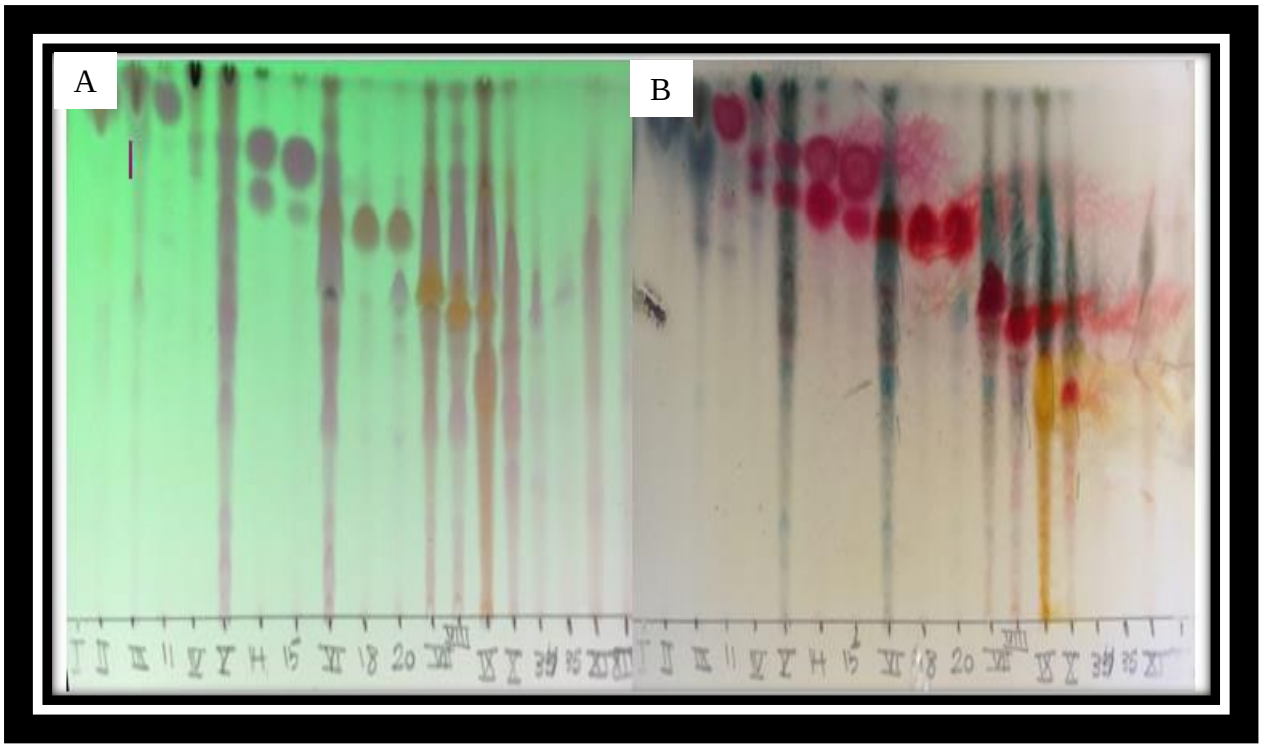


Figure 3.2: TLC plate (silica gel) of combined fractions under UV (254 nm; A), and after spraying with H_2SO_4/vanillin and then heated (B). TLC was developed using solvent system E [DCM - MeOH (9.5:0.5)]

3.5 Isolation of pure compounds

3.5.1 Isolation of compound 1 and 2

Main fraction 11 and 18 (Fig. 3.1) were collected as crystals. They were rinsed three times with hexane to get the crystals in their purest form, concentrated and re-spotted using solvent system C [Hex:EtOAc (7:3)] to further confirm the purity of the obtained fractions. The TLC plate showed only a single spot after development and confirmed the purity and labelled the compound as **1** (44.1 mg) and **2** (65.4 mg) as shown in Fig. 3.3.

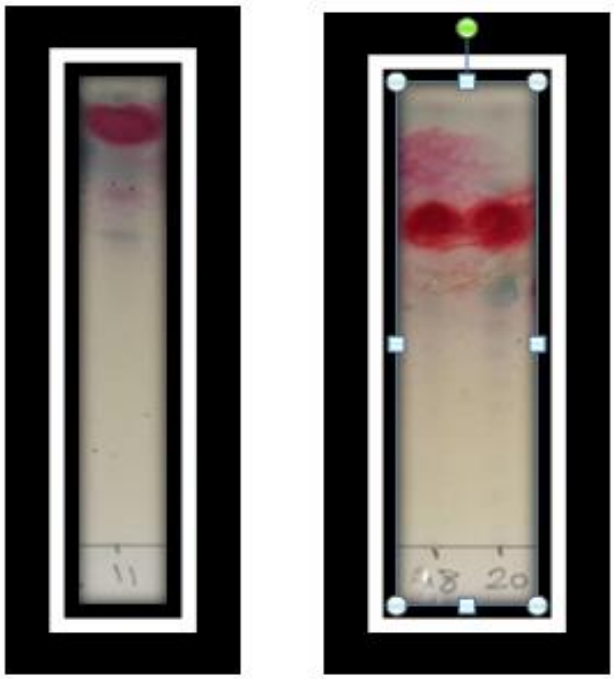


Figure 3.3: TLC plates of Br1-11 and Br1-18 (compounds **1** and **2**)

3.5.2 Isolation of compound 3

Main fraction XI (1582.2mg) was applied to silica gel column using Hex:EtOAc gradient (7:3 and 100%). Fractions of 100 mL were collected and developed on TLC using solvent system C [Hex:EtOAc (7:3)] and the fractions that exhibited the same profiles on the TLC plate were combined as shown in Table 3.4.

Table 3.4: Fractions obtained upon fractionation of fraction XI

Fraction	Weight (mg)	Assigned code
1-4	2.7	Br-XI-I
5-9	133.2	Br-XI-II
10-13	156.1	Br-XI-III
14-19	321.4	Br-XI-IV
20-27	211.2	Br-XI-V
28-35	55.2	Br-XI-VI
36-37	25.1	Br-XI-VII
38-41	333.6	Br-XI-VIII
42-44	41.2	Br-XI-IX
45-46	128.9	Br-XI-X
47-49	151.4	Br-XI-XI
50-53	236.8	Br-XI-XII
54-57	253.1	Br-XI-XIII
58-59	187.4	Br-XI-XIV
60-66	365.2	Br-XI-XV

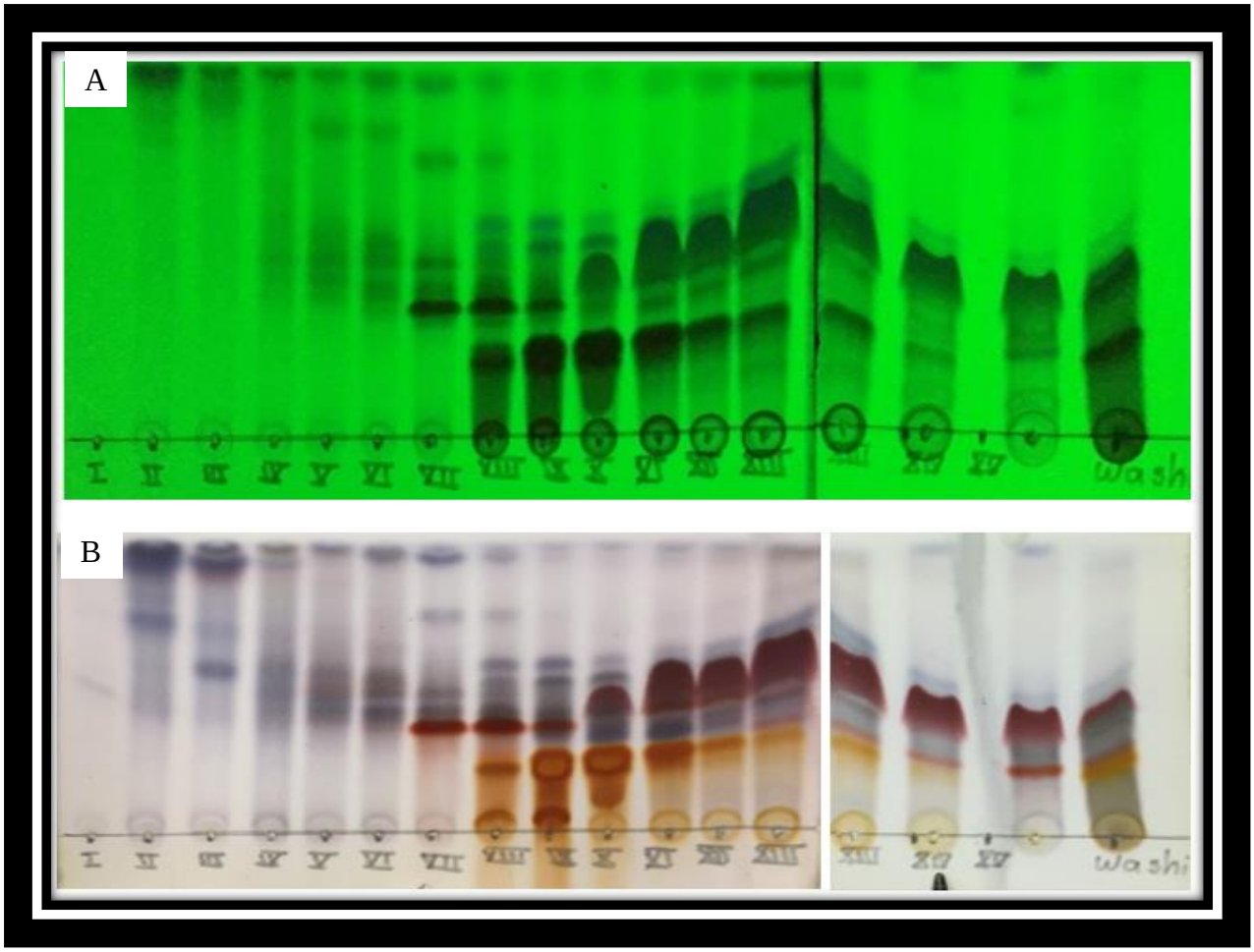


Figure 3.4: TLC plate (silica gel) of combined fractions of XI under UV (254 nm; A), and after spraying with H_2SO_4/vanillin and then heated (B). TLC was developed using solvent system C [Hex:EtOAc (7:3)]

The sub-fraction IX-XV (365 mg) was subjected then to Sephadex chromatography (using 90% aqueous ethanol). Fractions of 5 mL each were collected and concentrated using rotary evaporator. The collected fractions (79-92) were combined, evaporated and re-spotted using solvent system C [Hex:EtOAc (7:3)] to further confirm the purity of the combined fractions. The TLC plate showed only s single spot after development and confirmed the purity (Fig. 3.5) and labelled the compound as **3** (34.1 mg).

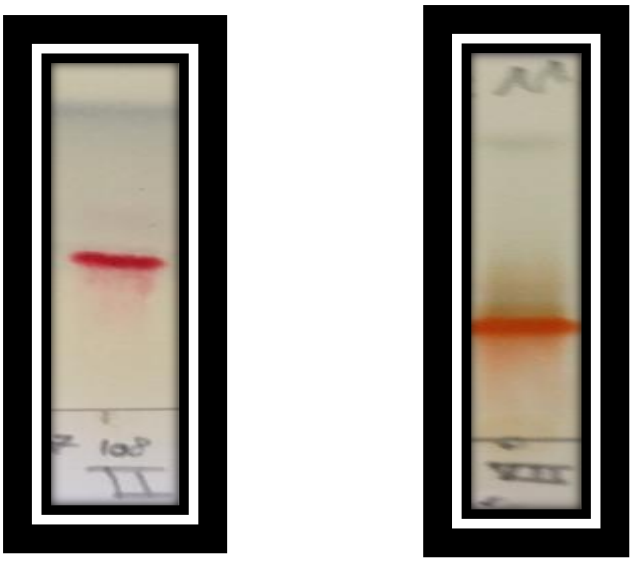


Figure 3.5: TLC plate of IX-XV (compound **3**)

3.5.3 Isolation of compound 4

The sub-fraction XI-XIV (187 mg) was subjected then to sephadex (using 90% aqueous ethanol). Fractions of 5 mL each were collected and concentrated using rotary evaporator and the collected fractions were spotted using solvent system E [DCM:MeOH (9.5:0.5)] as shown in Figure 3.6.

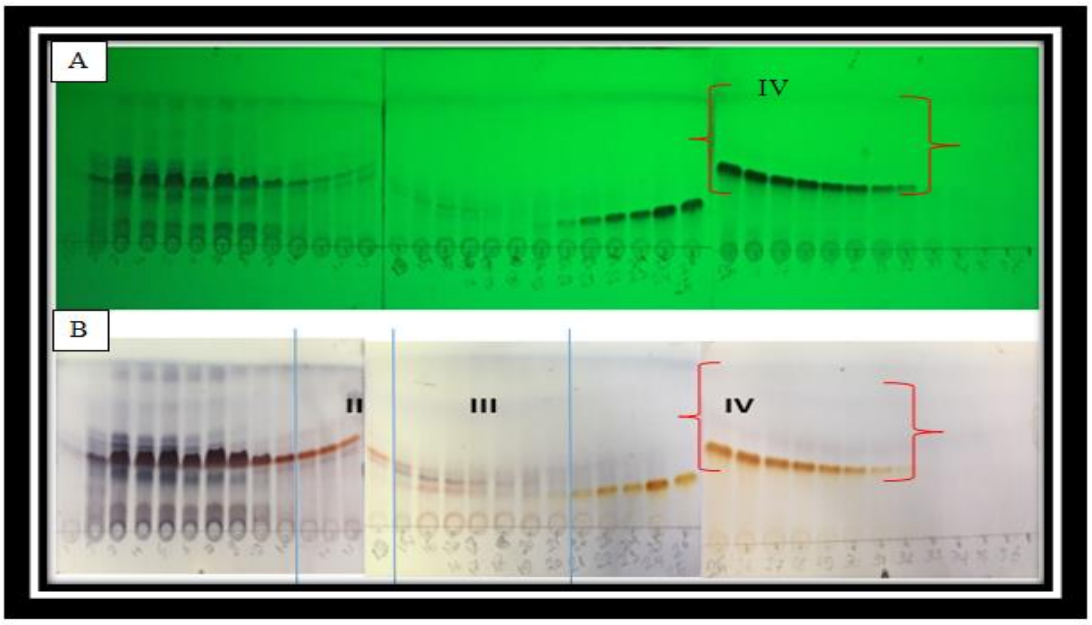


Figure 3.6: TLC plate (silica gel) of collected fractions under UV (254 nm; A&C), and after spraying with H_2SO_4/vanillin and then heated (B&D). TLC was developed using solvent system F [DCM:MeOH (9.5:0.5)]

The collected fractions IV (79-92) were combined, evaporated and re-spotted using solvent system E [DCM:MeOH (9.5:0.5)] to further confirm the purity of the combined fractions. The TLC plate showed only a single spot after development and confirmed the purity (Fig. 3.7) and labelled the compound as **4** (21.1 mg).

