STRATHCLYDE INSTITUTE OF PHARMACY & BIOMEDICAL SCIENCES

METABOLOMIC PROFILING OF CERTAIN *SALSOLA* SPECIES GROWING IN SAUDI ARABIA AND ISOLATION OF THEIR ACTIVE PRINCIPLES

A THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN

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BY

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DECLARATION

“I declare that, except where specifically indicated, this thesis is the result of the author’s original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.”

Signed: Samar Samir Murshid

Date:

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ABBREVIATIONS

µm	Micrometer, 10^{-6} m
°C	Degrees Celsius
G	Gram
µg	Microgram, 10^{-6}g
ng	Nano gram, 10^{-9}g
mg	Milligram, 10^{-3}g
L	Liter
mL	10^{-3} liter
ppm	Parts per million
Hz	Hertz
Min	Minute
δ	Chemical Shift
NMR	Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon Nuclear Magnetic Resonance
1D- and 2D-	One- and two-dimensional
^{1}H-^{1}H COSY	Homo Nuclear Correlation Spectroscopy
HMBC	Hetero Nuclear Multiple Bond Connectivity
HSQC	Hetero Nuclear Single Quantum Coherence
HMQC	Heteronuclear Multiple-Quantum Correlation
HPLC	High Pressure Liquid Chromatography
HR-LCMS	High Resolution Liquid Chromatography Mass Spectrometry
GC-MS	Gas Chromatography-Mass Spectroscopy
MS	Mass Spectrum

HRFABMS High Resolution Fast Atom Bombardment Mass Spectroscopy

UPLC-MS Ultra-Performance Liquid Chromatography coupled to Mass Spectrometry

UPLC-ESI-MS/MS Ultrahigh Performance Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry

UHPLC-QqQ-MS Ultrahigh Performance Liquid Chromatography method coupled with Triple Quadrupole Mass Spectrometry

LC-ESI-MS Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric

FABMS Fast Atom Bombardment Mass Spectroscopy

m/z Mass to charge ratio

J Nuclear spin-spin coupling constant

Mult. Multiplicity of a signal

m Multiplet

s Singlet

d Doublet

dd Doublet of Doublet

t Triplet

q Quartet

eq equatorial

brt Broad Triplet

brq Broad Quartet

PTLC Preparative Thin Layer Chromatography

O2PLS Modified Orthogonal Projections to Latent Structures

OPLS-DA Orthogonal Partial Least Square-Discriminant Analysis

PC Principal Component

PCA Principal Component Analysis

PLS-DA Partial Least Squares, or Projections to Latent Structures- Discriminant Analysis

Rt	Retention time
R_f	Relative to solvent front
SIMCA	Soft Independent Modelling by Class Analogy
LC	Liquid Chromatography
MPLC	Medium Pressure Liquid Chromatography
TLC	Thin Layer Chromatography
VLC	Vacuum Liquid Chromatography
CC	Column Chromatography
FC	Flash Chromatography
UV	Ultra Violet
$[M]^+$	Molecular Ion Peak
MWt	Molecular Weight
MF	Molecular Formula
$CHCl_3$	Chloroform
ABTS	2,2′-azino-bis-(-3 ethylbenzothiazoline-6-sulfononic acid)
DPPH	2,2-Diphenyl-1-picrylhyrazyl
DMSO-d_6	Deuterated Dimethyl Sulphoxide
DMSO	Dimethyl Sulphoxide
DCM	Dichloromethane
EtOAc	Ethyl Acetate
Hex	Hexane
FA	Formic acid
HOAC	Acetic acid
H_2SO_4	Sulphuric Acid
ACN	Acetonitril

MeOH	Methanol
BuOH	Butanol
ED_{50}	Effective Dose for 50 percent of the Group
LD_{50}	Dose that kills 50 percent of the Animals
IC_{50}	Half Maximal Inhibitory Concentration
MIC	Minimum Inhibitory Concentration
IZD	Inhibition Zone Diameter
SI	Selectivity Index
OS	Oxidative Stress
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
NOS	Nitric Oxide Synthase
SOD	Superoxide Dismutase
GPX-1	Glutathione Peroxidase-1
NPs	Natural Products
SS NPs	Semisynthetic natural products
NP-ADCs	Natural product-containing antibody drug conjugates
DNP	Dictionary of Natural Products
SAR	Structure Activity Relationship
NCEs	New Chemical Entities
HTS	High Throughput Screening
EMEA	European Agency for the Evaluation of Medicinal Products
FDA	Food and Drug Administration
NCI	National Cancer Institute (USA)
NCCLS	National Committee for Clinical Laboratories Standards

SCUBA	Self Contained Underwater Breathing Apparatus
BLAST	Basic Alignment Search Tool
SIPBS	Strathclyde Institute of Pharmacy and Biomedical Science
SIDR	Strathclyde Innovations for Drug Research
PO	Oral Route of Administration
IV	Intravenous Route of Administration
TOP	Topical Route of administration
et al	Et alia (and others)
UK	United Kingdom
KSA	Kingdom of Saudi Arabia
KAU	King Abdulaziz University
KE	*Salsola kali* etheyl acetate extract
KM	*Salsola kali* methanol extract
COX	Cyclooxygenase enzyme
lL-1*β*	Interleukin-1 beta also known as leukocytic pyrogen, leukocytic endogenous mediator
CBG	Corticosteroid-Binding Globulin
icIL-1*α*	Intracellular Interleukin-1 Alpha
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
MMPs	Matrix Metalloproteinases
DGP	Diabetic Gastroparesis
lL-6	Interleukin 6
IFN-γ	Interferon Gamma
TNF-*α*	Tumor Necrosis Factor Alpha
A549	Adenocarcinoma Human Alveolar Basal Epithelial Cell
C32	Amelanotic Melanoma Cell

LNCaP	Hormone-Dependant Prostate Carcinoma
MCF-7	Human Breast Adenocarcinoma Cell
PANC-1	Human Pancreatic Cancer cell
HepG2	Hepatic Cancer Cell
HCT-116	Colorectal Cancer cell
HT29	Human Colon Carcinoma
ACHN	Renal Adenocarcinoma Cell
COR-L23	Large Cell Lung Carcinoma
AChE	Acetylcholinesterase
CYP	Cytochrome
DNA	Deoxyribonucleic Acid
FA	Fatty acid
LPS	Lipopolysaccharides
BChE	Butyrylcholinesterase
AChE	Acetylcholinesterase
HMDB	Human Metabolome Database
BLAST	Basic Local Alignment Search Tool
MTT	3-(4,5- dimethylthiaxolyl-2)-2,5-diphenyltetrazoliumbromide
TM	Traditional medicine

ABSTRACT

Medicinal plants have long been recognized as a valuable source of bioactive compounds with potential therapeutic properties. Many of these plants contain a wide array of micro molecules, such as polyphenols, flavonoids, alkaloids, terpenoids, and other phytochemicals, which have been found to possess antioxidant and anticancer properties. Oxidative stress can play a significant role in the development and progression of cancer. Therefore, searching for antioxidant compounds is indeed step towards protection against cancer. The flora of Saudi Arabia offers a valuable resource of plants that have not been extensively studied for their chemical and biological activity. *Salsola kali* (Amaranthaceae), commonly known as prickly saltwort or tumbleweed, is found in the Saudi flora, possesses medicinal importance and is employed in traditional medicinal practices.

In this work, the biological activity of the aerial parts of *Salsola kali* total methanol extract (KM) as well as its ethyl acetate (EtOAc) (KE) fraction were tested for their antioxidant activity adopting DPPH assay. Moreover, their anticancer activities were assessed *in vitro* using brine shrimp assay and against different human cancer cell lines (HepG2, MCF-7, HCT-116).

The results showed promising antioxidant as well as anticancer activities especially against HepG2 with IC_{50} values of 104.5 µg/mL and 34.5 µg/mL for KM and KE, respectively. A metabolomics-guided approach was applied by using NMR and LC-HRMS as profiling tools to afford an intensive chemical profile of the *Salsola* to target the bioactive metabolites. The spectral data was processed using Xcalibur, MZmine 2.53, in-house MS-Excel macro, and Dictionary of Natural Products for dereplication studies. (KE) fractions were subjected to extensive metabolic studies. A supervised multivariate analysis was done by orthogonal partial least squares discriminant analysis (OPLS-DA) in SIMCA-P V 17.0 to predict and pinpoint the plausible bioactive components.

The result revealed that 43 hits were detected in the extract which fall under the chemical classes of phenolics, more especially flavonoids and their derivatives, organic acids, nitrogenous compounds, and sugar derivatives. Further, the EtOAc fraction of *Salsola kali* (KE) was fractionated on MPLC and the obtained 15 fractions tested again for their antioxidant activity. Higher anti-oxidant activities for the EtOAc crude extract as compared to its methanolic congruent. Several EtOAc fractions including; F4, F5, F6, F7-9, F52-55, F56-60, and F61-76 showed significant anti-oxidant activities as compared to ascorbic acid within the range from 41 % and up to almost 65 %. Major active compounds in the bioactive fractions were then isolated by different chromatographic techniques.

The five isolated compounds were characterized by NMR and identified as lupeol (**1**), *trans* 4-methoxy cinnamic acid (**2**), ferulic acid (**3**), 4-anisaldehyde (**4**) and isorhamnetin -3-*O*-*β*-D-galactopyranoside (**5**) which were reported for the first time in *S. kali*. Based on the presented data, our study would greatly add to the knowledge of identifying promising candidates of natural origin for drug discovery and development process.

Keywords: *Salsola* species, *Salsola kali*, spectroscopic techniques, metabolomics tools, anti-oxidant effect, anticancer effect

CHAPTER I: INTRODUCTION

1. General Introduction

1.1. A Historical Overview of Natural Products

Natural products (NPs), also known as secondary metabolites, are produced by different organisms. One third of them originated from plants that is over 37%, microbes as bacteria represent 33% or another one third, fungi sources consisted 26%, 2% were derived from marine organisms and 2% were from other origins such as animal sources (**Figure 1.1**) (Spainhour, 2005; Patridge et al., 2016). Since NPs have influenced the development of many of the most widely used medications in the world, it is expected that they will aid in the search for new therapeutic agents. NPs, which include fruits and vegetables, have been acknowledged as important sources of bioactive polyphenolic compounds that must be consumed on a daily basis throughout one's lifetime (Gosslau et al., 2011). Natural products (NPs), have been the most fruitful source of potential drug leads (Dias et al., 2012). As chemical materials, natural products include a diversity of compounds, including terpenoids, polyketides, peptides, alkaloids, etc. The occurrence of natural products is not merely by accidents or products of convenience of nature. They are more than probably natural expression of the increase in complexity of organism (Spainhour, 2005).

The earliest records of the natural products were shown or described on clay tablets in cuneiform from *Mesopotamia* (2600 B.C.) that recommended oils from *Cupressus semperyiens* (Cypruss) and *Commiphora* species (myrrh) that are yet used now to treat several ailments including inflammation, cold and cough (Cragg and Newman, 2005). The *Ebers Papyrus* (2900 B.C.), an Egyptian Pharmaceutical record, declared a number of plant-based drugs including pills, gargles, infusions and ointments (Dias et al., 2012). The Greeks (371–28 B.C.) came after them, and the Christian era sent the Romans their plant-based medical knowledge, which the Romans then passed on to the Arabs (Sayed, 1980). The Chinese Materia Medica (1100 B.C., Wu Shi Er Bing Fang, contains 52 prescriptions), Shennong Herbal (~100 B.C., 365 drugs) and the Tang Herbal (659 A.D., 850 drugs) comprise documented information concerning the uses of natural products (Cragg and Newman, 2005). Penicillin, cephalosporins, tetracyclines, aminoglycosides, rifamycin, chloramphenicol, and lipopeptides were discovered late in the 20th century as a result of the documentation of traditional knowledge on the medicinal uses of plants. This knowledge eventually reached the study of anti-bacterial drugs (Krause et al., 2013).

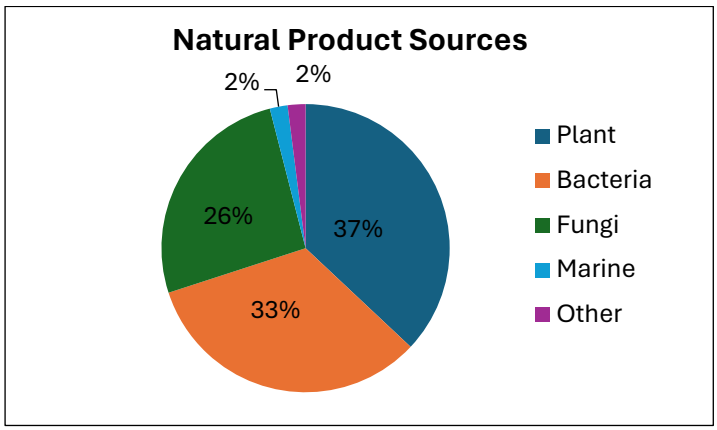


Figure 1.1: FDA approved NP separated by biological source (Patridge et al., 2016).

1.2. Drug Discovery

Drug discovery has one fundamental aim of finding potential novel drug analogues and new chemical entities (NCEs) that are nontoxic and effective in treating diseases. The key to achieving this goal is the urgent need to discover a potential new drug analog as quickly and cost-effectively as possible, ensuring the highest possible return on the investment (Gray et al., 2012). The substantial time and cost of developing new drugs (**Figure 1.2**) is between 10 to 15 years and requires between 800 million and 1.8 billion dollars bringing a new drug to market (Powers, 2014). Therefore, the need to utilize different analytical techniques such as metabolomics is crucial in the discovery of novel drugs due to its ability to identify flaws in new bioactive molecules earlier thereby reducing the cost of development failure at a late stage in the workflow (Kraljevic et al., 2004).

There are three distinct approaches to uncovering new medications, which vary based on the type, origin, and classes of compounds involved. Searching various natural sources is the first step towards discovering new bioactive compounds. These chemical compounds are produced by both macro and microorganisms via distinct biosynthetic pathways (Alvin et al., 2014). With computer-based drug design (CBDD), on the other hand, a chemical compound is designed *in-silico* by computer software to fit and interact with a specific receptor or active site (Mandal and Mandal, 2009). Combinatorial chemistry is then used to generate libraries of synthesised compounds that can be tested against specific targets. Next, data analysis is done to determine which medications among the various congeners are the most effective (Gallop et al., 1994; Liu et al., 2017).

In an effort to leverage the 2nd and 3rd pathways of drug discovery (CBDD and combinatorial drug discovery), pharmaceutical companies and research institutions have recently demonstrated

interest in utilising new technologies like computer-based tools, drug docking, and 3D X-ray crystallography to their advantage (Muller, 2009).

Modern drug discovery programs are aimed at producing drug molecules or hit molecules capable of interacting with molecular targets. These molecular targets could be in the form of enzymes, proteins, the human genome or sections of it (Hopkins and Groom, 2002), the DNA or segments of it, and other such units, complex or simple, capable of influencing the body´s activities with respect to diseases or diseased conditions (Tse, 2010). The rapid generation of large and diverse natural product libraries and databases optimized for high-throughput screening (HTS) has greatly enhanced the exploration of natural products in modern drug discovery programs (Bindseil et al., 2001). While there are commercially available natural products libraries, many of the varying number of compounds are held in universities and research centers. They may hold as little as hundreds of compounds to as much as millions in the libraries. With this large and structurally diverse number of compounds/ extracts available hits can be found for almost any screening or target activity. With the number of hits and structures known, a good structure activity relationship (SAR) can be developed, and lead optimization could commence. Some of the commonly used natural product libraries and databases are: Dictionary of Natural Product (DNP), Natural Products Alert (NAPRALERT), ChemSpider, AntiBase, and MarinLit, while repositories have been maintained by Strathclyde Innovations for Drug Research (SIDR), Albany Molecular Research Inc., Tim Tech Natural Products and Phytopure® (Gray et al., 2012).

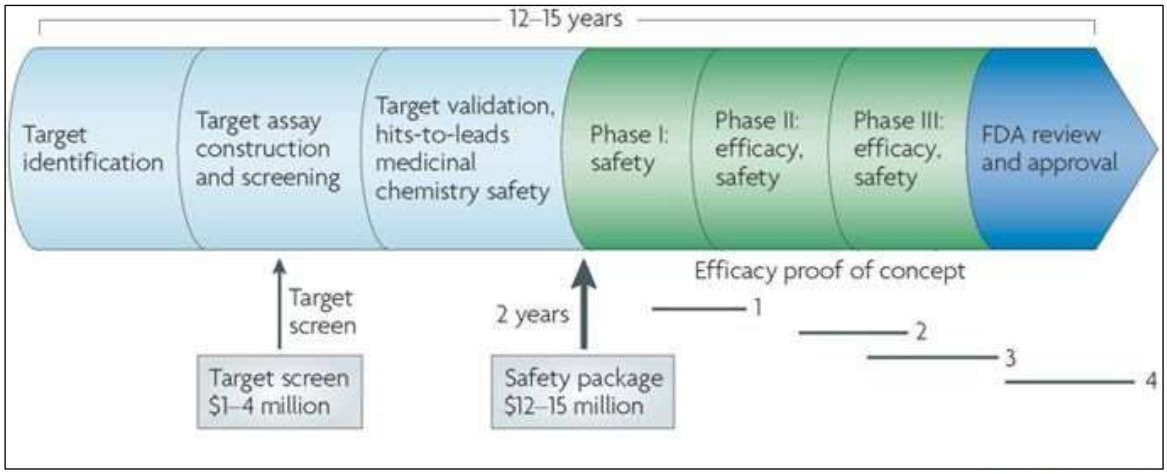


Figure 1.2: Representative pathway of drug discovery and development (Roses, 2008).

1.2.1. Natural Products and Drug Discovery

In the search for new medicines, plants are regarded as a latent treasure. They contain a wide range of secondary metabolites that modify the enzymes responsible for human diseases (Bader et al., 2015; D'Ambola et al., 2019).

Over half of the medications available on the market today are derived from natural products (Clark, 1996). Natural materials, especially plants, have been used for centuries to prevent and cure illnesses; this has led to the discovery of most contemporary pharmaceuticals (Cheuka et al., 2016).

These days, it is uncommon to use plants as raw samples. In today's pharmaceutical care, isolation and purification of the active ingredients are the recommended techniques instead. Isolated natural products exhibit a remarkable diversity of chemical structures in addition to having broad therapeutic spectra, including anti-cancer, anti-diabetic, and anti-bacteria properties (Cheuka et al., 2016). Approximately 50% of the natural products have a low log p value, hence they get absorbed easily and this complies with "Lipinski's Role of five" for orally available drugs (Ganesan, 2008).

Drug discovery has been closely combined with natural products from the early beginning. So, natural products have a significant role in pharmacy and health care research. Many drugs are entirely prepared from natural products while a great proportion (50%) of modern medicines has been directly or indirectly derived from them (Newman and Cragg, 2007). This might not be astonishing as herbal drug constituted the basis of traditional healthcare for a very long time. Very famous natural product-based drugs are penicillin, rifampicin and erythromycin (for treatment of bacterial infections); statins (cholesterol-decreasing drug); quinine and artimesinin (for treatment of malaria); paclitaxel, vincristin and vinblastin (anticancer); salicylic acid and non-addictive cocaine extracts (pain relief). About 80% of the drugs used for treatment of bacterial infections are either natural products or their derivatives, whereas 60% of anti-cancer drugs are either natural products or derivatives thereof (Butler, 2004; Newman and Cragg, 2007). Yet, natural products go further to provide incomparable variety of structures compared to standard combinatorial chemistry offering opportunities for finding chiefly novel low molecular weight lead compounds. It is worth mentioning that the world's biodiversity that had been assessed for significant biological activity does not exceed 10%, while many more natural lead compounds are still waiting for their discovery (Cragg and Newman, 2005). Throughout history, the overwhelming knowledge on natural product as source of medicine was built upon their usage as medicinal plants, which resulted from either by experimentations to treat diseases or palatability trials to maintain health through available food (Kinghorn et al., 2011). The basis of

most of the early medicines have been formed by traditional medical experiments, that were followed by chemical, pharmacological, and clinical studies (Butler, 2004). The most famous to date would be the synthesis of the anti-inflammatory agent, acetylsalicylic acid (aspirin). Aspirin is a derivative of salicin which was isolated from the bark of *Salix alba* L. Another example is the phytochemical study of *Papaver somniferum* L. (opium poppy) led to isolation of many alkaloids, including morphine (**Figure 1.3**), being first reported in 1803 (Dias et al., 2012). The *foxglove*-derived plant *Digitalis lanata* L. has also been used medicinally in the past; in the early 16th century (Krause et al., 2013). Conversely, bioactive metabolites, like digoxin and digitoxin, were not used until the 1700s and the 1930s, respectively, after it was discovered that they improved heart failure (Dias et al., 2012; Lahlou, 2013; Patridge et al., 2016; Ward and Pasinetti, 2016). Galantamine is a natural product discovered through an ethnobotanical lead and first isolated from *Galanthus woronowii* Losinsk (Amaryllidaceae) in Russia in the early 1950s. Galantamine is now approved for the treatment of Alzheimer's disease. It slows the process of neurological degeneration by inhibiting acetyl cholinesterase as well as binding to and modulating the nicotinic acetylcholine receptor (Heinrich and Teoh, 2004). Tiotropium has recently been released in the United States for treatment of chronic obstructive pulmonary disease (Barnes, 2002). It is an inhaled anticholinergic bronchodilator, based on ipratropium, a derivative of atropine isolated from *Atropa belladonna* L. and other members of the *Solanaceae* family (Barnes et al., 1995). Tiotropium has shown increased efficacy and longer lasting effects when compared with other available chronic obstructive pulmonary disease medications (Barnes, 2000).

As synthetic drugs gained popularity, it was preferred to produce compounds from natural sources because the amount of chemicals in plants can vary greatly depending on the season, climate, and time of year that they are harvested. Despite the fact that the medical community prefers synthetic medications for practical reasons, about 40% of these are still derived from herbs to treat cancer and cardiovascular diseases (Gurib-Fakim, 2006). Throughout history, both the Sumerians and ancient Greeks have proven the application of poppy extracts in medicines. Nevertheless, the Arabs have described opium as addictive. In the same manner, so many instances have been reported verifying the significance and importance of drugs of natural origins (Dias et al., 2012).

The number of people died from cancer has increased in the recent times (Torre et al., 2015). This exponential rise resulted from the increasing occurrence of carcinogens in the environment. So, several types of plants have been investigated to study anti-cancer activity proving to be useful in the treatment and prophylaxis of cancer. Moreover, the active compounds from the

plants seem to be safe and effective in different types of cancer (Khan et al., 2019). For example, *Phaleria macrocarpa* (also known as *Mahkota dewa*) and *Fagonia indica* (also known as *Dhamasa*) have historically been employed for the anticancer characteristics of the active compounds in these plants (Faried et al., 2007; Shehab et al., 2011). In cancer cells, apoptosis may be induced by using metabolites that have been isolated from the plant material. Gallic acid, which serves as the active component, was isolated from the fruit extract of *Phaleria macrocarpa* and has been shown to have a role in the activation of apoptosis in cell lines derived from lung cancer, leukaemia, and colon adenocarcinoma (Inoue et al., 1994; Sohi et al., 2003).

In addition, numerous other plant-derived drugs are currently undergoing clinical tests. Screening of more medicinal plants is important for finding potential new compounds for therapeutic uses. Thus, researches based on ethnopharmacological information generally consider an appropriate approach in the discovery of new effective agents from higher plants (Kloucek et al., 2005). It is widely acknowledged that extensive research still needs to be carried out and a cause of major concern is that some species are becoming extinct with their potential properties unknown (Jachak and Saklani, 2007). Understanding the effectiveness of natural products responsible for the actions of these medicinal plants would be a good development.

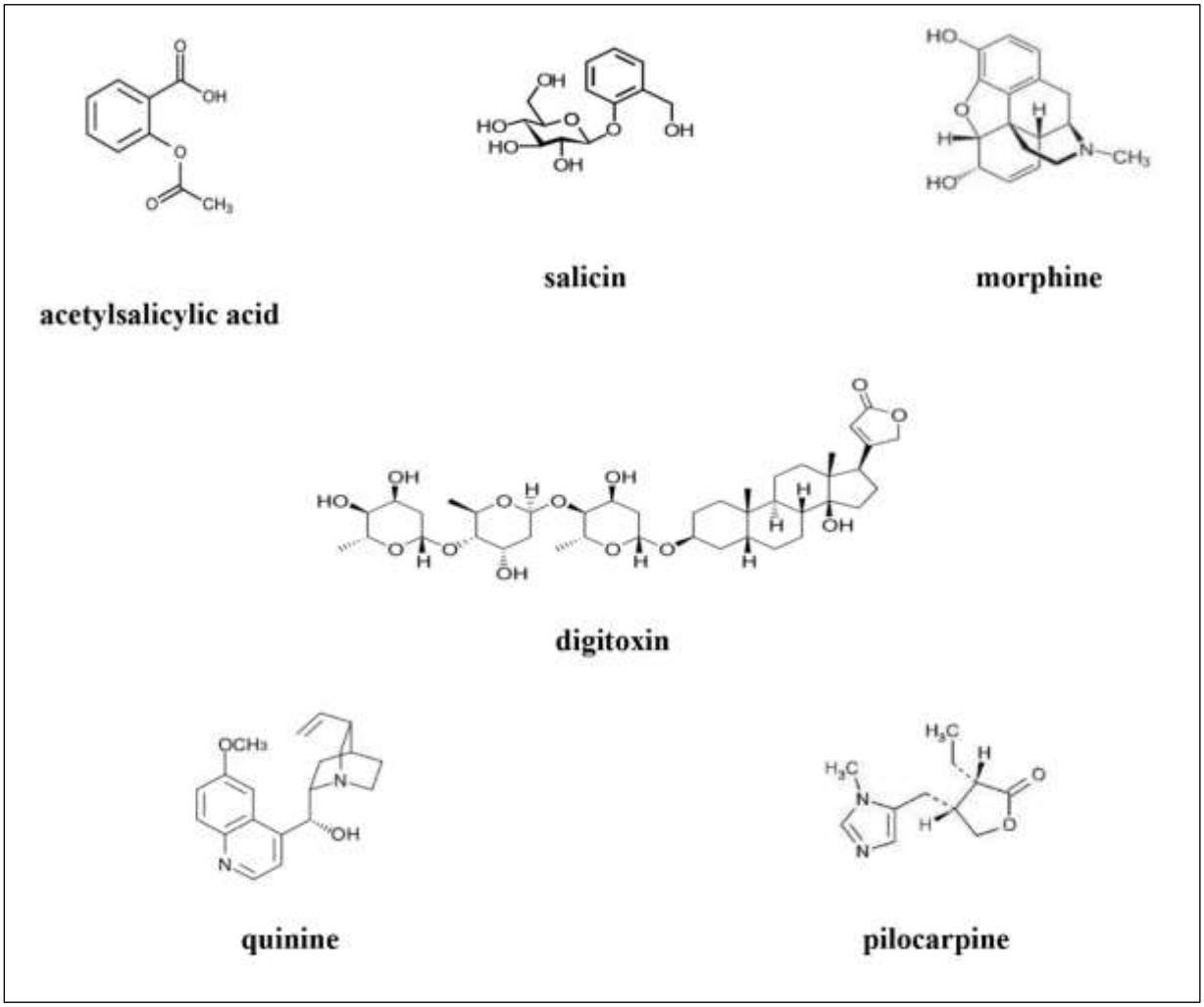


Figure 1.3: Commercially important drugs first reported in 1803 (Dias et al., 2012).

Data analysis in the US from 1959 to 1980, indicated that about 25% of the prescriptions contained either plant extracts or their active principles (Farnsworth et al., 1985). Currently, 119 chemical substances described then from 90 plant species, could be considered as an important drug in usage in at least one country all over the world. As a result of phytochemical studies based on traditional use of plants, 80% of those drugs have been discovered and are now being used as medicines (Patridge et al., 2016). Several compounds isolated from soil microorganisms and actinomycetes were discovered and approved as drugs (**Table 1.1, Figure 1.4**) (Ganesan, 2008).

It was declared by a study, using US based prescription data from 1993, that natural products were still performing a key role in treatment of diseases (Farnsworth et al., 1985; Spainhour, 2005; Nasim et al., 2022). So, today natural products research is most likely to continue to exist and develop to become even more valuable as generators of new drug leads. This could also be

attributed to the broad chemical diversity available in natural products and the degree of their novelty (Spainhour, 2005).

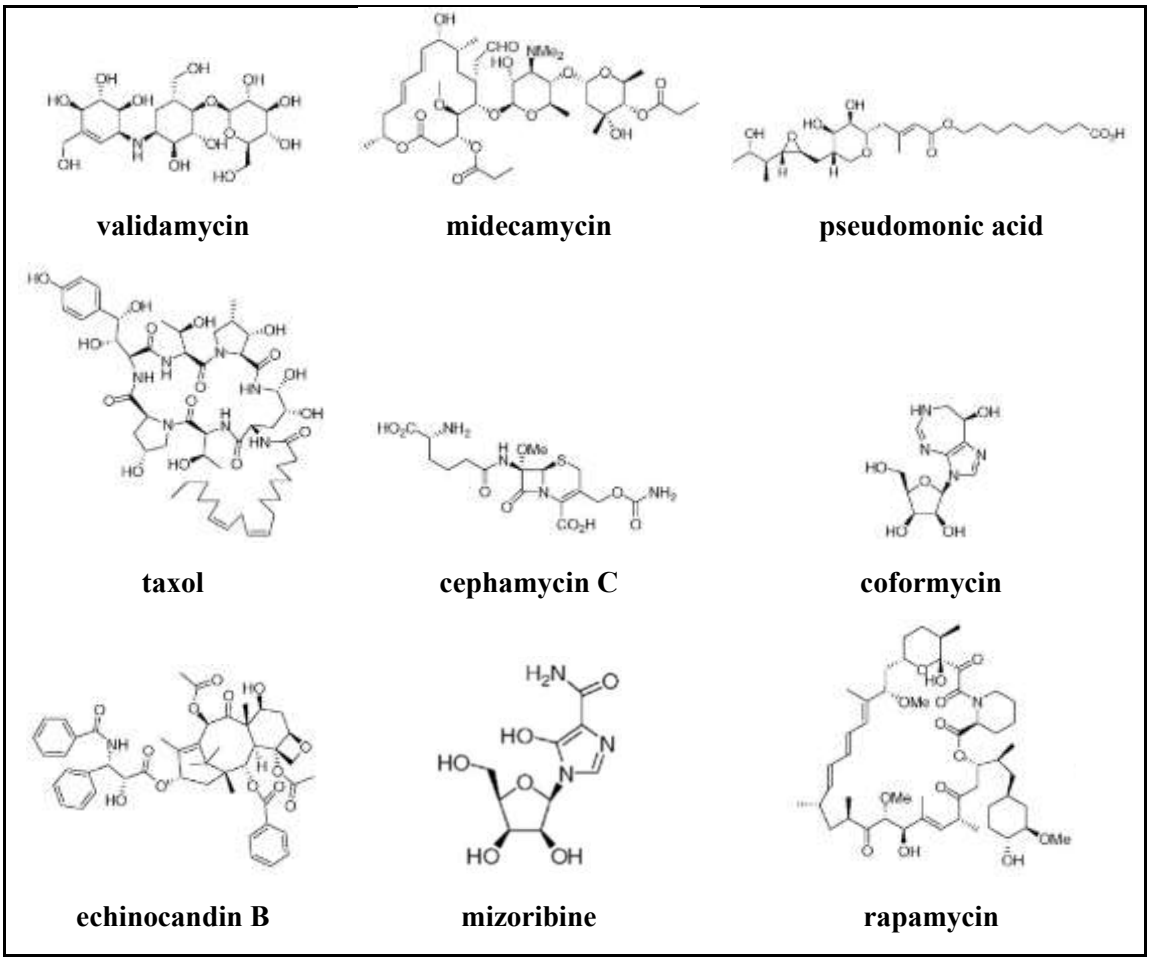


Figure 1.4: Representative chemical structures of different natural products discovered since 1970 that leads to an approved drugs (Ganesan, 2008).

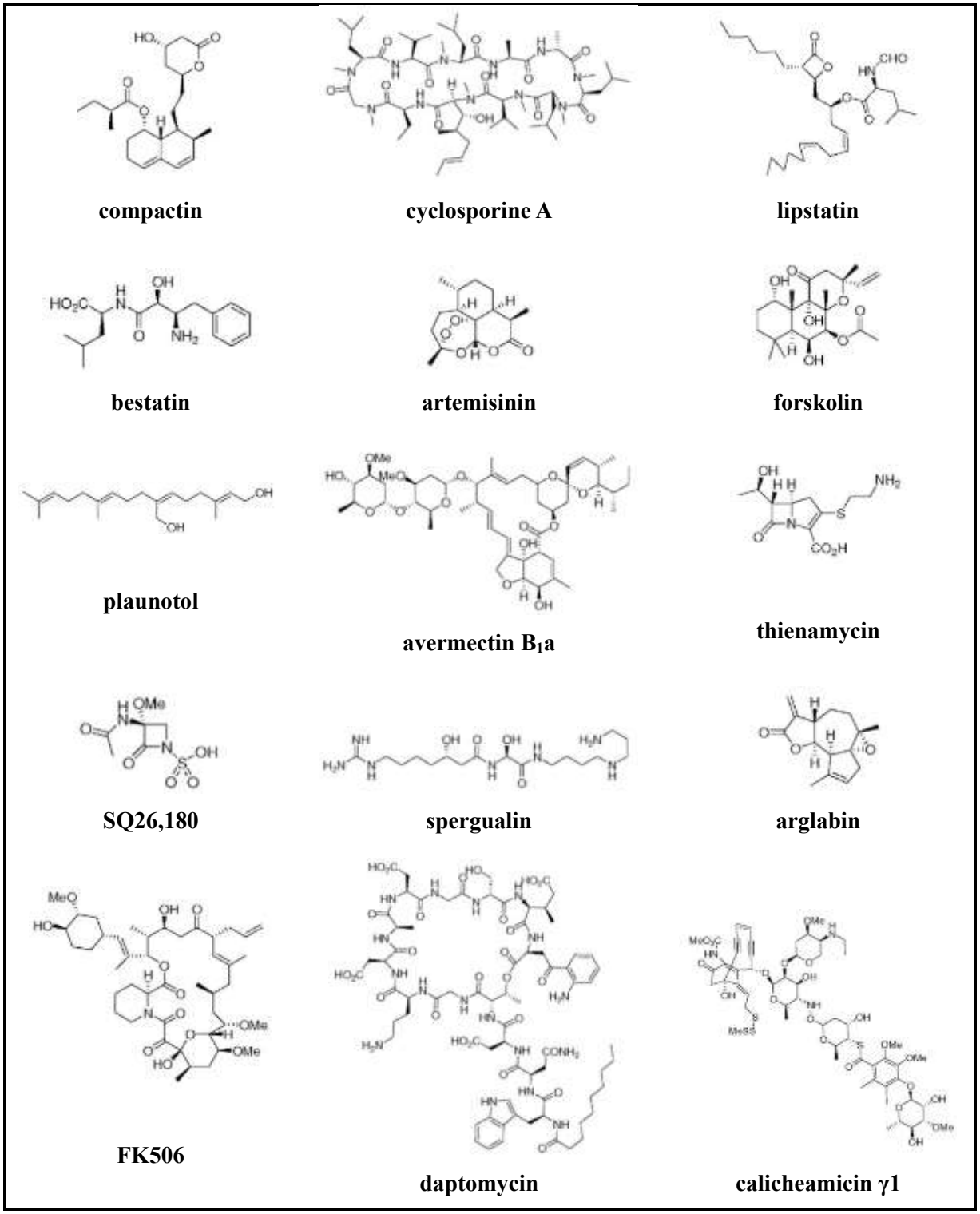


Figure 1.4 (Cont.): Representative chemical structures of different natural products discovered since 1970 that leads to an approved drugs (Ganesan, 2008).

Table 1.1: Different natural products discovered since 1970 that leads to an approved drugs (Ganesan, 2008; Patridge et al., 2016).