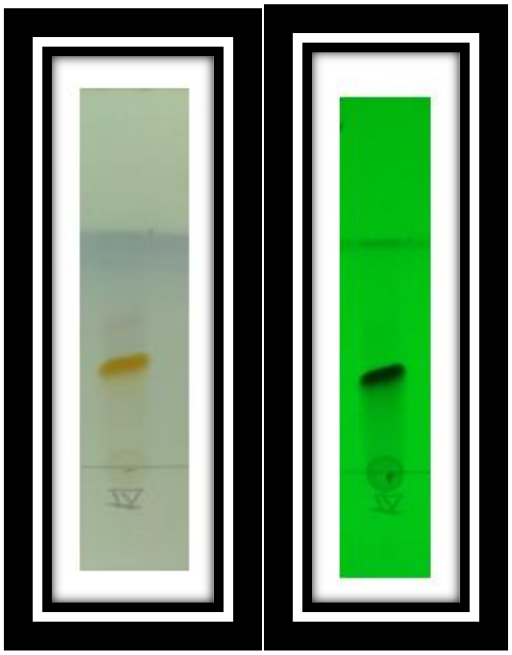


Figure 3.7: TLC plate of IX-XIV-IV (compound **4**)

3.5.4 Isolation of compound 5

The sub-fraction VII (628 mg) was subjected to sephadex (using 90% aqueous ethanol). Fractions of 5 mL each were collected and concentrated using rotary evaporator. The collected fractions were spotted using solvent E [DCM:MeOH (9.5:0.5)] as shown in Figure 3.8.

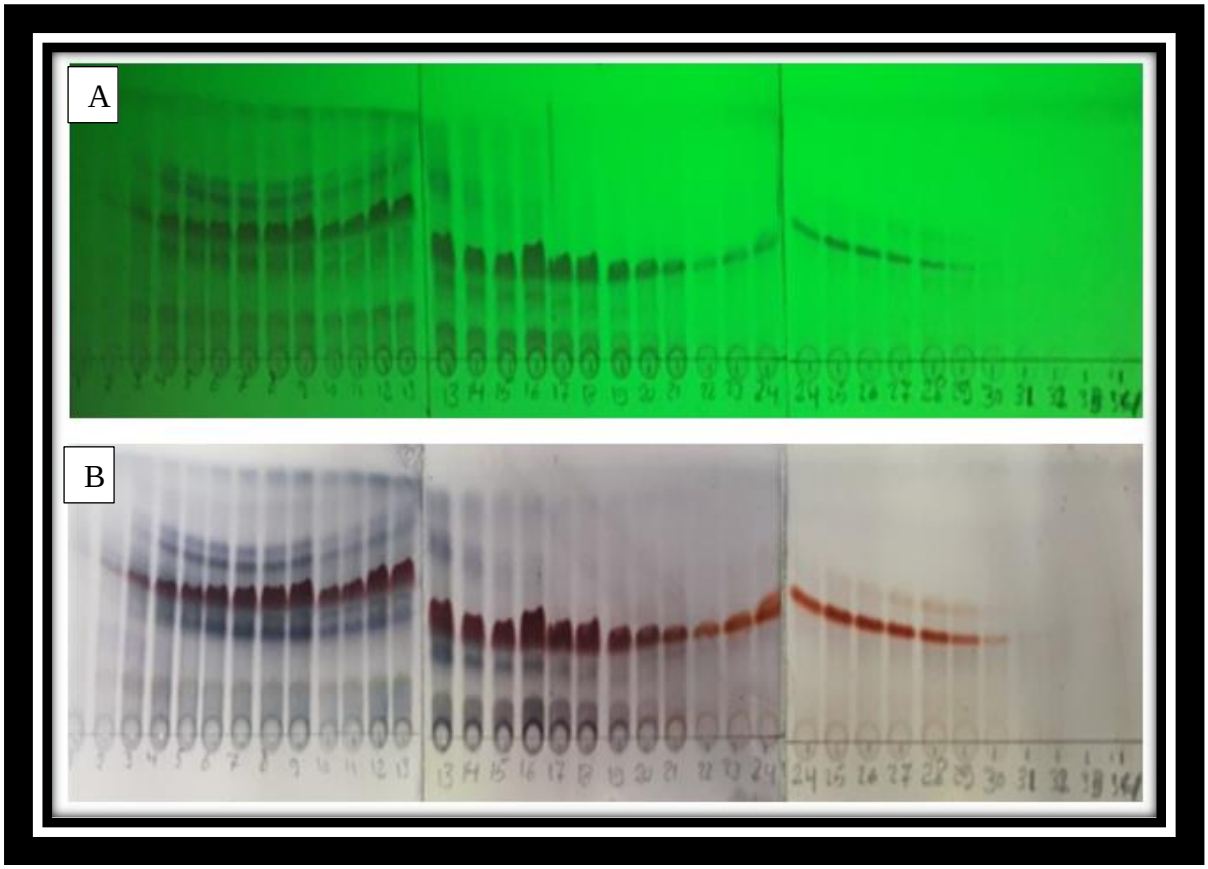


Figure 3.8: TLC plate (silica gel) of combined fractions under UV (254 nm; A), and after spraying with H_2SO_4/vanillin and then heated (B). TLC was developed using solvent system E

The collected fractions (24-30) were pooled together, evaporated and re-subjected to sephadex for further purification using 90% aqueous ethanol. The collected fractions were spotted using solvent system E [DCM:MeOH (9.5:0.5)] and similar fractions were combined accordingly. The TLC plate showed only a single spot after development and confirmed the purity (Fig. 3.9) and labelled the compound as **5** (14.1 mg).

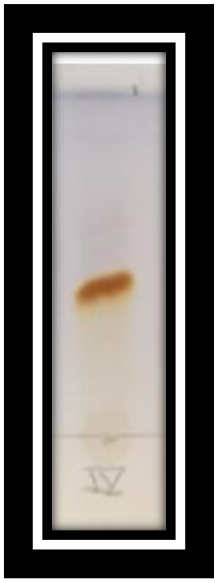


Figure 3.9: TLC plate of VII-4 (compound **5**)

3.5.5 Isolation of compound 6 and 7

The **fraction IX** (230 mg) was subjected to silica gel using Hex-EtOAc gradient (7:3 and 100%). Fraction of 50 mL was collected and concentrated using rotary evaporator. Fractions obtained were developed on TLC plate using solvent system C [Hex:EtOAc (7:3)] and the fractions that shown the same profiles on the TLC plate were combined as indicated in Table 3.5.

Table 3.5: Fractions obtained upon fractionation of fraction IX

Fraction	Weight (mg)	Assigned code
1-4	2.7	Br-IX-1
5-10	33.2	Br-IX-2
11-17	36.1	Br-IX-3
18-19	21.4	Br-IX-4
20-38	125.2	Br-IX-5
39-41	13.8	Br-IX-6

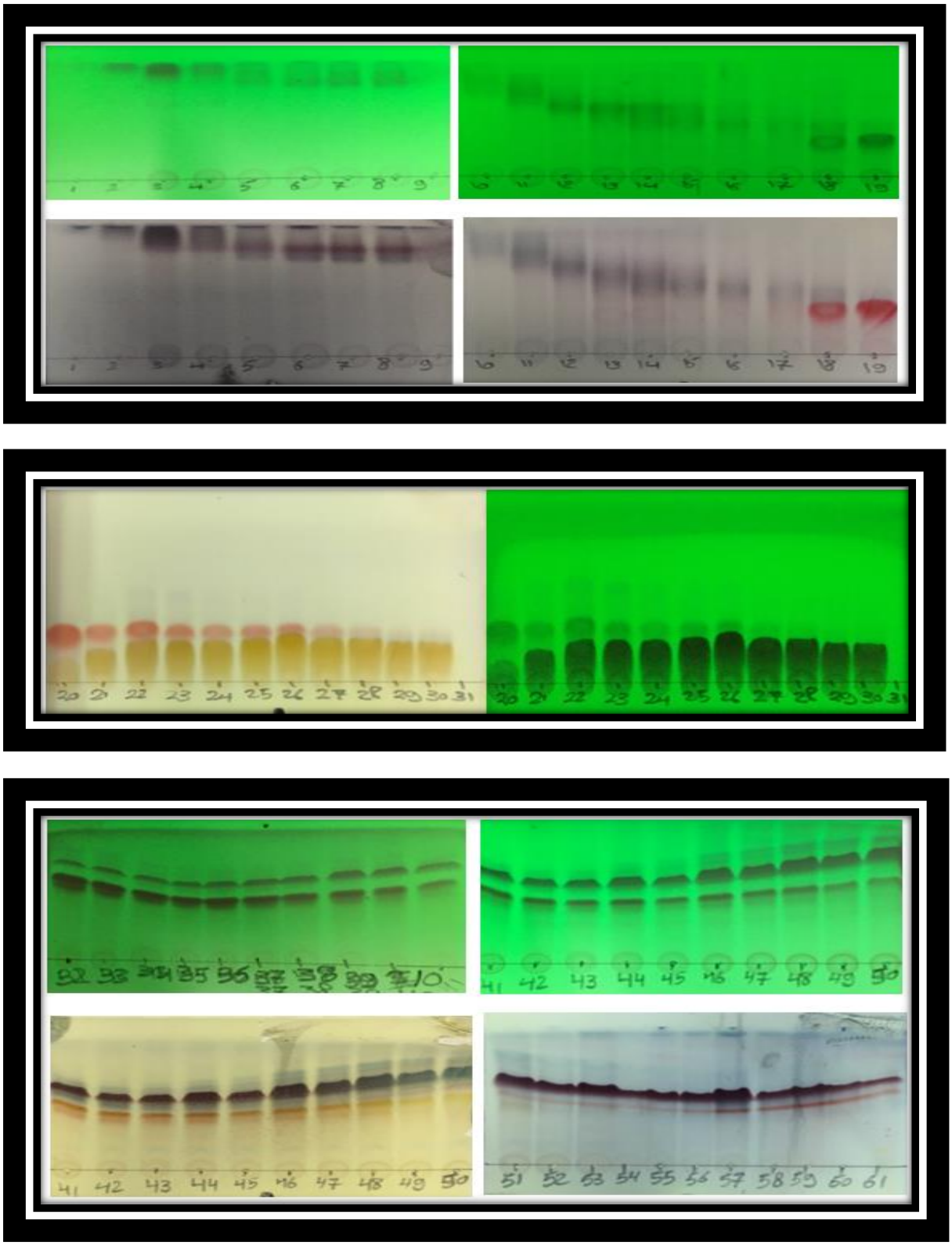


Figure 3.10: TLC plate (silica gel) of combined fractions under UV (254 nm; A&C), and after spraying with H_2SO_4/vanillin and then heated (B&D). TLC was developed using solvent system C [Hex:EtOAc (7:3)].

The collected fractions (38-50) were subjected to the preparative TLC using solvent C as shown in Figure 3.11. After spotting and developing the prep TLC using solvent C [Hex:EtOAc (7:3)], two layers were observed and cut accordingly.

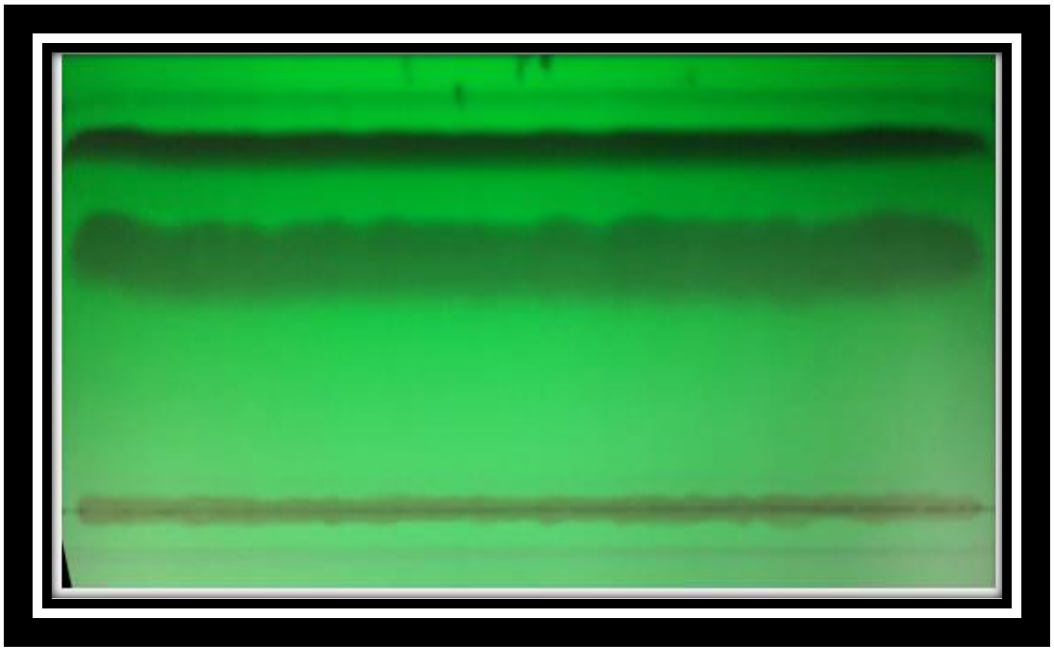


Figure 3.11: Preparative TLC plate of BR-IX

The collected silica gel fractions were filtered using methanol and evaporated using rotavapor. The fractions were re-spotted using solvent system C [Hex:EtOAc (7:3)] to further confirm their purity. The TLC plate showed only a single spot after development and confirmed the purity (Fig. 3.12) and labelled the compound as BR-IX-4 (14.1 mg) and BR-IX-5 (13.1 mg).