Lead, Year Structural class	Origin	Discoverer	Drug, Year	Route
validamycin, 1970 (Oligosaccharide)	Actinomycete *(Streptomyces hygroscopicus)*	Takeda (JAP)	acarbose, 1990 voglibose, 1994	PO PO
midecamycin, 1971 (Macrolide)	Actinomycete *(Streptomyces mycarofaciens)*	Meiji (JAP)	miocamycin, 1985	PO
pseudomonic acid, 1971 (Polyketide)	Bacteria *(Pseudomonas fluorescens*)	Beecham (UK)	mupirocin, 1995	TOP
taxol, 1971 (Diterpene)	Plant *(Taxus brevifolia)*	Res Triangle Inst/NIH (USA)	paclitaxel, 1993 docetaxel, 1995	IV IV
cephamycin C, 1971 (*β*-lactam)	Actinomycete *(Streptomyces clavuligerus)*	Lilly (USA)	moxalactam, 1982 cefotetan, 1984 cefbuperazone, 1985	IV IV IV
coformycin, 1974 (Nucleoside)	Actinomycete *(Streptomyces kaniharaensis)*	Inst Microbial Chem (JAP)	pentostatin, 1992	IV
echinocandin B, 1974 (Cyclopeptide)	Fungus *(Aspergillus nidulans)*	Ciba-Geigy (SWI)	caspofungin, 2001 micafungin, 2002 anidulafungin, 2006	IV IV IV
mizoribine, 1974 (Nucleoside)	Fungus *(Eupenicillium brefeldianum)*	Toyo (JAP)	mizoribine, 1984	PO
rapamycin, 1974 (Polyketide)	Actinomycete *(Streptomyces hygroscopicus*)	Ayerst (CAN)	sirolimus, 1999 everolimus, 2004 zotarolimus, 2005	PO PO PO
compactin, 1975 (Polyketide)	Fungus *(Penicillium citrinum)*	Sankyo (JAP)	lovastatin, 1984 simvastatin, 1988 pravastatin, 1989 fluvastatin, 1994 atorvastatin, 1997 cerivastatin, 1997 pitavastatin, 2003 rosuvastatin, 2003	PO PO PO PO PO PO PO PO
cyclosporine A, 1975 (Cyclopeptide)	Fungus *(Tolypocladium inflatum)*	Sandoz (SWI)	ciclosporin, 1983	PO
lipstatin, 1975 (Polyketide)	Actinomycete *(Streptomyces toxytricini)*	Roche (SWI)	orlistat, 1987	PO
bestatin, 1976 (Peptide)	Actinomycete *(Streptomyces olivoreticuli)*	Inst Microbial Chem (JAP)	ubenimex, 1987	PO

Table 1.1: Different natural products discovered since 1970 that leads to an approved drugs (Ganesan, 2008; Patridge et al., 2016).

Lead, Year Structural class	Origin	Discoverer	Drug, Year	Route
thienamycin, 1976 *β*-lactam	Actinomycete *(Streptomyces cattleya)*	Merck (USA)	imipenem, 1985 meropenem, 1994 panipenem, 1994 faropenem, 1997 biapenem, 2002 ertapenem, 2002 doripenem, 2005	IV IV PO IV IV IV IV
artemisinin, 1977 Sesquiterpene	Plant *(Artemisia annua P)*	Qinghaosu Res Grp (PRC)	artemisinin, 1987 artemether, 1987 artenusate, 1987 arteether, 2000	PO PO PO PO
forskolin, 1977 Diterpene	Plant *(Coleus forskohlii)*	Hoechst (IND)	colforsin, 1999	IV
plaunotol, 1977 Diterpene	Plant *(Croton sublyratus)*	Sankyo (JAP)	plaunotol, 1987	PO
avermectin B_1a, 1979 Polyketide	Actinomycete *(Streptomyces avermitilis)*	Kitastato Inst (JAP)/Merck (USA)	ivermectin, 1987	PO
SQ26,180, 1981 *β*-lactam	Actinomycete *(Chromobacterium violaceum)*	Squibb (USA)	aztreonam, 1984 carumonam, 1988	IV IV
spergualin, 1981 Peptide	Bacteria *(Bacillus laterosporus)*	Inst Microbial Chem (JAP)	gusperimus, 1994	IV
arglabin, 1982 Sesquiterpene	Plant *(Artemisia glutinosa)*	Inst Phytochem (USSR)	arglabin, 1999	PO
FK506, 1984 Polyketide	Actinomycete *(Streptomyces tsukubaensis)*	Fujisawa (JAP)	tacrolimus, 1993	PO
daptomycin, 1986 Cyclodepsipeptide	Actinomycete *(Streptomyces roseosporus)*	Lilly (USA)	daptomycin, 2003	IV
calicheamicin γ_1, 1988 Polyketide	Actinomycete *(Micromonospora echinospora)*	Lederle (USA)	gemtuzumab, 2000	IV
Mevastatin, 1978	Fungi (*Aspergillus terrus*)	Merck (USA)	Lovastatin, 1999	PO
Mevastatin, 1978	Fungi (*Aspergillus terrus*)	Merck (USA)	Rosovastatin, 2016	PO

1.2.2. Natural Products Based Drugs Approved by the FDA

The dominant concern of the drug industry is to improve the capability to increase the speed of discovering new drugs (Spainhour, 2005).

Between 1981 and 2010, 34% of the drugs approved by the FDA were based on small-molecule NPs and their derivatives (Harvey et al., 2015). Between the 1999 and 2013, 31 drugs, which is 28% of among those 112 of the FDA-approved drugs (**Figure 1.5**) were NP-derived, while the proportions of the three different FDA-approved drug categories varied (Ding et al., 2016).

Moreover, during the period between 2005 and 2010, 19 natural products based drugs were approved for the market (**Figure 1.6**), which included semisynthetic natural products as well (Mishra and Tiwari, 2011).

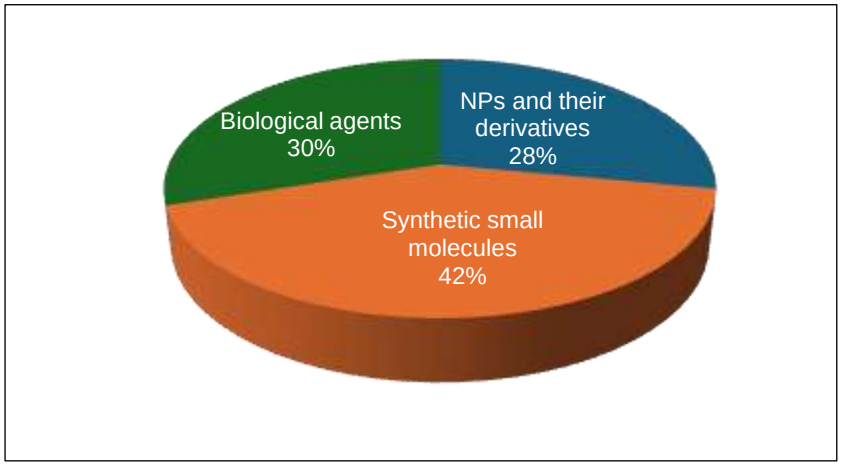


Figure 1.5: A detailed analysis of the 112 FDA-approved drugs between 1999 and 2013 revealed that 28% of those (31 drugs) were derived from NPs and their derivatives, in comparison to 42% (47 drugs) that were synthetic small molecules, and 30% (34 drugs) that were biological agents (Ding et al., 2016).

The following list includes drugs from natural products approved by FDA

Anidulafungin (**Eraxis™** in the US, **Ecalta™** in Europe), obtained by semisynthesis of echinocandin B (Debono et al., 1995). Anidulafungin is effective against invasive and oesophageal candidiasis and candidemia. FDA approved it in February 2006 (Mishra and Tiwari, 2011).

Arterolane (**Synriam™**) was approved in 2012 for the treatment of uncomplicated malaria in adults caused by *Plasmodium vivax* (Butler et al., 2014).

Aztreonam lysine (**Cayston™**) is an inhaled formulation composed of lysine salt (Retsch-Bogart et al., 2008). It was approved by FDA for use in cystic fibrosis (CF) patients having a pulmonary

infection by *Pseudomonas aeruginosa* in February 2010 (McDevitt and Rosenberg, 2001; Mishra and Tiwari, 2011).

Cabazitaxel (Jevtana®) a paclitaxel derivative, was approved by the FDA in 2010 for the treatment of hormone-refractory metastatic prostate cancer (Malhotra et al., 2013).

Canagliflozin (Invokana®) is derived from phlorizin. It was the first selective sodium-D-glucose cotransporter-2 (SGLT2) inhibitor to be approved in March 2013 by the FDA for the treatment of type 2 diabetes as adjunct to diet and exercise (FDA, 2014b).

Capsaicin (Qutenza®) was isolated for the first time in 1876 as an active component of chili peppers (genus *Capsicum*) (Caterina et al., 1997). Its burning sensation is attributed to binding with ion channel receptor vanilloid receptor subtype 1 (VR 1) (Mori et al., 2006; Kissin, 2008). In November 2009, it was approved for treatment of neuropathic pain combined with post-therapeutic neuralgia (Mishra and Tiwari, 2011).

Dapagliflozin (Farxiga®) is derived from phlorizin. It is a selective sodium-D-glucose cotransporter-2 (SGLT2) approved by FDA in January 2014 to improve glycaemic control in type 2 diabetic patients. It was first isolated from the bark of apple trees (FDA, 2014a).

Doripenem (Finibax®, Doribax®) is considered as a broad-spectrum antibiotic. It was formally approved by FDA in October 2007 for the usage in urinary tract and intra- abdominal infections (Mishra and Tiwari, 2011).

Everolimus (Afinitor®) was approved by FDA in 2009 as an immunosuppressant against advanced renal cell carcinoma (Mishra and Tiwari, 2011).

Exenatide (Byetta®) composed of 39 amino acids that was isolated from the oral secretions of the poisonous lizard *Heloderma suspectum* (Gila monster) (De La Torre and Reboli, 2007; Wolfson, 2007). It gained the FDA approval in April 2005 for treatment of type 2 diabetes mellitus (Barnett, 2007; Cvetković and Plosker, 2007).

Fumagillin (Flinsint®), was approved in September 2005. It is an antimicrobial lead capable of inhabiting the proliferation of endothelial cells. It was derived from *Aspergillus fumigatus* (Lanternier et al., 2009; Mishra and Tiwari, 2011).

Ingenol mebutate (Picato®) was first isolated from *Euphorbia paralias*. It is substrate for the Epidermal Multidrug Transporter (ABCB1) that targets tumor vasculature, reduces lesions and removes mutant p53 patches. It was approved by FDA in January 2012 as a topical treatment of actinic keratosis (Ogbourne et al., 2004; Gras, 2013).

Ixabepilone (Ixempra™) is a semisynthetic derivative of epothilone B which was obtained from *Sorangium cellulosum* (Conlin et al., 2007). It is effective as an anticancer drug which binds with *β*-tubulin subunits causing blocking of cells in the mitotic phase. The drug was approved in October 2007 by FDA (Reichle and Conzen, 2008).

Lisdexamfetamine (Vyvanse™) is a drug designed to help Attention-Deficit Hyperactivity Disorder (ADHD). It consists of dextroamphetamine coupled with the amino acid L-lysine (Jasinski and Krishnan, 2009), which was approved in February 2007 by FDA (Mishra and Tiwari, 2011).

Methylnaltrexone bromide (Relistor®) an opioid receptor antagonist, was approved for the treatment of opioid-induced constipation (Bader et al., 2011). Methylnaltrexone is an *N*-methyl derivative of naltrexone (Yuan, 2007). It acts through blocking peripheral opioid receptors activated by opioids. The drug is effective in management of alcohol and opioid dependence, approved by FDA in April 2008 (Mishra and Tiwari, 2011).

Omacetaxine mepesuccinate (Synribo®) was approved by FDA in October 2012 for the treatment of chronic myelogenous leukemia (CML) with resistance and intolerance to two or more tyrosine kinase inhibitors. It was isolated from *Cephalotaxus harringtonia* and acts due to its protein translation inhibition activity (Gandhi et al., 2014; Visani and Isidori, 2014).

Pleuromutilin is a metabolite of fungal origin showing antibacterial activity by inhibiting bacterial protein synthesis (Daum et al., 2007).

Retapamulin (Altabax™ in the US and **Altargo™** in Europe) is used for topical treatment of impetigo caused by Gram-positive *Staphylococcus aureus* or *Streptococcus pyogenes*. It is a semi-synthetic derivative of pleuromutilin antibiotics (Thomson and Moland, 2004; Kobayashi et al., 2005). It was approved by FDA in April 2007 (Jacobs, 2007).

Romidepsin (Istodax®) is a cyclic depsipeptide obtained from *Chromobacterium violaceum* (Woo et al., 2009) and approved by FDA in November 2009 to be used against selective cutaneous T-cell lymphoma (CTCL) (Kim et al., 2012c) and in June 2011 for peripheral T-cell lymphoma (PTCL) (Mishra and Tiwari, 2011; Coiffier et al., 2012).

Sativex® is a mixture of dronabinol and cannabidol, derived from cannabis. It was launched for multiple sclerosis to relieve neuropathic pain. It was approved by Health Canada as well in August 2007 as an adjunctive analgesic for severe pain in the cases of advanced cancer patients (Johnson et al., 2010; Mishra and Tiwari, 2011).

Sirolimus (Rapamune®) was isolated from the bacterium *Steptomyces hygroscopicus* and proved to be as a significant antifungal drug (Pritchard, 2005). Additionally, it was used with coronary stents to prevent restenosis of coronary arteries due to balloon angioplasty. FDA approved this drug in February 2008 (Mishra and Tiwari, 2011).

Telavancin (Vibativ™) is a semisynthetic analogue of the antibiotic vancomycin (Laohavaleeson et al., 2007). In September 2009, telavancin was approved by the FDA for treatment of nosocomial pneumonia or hospital aquired pneumonia (Mishra and Tiwari, 2011).

Temsirolimus (Torisel®) is a semisynthetic derivative of Sirolimus (Hudes et al., 2007), used as an intravenous drug against renal cell carcinoma. It was approved by the FDA in May 2007 (Wan and Helman, 2007).

Tigecycline (Tygacil®) is classified among the new generation of antibiotics (glycylcyclines) granting broad spectrum activity by inhibiting the protein translation (Slover et al., 2007). In June 2005, the FDA approved it for the treatment of complicated intra-abdominal and skin infections (Breedt et al., 2005).

Trabectedin (Yondelis®) is obtained from the marine tunicate *Ecteinascidia turbinata*. It is a tetrahydroisoquinoline alkaloid effective in the treatment of advanced soft tissue sarcoma and approved in September 2007. In November 2009, it was authorized additionally by the European Commission for the treatment of relapsed platinum-sensitive ovarian cancer combined with DOXIL®/ Caelyx®. Moreover, it is now in Phase II trials for treatment of breast and prostate cancers and pediatric sarcomas (Mishra and Tiwari, 2011).

Varenicline (Chantix®/Champix®) was approved in 2006 for the treatment of tobacco dependence. It is a partial agonist of the nicotinic acetylcholine receptor subtype $\alpha_4\beta_2$ (Coe et al., 2005a; Coe et al., 2005b; Rollema et al., 2007).

Veregen™ (Polyphenon® E ointment) was used for treatment of genital warts and composed of a mixture of catechins obtained from green tea, which was received FDA approval in 2006 (Mishra and Tiwari, 2011).

Vinflunine (Javlor®) is a fluorine-containing derivative of *vinca* alkaloid vinorelbine, was approved in September 2009 for the treatment of advanced urothelial cancer (Aparicio et al., 2012).

Zinconotide (Prialt®) is a sensitive calcium channel blocker used to relief pain. It is a synthetic form of the peptide ω-conotoxin that is derived from the toxin of *Conus magusso*. The FDA

approved it in December 2004, given as an infusion using an intrathecal pump into the cerebrospinal fluid (Mishra and Tiwari, 2011).

Zotarolimus is a derivative of Sirolimus and acts as a cell proliferation inhibitor and it prevents scar tissue formation and minimizes restenosis in angioplasty patients (Chen et al., 2007).

Zucapsaicin (Zuacta®) was approved in July 2010 for treatment of severe pain in adults with osteoarthritis of the knee. It is derived from capsaicin (Szolcsányi and Pintér, 2013).

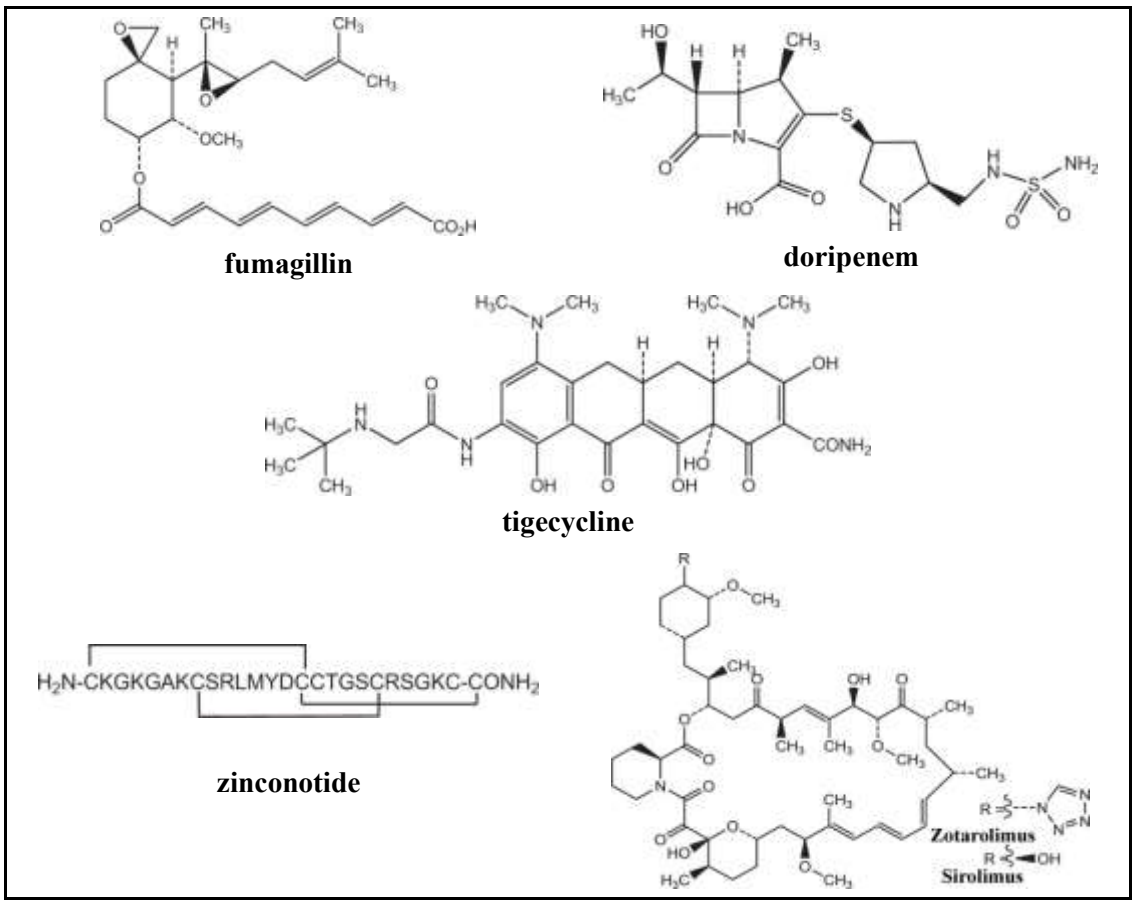


Figure 1.6: Representative chemical structure of drugs approved by FDA (Mishra and Tiwari, 2011).

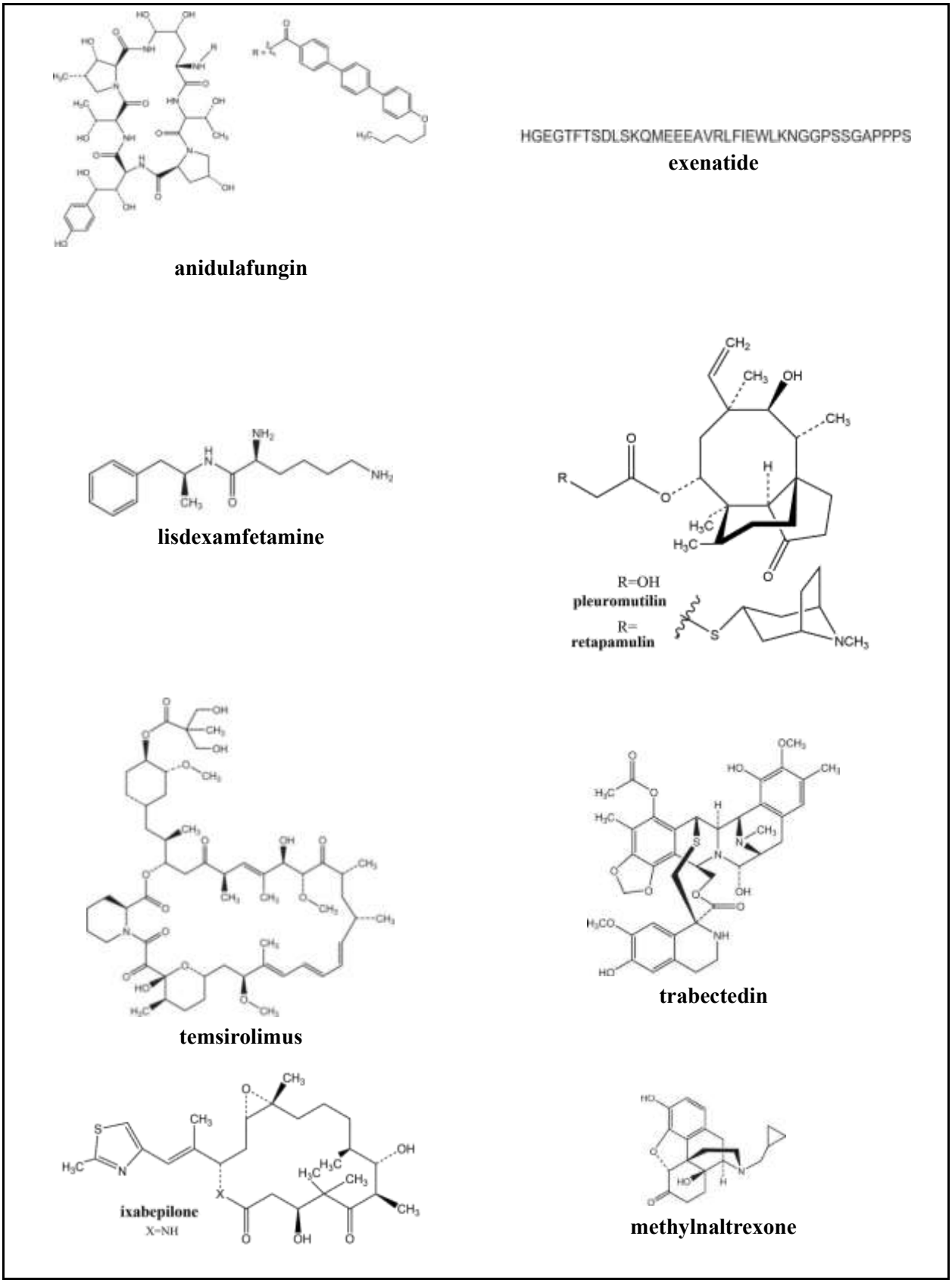


Figure 1.6 (Cont.): Representative chemical structure of drugs approved by FDA (Mishra and Tiwari, 2011).

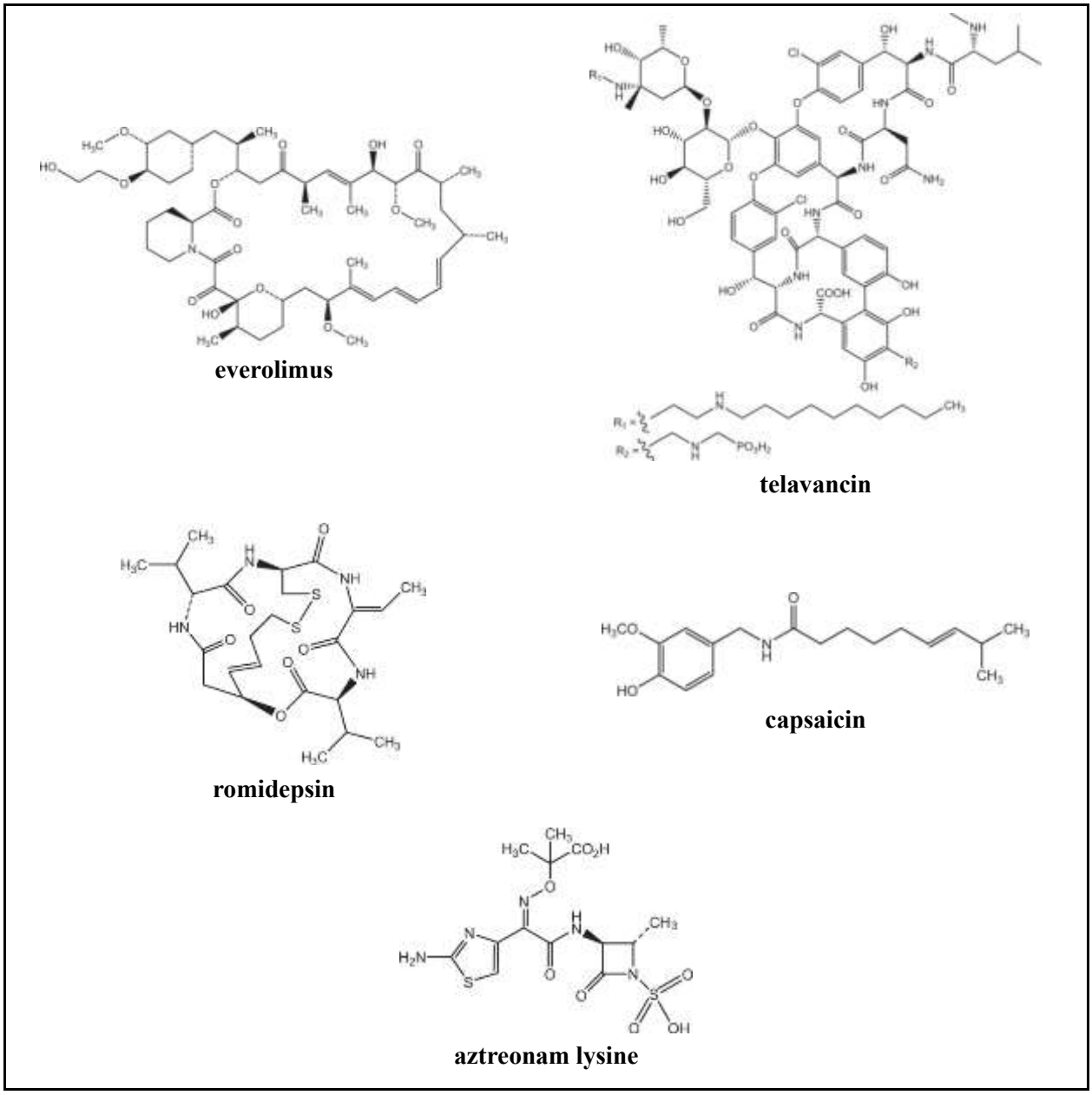


Figure 1.6 (Cont.): Representative chemical structure of drugs approved by FDA (Mishra and Tiwari, 2011).

Several natural product drugs of plant origin have recently been approved for the whole world market (**Figure 1.7**), including ingenol mebutate, omacetaxine mepesuccinate, dapagliflozin, zucapsaicin, methylnaltrexone, cabazitaxel, arterolane, vinflunine, while a large number of plant-derived natural products are currently involved in early/late-phase clinical trials (Krause et al., 2013).

As reviewed by (Butler et al., 2014), about 25 natural products and NP-derived drugs were approved for the market from January 2008 to December 2013 with five being classified as natural products (NPs), ten as semi-synthetic natural products (SS NPs), and ten as natural product-derived drugs, which include two natural product-containing antibody drug conjugates (NP-ADCs) (**Table 1.2**).

By the end of 2013, 547 NPs and their derivatives were FDA-approved drugs representing more than a third (38%) of all FDA-approved drugs (Patridge et al., 2016).

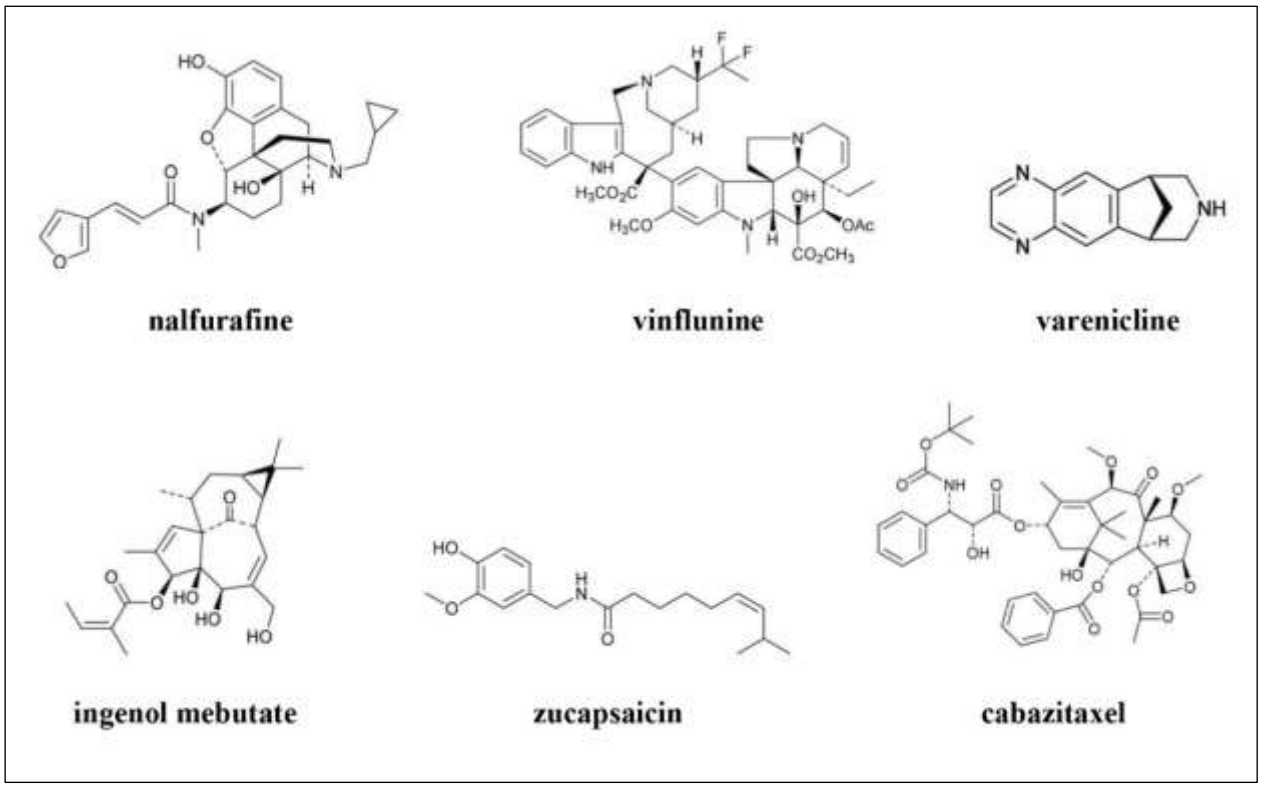


Figure 1.7: Representative natural product drugs of plant origin approved by FDA (Butler et al., 2014).

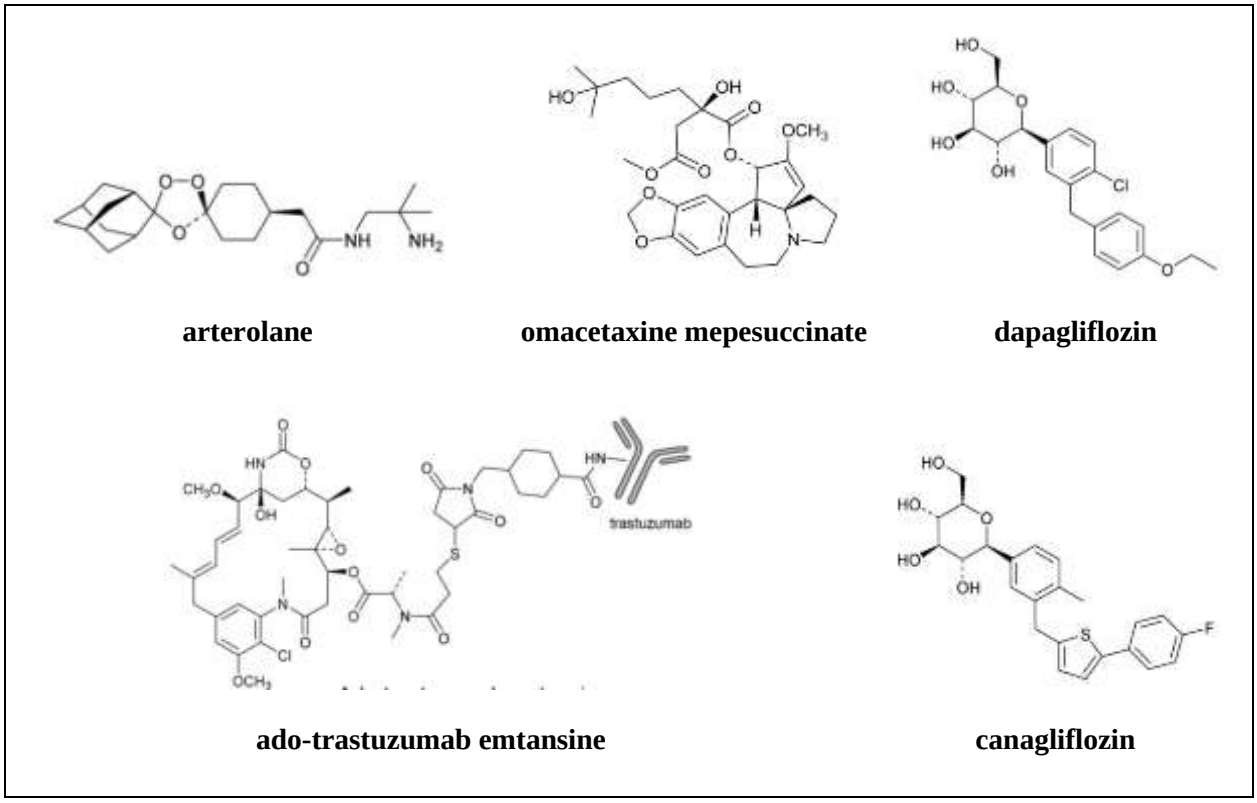


Figure 1.7 (Cont.): Representative natural product drugs of plant origin approved by FDA (Butler et al., 2014).

Table 1.2: Natural products derived drugs launched with reference to their source and disease area (Butler et al., 2014; de la Torre and Albericio, 2022).

Year	Generic name (Trade name)	Lead compound (source)	Disease area
2008	ceftobiprole medocaril (Zeftera®, Zevtera™)	cephalosporin C (fungus)	Bacterial infection
2008	umirolimus (Biomatrix™)	sirolimus (actionmycetes)	Cardiovascular surgery
2008	methylnaltrexone (Relistor®)	morphine (plant)	Opioid-induced constipation
2009	tebipenem pivoxil (Orapenem®)	thienamycin (actionmycetes)	Bacterial infection
2009	telavancin (Vibativ®)	vancomycin (actionmycetes)	Bacterial infection
2009	romidepsin (Istodax®)	romidepsin (bacteria)	Cancer
2009	vinflunine (Javlor®)	vinblastine (plant)	Cancer
2009	nalfurafine (Remitch®)	morphine (plant)	Pruritus
2009	everolimus (Afinitor®)	*streptomyces hygroscopicus* (bacteria)	Cancer
2009	artemether (Riamet®)	artemisia (plant)	Parasitic infection
2009	capsaicin (Qutenza®)	capsicum (plant)	Topical pain
2010	cabazitaxel (Jevtana®)	paclitaxel (plant)	Cancer
2010	fingolimod (Gilenya®)	myricocin (fungus)	Multiple sclerosis
2010	ceftaroline fosamil (Teflaro®)	cephalosporin C (fungus)	Bacterial infection
2010	eribulin (Halaven®)	halichondrin B (sponge)	Cancer
2010	mifamurtide (Mepact®)	muramyl dipeptide (bacteria)	Cancer
2010	zucapsaicin (Zuacta®)	capsaicin (plant)	Pain
2011	fidaxomicin (Dificid®)	fidaxomicin (action)	Bacterial infection
2011	spinosad (Natroba™)	spinosyn A (action)	Parasitic infection
2011	brentuximab vedotin (Adcetris®)	dolastatin (cyanobacteria)	Cancer
2011	vandetanib (Caprelsa®)	zea (plant)	Cancer
2012	ingenol mebutate (Picato®)	ingenol mebutate (plant)	Actinic keratosis
2012	dapagliflozin (Forxiga®)	phlorizin (plant)	Type 2 diabetes
2012	omacetaxine mepesuccinate (Synribo®)	omacetaxine mepesuccinate (plant)	Cancer
2012	carfilzomib (Kyprolis®)	epoxomicin (actionmycetes)	Cancer
2012	arterolane /piperaquine (Synriam™)	artemisinin (plant)	Parasitic infection
2012	novolimus (Desyne™)	sirolimus (actionmycetes)	Cardiovascular surgery
2013	canagliflozin (Invokana®)	phlorizin (plant)	Type 2 diabetes
2013	ado-trastuzumab emtansine (Kadcyla®)	maytansine (plant)	Cancer
2021	voclosporin (Lupkynis™)	cyclosporine (fungus)	Immunosuppressant, treatment of lupus nephritis.
2021	ibrexafungerp (Brexafemme™)	triterpenoid (fungus)	Antifungal indicated for vulvovaginal candidiasis (vaginal yeast infection).