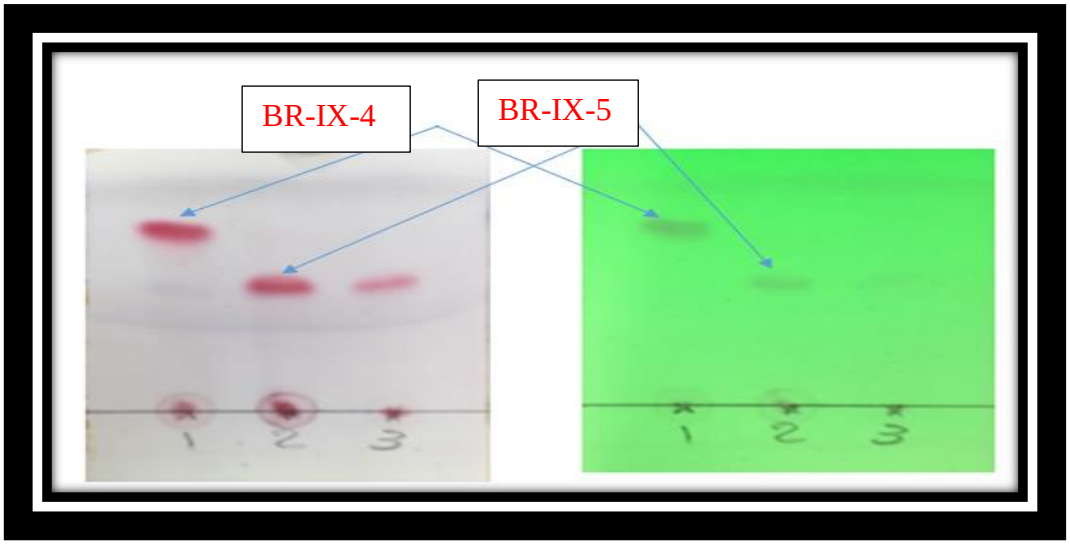


Figure 3.12: TLC plate of BR-IX-4 and BR-IX-5 (compound **6** and **7**)

3.6 Spectroscopic data of the isolated compounds (1-7)

5,8-dihydroxy-7-methoxyflavone (1): ^{1}H NMR (400 MHz, $CDCl_3$): 11.86 (1H, *s*, 5-OH), 7.42 (5H, *m*, H-2'. H-3', H-4', H-5', H-6'), 6.06 (1H, *s*, H-6), 5.44 (1H, *dd*, *J* = 1.9, 12.7 Hz, H-2), 3.79 (3H, *s*), 3.05 (1H, *q*, *J* = 4.4 Hz each, trans), 2.82 (1H, *dd*, *J* = 2.7, 17.2 Hz, H-3 cis).

Pinostrobin (2): ^{1}H NMR (400 MHz, $CDCl_3$): 7.30 (5H, *m*, H-2'. H-3', H-4', H-5', H-6'), 5.95 (2H, *d*, *J* = 2.7 Hz, H-6, H-8), 5.26 (1H, *dd*, *J*= 12.8, 2.8 Hz, H-2), 3.72 (3H, *s*, 7-OMe), 3.08 (1H, dd, *J* = 15.1, 12.8 Hz, H-3β), 2.63 (1H, dd, *J* = 14.7, 2.8 Hz, H-3α).

Dihydrobaicalein (3): ^{1}H NMR (400 MHz, $CDCl_3$): 11.93 (1H, *s*, 5-OH), 7.46 (5H, *m*, H-2'. H-3', H-4', H-5', H-6'), 6.58 (1H, *s*, H-8), 6.18 (1H, *s*, H-2), 3.90 (2H, *s*, H-3αβ).

Glabranin (4): ^{1}H NMR (400 MHz, $CDCl_3$): 12.18 (1H, *s*, 5-OH), 7.40 (5H, *br.s*), 5.96 (1H, *s*, H-6), 5.19 (1H, *br.t*, *J* = 7.1 Hz, =CH)), 3.23 (2H, *br. d*, *J* = 7 Hz, Ar-CH2), 3.02 (1H, *dd*, *J* = 12.*0* and 17.5 *Hz*), 2.73 (1H, *dd*, *J* = 4.5 and 17.5 Hz), 1.70 (6H, *br.s*, =C(CH_3)$_2$).

Allopatuletin (5): ^{1}H NMR (400 MHz, $CDCl_3$): 7.63 (1H, *d*, *J* = 2.1 Hz, H-1'), 7.53 (1H, *dd*, *J* = 2.1, 8.5 Hz, H-5'), 6.91 (1H, *d*, *J* = 8.5 Hz, H-6'), 6.23 (1H, *s*, H-8), 3.74 (3H, *s*).

Pinostrobin chalcone (6): ^{1}H NMR (400 MHz, $CDCl_3$): 7.83 (1H, *d*, *J* = 15.5 Hz), 7.67 (1H, *d*, *J* = 15.5 Hz), 7.53 (2H, *d*, *J* = 7.0 Hz, H-2, H-6), 7.33 (2H, *d*, *J* = 7.0 Hz, H-3, H-5), 7.2 (1H, *m*, H-4), 5.94 (1H, *s*, H-3'), 5.89 (1H, *s*, H-5'), 3.84 (3H, *s*, MeOH).

Helichrysetin (7): ^{1}H NMR (400 MHz, $CDCl_3$): 7.88 (1H, *d*, *J* = 15.5 Hz, H-7), 7.75 (1H, *d*, *J* = 15.5 Hz, H-8), 7.61 (2H, *d*, *J* = 8.6 Hz, H-2, H-6), 6.93 (2H, *d*, *J* = 7.0 Hz, H-3, H-5), 6.09 (1H, *d*, *J* = 1.6 Hz, H-3'), 6.02 (1H, *d*, *J* = 2.2 Hz, H-5'), 3.97 (3H, *s*, OMe).

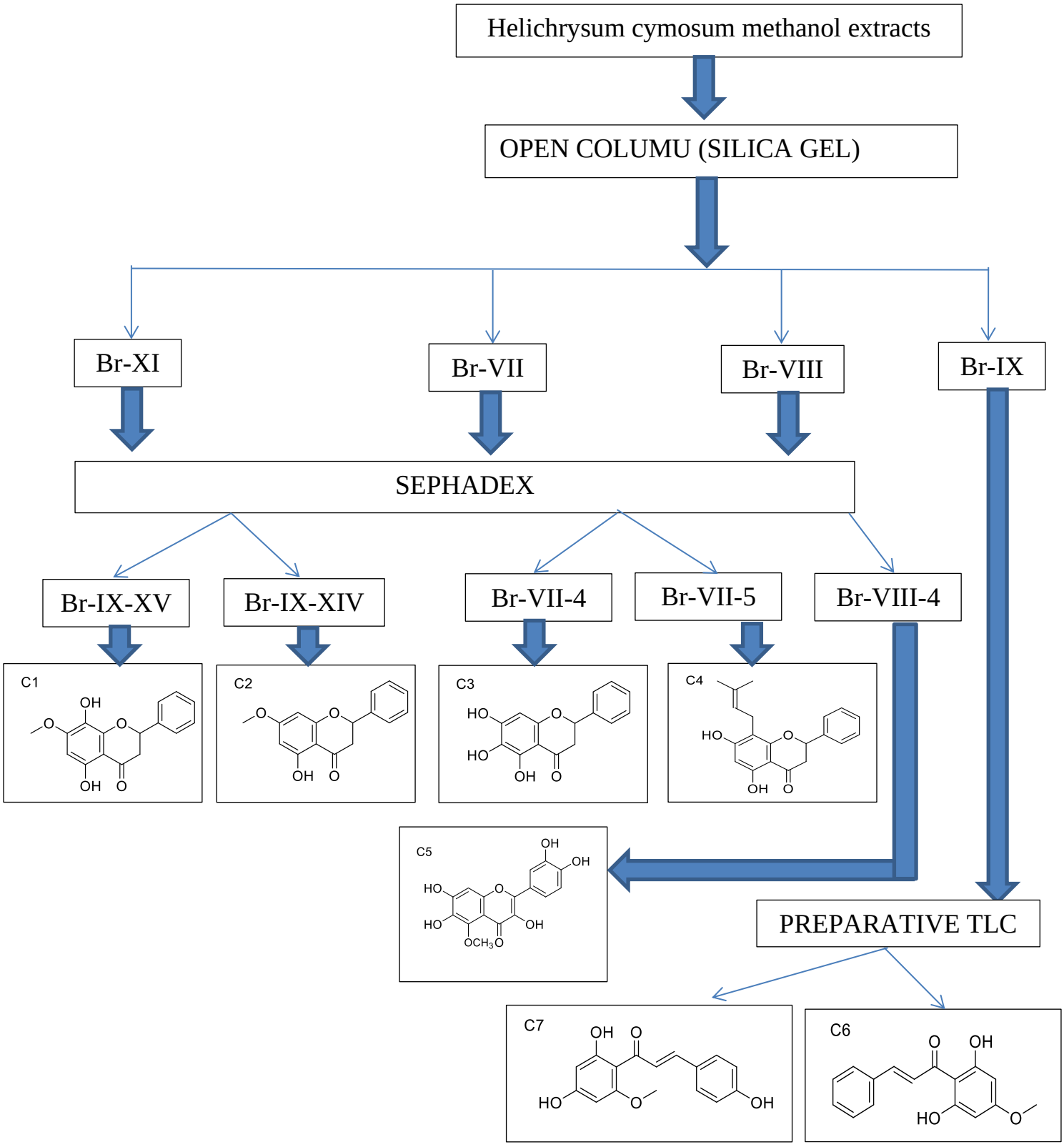
3.7 Statistical Analysis

All the measurements were repeated three times and the IC_{50} was calculated utilizing GraphPad Prism 5 version 5.01 statistical software. The data presented are means ± SD obtained from 96-well plate readers for all in vitro experiments.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Isolation of pure compounds

Compounds **1-7** were isolated and purified using a series of silica gel and LH20 column chromatography and preparative thin layer chromatography.



Scheme 4.1: A flow diagram of experimental procedure for the isolation of compounds

4.2 Results and discussion: Chemical characterization

4.2.1 Structure elucidation of 5,8-dihydroxy-7-methoxyflavone (1)

Compound **1** was isolated as a yellow powder as described in scheme 4.1. The ^{1}H-NMR spectrum showed characteristic resonances of aromatic protons at 7.42 (5H, *m*, H-2'. H-3', H-4', H-5', H-6') and 6.06 (1H, *s*, H-6). The presence of a doublet of doublet at 2.82 (1H, *dd*, *J* = 2.7, 17.2 Hz, H-3 cis) and quartet at 3.05 (1H, *q*, *J* = 4.4 Hz each, trans) were observed, in addition to another doublet of doublet at 5.44 (1H, *dd*, *J* = 1.9, 12.7 Hz, H-2), which can be attributed to the coupling of H-2 with the two proton located at position 3. The appearance of singles at 3.79 (3H, *s*), corresponding to a methoxy group and 11.86 (1H, *s*, 5-OH). The ^{13}C NMR and DEPT showed the presence of 11 carbons divided as one methylene (43.3/C-3), one methoxy group, and one carbonyl group (195.7/C-4), in addition to seven quaternary carbons and methine carbons. The ^{13}C signals at 195.7 and 61.3 correspond to the carbonyl group and the methoxy group respectively. The presence of a CH_2 at position 2 was confirmed by DEPT (43.3/C-3). Finally, the obtained data (NMR data) in comparison with those published in literature confirmed the structure of compound **1** as 5,8-dihydroxy-7-methoxyflavone (Meng Bai, 2018). This compound was isolated for the first time from *H. cymosum.*

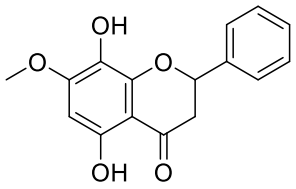


Figure 4.1: Chemical structure of 5,8-dihydroxy-7-methoxyflavone

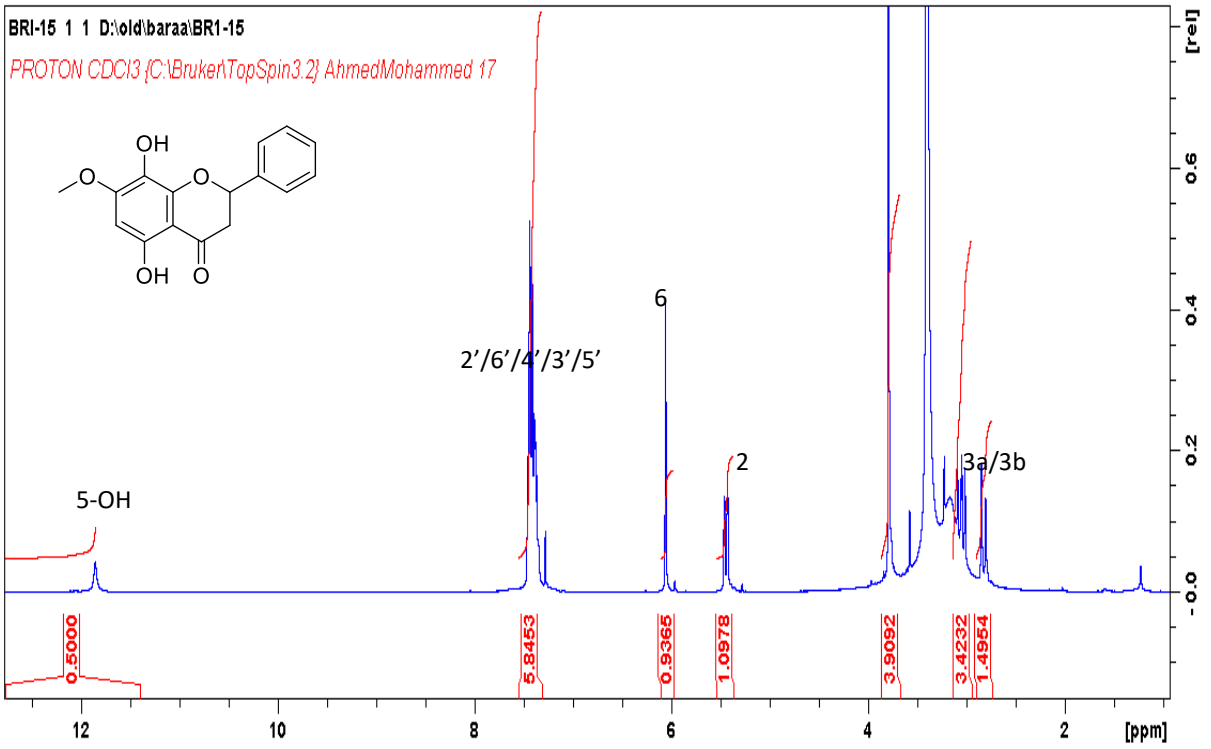


Figure 4.2: ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of **1**

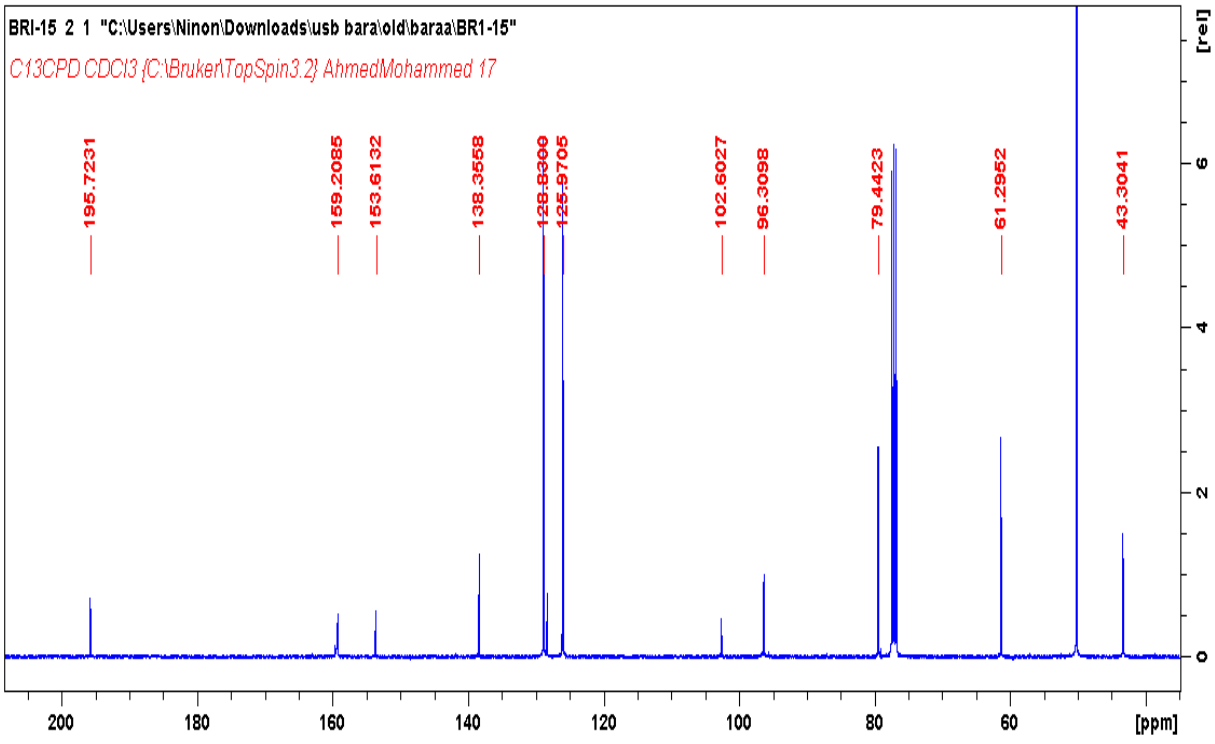


Figure 4.3: ^{13}C-NMR NMR (400 MHz, $CDCl_3$) spectrum of **1**

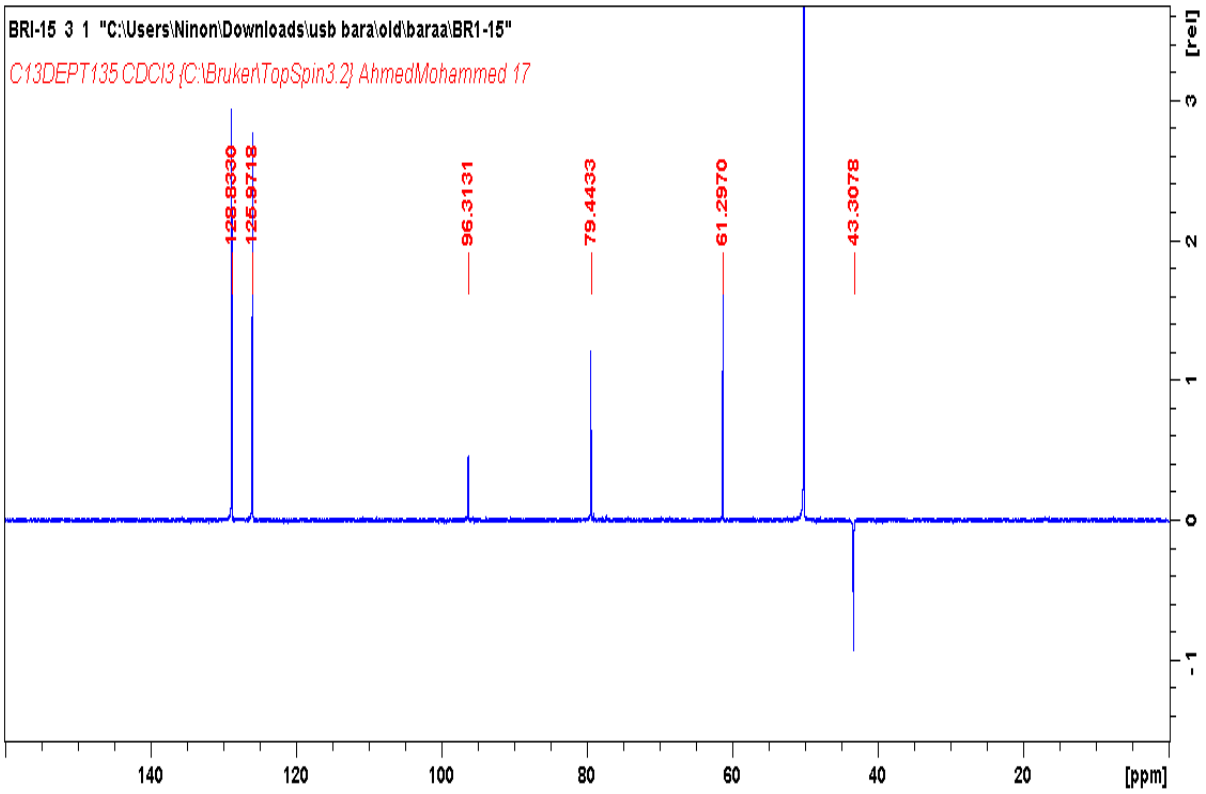


Figure 4.4: DEPT-NMR (400 MHz, $CDCl_3$) spectrum of **1**

4.2.2 Structure elucidation of pinostrobin (2)

Compound **2** was isolated as an orange powder as describe in scheme 4.1. It was identified as pinostrobin from its NMR data, which was very similar to that of compound **1** except for the absence of the OH group at position 8. The ^{1}H NMR showed the presence of signals at 7.30 (5H, *m*, H-2'. H-3', H-4', H-5', H-6'), 5.95 (2H, H-6, H-8), 5.26 (1H, *dd*, *J*= 12.8, 2.8 Hz, H-2), 3.72 (3H, *s*, 7-OMe), 3.08 (1H, *dd*, *J* = 15.1, 12.8 Hz, H-3β), 2.63 (1H, *dd*, *J* = 14.7, 2.8 Hz, H-3α). The ^{13}C NMR spectrum displayed fourteen signals, which were divided by DEPT into six quaternary carbons 190.3/C-4, 165.1/C-7, 138.3/C-1', 164.8/C-9, 104.7/C-10, 162.7/C-5, one methoxy group 55.6/C-7OMe), one methylene 45.2/C-3 one methine at 78.8/C-2 and aromatic methine carbons at 125.9/C-2', C-3', C-5', C-6', 128.6/C-4', 96.2/C-6 and 93.3/C-8. The obtained data (NMR data) in comparison with those published in literature confirmed the

structure of compound **2** as pinostrobin (Meng Bai, 2018; Ching, et al., 2007; Tanjung, et al., 2013). This compound was isolated for the first time from *H. cymosum.*