By the end of 2015, 279 NP based drugs have been approved by the FDA representing 18.4% of the total 1515 NCEs approved by the FDA in this time (Kinch et al., 2014; FDA, 2015b). The data included NP and NP-derived drugs approved by the FDA (**Table 1.3**) was obtained from published reviews and other references obtained by literature search, that was then compared, corrected and supplemented with information from FDA (FDA, 2015c).

Table 1.3: NP and NP-derived drugs approved by the FDA

Generic name	Indication	Approval	Classification	Source	Species	References
oritavancin	Antibacterial	2014	SS-NP	Bacteria	*Amycolatopsis orientalis*	(Zhu et al., 2011; Butler et al., 2014; Katz and Baltz, 2016; Newman and Cragg, 2016)
dalbavancin	Antibacterial	2014	SS-NP	Bacteria	*Nonomuraea*	(Butler et al., 2014; Katz and Baltz, 2016; Newman and Cragg, 2016)
ceftolozane	Antibacterial	2014	SS-NP	Fungi	*Acremonium*	(Katz and Baltz, 2016; Newman and Cragg, 2016)
tazobactam	Antibacterial	2014	SS-NP	Fungi	*Penicillium*	(Zhu et al., 2011; Butler et al., 2014; Newman and Cragg, 2016)
vorapaxar	Anti-thrombotic	2014	NP derived	Plant	*Galbulimima*	(Butler et al., 2014; Newman and Cragg, 2016)
dapagliflozin	Type 2 Diabetes	2014	NP derived	Plant	*Malus*	(Zhu et al., 2011; Butler et al., 2014; Newman and Cragg, 2016)
empagliflozin	Type 2 Diabetes	2014	NP derived	Plant	*Malus*	(Butler et al., 2014; Newman and Cragg, 2016)
naloxegol	Opioid-induced constipation	2014	SS-NP	Plant	*Papaver somniferum*	(Butler et al., 2014; Newman and Cragg, 2016)
sugammadex	Antidote	2015	NP derived	Bacteria	*Bacillus macerans*	(Kurkov and Loftsson, 2013; Newman and Cragg, 2016)

1.2.3. Pre-clinical and Clinical Studies

Pre-clinical and clinical studies are done *in vitro*, *in vivo* and *in silico* and include: the development of large-scale synthesis of the lead compound; animal safety studies; carcinogenicity studies; drug delivery, elimination, and metabolism studies; formulation experiments; and dose-ratio studies. Those candidates that have promising outcomes in pre-clinical studies can then proceed to clinical human trials. Clinical trials are divided into four phases where safety, tolerability, pharmacodynamics, and pharmacokinetic properties of the drug are tested as well as the effectiveness of the drug is tested and compared with standard therapy to establish its relevance in treatment and monitor side effects. Following successful phase 1, 2 and 3 clinical trials the drug can be made available for the market under intial probation if approved by relevant authorities such as the FDA in the United States and the European Agency for the Evaluation of Medicinal Products (EMEA) in Europe. Phase 4 begins when the drug reaches the market and involves monitoring safety and detecting rare or long-term side effects in a large population and time period (Taylor, 2015; Umashankar and Gurunathan, 2015). Several plant-derived natural compounds and scaffolds were evaluated and have passed clinical trials with a majority being in the field of oncology such as the *vinca* alkaloids, vincristine and vinblastine isolated from *Catharanthus roseus* (Leggans et al., 2013), together with paclitaxel or taxol isolated from *Taxus brevifolia* (Fitzgerald et al., 2012); combretastatin A4, a stilbene, isolated from the root bark of *Combretum caffrum* (Pettit et al., 1995; Nowak et al., 2013); curcumin, a diarylheptanoid, is a major metabolite of turmeric (*Curcuma longa*) (Alberti et al., 2018); genistein is an isoflavone commonly found in many plants as *Genista tinctoria*, *Glycine max*, and *Lupinus albus* (Mahmoud et al., 2014); rohitukine, a chromane alkaloid which was first isolated from *Amoora rohituka* (Roxb.) and from an Indian medicinal plant, *Dysoxylum binectariferum* (Bose et al., 2013); and lastly resveratrol (Gescher et al., 2013), is a stilbenoid, a type of natural phenol, and a phytoalexin produced by several plants in response to injury or, when the plant is under attack by pathogens isolated from *Festuca versuta* seed (**Table 1.4**).

Table 1.4: Plant derived compounds in oncology clinical trials (Butler et al., 2014).

Lead compound (Generic name)	Mode of action	Derivation	Development status (administration)	Developer
combretastatin A4 (fosbretabulin, BNC105P)	Tubulin binding	SS-NP, NP-derived	Phase II/III (IV) Phase I/II (IV)	OXiGENE Bionomics
paclitaxel (ortataxel, TPI-287)	Tubulin stabilisation	SS-NP	Phase II (IV)	Cortice Biosciences
curcumin (curcumin)	Anti-inflammatory antioxidant	NP	Phase I/II (oral)	NCI
camptothecin (gimatecan, karenitecin, CZ48)	Topoisomerase I	NP-derived	Phase I (oral) Phase II completed (oral) Phase III (IV) Phase I (oral)	Novartis Sigma-Tau BioNumerik CHRISTUS Stehlin Foundation for Cancer Research
gossypol (-)-gossypol	Bcl-2-inhibitor	NP	Phase I/II (oral)	Ascenta Therapeutics
genistein (ME-143, ME-344) (genistein)	NADH-oxidase Protein-tyrosine kinase-inhibitor, antioxidant	NP-derived NP	Phase I completed Phase I (IV) Phase I/II (oral)	MEI Pharma MEI Pharma
picropodophyllotoxin (AXL 1717)	Tubulin binding / IGF-IR inhibitor	NP	Phase I/II (oral)	Axelar AB
resveratrol (resveratrol)	Various biological Activities	NP	Phase I/II (oral)	
rohitukine (alvocidib, riviciclib, voruciblib)	Cyclin-dependent kinase inhibition	NP-derived	Phase II (IV) Phase I/II (IV) Phase I/II (oral)	NCI (Sanofi Aventis) Piramal Enterprises Piramal Enterprises
vinblastine (vintafolide)	Transcription inhibitor	NP-derived	Phase II/III (IV)	Merck (Endocyte)

The number of FDA approved new chemical entities (NCEs) in 10-year periods, separated by biological source is presented in **Figure 1.8**. It is noticeable that plant products are the most dominant for the first half of the 20th century. Since 1946, an average of 0.9-1.6 plant product NCEs have been approved each year. From 2006 to 2015 an average of 1.5 plant based NCEs were approved annually which indicates that plants are still an important source for drug discovery.

It is clear that, throughout the 20th century, FDA approval has recognized NPs are getting better in terms of their characterization, although some remain as complex mixtures, including *Veratrum viride* root, *ergoloid mesylates* and *sinecatechins* plants (Patridge et al., 2016). In spite of the complexity of some plant types, the World Health Organisation (WHO) documented that between 70 and 80% of the population in developing countries still relies on herbal medicines, that included plants and plant extracts, as part of their primary healthcare resources in order to ameliorate illnesses and promote healing (Panday and Rauniar, 2016; Rahman et al., 2016).

Researchers studying NPs have focused on various lead micro-molecule structures in the contemporary era (Galanakis, 2015) and how they interact with biological macro-molecule activities (Lahlou, 2013; Abbasi et al., 2015). NPs, especially those found in plants, have thus always been and will remain essential sources of novel pharmaceutical compounds.

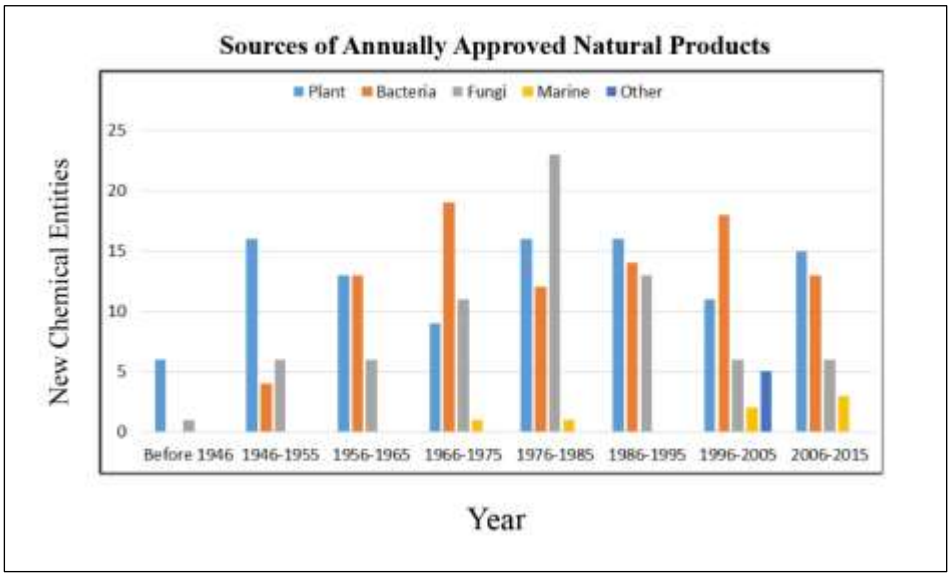


Figure 1.8: Number of FDA approved NCEs in 10-year periods, separated by biological source (Dias et al., 2012).

Overall, it is clear that medicinal plants continue to represent a rich and largely untapped resource of new chemical entities (NCEs) for the discovery of drugs (Katiyar et al., 2012). However, the isolation and characterization of bioactive molecules is not a simple process and is beset with many challenges. Despite tremendous development in separation science, the isolation of these molecules is still empirical. Nowadays with advent of hyphenated instrumentation like LC-Mass-NMR, the dereplication process has helped to analyze the extracts very fast and is becoming the leading platforms in drug discovery (Lahlou, 2013).

1.3. Traditional Medicine

Traditional medicine (TM) is a long-standing, non-conventional approach to treating a wide range of illnesses in various Eastern cultures. Its potential benefit is now acknowledged globally. It refers to methods, approaches, knowledge, and beliefs related to health that include manual techniques, exercises, spiritual therapies, and medicines derived from plants, animals, and minerals that can be used alone or in combination to treat, diagnose, and prevent diseases or preserve wellbeing (WHO, 2000). Since TM practices are influenced by a variety of factors, including culture, history, individual attitudes, and philosophy, they generally differ significantly between countries and regions (WHO, 2002). TM is used in several Latin American, Asian, and African nations to help with basic healthcare needs. Up to 80% of people in Africa receive their primary care from TM, whereas in industrialised nations, TM adaptations are known as "Complementary" or "Alternative" medicine (CAM) (WHO, 2000).

1.3.1. Reasons for Promoting the Use of Traditional Medicine

Essential medicines are inaccessible to more than one-third of the population in developing countries. For thousands of years, many TM systems around the world have been based on plants. Nearly 80% of people in developing countries rely primarily on plants for medical care (Uniyal et al., 2006; Hasan et al., 2016). Many significant pharmaceuticals have been developed because of the documentation of traditional knowledge regarding the medicinal uses of plants. As per the World Health Organisation (WHO, 2002), the availability of safe and efficacious complementary and alternative medicine (CAM) therapies may turn into a vital instrument for enhancing healthcare accessibility. The potential exists, though, that an expanding market for herbal products and their significant economic advantages could endanger biodiversity due to overharvesting of the raw material. If these practises are not stopped, they could result in the extinction of endangered species as well as the devastation of natural resources and habitats. One other related issue is that most national conventional patent laws and international standards for patent law currently do not provide enough protection for traditional knowledge (WHO, 2000; 2023).

1.3.2. Use of Traditional Medicine in Saudi Arabia

Patients receiving primary care in Saudi Arabia frequently employ traditional indigenous therapies, particularly those that are based on culture (Al-Bedah et al., 2017). Even though traditional medicinal plants are becoming more and more popular in Saudi Arabia, the majority of population does not rely on native plants because of the country's excellent healthcare system; instead, they only visit the official herbal stores in towns (El-Ghazali et al., 2010).

Covering around 2.25 million km^2, Saudi Arabia accounts for nearly 80% of the Arabian Peninsula (Almazroui et al., 2012). It extends between latitude 16´83° N 32´43°N and longitude 34´ 36° E 56°E (Meelad, 2006). The folra in Saudi Arabia Comprise approximately 2250 species, it is a significant contributor to biodiversity. Furthermore, there are nearly 2300 species, up from about 1500 (Alfarhan et al., 2005; Masrahi et al., 2012; Al-Khulaidi et al., 2023). Saudi Arabia possesses valuable medicinal plants, and the country's natural stress conditions—heat and drought—are thought to be beneficial for medicinal plants (El-Ghazali et al., 2010). Due to the arid climate, the flora of Saudi Arabia and the other countries on the peninsula has long been neglected. But conventional medicine still plays a big role in Saudi Arabia's history and is still used extensively today (Al-Essa et al., 1998; Ullah et al., 2020). A primary factor contributing to the rising usage of traditional medicine is the growing trend among patients to seek out various forms of self-care and to be more proactive with their own health. With the belief that "natural means safe" many consumers have resorted to natural traditional medicinal products and practises during this process. Still, this isn't always the case. Examples of improper use of traditional medicines by patients have been documented in several reports. These include overdose incidents, unreported use of suspected or counterfeit herbal remedies, and accidental injuries from unauthorised practitioners (WHO, 1998). Herbs continue to serve as the basis for many commercial drugs used to treat conditions like heart disease, blood pressure regulation, pain management, asthma, and other illnesses, which further supports the value of herbal medicine (Calixto et al., 2000; Okigbo and Mmeka, 2006; Wardle, 2020).

1.4. Metabolomics Approach

1.4.1. Introduction to Metabolomics

The term "metabolome" was first used in 1998, according to historical records (Oliver et al., 1998). High Resolution Liquid Chromatography Mass Spectrometry (HR-LCMS), Gas Chromatography Mass Spectrometry (GC-MS), and Liquid Chromatography Nuclear Magnetic Resonance (LC-NMR) are hyphenated analytical techniques used in metabolomics research (Dunn et al., 2005). These techniques are combined with available databases such as The Human Metabolome Database (HMDB), Metabolite Mass Spectral Database (METLIN), the Golm Metabolome Databases in conjunction with cutting-edge chemometric tools such as MZmine, MZmatch, and MetaboLab. Data is statistically analysed using multivariate software such as SIMCA or a freeware like Metaboanalyst, which allows simple pattern recognition (Smith et al., 2005; Hummel et al., 2007; Wishart et al., 2007; Kind et al., 2009; Horai et al., 2010). Integration of these approaches and methodologies has shown metabolomics to be an efficient and robust tool for drug discovery research.

Metabolomics is a discipline that focuses on identifying and fully analysing the changes in low-molecular-weight metabolites in biological systems. These metabolites are a diverse set of compounds that serve as building blocks for a variety of biological components or play critical roles in various cellular functions, including enzyme regulation and cell signalling. Metabolomics is extensively used to discover biomarkers for medical diagnostics and prognosis, to understand disease evolution processes, and to identify new therapeutic targets (Lin et al., 2010; Johnson et al., 2016).

Metabolomics was classified as either targeted or untargeted. Targeted metabolomics is aimed to investigate a pathway and analyze at length the impact of a target metabolite as a biomarker (Griffiths et al., 2010; Patti et al., 2012). An untargeted approach, on the other hand, examines as many metabolites as possible in a biological sample without prioritizing any certain pathway, enzyme, or biomarker (**Figure 1.9**) (Patti et al., 2012). Metabolomics has been adopted as an analytical platform in a wide range of research fields, including ecology (Lankadurai et al., 2013), biomedical and systems biology (Weckwerth, 2003), toxicology (Ramirez et al., 2013), nutrition and food science (Wishart, 2008), herbal products (Sun et al., 2012), and most recently in natural products research (Robinette et al., 2012; Tawfike et al., 2013).

The metabolome is a genomic end-product that defines the complete quantitative group of small molecular weight compounds (metabolites) present in a cell or organism that participate in metabolic processes (Dunn et al., 2005).

Metabolomics is one of the omics cascades which includes genomics, proteomics, and transcriptomics that intends to qualitatively and quantitatively characterize the metabolome by using different analytical platforms and methodologies (McMurray et al., 1989). In addition to the other omics techniques, metabolomics plays a crucial role in integrating genotype-phenotype data (Lopes et al., 2017).

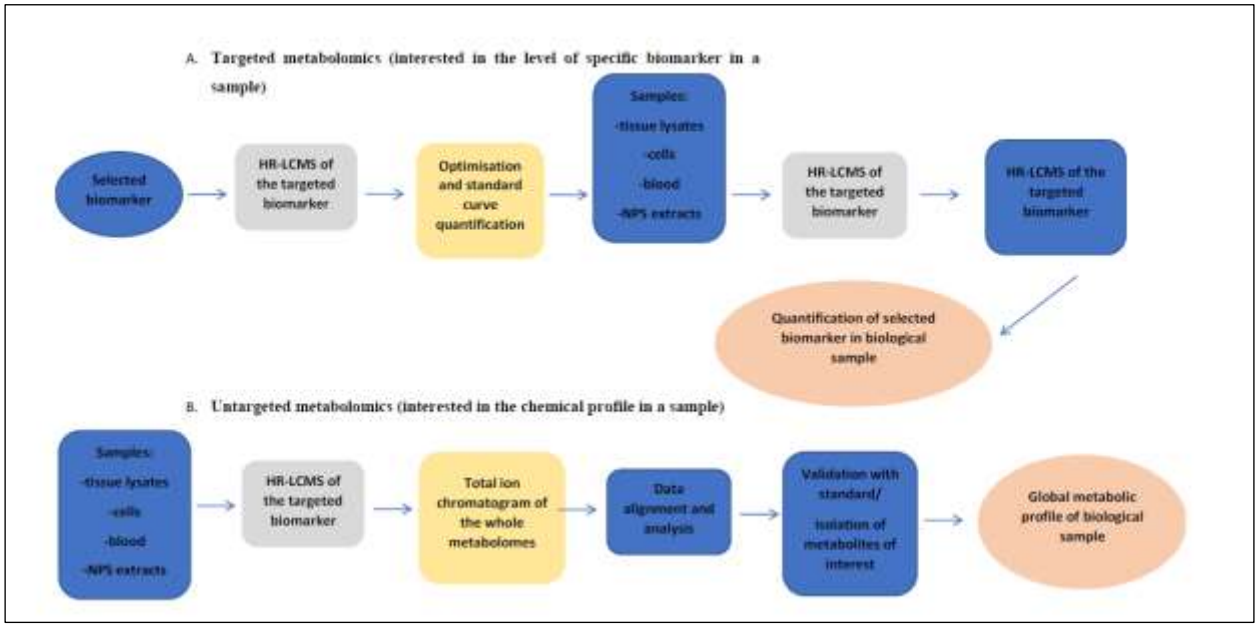


Figure 1.9: Schematic diagram for metabolomics approach (**A**) metabolomic targeted approach (**B**) metabolomic untargeted approach. Figured adapted from (Patti et al., 2012).