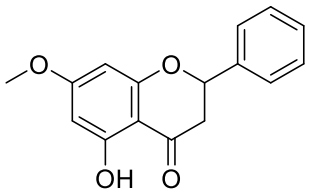


Figure 4.5: Chemical structure of pinostrobin

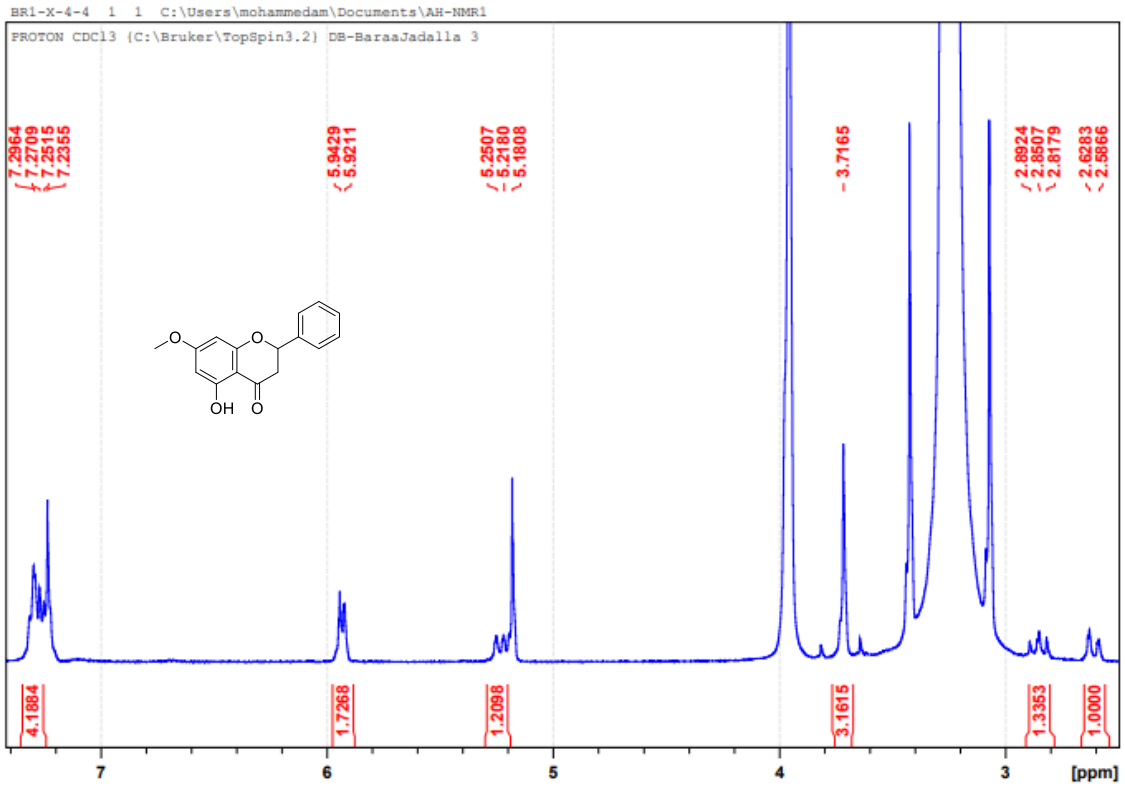


Figure 4.6: ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of **2**

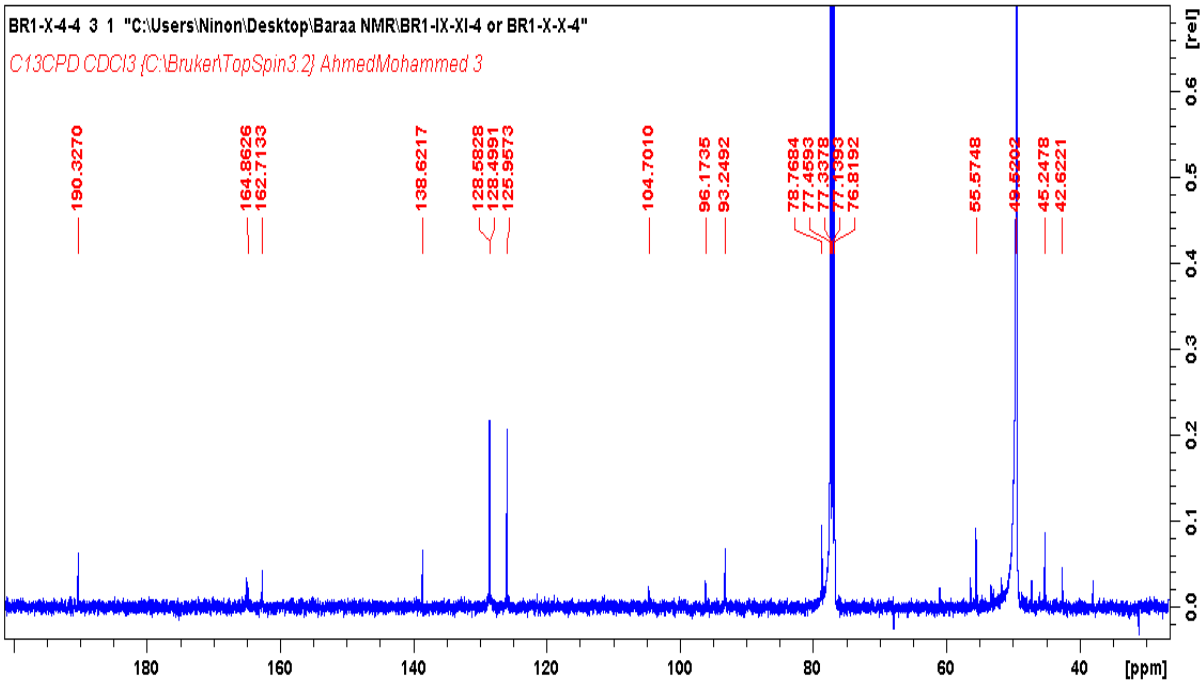


Figure 4.7: ^{13}C-NMR (400 MHz, $CDCl_3$) spectrum of **2**

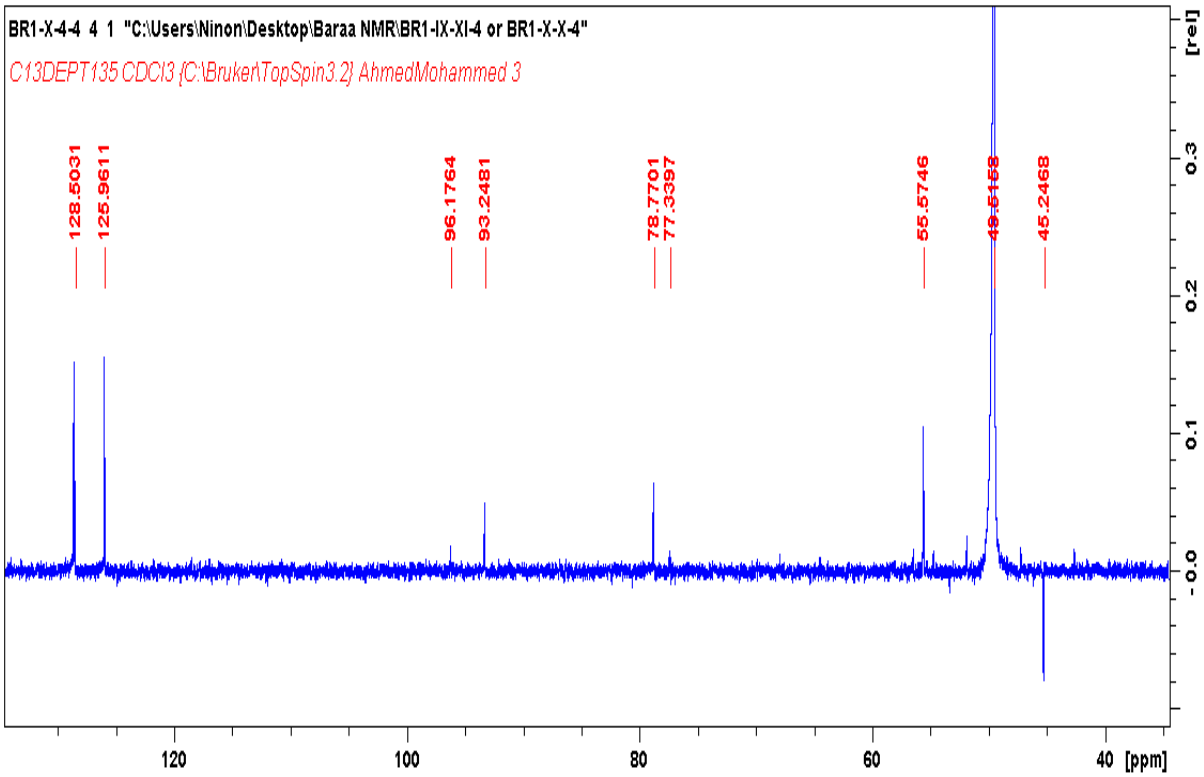


Figure 4.8: DEPT-NMR (400 MHz, $CDCl_3$) spectrum of **2**

4.2.3 Structure elucidation of dihydrobaicalein (3)

Compound **3** was isolated as a yellow powder as describe in scheme 4.1. It was identified as dihydrobaicalein from its NMR data, which was similar to that of compound **2** except the presence an extra OH group at position 6, which appears as an oxygenated proton at 6.59 and the absence of the methoxy group, which can be observed from the ^{1}H NMR. The obtained data (NMR data) in comparison with those published in literature, confirmed the structure of compound **3** as dihydrobaicalein (Piseth Nhoek, 2018). This compound was isolated for the first time from *H. cymosum.*

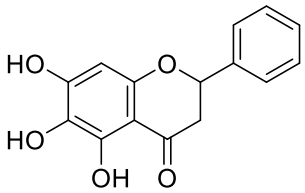


Figure 4.9: Chemical structure of dihydrobaicalein

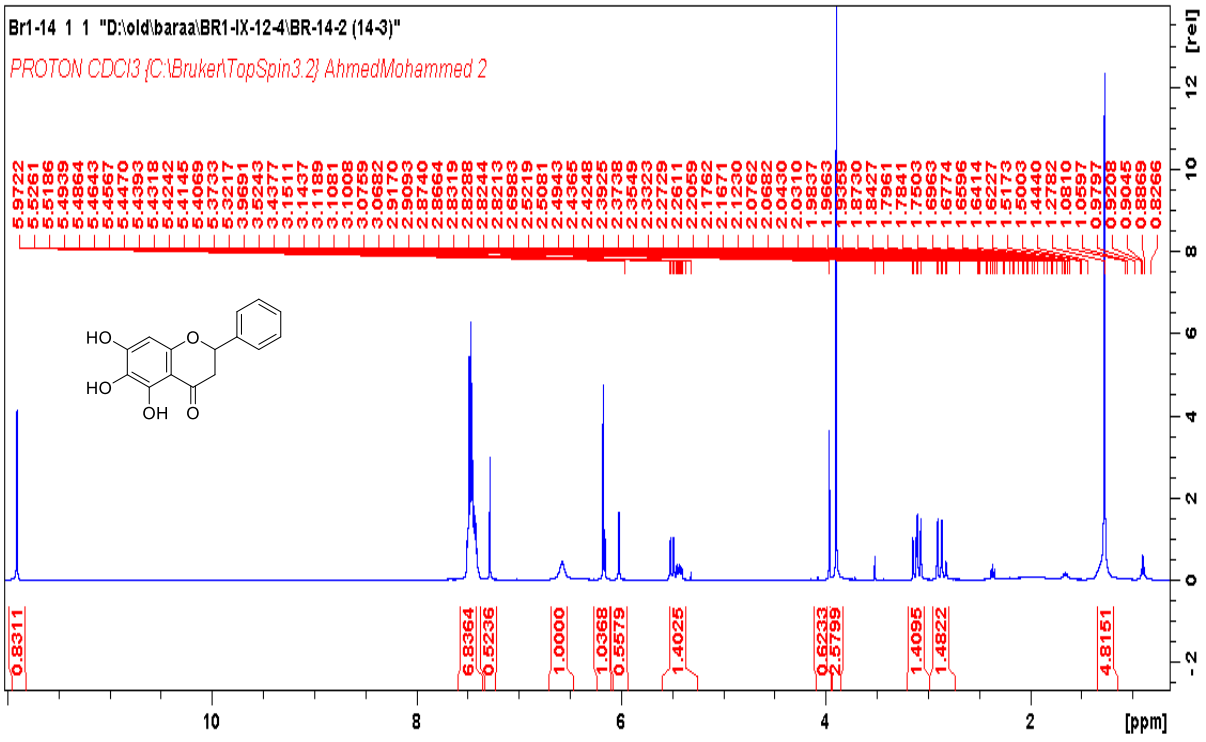


Figure 4.10: ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of **3**

4.2.4 Structure elucidation of glabranin (4)

Compound **4** was isolated as an orange powder as describe in scheme 4.1. It was identified as glabranin from its NMR data. The ^{1}H NMR spectrum showed the signal of the γ, γ dimethylallyl fragment at 1.70 (6H, *br.s*, =C(CH3)2), 3.23 (2H, *br. d*, *J* = 7 Hz, Ar-CH2) and 5.19 (1H, *br.t*, *J* = 7.1 Hz, =CH), the signal of the proton of the monosubstituted benzene B ring at 7.4 (5H, *br.s*). The signal of the aromatic proton of the ring A at 5.96 (1H, *s*, H-6). The signal of the H-bound proton of the OH group at 12.18 (1H, *s*, 5-OH) and the signals at 2.73 (1H, *dd*, *J* = 4.5 and 17.5 Hz), 3.02 (1H, *dd*, *J* = 12.0 and 17.5 Hz). The presence of an olefinic group was confirmed with a signal at 122.3 and 138.5 pm on the ^{13}C NMR spectra and DEPT showed the presence of two methylene groups at 21.0/C-6 and 43.2/C-3 and two methyl groups at 17.7 and 27.7 pm. The obtained data (NMR data) in comparison with those published in literature, confirmed the structure of compound **4** as glabranin. (Jakupovic, et al., 1988; Yuldashev, et al., 2000; Frattaruolo, et al., 2019). This compound was previously isolated from *H. cymosum* (Jakupovic, et al., 1988).

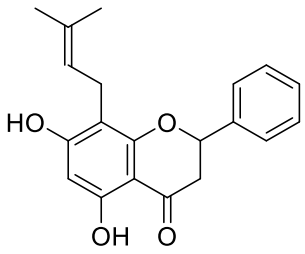


Figure 4.11: Chemical structure of glabranin

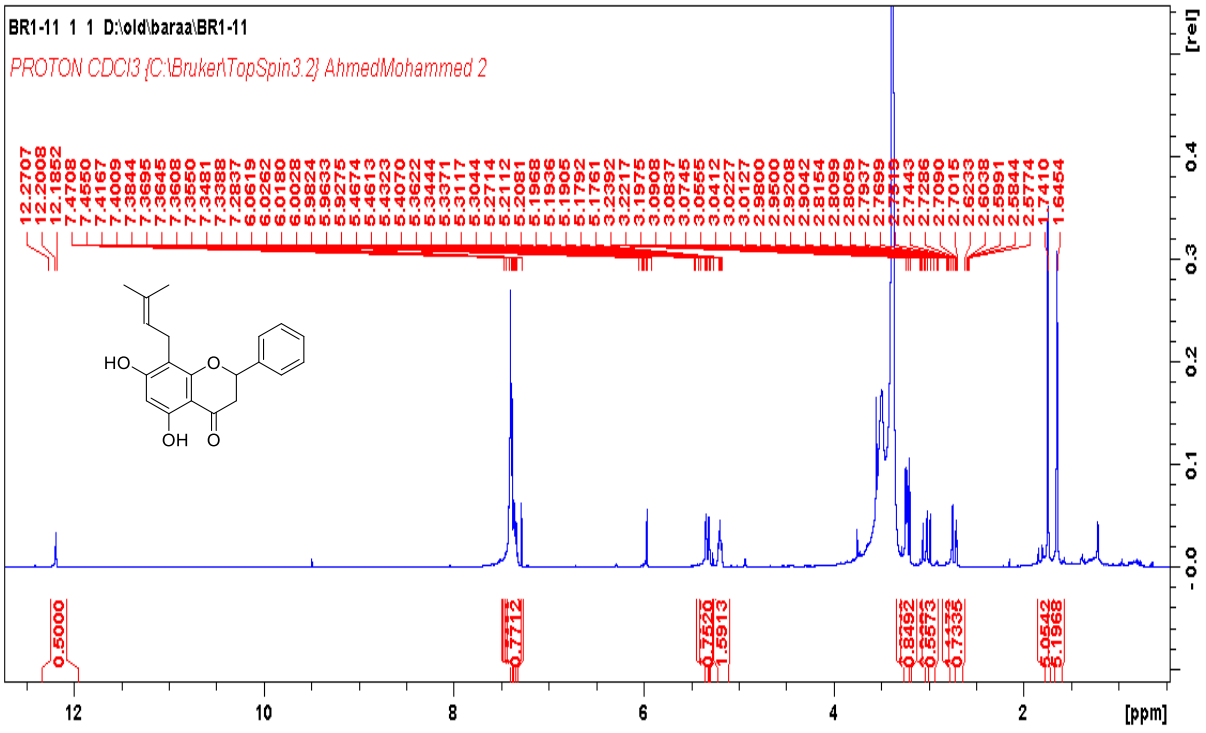


Figure 4.12: ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of **4**

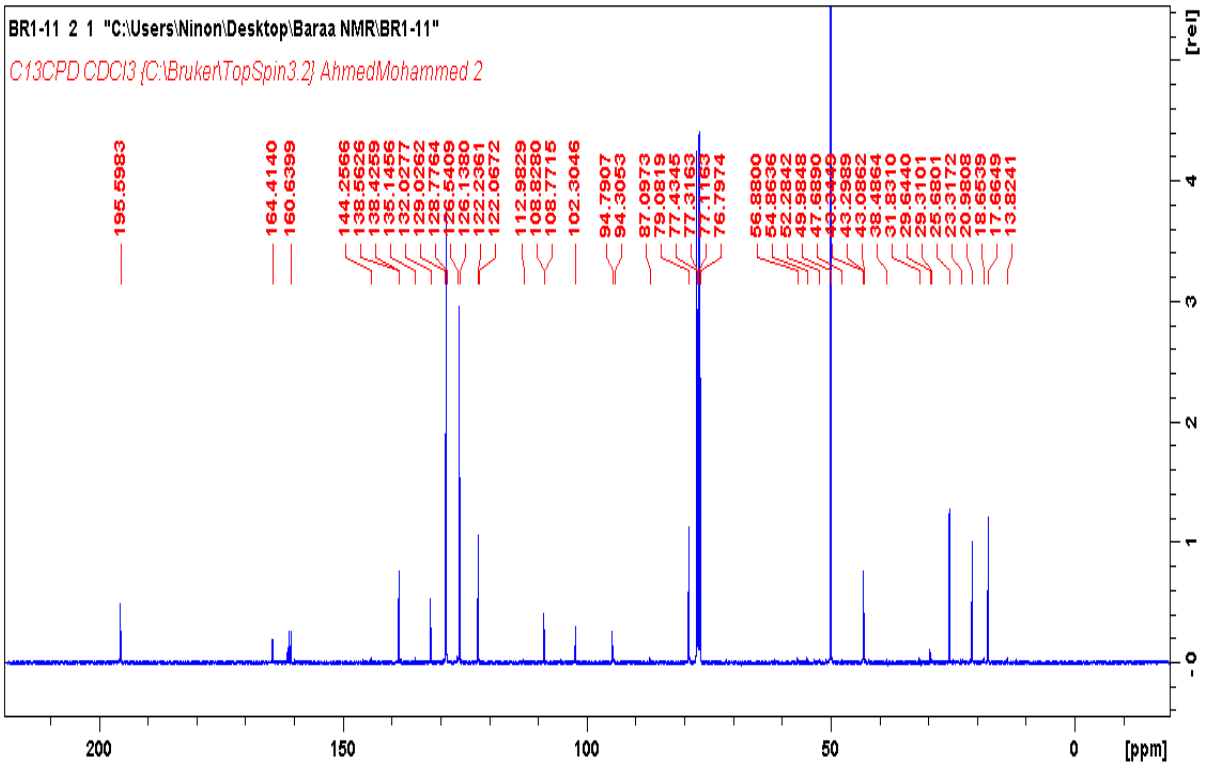


Figure 4.13: ^{13}C-NMR (400 MHz, $CDCl_3$) spectrum of **4**

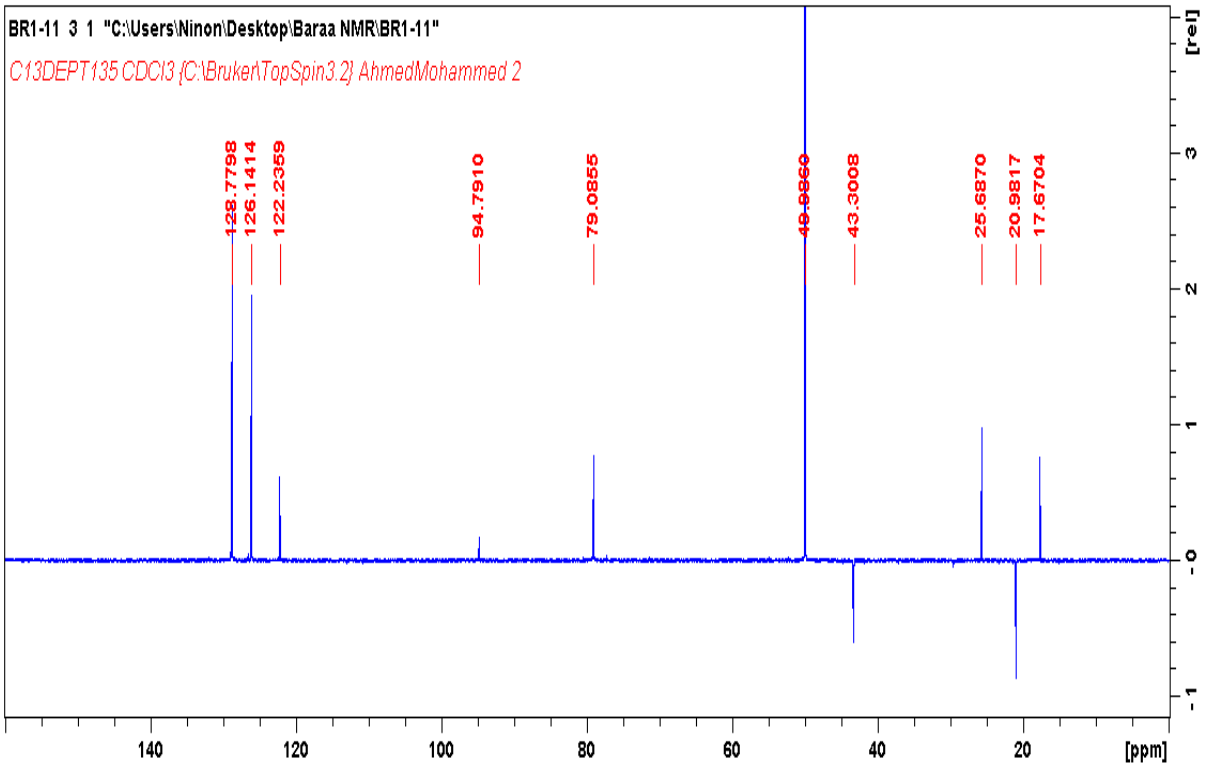


Figure 4.14: DEPT-NMR (400 MHz, $CDCl_3$) spectrum of **4**

4.2.5 Structure elucidation of allopatuletin (5)

Compound **5** was isolated as a yellow-brown powder as describe in scheme 4.1. It was identified as allopatuletin from its NMR data, which was similar to that of compound **3** except the substitution of the OH group at position 5 by the methoxy group and the appearance of two extra OH groups at position 3' and 4' in ring B as well as an olefinic group at 122.2/C-3 and 147.8/C-2, indicating the presence of a double bond. The obtained data (NMR data) in comparison with those published in literature, confirmed the structure of compound 5 as allopatuletin. This compound was previously isolated from *Xanthium strumarium*, which belongs to the same family Asteraceae as *H. cymosum* (Bhardwaj, 1980).

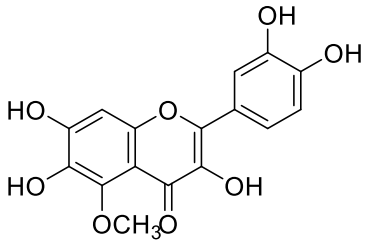


Figure 4.15: Chemical structure of allopatuletin

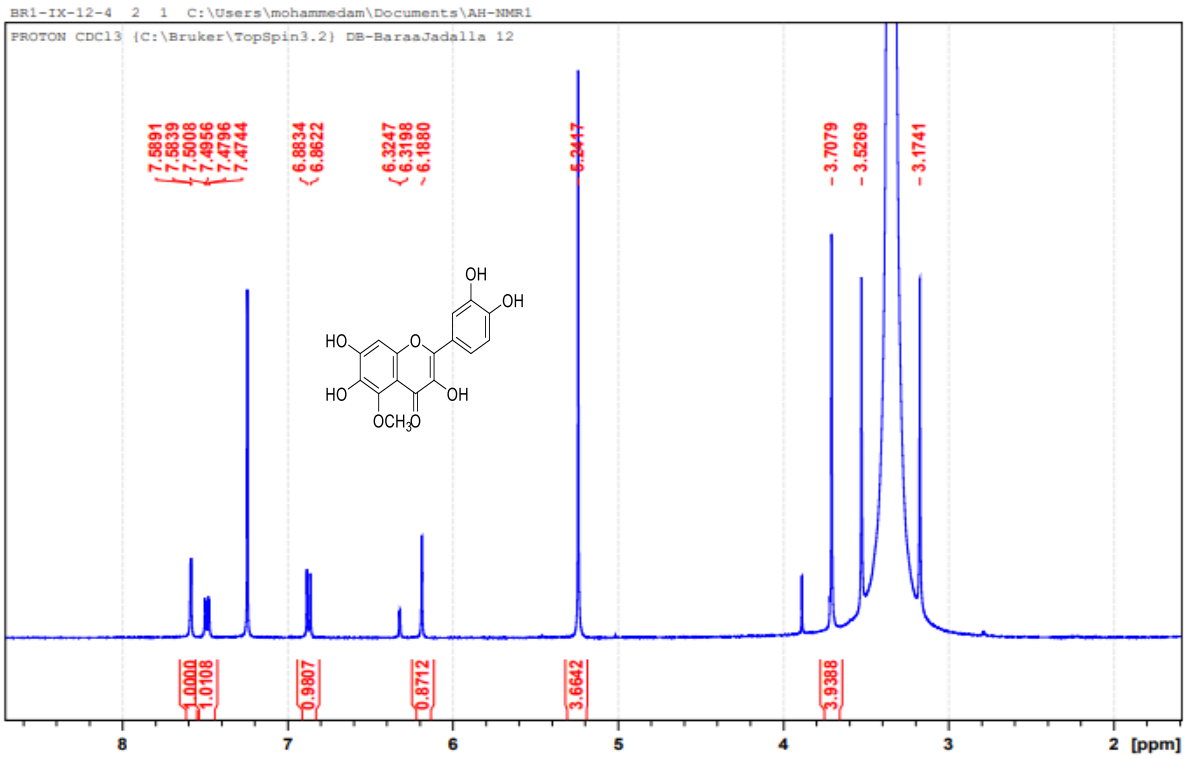


Figure 4.16: ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of **5**

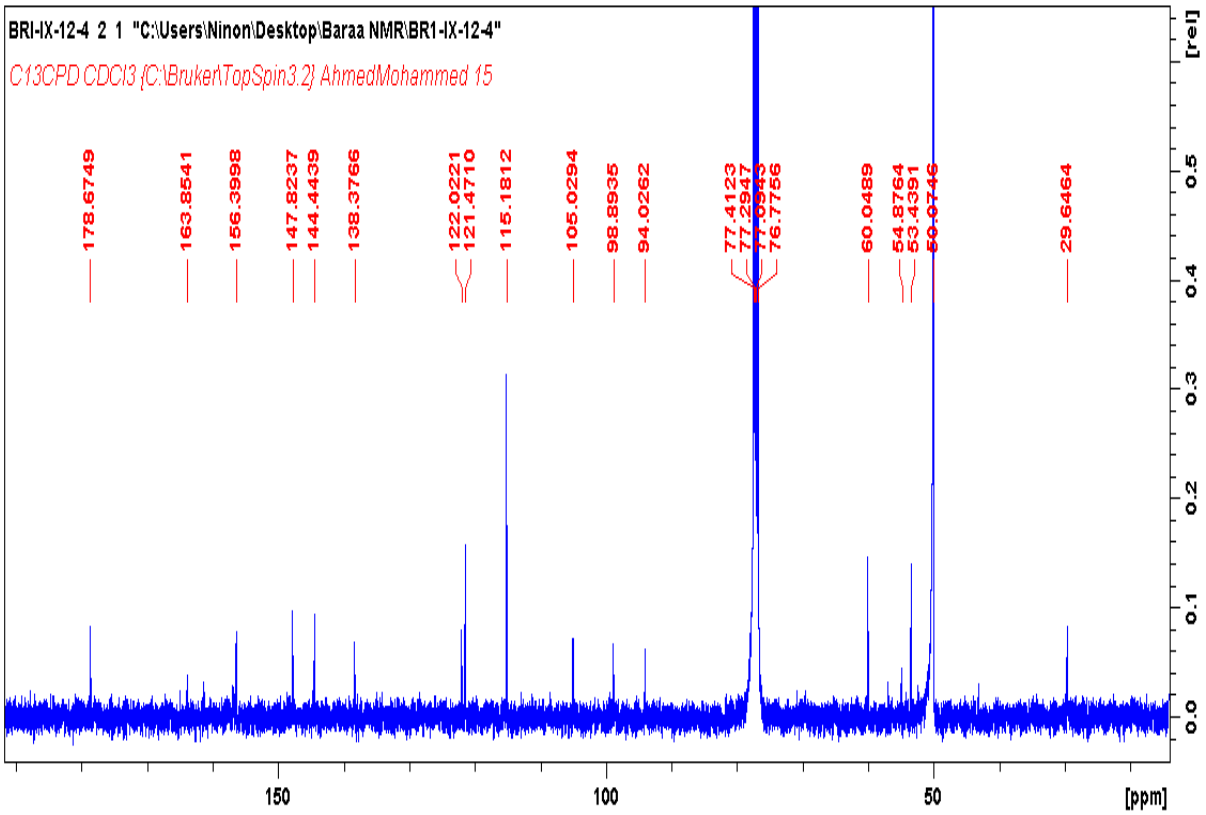


Figure 4.17: ^{13}C-NMR (400 MHz, $CDCl_3$) spectrum of **5**

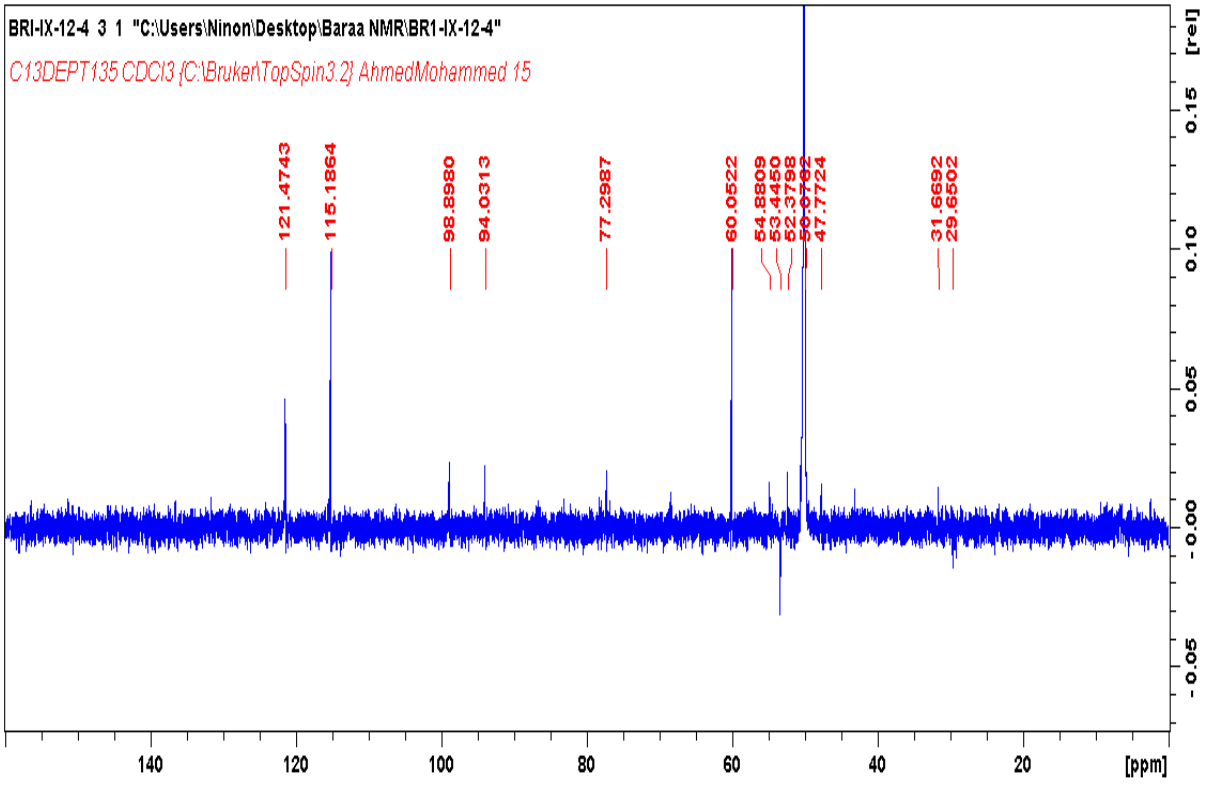


Figure 4.18: DEPT-NMR (400 MHz, $CDCl_3$) spectrum of **5**

4.2.6 Structure elucidation of pinostrobin chalcone (6)

Compound **6** was isolated as an orange powder as describe in scheme 4.1. It was identified as pinostrobin chalcone from its NMR data. The ^{1}H NMR showed two distinct sets of downfield doublets at 7.67 (1H, *d, J* = 15.5 Hz) which was assigned to proton at α-carbon and another doublet observed at 7.83 (*d, J* = 15.55 Hz) was assigned to proton at β-carbon. Another set of doublets was observed at 7.53 (d, *J* = 7.0 Hz, H-2, H-6) and 7.33 (*d, J* = 7.0 Hz, H-3, H-5) and a multiplet observed between 7.2-7.1 was attributed to the proton at H-4. Two singlets that appeared at 5.89 (*d, J* = 2.46 Hz) and 5.94 were assigned to C-5′ and C-3′ protons, respectively and also a singlet was observed at δ 3.84, which was assigned to the methoxy protons at C-4′.