1.4.2. Tools of Spectral Analysis

It is difficult to identify and quantify metabolites in natural product extracts (Kjer et al., 2010). This is because the secondary metabolites have various atomic arrangements, resulting in different chemical and physical characteristics. Identifying and measuring different chemical groups of natural products requires reliable, robust, selective, and high-resolution analytical tools. Liquid chromatography-mass spectroscopy (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy are complimentary analytical methods that are used as metabolomics tools. Mass spectrometry is sensitive, but it may not be reproducible depending on the instrument type and ionization capabilities of the metabolites. It is also advantageous for elucidating unknowns by multiple MS fragmentation. Whereas, NMR data are more reproducible, but it may not be sensitive enough to identify metabolites at low quantities (Tawfike et al., 2013). The most common metabolomics approach are LCMS and NMR (Villas-Bôas et al., 2005) and the obtained spectral dataset is further processed using different software such as MZmine, MetAlign, MestReNova, XCMS, MS-DIAL and MathDAMP (Tsugawa et al., 2015). Respective software can be coupled with online or commercially available libraries and databases like the Dictionary of Natural Products has over 260 000 metabolites and the Dictionary of Marine Natural Products contains over 48 000 (Informa, 2021), ChemSpider (Pence and Williams, 2010), MarinLit (Blunt et al., 2012), which has about 58 000 compounds for marine natural products. AntiBase (Laatsch, 2014) is a database used for microbial metabolites and supported the dereplication strategy or an in-house custom database to dereplicate secondary metabolites (Macintyre et al., 2014; Harvey et

al., 2015). Then the processed data is further subjected to multivariate statistical analysis applying either unsupervised clustering such as partial component analysis (PCA) or supervised clustering such as orthogonal partial least squares discriminant analysis (OPLS-DA) to provide information on the putative bioactive metabolite at the first fractionation step or detect putative biomarkers in a cellular process (Morlock et al., 2014; Nejadgholi and Bolic, 2015; Robotti and Marengo, 2016). An overview of the more common advantages and disadvantages of analytical techniques is given in **Table 1.5**

1.4.3. Mass Spectrometry (MS)

Mass spectrometry (MS) is the preferred analytical approach in metabolomics for identification and/or quantification of different classes of metabolites and characterized by their mass to charge ratio (*m/z*) (Becker et al., 2012). A mass spectrometer consists of a sample inlet, an ion source, a mass analyser, and a detector with a functions that introduce the sample into the mass spectrometer, generating gas- phase ions through an ionisation technique, separating the ions according to their mass-to-charge ratio (*m/z*), and generating an electric current proportional to the abundances of the incident ions (Becker et al., 2012). Metabolite identification and quantification are now possible with the development of capillary electrophoresis (CE), gas chromatography (GC), and high-performance liquid chromatography (HPLC). The use of MS in metabolomics has several important benefits. It is highly sensitive to detect and identify metabolites from a small volume of samples. Employing high resolution mass measurements afford ease in mass spectral data interpretation and molecular formula prediction (Lindon et al., 2000). In addition to this, the integration of MS with separation methods broadens the concept of chemical analysis that may be performed on very complicated biological materials (Villas-Bôas et al., 2005). In contrast to NMR spectroscopy, however, mass spectrometry is a slow process that is also destructive, meaning that the sample cannot be recovered once it has been analysed (Wishart et al., 2009).

1.4.4. Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) spectroscopy is a method that is frequently applied in metabolomics research. This method has many advantages that include the following: it is specific while at the same time non-selective; it is non-destructive; it does not require separation or derivatization; it is quick; and it provides highly reproducible and quantitative results (Wishart, 2016). The NMR spectrum is unique to each compound and provides unique structural information about the parts of the sample being studied by combining the information on chemical shift (nature of chemical environment), signal multiplicities (neighbouring signals), homonuclear and heteronuclear coupling constants, integral of the signal (number of protons),

spin-spin coupling (number and nature of neighbours and connectivity information) (Dona et al., 2016).

Although the one-dimensional proton (1D-^{1}H) NMR is the most common type of nuclear magnetic resonance (NMR) experiment; there are currently other NMR techniques that can be performed that provide additional chemical and structural information, which include Homo Nuclear Correlation Spectroscopy (COSY), Hetero Nuclear Single Quantum Coherence (HSQC), Hetero Nuclear Multiple Bond Connectivity (HMBC), and Rotating frame Overhause Effect Spectroscopy (ROESY) spectral data. These techniques are useful in situations where the ^{1}H NMR is unable to provide enough data to completely characterize the metabolite (Villas-Bôas et al., 2005).

Table 1.5: A summary of common analytical techniques used in metabolomics

Analytical technique	Advantages	Disadvantages
NMR	• Robust and reproducible • Minimal sample preparation required. • Sample analysis is fast and robust. • Non-destructive	• Low analytical sensitivity • More than one peak per compound in most cases meaning spectra are often complex. • Does not analyse fats and lipids well. • NMR spectrometers can take up a lot of space
GC-MS	• High analytical sensitivity • Robust and reproducible technique • Large dynamic range • Compound identification is facilitated by large, well-established mass spectral libraries	• Sample analysis can be slow. • The similarity of isomers can make it difficult to identify compounds. • Many metabolites are non-volatile and must be derivatized prior to analysis. • Many large molecules (e.g. proteins) cannot be measured
LC-MS	• High analytical sensitivity • Robust technique • Large dynamic range • No derivatization required (usually)	• Analysis can be slow. • Reproducibility issues and matrix effects can hinder compound identification. • There are very few commercial libraries

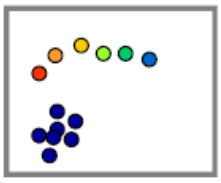
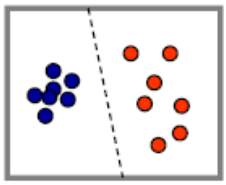
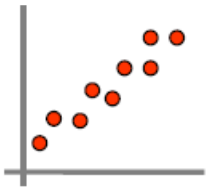
1.4.5. Chemometrics and Multivariate Analysis

A multivariate statistical analysis of the processed data could be performed for data pattern visualisation. The analysis was done by using unsupervised clustering such as partial component analysis (PCA), or hierarchical clustering (HCA), soft independent modelling by class analogy (SIMCA), or supervised clustering such as partial least squares (PLS), partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis

(OPLS-DA) as well as modified orthogonal projections to latent structures (O2PLS) (Wiklund, 2008; Morlock et al., 2014; Nejadgholi and Bolic, 2015; Robotti and Marengo, 2016).

PLS can be used to discriminate between two classes such as healthy versus diseased states to target biomarkers for diagnosis and treatment. It can also be used to discriminate between bioactive versus inactive natural products to pinpoint secondary metabolites responsible for specific bioactivity. This approach is an expansion of the supervised PLS regression method that incorporates a new component, the orthogonal signal correction filter (OSC), which allows systemic variation in X that is orthogonal to or uncorrelated to Y to be recognized OPLS-DA give the information on the putative bioactive metabolite either at the first fractionation step or in a cellular process (Wold et al., 1987). All the multivariate approaches are summarised in **Table 1.6** and **Figure 1.10.**

Table 1.6: Some of the approaches used in multivariate analysis (Wiklund, 2008).

PCA: Overview	SIMCA: Classification	PLS-DA and OPLS-DA: Discrimination	O2-PLS: Regression
Trends	Pattern recognition	Discriminating between groups	Comparing blocks of omics data
Outliers	Diagnostics		
Quality control	Healthy/diseased	Biomarker candidates	Metabolomic vs proteomic vs genomic
Biological diversity	Toxicity mechanisms	Comparing studies or instrumentation	
Patient monitoring	Disease progression		Correlation spectroscopy
			

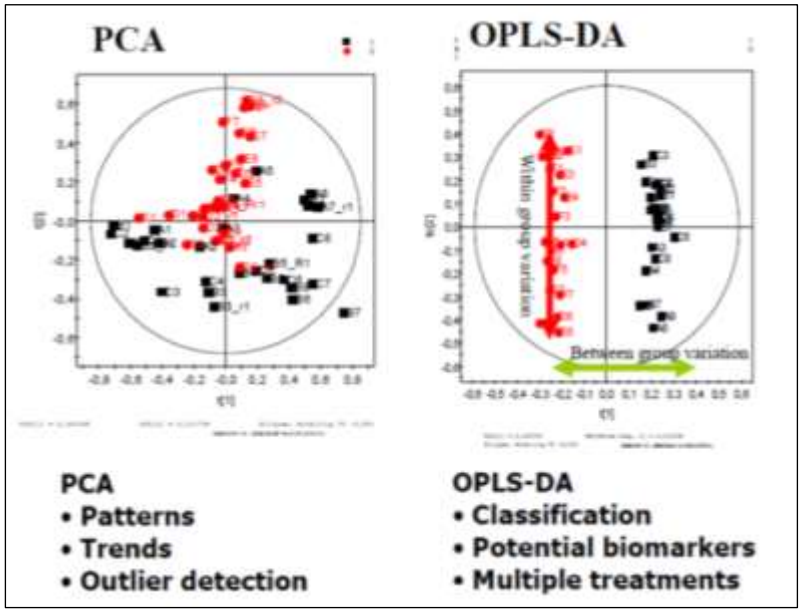


Figure 1.10: Score plots for PCA and OPLS-DA approaches.

The processing of MS and NMR data yields a matrix of signal intensities, which can be utilized with statistical analytical tools (Spicer et al., 2017). Using principal component analysis (PCA) is considered the first step in metabolomics data analysis and an exploratory and visualization method. This analysis provides an overview of the variability of the dataset when the samples are grouped based on similarity or differences within the group of samples, allowing for more accurate interpretation of the results. This makes it possible to identify patterns, groups, and outliers (Dona et al., 2016).

PCA uses two types of plots, the scores plot and the loadings plot. The scores plot summarizes the observations (crude extract or fractions) (each point represents individual sample). Whereas the loadings plot provides the information about the variable (mass ion peak, *m/z* or chemical shifts, ppm) that have the greatest impact on the positioning of the samples in the scores plot and are responsible for the clustering of samples, as shown in **Figure 1.11**

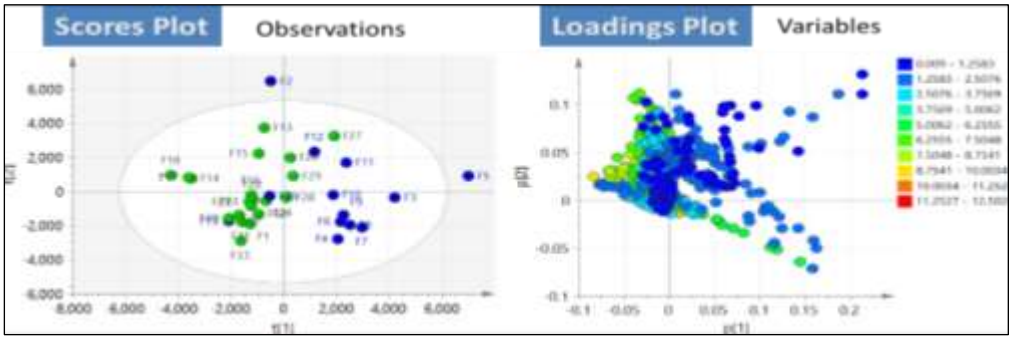


Figure 1.11: PCA scores and the loadings plots (Geladi, 2003).

PCA is also known as an unsupervised analysis that doesn't make assumptions about the data and identifies the sources of variation among the observations, and thus, observations are classified in scores plot depending on the variables of the loadings plot (Covington et al., 2017). On the other hand, the PLS-DA approach is supervised. Thus, separate groups of observations are defined by the user and are accordingly clustered in the scores plot, whereas the variables in the loadings plot are grouped so they are responsible for the observations separation in scores plot (Covington et al., 2017).

Supervised pattern recognition approach is used after PCA analysis to maximize separation between different groups of samples and to find metabolic signs that contribute to classification (Dona et al., 2016). OPLS-DA is the most widely used supervised technique, it gives the same predictive power as PLS but a better interpretation of the important variables than PLS. This technique gives information on the factors that contribute to class separation and is used thus improve separation between predictive and nonpredictive variation (Bylesjö et al., 2006).

Therefore, the greatest advantage of implementing metabolomics tools is it could save time and resources, thus provide a guideline in decision making for further isolation work or in determining biomarkers. In details, (**Figure 1.12**) illustrated a series of metabolomics tool in bioassay-guided fractionation in natural products research (Macintyre et al., 2014). The processed data from multivariate analysis is then plotted in S-plot for recognition pattern of active versus inactive, as well as known versus novel metabolites. The result from multivariate analysis is cross matched with dereplication database, which provide aid information for the next step of purification. Structural confirmation is obtained *via* 2D NMR spectroscopy and tandem MS-MS spectrometry.

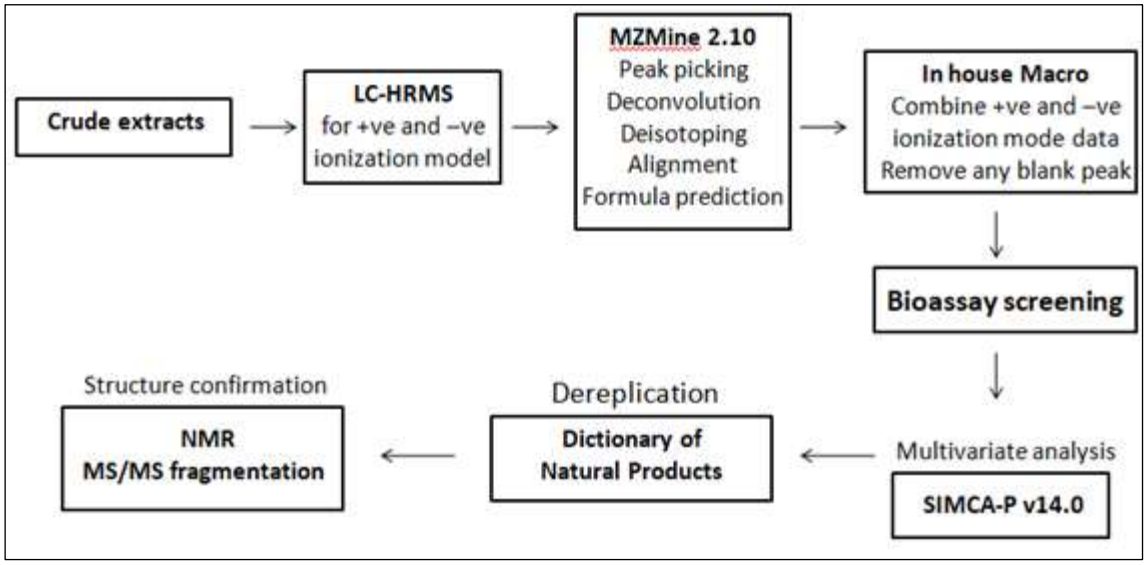


Figure 1.12: Metabolomics work flow in natural product study (Macintyre et al., 2014).

The manual-based structural elucidation approach is a sophisticated, time-consuming, and difficult process. The information gained from 1D and 2D NMR data, as well as information from metabolite databases such as DNP and AntiMarin, is combined to accomplish reliable metabolite identification in this manner (Dona et al., 2016). The majority of the metabolites detected by NMR spectroscopy and mass spectrometry were identified using information accessible in a variety of databases; however, some of the metabolites were unknown, unidentified, or really unique, which made the elucidation process more challenging (Dona et al., 2016).

1.4.6. Applications of Metabolomics in Natural Products Research

Metabolomics has made significant contributions in finding natural products for drug discovery. Metabolomics has brought several crucial applications, such as tracking novel compounds and active metabolites or optimizing the production of secondary metabolites. It is possible to employ both PCA and OPLS-DA to guide the separation of compounds and the prioritization of fractions for future work isolation, so saving time and resources while directing the effort toward the isolation of novel and bioactive compounds (Tawfike et al., 2013; Harvey et al., 2015; Covington et al., 2017). Furthermore, the capacity of metabolomics to compare and screen secondary metabolites, identifying outliers, differences, and biomarkers across experimental groups, whether they were from different sources or from different fractions associated to the same source. This will aid in the tracking novel and bioactive target metabolites and/or biomarkers that become accessible at the earlier stages of the research (Tawfike et al., 2013; Wu et al., 2015). Later, this biomarker is separated, and its structural information provided by the NMR or LC-HRMS is used to compare it with the dereplication database (Wu et al., 2015). 2D NMR and fragmentation patterns in MS/MS spectra in conjunction with molecular networking assessment might be utilized to identify the chemical if the core structure is known but the functional groups are unknown. Even if its nucleus was unique, a complete de novo NMR structural analysis is required (Tawfike et al., 2013).

The traditional approach of bioassay-guided isolation of natural products is being modified nowadays to make use of technological advances, to adapt current understanding in medicinal chemistry and to utilize cheminformatics approaches in designing libraries to explore biologically relevant chemical space. Advances such as the use of pre-fractionation high-throughput strategies has improved the applicability of natural product-based screening collections and eased the comprehensive removal of compounds that are likely to cause artefacts and interferences (Harvey et al., 2015). The pharmaceutical company, Wyeth, made use of the high-throughput screening (HTS) that was introduced into the drug discovery program created a pre-fractionated natural product library for drug or lead likeness (Wagenaar, 2008). Metabolomics might potentially be utilized as a technique for phytomedicine quality control. Variations across species, adulteration, environmental changes, post-harvest treatment, and extraction may all result in a distinct metabolite profile and have a considerable impact on the effectiveness of phytomedicines. All these modifications might be recognized using PCA (Yuliana et al., 2011).

Moreover, metabolomics can be utilized to correlate the chemical profile and bioactivity pattern of some phytomedicines in which the activity is the consequence of synergy between several

individually inactive chemical constituents. The bulk of chemical constituents is monitored to ensure that biological activity is maintained (Yuliana et al., 2011). In addition, it is possible to mine metabolomics data to discover biosynthetic precursors that may be employed to promote the synthesis of a certain functional novel product (Harvey et al., 2015).

1.5. Motivation of the Study or Hypothesis

Humans have relied on nature from the beginning for their basic needs and natural products have been a very important source of medicine. Thus, natural products play a vital role in the discovery of lead structures for drug development.