The ^{13}C NMR and DEPT showed twelve carbons δ_C 142.0 (C-β), 135.5 (C-1), 128.3 (C-2/C-6), 128.8 (C-3/C-5), 129.9 (C-4), 127.7 (C- α), 105.7 (C-1‘), 163.3 (C-2‘), 96.2 (C-3‘), 164.8 (C-4‘), 91.6 (C-5‘), 167.6 (C-6‘), 192.2 (C=O), 55.5 (MeOH). The obtained data (NMR data) in comparison with those published in literature, confirmed the structure of compound **6** as pinostrobin chalcone (Mohammed, et al., 2018). This compound was isolated for the first time from *H. cymosum.*

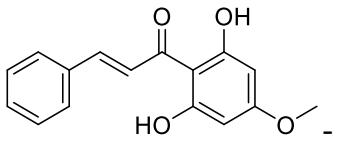


Figure 4.19: Chemical structure of pinostrobin chalcone

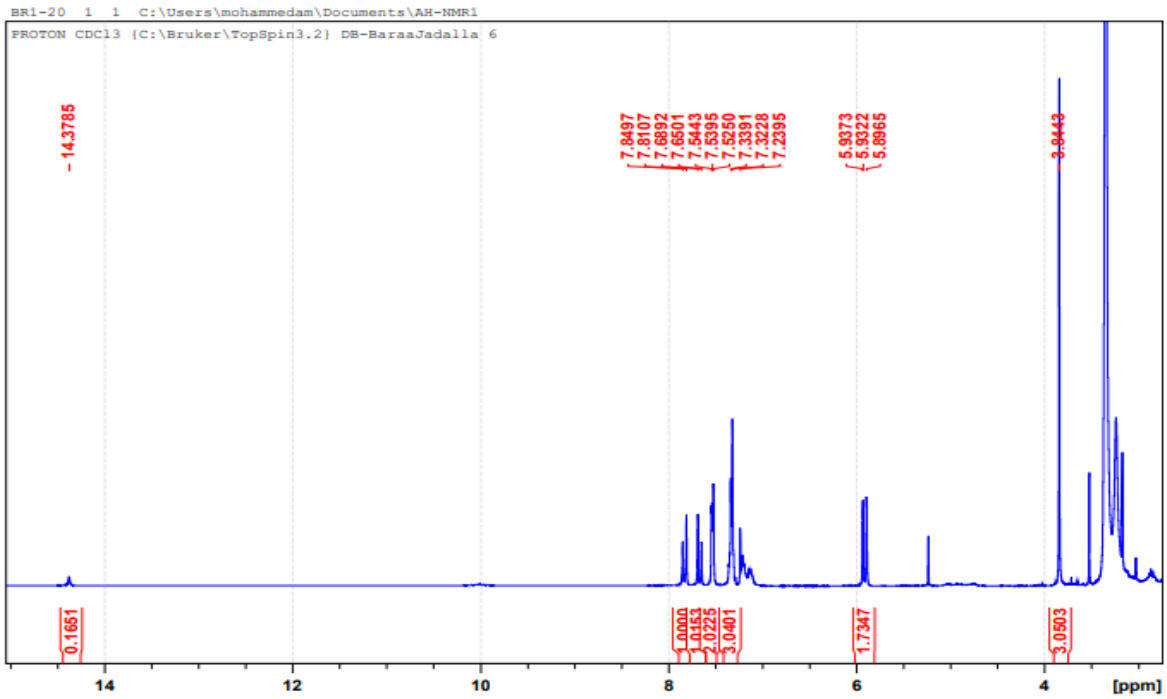


Figure 4.20: ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of **6**

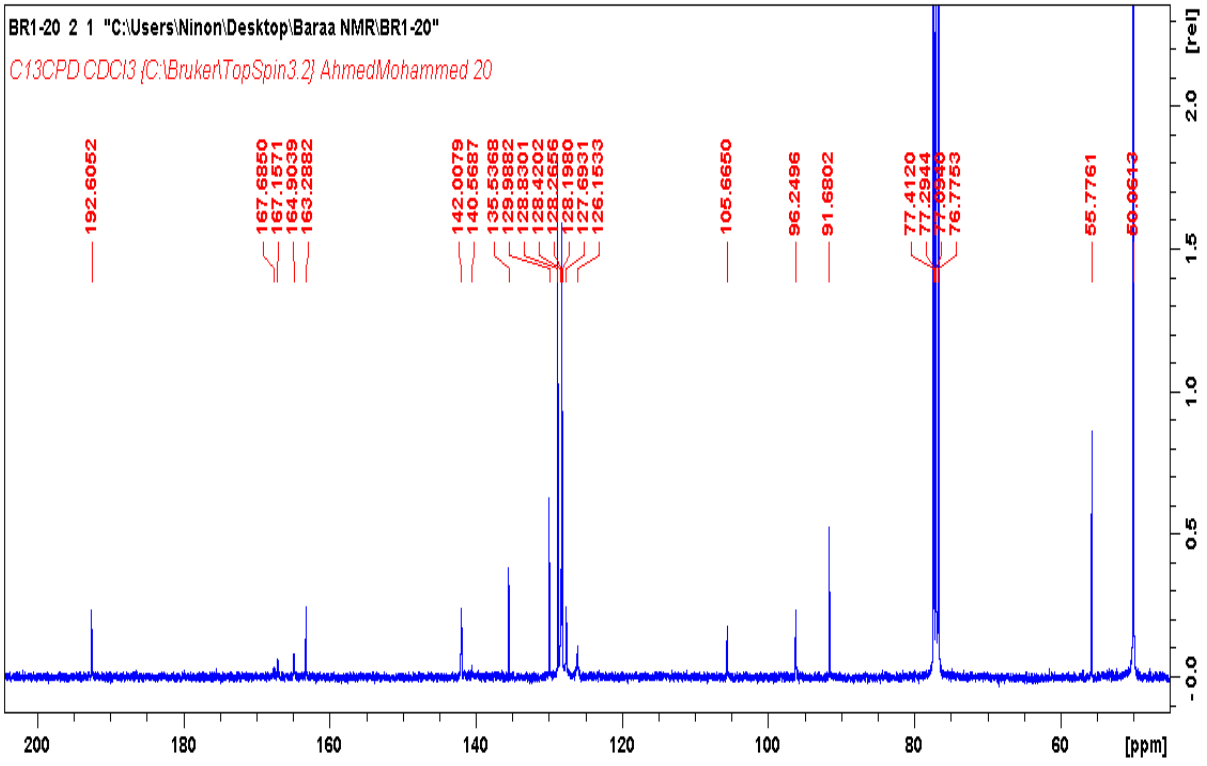


Figure 4.21: ^{13}C-NMR (400 MHz, $CDCl_3$) spectrum of **6**

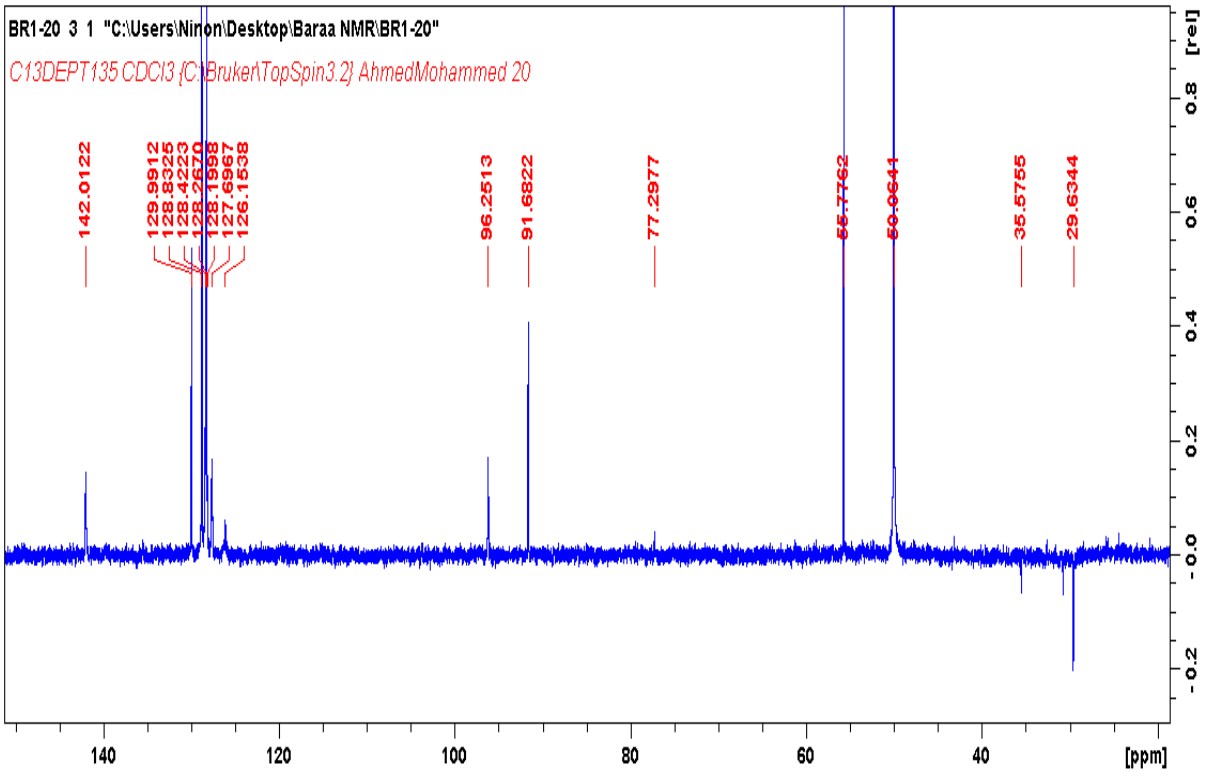


Figure 4.22: DEPT-NMR (400 MHz, $CDCl_3$) spectrum of **6**

4.2.7 Structure elucidation of helichrysetin (7)

Compound **7** was isolated as an orange powder as describe in scheme 4.1. It was identified as helichrysetin from its NMR data, which was similar to that of compound **6** except that compound 7 has an extra OH group at position 4, and the appearance of the methoxy group at position 2' (3.84/55.8), which can be observed on the ^{1}H NMR. The obtained data (NMR data) in comparison with those published in literature confirmed the structure of compound **7** as helichrysetin (Chatsumpun, et al., 2017). This compound was isolated for the first time from *H. cymosum.*

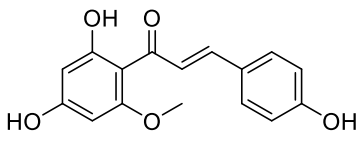


Figure 4.23: Chemical structure of helichrysetin

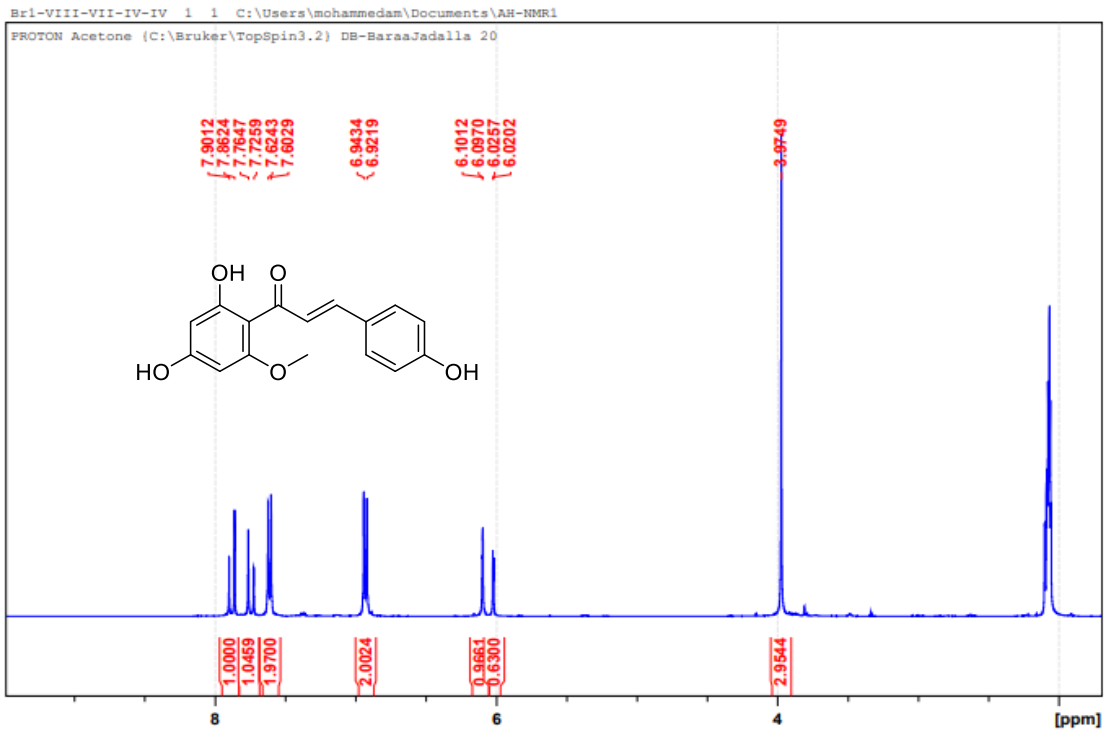


Figure 4.24: ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of **7**

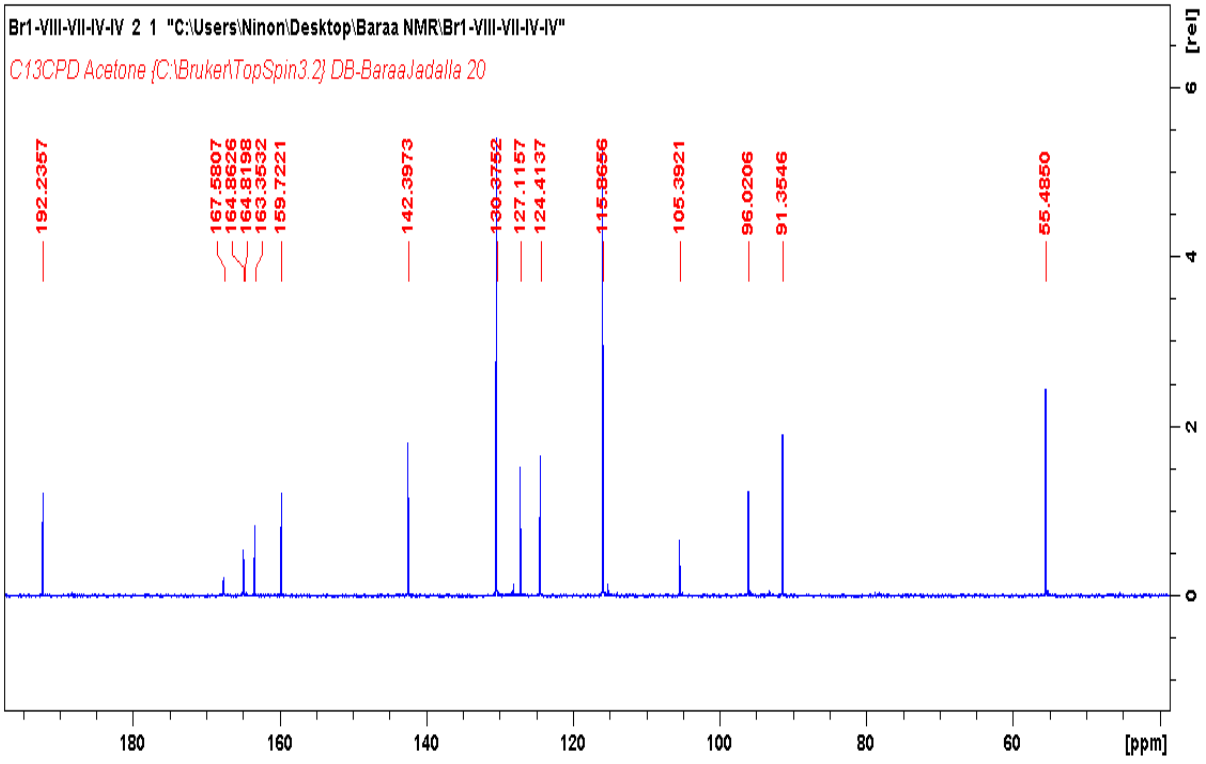


Figure 4.25: ^{13}C-NMR (400 MHz, $CDCl_3$) spectrum of **7**

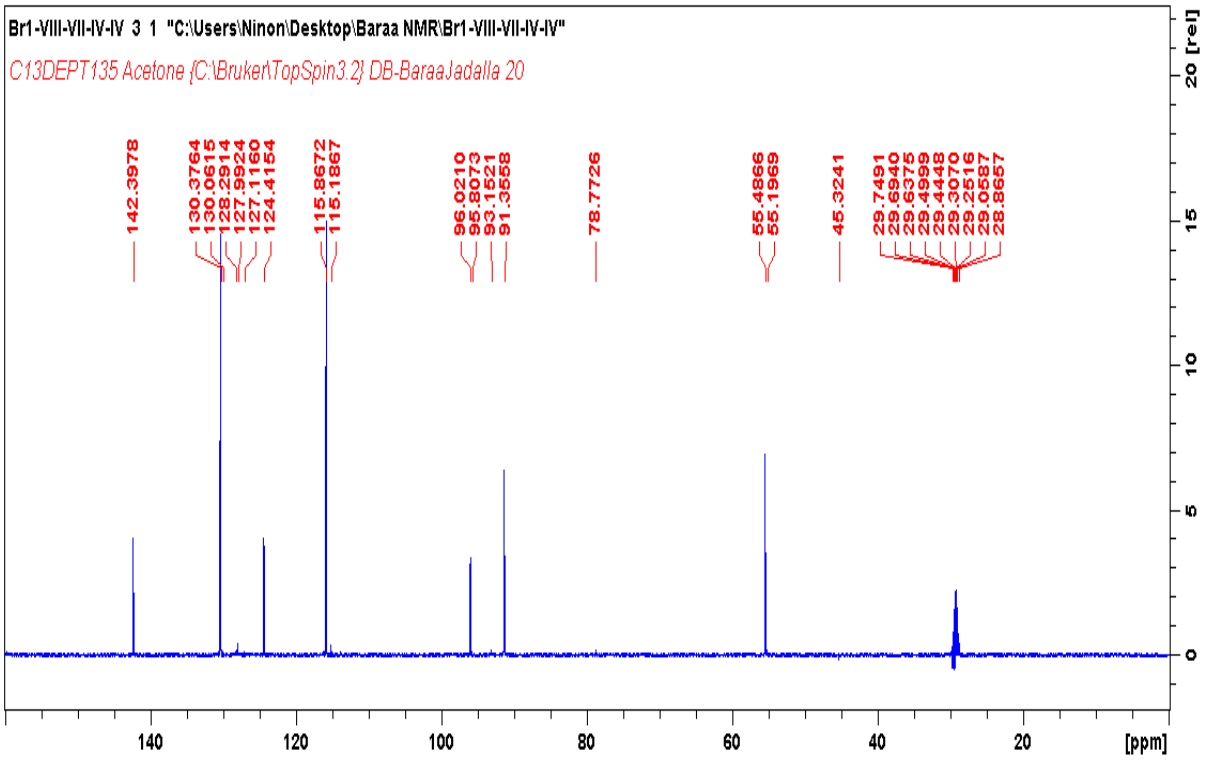


Figure 4.26: DEPT-NMR (400 MHz, $CDCl_3$) spectrum of **7**

4.3 Results and discussion: Biological evaluation

4.3.1 Alpha-glucosidase and alpha-amylase activities

Alpha-glucosidase enzyme is situated in the brush boundary of the small intestine epithelium. It directly involved in the catalytic reaction of the breaking down of disaccharides and starch to glucose (Gericke, et al., 2017). Alpha-amylase enzyme is directly implicated in the breaking down of starch to simpler sugars (Nickavar, et al., 2008).

In the research of prominent antidiabetic candidates from natural sources, the bio-evaluation of plants has been increased, and a significant research works are being conducted to identify plants/secondary metabolites with antidiabetic potentials with the main focus on the inhibition of alpha-glucosidase and alpha-amylase enzymes. In this research study, the inhibitory activity of *H. cymosum* and its isolates were investigated and the results shown that **5** exhibited the highest alpha-glucosidase inhibitory activity with IC_{50} value of 13 µM, followed by **7** and **3** with IC_{50} values of 18.16 µM and 44.4 µM respectively as indicated in Table 4.1. Helichrysetin (**7**) has not been reported to demonstrate antidiabetic activity. However, phloretin, which belongs to the same class of compounds with **7**, has been reported to exhibit strong reversible alpha-glucosidase inhibitory activity in a mixed-type manner with IC_{50} value of 114 µM (Han, et al., 2017). Its glucoside (phlorizin) inhibits the sodium-dependent glucose transporters (SGLT1 and SGLT2) resulting in the reduction of the re-uptake of glucose from the small intestine and kidneys (Liu, et al., 2012). Synthetic derivatives of phlorizin are clinically used in the treatment of diabetes (Liu & Lee, 2011). Compound **6** has also been reported to exert hypoglycemic activity in streptozotocin-induced diabetic rats and ameliorates insulin resistance (Shen, et al., 2017).

Pinostrobin chalcone (**6**) is one of the secondary metabolites that has been reported to be implicated/involved for the blood glucose levels reduction in rats with streptozotocin-induced

diabetes (Marques, et al., 2015). Unfortunately, this compound showed moderate activity when tested for alpha glucosidase as shown in Table 4.1. Among the tested compounds, none of them demonstrated alpha amylase inhibitory activity at a minimum concentration of 8.33 µg/mL as indicated in Table 4.1. Remarkably, the structure-activity relationship (SAR) demonstrated that the more polar compounds showed the highest activities.

Table 4.1: Inhibitory activities of *H. cymosum* and its isolates on alpha-glucosidase and alpha-amylase

Items	**Alpha-glucosidase**	**Alpha-amylase**
	IC_{50} (µM)	IC_{50} (µM)
1	NA	NA
2	204.23	NA
3	44.4	NA
4	NA	NA
5	13	NA
6	206.4	NA
7	18.16	NA
Acarbose	945.47	15.8

NA not active at the tested concentrations. The results are presented as mean ±SEM for n = 3

In general, flavonoids have been reported to possess significant antidiabetic activity due to the OH group allocated at C-3 on the C-ring and additional OH groups attached to the A and B-ring, which play a vital role in the inhibition process of alpha-glucosidase and-amylase enzymes as well as hypoglycaemia, insulin activation, glucose uptake activation (Wang, et al., 2010).

Additionally, flavonoids are frequently used in antidiabetic diets and many of them including catechins, kaempferol, fisetin, naringenin, luteolin, quercetinmorin, rutin, silymarin, baicalein,

chrysin, icariin, diosmin, isoliquiritigenin, isoangustone A, genistein were reported to be active for their antidiabetic properties (Hartogh & Tsiani, 2019).

4.3.2 Antioxidant activity

Oxidative stress is well known as one of the principal causes of diabetes complications due to over-accumulation of free radicals, which cause mitochondrial superoxide overproduction, insulin resistance, diabetes tissue injury, weakened glucose tolerance, β-cell dysfunction, and (Yatoo, et al., 2016). The *in vitro* antioxidant activity of the isolated compounds from *H. cymosum* were investigated by evaluating their ORAC as well as FRAP activities. ORAC is an assay based on a hydrogen atom transfer (HAT) mechanism (Apak, et al., 2016), while FRAP is based on a single electron transfer (SET) mechanism (Pérez-Fons, et al., 2010). The results of the in vitro antioxidant demonstrated that **6** and **2** exhibited strong activity on ORAC (122.86 ± 0.7 and 91.70 ± 0.4) µM TE/mL respectively. Compounds **5** and **7** showed strong activity on FRAP (1006.34 ± 1.7; 977.79 ± 0.8) µM AAE/g, followed by compound 1 that showed a moderate activity (648.75 ± 1.1) µM AAE/g as shown in Table 4.2. Those two compounds have been already reported to be good candidates to serve as safe antioxidant (Shindo, et al., 2006). The crude extract of *H. cymosum* has been reported to possess antioxidant activity with SC50 value of 6.3 g/L (Süntar, et al., 2013; Maroyi, 2019). Phenolic compounds are in charge of the antioxidant activity of many plant species by neutralization of free radicals via hydrogen atom transfer or electron transfer or by metal ion complexation amongst other mechanisms. However, there are other aspects also that can be taken in consideration including the presence of vicinal OH groups, which is very crucial in a pronounced antioxidant activity (Souza de Oliveira, et al., 2015).

Table 4.2: Antioxidant activities of *H. cymosum* constituents

Items	ORAC (µmol TE/mL)	FRAP(µM AAE/g)
1	14.34 ± 0.2	648.75 ± 1.1
2	91.70 ± 0.4	197.36 ± 0.7
3	56.93 ± 1.1	31.00 ± 0.24
4	25.19 ± 0.4	36.21 ± 0.9
5	28.12 ± 0.5	1006.34 ± 1.7
6	122.86 ± 0.7	93.7 ± 0.6
7	29.63 ± 0.6	977.79 ± 0.8

The results are expressed as mean ±SEM for n = 3

Flavonoids represent a very large class of phytochemicals found in a wide range of vegetables, fruits, and herbs that can play a prominent role as natural antioxidants, due to the existence of hydroxy groups and aromatic rings in their chemical structures (Panche, et al., 2016). In general, the presence of a hydroxy group on the B-ring is the most important active group, for the donation of hydrogen to peroxyl/hydroxyl radicals, thereby neutralizing or stabilizing them and giving rise to a very stable flavonoid radical (Wolfe & Liu, 2008). Our result is therefore an indication that **7** and **2** have the great capacities of donating hydrogen atom(s) from β-OH groups to stabilize free radicals.

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

Considering the fact that an extensive amount of phytochemical and biological research has been conducted on the *Helichrysum* species, few of them have investigated *H. cymosum*. Its selection was based on the non-availability of scientific research study conducted on the phytochemical isolation of different secondary metabolites present within the plant extract as well as the pharmaceutical application to support their respective traditional uses. This research work therefore is the first scientific report on the extensive phytochemical analyses of *H. cymosum* and their possible biological application as antidiabetics as well as antioxidant candidates for the management of diabetes and oxidative stress related diabetes.

The preliminary phytochemical investigation using thin layer chromatography (TLC) displayed that extracts of HC (results not shown) exhibited yellow/brown spots of chemical constituents after sprayed with vanillin/H_2SO_4. The TLC results indicated that those extracts might contain interesting phenolic compounds especially flavonoids.

The structure elucidation of the isolated compounds was carried out using spectroscopic techniques such as 1D NMR.

Table 5.1: Summarized the extensive phytochemical analyses of the isolated compounds from *H. cymosum*

Code	Name	Remark
1	5,8-dihydroxy-7-methoxyflavone	Known*
2	pinostrobin	Known*
3	dihydrobaicalein	Known*
4	glabranin	Known
5	allopatuletin	Known*

6	pinostrobin chalcone	Known*
7	helichrysetin	Known*

*Compounds isolated for the first time from this plant

The in vitro bio-evaluation of alpha glucosidase and alpha amylase as well as the in vitro antioxidant capacity were performed.

The phytochemical investigation of *H. cymosum* afforded seven known compounds, which were isolated for the first time from this plant. The bio-evaluation of *H. cymosum* against alpha glucosidase shown that **5** exhibited the highest alpha-glucosidase inhibitory activity with IC_{50} value of 13 µM, followed by **7** and **3** with IC_{50} values of 18.16 µM and 44.4 µM respectively. None of the tested compounds shown alpha amylase inhibitory activity.

The results of the in vitro antioxidant demonstrated that **6** and **2** exhibited strong activity on ORAC (122.86 and 91.70 µM TE/mL) respectively. Compounds **5** and **7** demonstrated strong activity on FRAP (1006.34 ± 1.7; 977.79 ± 0.8) µM AAE/g, followed by compound 1 that showed a moderate activity (648.75 ± 1.1) µM AAE/g.

The significant biological activities demonstrated by **5**, **7** and **3** nominated them and *H. cymosum* as good candidate for the management of diabetes and might be submitted for further in vivo studies as well as clinical trials. These findings on alpha-glucosidase inhibitory activities and antioxidant capacities of *H. cymosum* and its isolates will be published in an international well-reputed Journal with this proposed title ‘Evaluation of alpha glucosidase inhibitory activity and antioxidant capacity of *H. cymosum* chemical constituents.

From the above, it is recommended that

1. Extensive biological studies such as glucose uptake to be conducted for the endogenous Southern African plant H.*Cymosum* for its bioactive compounds to explore their full bioactivity and mechanism of action.
2. Further biological investigations in animal models and clinical trials need to be done for compound **5** and **7** for their promising potential in controlling postprandial blood-glucose level.
3. Additional cytotoxicity studies should be done on the H. *Cymosum* and consumers should be counselled of its effects particularly of the already existing commercialized products.

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