In recent years, there has been a phenomenal rise in the interest of the scientific community to explore the pharmacological actions of herbs or to confirm the claims made about them in the official books of herbal traditional medicine. The emerging importance of biologically active medicinal plants and their constituents as possible therapeutic measures has become a subject of active scientific investigation.

Medicinal plant diversity and climate variation make Saudi Arabia a rich source of these plants; the flora comprises about 2300 species in 855 genera from which a high percentage of rare species can be found. A significant proportion of these plants have never been studied in terms of biology or medicine. Our floristic wealth is rich and diverse, yet it is rarely studied. Phytochemistry can play a major role in supporting scientific validation of our rich plant utilisation history and produce benefits for humankind's welfare. A new wave of interest in plant-based industries has been sparked by the growing awareness of natural options in all spheres of life. Herbal Technology (HT) is one such area, with a bright future for phytochemistry in Saudi Arabia (Al-Khulaidi et al., 2023).

A vital component of the history of the herbal folk material medica throughout the Arab Peninsula and the Gulf region is the use of indigenous herbal folk medicine. Traditional medicine occupies an important measure of Saudi Arabia’s culture, and it is commonly practiced until now. Selection of plants based on ethnobotanical information resulted in the recognition of promising new molecules with potential activities against various illnesses. The chemical composition, analysis, conservation, sustainable production of medicinal plants belonging to Saudi flora and determination of phytoconstituents responsible for the bioactivities are of great importance and need investigation as sufficient information is not yet available (Sher and Alyemeni, 2011; Almehdar et al., 2012).

Plants are chemical warehouses that contain a wide variety of chemical compounds that provide defence against harmful microbes and free radicals. Because of this, plants are an excellent source of organic antioxidants and antimicrobial substances. In most cases, these compounds are created as secondary metabolites. The study of plants is being done to find novel compound(s) that can be used as antibiotics and/or antioxidants because of the rise in illnesses linked to the presence of free radicals and the microorganisms' resistance to antibiotics. Traditional medical professionals frequently utilise the medicinal plant that was chosen for this study to treat a range of medical conditions.

Investment in research and development increased after the Second World War, a wide range of disease conditions required treatments and large-scale production of analgesics and antibiotics began. Attention on metabolomics has grown tremendously as indicated by the exponential increase in the number of publications in the field between the years (2000 – 2024) as recorded in PubMed (**Figure 1.13**).

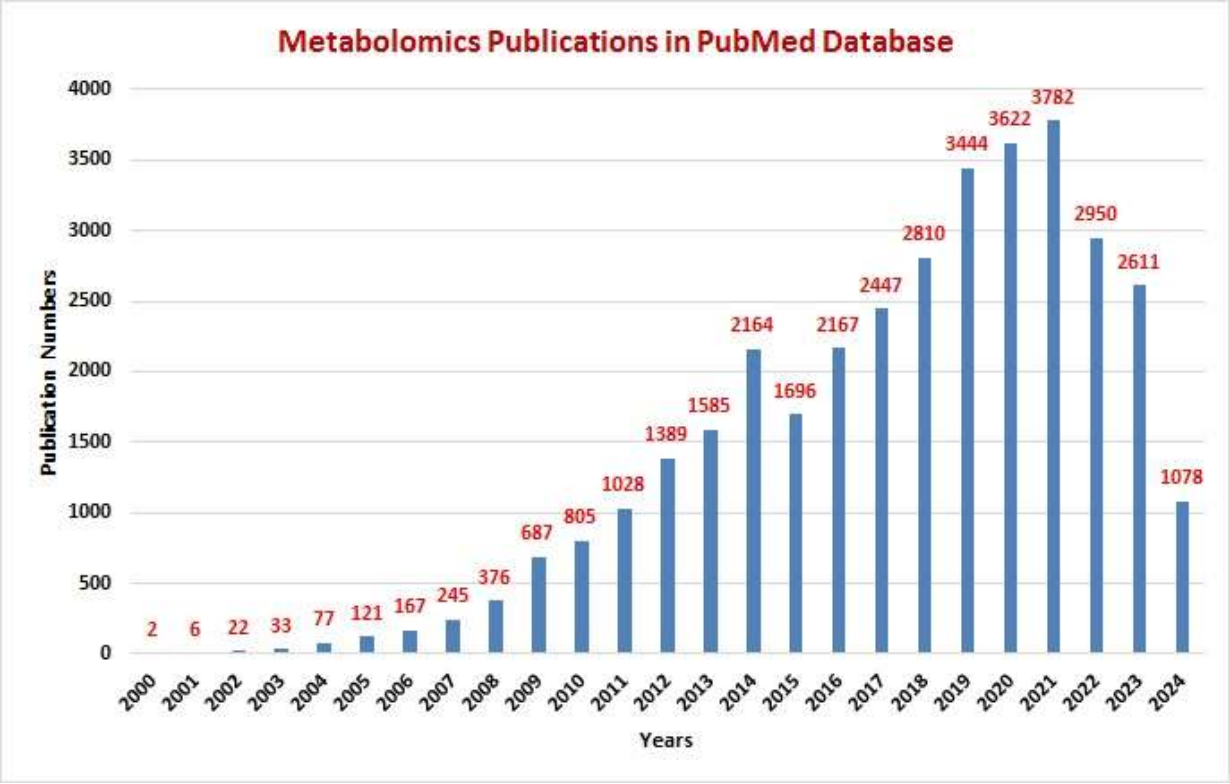


Figure 1.13: Number of metabolomics publications recorded in PubMed according to year

1.6. Aim of the Study

The scope of this study is to investigate one of the most commonly used Saudi medicinal plants belonging to the *Salsola* genus and discover a new potential drug with promising therapeutic and clinical activities targeting healthcare- pressing disease in KSA and the globe. *Salsola kali*, also known as prickly saltwort or tumbleweed, belongs to the Amaranthaceae family and can be found in the Saudi flora. This plant holds medicinal significance and is utilized in traditional medicinal practices. The current study is intended to focus on investigating the secondary metabolites of *Salsola kali* and exploring the biological activities of these metabolites.

Out of this scope, the specific aims of the presented study were:

1. To evaluate the antioxidant activity of the crude extract of *S. kali* (**KM**) and EtOAc fractions (**KE**) utilizing the DPPH assay.
2. To evaluate the cytotoxic activity of the crude extract of *S. kali* (**KM**) and EtOAc fraction (**KE**) against KSA prevalent tumors utilizing the MTT as well as Brine shrimp assays.
3. To identify the bioactive metabolites within the crude extract of *S. kali*, adopting extensive metabolomics-guided approach (LC-MS, MZmine 2.53, in-house MS-Excel macro, and Dictionary of Natural Products) to explore the phytochemical space and diversity.
4. To isolate and characterize the **KE** major constituents utilizing several chromatographic approaches and 1D/2D-NMR techniques.

1.7. Objectives of the Study

i. Collection and identification of plant material.

ii. Appropriate drying of collected samples.

iii. Perform the extraction of *Salsola kali* and fractionation of the obtained extract *via* modern chromatographic methods, including normal phase Flash® chromatography, and concisely analyse it using Thin-Layer Chromatography (TLC).

iv. Biological assays of the different fractions of *S. kali.*

v. Explore new insights for *S. kali* by 'chemical fingerprinting' using liquid chromatography-mass spectrometry (LC-MS) data in both negative and positive ionization mode as well as NMR spectroscopy.

vi. Integrate the chemical fingerprint of de-replicated metabolites of *S. kali* from the DNP with the results of the HTS screening.

vii. Identify the active 'target' compounds of *S. kali* obtained from by 'multivariance analysis (MVA)' including Principal Component Analysis (PCA), Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA), and S-plot analysis as methods of HTS screening under "omics".

viii. Phytochemical study of bioactive fractions.

ix. Use different chromatographic techniques to isolate the active 'target' compounds of *S. kali* as defined by OPLS-DA in terms of the bioassay results.

CHAPTER II: A REVIEW OF LITERATURE

2. Literature Background

2.1. Characterisation of Family Amaranthaceae (Chenopodiaceae)

The search for undiscovered plant flora is encouraged by growing interest in the expanded roles that phytomedicines and natural product remedies play in contemporary health care and the development of novel drugs. Taxonomic research and floral explorations can yield useful and effective information about the names, distribution, ecology, and applications of a variety of plant species, and consequently about an eco-system (Subramanayam and Nayar, 1971; Wainwright et al., 2022). Studies on the flora and fauna of any region are considered prerequisite for biodiversity assessment and setting strategic plans for land management, especially in arid and semi-arid regions.

One of the main centrospermous families is the Chenopodiaceae family, also referred to as the "Goosefoot" family. It is distributed throughout the world and includes roughly 122 genera and 1500 species, most of which are found in xerophytic and saline environments in the Arabian desert and are frequently reported in floristic works of the region (Al-Saleh et al., 1997). In order to survive in these conditions, the majority of Chenopodiaceae plants are succulents, halophytes, xerohalophytes, or xerophytes, with well-developed or much reduced leaves. They also typically grow as weeds, and some are food plants such as spinach, chard, beets, sugar beets, and quinoa (Le Houerou, 1993; Perveen and Qaiser, 2012).

The Kingdom of Saudi Arabia's main flora is comprised of 23 genera that belong to the family Chenopodiaceae (Chaudhary, 1999). On the other hand, (Al-Turki, 1997) listed 29 species from 14 genera. It was reported that a total number of 38 species belonging to 15 genera of the family Chenopodiaceae were encountered in the Qassim region, Saudi Arabia (Abdalla et al., 2016).

2.2. Introduction of *Salsola* Genus

The genus *Salsola* L. (Russian thistle, Saltwort), a genus of semi-dwarf to dwarf shrubs and woody tree species, is a halophyte plant which considers one of the largest genera in the family Amaranthaceae previously Chenopodiaceae. The genus can also help with the restoration and reclamation of degraded salty areas and saline soils (Botschantzev, 1974; Mabberley, 1997; Pyankov et al., 2000; Chauhan et al., 2018; Altay and Ozturk, 2020). The genus name derives from the Latin word salsus, which means "salty" in reference to the salt tolerant plants (Reimann and Breckle, 1995; Idzikowska, 2005). Moreover, this genus is recognized as a cosmopolitan

group of plants which are distributed and naturalized worldwide. The exact number of species that belong to this genus has yet to be determined. It is reported that over 64 species which are widespread in arid and semi-arid regions of Central Asia, Middle East, Africa, and Europe (Mosyakin, 1996; Pyankov et al., 2000; Pyankov et al., 2001; Toderich et al., 2012). *Salsola* species have a variety of features that contribute to their achievement as a potential forage species in semi-arid to dry settings along sea beaches, such as extensive seed production, resistance to extreme climatic conditions including high temperature and extended drought conditions and around 45% of the desert communities (Farmer et al., 1976; Khan et al., 2002; Toderich et al., 2012; Altay and Ozturk, 2020). These are well-known common traditional forage plants that grow in a variety of saline and alkaline environments worldwide (Batanouny, 1995; Öztürk et al., 2019). These plants typically grow on flat, generally dry, and/or slightly saline soils, with some species occurring in salt marshes. Easy-to-grow plants on dry soil are resistant to pH shifts and harsh weather. They are planted commercially in many locations and even sold in markets across different countries (Altay and Ozturk, 2020). *Salsola* is found to be an allelopathically[1] active species that inhibits the growth of a number of related species as it decays (Sokolowska-Krzaczek et al., 2009). It is autotoxic[2], but none of the identified phytotoxins administered to it prevents it from germinating (Lodhi, 1979).

The genus is rich in vast classes of phytoconstituents mainly flavonoids, phenolic compounds, nitrogenous compounds, saponins, triterpenes, sterols, volatile constituents, lignans, coumarins and cardiac glycosides (ElNaggar et al., 2022). Moreover, it showed different biological activities including analgesic, anti-inflammatory, antiviral, antibacterial, anticancer, cardioprotective and hepatoprotective activities. Numerous species within the genus have also been utilized in various industrial applications. For instance, *S. soda* and *S. kali* have been employed in the production of soap and glass (Gheraissa et al., 2024). Additionally, these species are utilized as a source of sodium carbonate for linen bleaching in the cotton industry (Wisniak, 2003; Tite et al., 2006; Hanif et al., 2018).

The genus *Salsola* is frequently overlooked, and few people are aware of its significance. The majority of the studies focus on pollen morphology (Boulos, 1991) and species identification (Toderich et al., 2010). Meanwhile, little research was done on its phytochemical makeup or biological effects. There is very little information on the adaptation characteristics of *Salsola*

[1] Allelopathy refers to the beneficial or harmful effects of one plant on another plant from the release of boichemicals knows as allelochemicals.
[2] Autotoxicity, meaning self-toxiticy, is a biological phenomenon whereby a species inhibits growth or reproduction of other members of its species through the production of chemicals released into the environment.

plants for their efficient use in drought-prone semi-arid to arid settings, as well as to remediate degraded salt soils.

Therefore, this review aims to cover all aspects of genus *Salsola*, including taxonomy, distribution, chemical constituents and reported biological activities. This review is based on literature obtained through computer search in different data bases including ScinceDirect, Web of Knowledge, SCOPUS, PubMed, and Google Scholar using keywords "*Salsola* and chemistry", "*Salsola* and phytoconstituents", "*Salsola* and taxonomy" and "*Salsola* and biological activities", from 2010 to 2023.

2.2.1. Morphology, Taxonomy and Distribution

From the taxonomic point of view, *Salsola* belongs to tribe Salsoleae of subfamily Salsoloideae in family Amaranthaceae (Akhani et al., 2007). It is classified now as one of the Amaranthaceae genera after merging family Chenopodiaceae with the family Amaranthaceae according to the angiosperm phylogeny group (AGP-IV) (Nazish et al., 2019; Rudov et al., 2020). The taxonomy of *Salsola* sp. is debatable and confusing due to their diversity and physical similarity between many species (64 species, **Table 2.1**) (Turki, 1999; POWO, 2022).

Plants belonging to the genus *Salsola* have the following taxonomic classification (Akhani et al., 2014):

Kingdom: Plantae - Plants
Subkingdom: Tracheobionta - Vascular plants
Superdivision: Spermatophyta - Seed plants
Division: Magnoliophyta - Flowering plants
Class: Magnoliopsida - Dicotyledons
Subclass: Caryophyllidae
Order: Caryophyllales
Family: Amaranthaceae (previously, Chenopodiaceae)
Subfamily: Salsoloideae
Tribe: Salsoleae
Genus: *Salsola*

Many writers researched the anatomy of the genus *Salsola,* however they all focused on C3–C4 Kranz anatomy in the genus's and allied genera's leaves (Jahromi et al., 2019). Mostly, *Salsola* species are shrubs, subshrubs, or trees, annual or perennial herbs. Most of them have bisexual axillary flowers, with five petals, five stamens, and a pistil with two stigmas that can be solitary or clustered to form loose or dense spikes (**Figure 2.1**). Each flower is subtended by two prominent bracteoles, with a frequently hard 5-segmented perianth (often winged in fruit), and a superior ovary. Fruit is spherical, carrying seeds with a spiral embryo. Seeds are horizontal, subglobose (Mosyakin, 1996; Borger et al., 2008; Altay and Ozturk, 2020). The leaves are

alternate, small, simple, entire, and sessile. They're usually succulent, hairy, and thickly packed, which helps to protect the branches (Klopper and Van Wyk, 2001). Furthermore, *Salsola* leaves are classified into two anatomical types: Salsoloid type leaf; with continuous layers of chlorenchymatous cells with vascular bundle at the center of the leaf and small peripheral vascular bundles adhere to chlorenchyma (Carolin et al., 1982), and Sympegmoid type leaves; with three or two palisade layers and a discontinuous layer of indistinctive bundle sheath cells (typ-ically non-Kranz) around water-storage tissue (Carolin et al., 1982). The genus' stem anatomy was unusual and performed on few species like *S. kali* and *S. crassa* (synonym of *Climacoptera crass*) (Bercu and Bavaru, 2004; Jahromi et al., 2019; POWO, 2022). This is because of the difficulties of sectioning the woody, hard stem, as well as the aberrant secondary growth seen in many Amaranthaceae species (Carolin et al., 1982).

The genus resists soil salinity therefore it is known to grow in hypersaline, arid and semiarid regions (Kühn et al., 1993). The genus is native to Africa (Mediterranean region), Euro Asia, California, and Australia (**Figure 2.2**) (POWO, 2022). It was introduced to South Africa, and some territories in North and South America (Chauhan et al., 2018).

Table 2.1: List of accepted species in genus *Salsola* and their synonyms (Akhani et al., 2007; POWO, 2022).

Accepted species in genus *Salsola*	Synonyms
Salsola acanthoclada Botsch.	*Nitrosalsola acanthoclada* (Botsch.) Theodorova
Salsola africana (Brenan) Botsch.	*Salsola dendroides* var. *africana* Brenan
Salsola algeriensis Botsch.	*Nitrosalsola algeriensis* (Botsch.) Theodorova
Salsola angusta Botsch.	-
Salsola arbusculiformis Drobow	-
Salsola australis R.Br.	*Kali australe* (R.Br.) Akhani & Roalson *Kali macrophyllum* (R.Br.) Galasso & Bartolucci *Salsola macrophylla* R.Br. *Salsola tragus* var. *australis* (R.Br.) Bég.
Salsola austroiranica Akhani	-
Salsola austrotibetica Sukhor.	-
Salsola baranovii Iljin	-
Salsola brevifolia Desf.	*Nitrosalsola brevifolia* (Desf.) Theodorova
Salsola chellalensis Botsch.	*Nitrosalsola chellalensis* (Botsch.) Theodorova
Salsola chinghaiensis A.J.Li	-
Salsola collina Pall.	*Kali collinum* (Pall.) Akhani & Roalson *Salsola chinensis* Gand. *Salsola erubescens* Schrad. *Salsola ircutiana* Gand. *Salsola kali* subsp. *collina* (Pall.) O.Bolòs & Vigo
Salsola cruciata L.Chevall. ex Batt. & Trab.	*Darniella cruciata* (L.Chevall. ex Batt. & Trab.) Brullo
Salsola cyrenaica (Maire & Weiller) Brullo	*Darniella cyrenaica* Maire & Weiller *Salsola sieberi* subsp. *cyrenaica* (Maire & Weiller) Brullo & Furnari

Table 2.1: List of accepted species in genus *Salsola* and their synonyms (Akhani et al., 2007; POWO, 2022).

Accepted species in genus *Salsola*	Synonyms
Salsola daghestanica (Turcz. ex Bunge) Lipsky	*Noaea daghestanica* Turcz. ex Bunge
Salsola divaricata Masson ex Link	*Salsola capensis* Botsch. *Salsola divaricata* (Moq.) Ulbr.
Salsola drummondii Ulbr.	*Salsola obpyrifolia* Botsch. & Akhani
Salsola euryphylla Botsch.	-
Salsola foliosa (L.) Schrad. ex Schult.	*Anabasis c*lavata S.G.Gmel. *Anabasis foliata* Pall. ex Bunge *Anabasis foliosa* L. *Caspia foliosa* (L.) Galushko *Micropeplis foliosa* (L.) G.L.Chu *Neocaspia foliosa* (L.) Tzvelev *Salsola baccifera* Pall. *Salsola clavifolia* Pall.
Salsola glomerata (Maire) Brullo	*Darniella glomerata* (Maire) Brullo
Salsola gobicola Iljin	*Kali gobicola* (Iljin) Brullo & Hrusa
Salsola grandis Freitag, Vural & Adigüzel	-
Salsola griffithii (Bunge) Freitag & Khani	*Kali griffithii* (Bunge) Akhani & Roalson *Noaea griffithii* Bunge
Salsola gymnomaschala Maire	*Darniella gymnomaschala* (Maire) Brullo *Seidlitzia gymnomaschala* (Maire) Iljin
Salsola gypsacea Botsch.	
Salsola halimocnemis Botsch.	*Nitrosalsola gypsacea* (Botsch.) Theodorova
Salsola hartmannii Sukhor.	-
Salsola ikonnikovii Iljin	*Kali ikonnikovii* (Iljin) Akhani & Roalson *Salsola beticolor* Iljin *Salsola centralasiatica* Iljin *Salsola potaninii* Iljin
Salsola jacquemontii Moq.	*Kali jacquemontii* (Moq.) Akhani & Roalson *Kali nepalensis* (Grubov) Brullo, Giusso & Hrusa *Salsola nepalensis* Grubov
Salsola junatovii Botsch.	-
Salsola kali L.	*Corispermum pilosum* Raf. *Kali soda* Moench *Kali turgidum* (Dumort.) Gutermann *Salsola acicularis* Salisb. *Salsola aptera* Iljin *Salsola decumbens* Lam. *Salsola gmelinii* Rouy *Salsola kali* var. *apula* Ten. *Salsola kali* subsp. *austroafricana* Aellen *Salsola kali* var. *hirta* Ten. *Salsola kali* var. *mixta* W.D.J.Koch *Salsola kali* var. *rosacea* Pall. *Salsola kali* var. *rosacea* Moq. *Salsola kali* var. *rubella* Moq. *Salsola kali* var. *vulgaris* W.D.J.Koch *Salsola scariosa* Stokes *Salsola spinosa* Lam. *Salsola turgida* Dumort.
Salsola kerneri (Wol.) Botsch.	-

Table 2.1: List of accepted species in genus *Salsola* and their synonyms (Akhani et al., 2007; POWO, 2022).

Accepted species in genus *Salsola*	Synonyms
Salsola komarovii Iljin	*Kali komarovii* (Iljin) Akhani & Roalson
Salsola laricifolia Litv. ex Drobow	-
Salsola longifolia Forssk.	*Darniella longifolia* (Forssk.) Brullo *Darniella sinaica* (Brullo) Brullo *Salsola fruticosa* Cav. *Salsola longiflora* J.F.Gmel. *Salsola oppositifolia* Sieber ex Moq. *Salsola sieberi* C.Presl *Salsola sinaica* Brullo *Seidlitzia longifolia* (Forssk.) Iljin
Salsola mairei Botsch.	*Nitrosalsola mairei* (Botsch.) Theodorova
Salsola makranica Freitag	-
Salsola masclansii G.Monts. & D.Gómez	-
Salsola melitensis Botsch.	*Darniella melitensis* (Botsch.) Brullo
Salsola monoptera Bunge	*Kali monopterum* (Bunge) Lomon.
Salsola omanensis Boulos	-
Salsola oppositifolia Desf.	*Petrosimonia sibirica* (Pall.) Bunge
Salsola pachyphylla Botsch.	-
Salsola papillosa (Coss.) Willk.	*Salsola angularis* Sennen
Salsola paulsenii Litv.	*Kali paulsenii* (Litv.) Akhani & Roalson *Kali pellucidum* (Litv.) Brullo, Giusso & Hrusa *Salsola pellucida* Litv.
Salsola pontica (Pall.) Iliin	*Kali ponticum* (Pall.) Sukhor. *Kali tragus* subsp. *ponticum* (Pall.) Mosyakin *Salsola kali* var. *pontica* Pall. *Salsola kali* subsp. *pontica* (Pall.) Mosyakin *Salsola pontica* var. *glabra* Tzvelev *Salsola squarrosa* subsp. *pontica* (Pall.) Mosyakin *Salsola tragus* subsp. *pontica* (Pall.) Rilke
Salsola praecox (Litv.) Litv.	*Kali praecox* (Litv.) Sukhor. *Salsola elegantissima* Iljin *Salsola kali* var. *praecox* Litv. *Salsola paulsenii* subsp. *praecox* (Litv.) Rilke
Salsola praemontana Botsch.	*Nitrosalsola praemontana* (Botsch.) Theodorova
Salsola ryanii Hrusa & Gaskin	*Kali ryanii* (Hrusa & Gaskin) Brullo & Hrusa
Salsola sabrinae Mosyakin	*Salsola tragus* subsp. *grandiflora* Rilke
Salsola schweinfurthii Solms	*Darniella schweinfurthii* (Solms) Brullo
Salsola sinkiangensis A.J.Li	*Kali sinkiangense* (A.J.Li) Brullo, Giusso & Hrusa
Salsola squarrosa Steven ex Moq.	*Kali dodecanesicum* C.Brullo, Brullo, Giusso & Ilardi *Salsola controversa* Tod. ex Lojac. *Salsola squarrosa* subsp. *controversa* (Tod. ex Lojac.) Mosyakin
Salsola strobilifera (Benth.) Mosyakin	*Salsola australis* var. *strobilifera* (Benth.) Domin *Salsola kali* var. *strobilifera* Benth.
Salsola subglabra Botsch.	*Nitrosalsola subglabra* (Botsch.) Theodorova
Salsola tamamschjanae Iljin	*Kali tamamschjanae* (Iljin) Akhani & Roalson

Table 2.1: List of accepted species in genus *Salsola* and their synonyms (Akhani et al., 2007; POWO, 2022).

Accepted species in genus ***Salsola***	**Synonyms**
Salsola tamariscina Pall.	*Caroxylon tamariscinum* (Pall.) Moq. *Kali tamariscinum* (Pall.) Akhani & Roalson *Salsola tamariscifolia* Falk *Salsola tenuifolia* Falk
Salsola tragus L.	*Kali tragus* (L.) Scop. *Salsola altaica* (C.A.Mey.) Iljin *Salsola brachypteris* Moq. *Salsola caroliniana* Walter *Salsola dichracantha* Kitag. *Salsola iberica* (Sennen & Pau) Botsch. ex Czerep. *Salsola kali* var. *brachypteris* (Moq.) Benth. *Salsola kali* var. *brevimarginata* W.D.J.Koch *Salsola kali* var. *caroliniana* (Walter) Nutt. *Salsola kali* var. *glabra* Ten. *Salsola kali* subsp. *iberica* (Sennen & Pau) Rilke *Salsola kali* var. *leptophylla* Benth. *Salsola kali* subsp. *ruthenica* (Iljin) Soó *Salsola kali* var. *tenuifolia* Tausch *Salsola kali* var. *tragus* (L.) Moq. *Salsola pestifer* A.Nelson *Salsola pseudotragus* (Beck) Iljin *Salsola ruthenica* Iljin *Salsola ruthenica* var. *filifolia* A.J.Li *Salsola ruthenica* var. *tragus* (L.) Morariu *Salsola tragus* subsp. *iberica* Sennen & Pau *Salsola tragus* var. *pseudocollina* Tzvelev *Salsola tragus* var. *tenuifolia* (Tausch) Tzvelev
Salsola tunetana Brullo	*Darniella tunetana* (Brullo) Brullo
Salsola turcica Yild.	-
Salsola verticillata Schousb.	*Darniella verticillata* (Schousb.) Brullo *Salsola deschaseauxiana* Litard. & Maire *Seidlitzia verticillata* (Schousb.) Iljin
Salsola webbii Moq.	*Anabasis tamariscifolia* Webb *Salsola ericoides* Lag. ex Willk. & Lange
Salsola zaidamica Iljin	*Kali zaidamicum* (Iljin) Akhani & Roalson
Salsola zygophylla Batt.	*Darniella zygophylla* (Batt.) Brullo

Figure 2.1: Photographs of selected *Salsola* spp.; **a**. *S. kali*, **b**. *S. collina,* **c**. *S. tragus*, **d**. *S. imbricate*, **e.** *S. komarovii*, **f**. *S. oppositifolia* Desf., **g**. *S. soda*, **h**. *S. laricifolia* (ElNaggar et al., 2022).

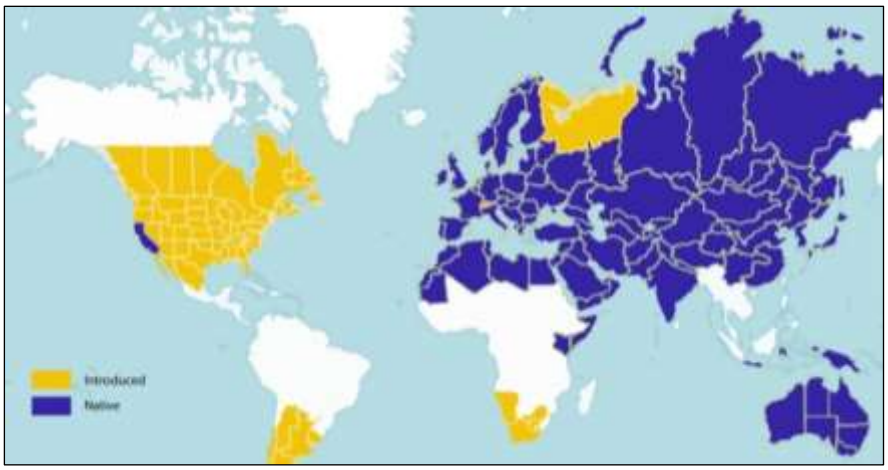


Figure 2.2: Distribution of genus *Salsola* in different regions of the world (Murshid et al., 2022).

2.2.2. Traditional Uses of Genus *Salsola*

Plants from the genus *Salsola* are known to be used in traditional medicine in the treatment of different aliments. *S. somalensis* is frequently used in traditional medicine to treat variety of conditions as skin diseases and cure tape worm infestation (Woldu and Abegaz, 1990; Abegaz and Woldu, 1991; Rasheed et al., 2013). In addition, the dried roots of *S. somalensis* are sold as anthelmintic by conventional medication distributors in a variety of markets in Ethiopia (Abegaz

and Dagne, 1978). The buds of *S. soda*; are the main edible parts of the plant; are consumed as vegetables in Italy and called "agretti" or "barba di frate". Other species like *S. tragus* and *S. baryosoma* are utilized as livestock fodder in arid and dry areas (Chauhan et al., 2018). The whole plant of *S. kali* is used as infusion by indigenous people residing in the Rif region, Northern Morocco to treat digestive system disorders (Chaachouay et al., 2022). The local population in the Mongolian People's Republic used *S. laricifolia* herb traditionally for the treatment of stomach diseases, fractured bones, healing wounds, itching, and swelling joints (Narantuyaa et al., 1986; Samdan and Batsukh, 2020). In sheep, some members of the *Salsola* genus produce prolonged gestation (pregnancy), and in female rats, they cause contraception (birth control) (Basson et al., 1969). Bushmen women in Namibia and South Africa consume the aqueous extracts (tea infusion) of *S. tuberculatiformis* (synonym of *Caroxylon tuberculatiforme* Botsch. Mucina) as an oral contraceptive in traditional medicine by inhibiting the adrenal cytochrome P-450 specific for 11 beta- Hydroxylase (P450c11) and reducing the biosynthesis of corticosteroids (Swart et al., 2003). Meanwhile, in the Cholistan desert, Southern Punjab, Pakistan, *S. baryosma* has a folklore reputation for treating indigestion, diarrhea, dysentery, itching, sores, colds, improve maleness, asthma, migraine, headache, and inflammations (Boulos, 1991; Turki, 1999; Klopper and Van Wyk, 2001). Moreover, in the Middle East, *S. baryosma* is used against some inflammatory diseases (Al-Saleh et al., 1993). *S. imbricata* has several folk medicinal applications in the treatment of painful and inflammatory conditions (Carolin et al., 1982), gastrointestinal disorders including poor digestion, vomiting, piles, dyspepsia, and abdominal distention (Ahmad et al., 2014). It is also used in the management and treatment of headach, migraine, vertigo, scabies, eruption and wounds (Wariss et al., 2017), where the bark extract showed higher potency than fruit extract as anthelmintic (Ajaib et al., 2019). In China and Korea (Taia et al., 2018; Ajaib et al., 2019; Mohammed et al., 2021), the whole fresh herb of *S. collina* is widely used to treat hypertension (Taia et al., 2018), headache, insomnia, constipation (Tundis et al., 2007; Hong et al., 2014) and vertigo (Mollaei et al., 2021b) as a herbal drink or medicine (Taia et al., 2018; Ajaib et al., 2019). While in Russia, *S. collina* was a component of the biologically active food additive “Heparon” recommended as a hepatoprotective when the hepatic cells are exposed to alcohol, medications and various toxins as well as it has anti-inflammatory and mild cholagogue effect (Tundis et al., 2007). Hyperpyrexia, hypertension, inflammation, jaundice, and gastrointestinal illnesses have all been treated using *S. komarovii* in the old days (Hong et al., 2014). In Southwest Asia, a species called *S. vermiculata* is used to cure gastrointestinal problems (Mollaei et al., 2021b). In addition, Bedouins and locals alike are familiar with *S. cyclophylla*, an edible halophyte, for its traditional medical usage in the treatment of inflammation and pain (Mohammed et al., 2021) and other health benefits, including

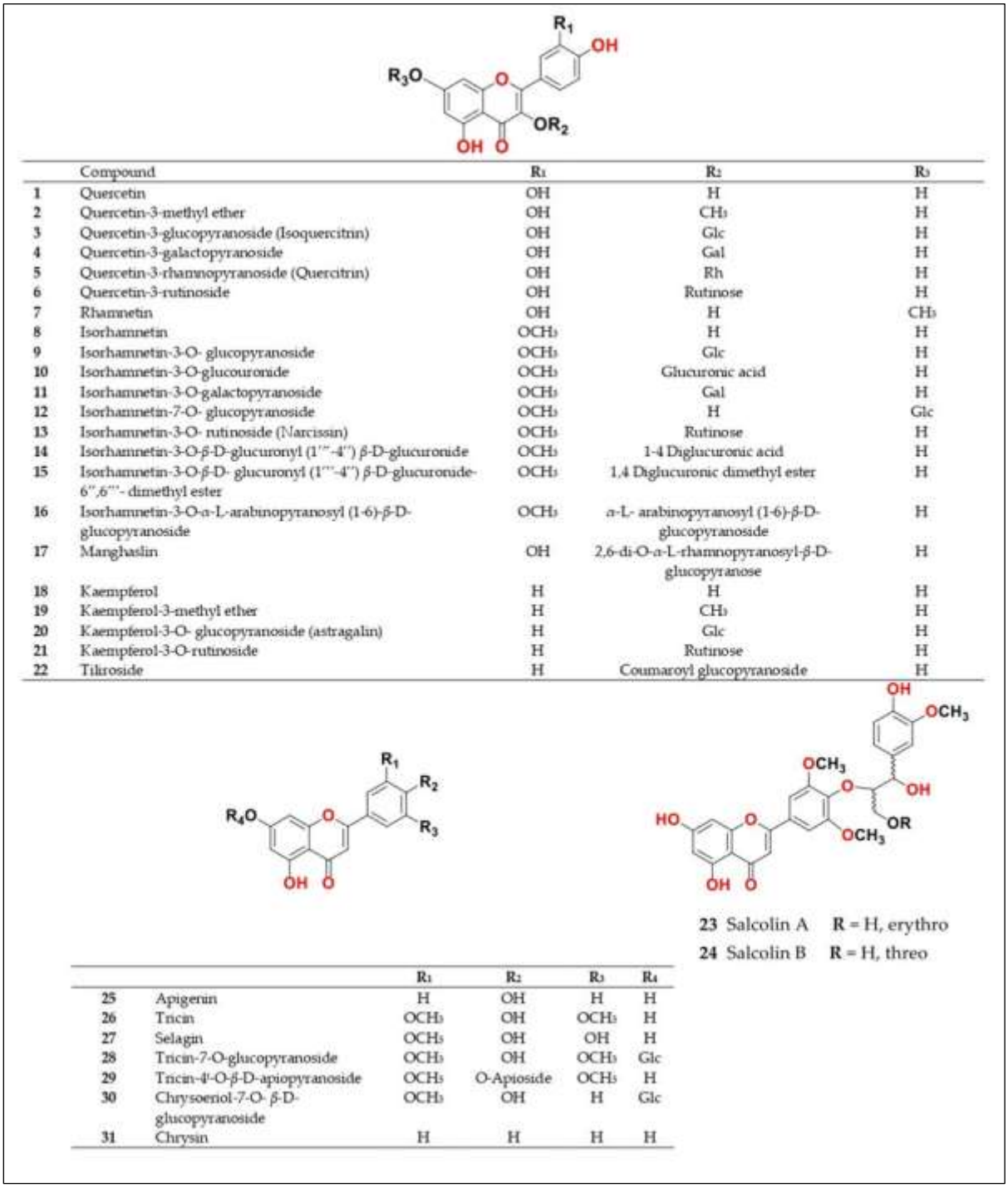
nutritional values (Mohammed et al., 2019; Mohammed et al., 2021). Thus, the plant is used as a tea and concoction for medicinal purposes by tribes and traditional healers alike to treat many diseases, particularly inflammation and pain. The plant is also used as a diuretic, laxative, and anthelmintic by the locals (Al-Jaloud et al., 1994). Moreover, *S. tetragona* leaves and aerial parts have been traditionally used to treat various ailments, including indigestion, constipation, abdominal and gastric pain, hypertension, kidney disease, and diabetes (Lakhdari et al., 2016). The powder or decoction of *S. tetragona* leaves can alleviate gastrointestinal pains, gastric pains, intestinal worms, microbial infections, cancer, and arrhythmia and can be used as a purgative (Daoud et al., 2016). The powder or decoction of leaves, roots, bark and fruit are used in Morocco to treat diabetes (Ghourri et al., 2013).

To find novel medications from this identified genus, more phytochemical, pharmacological, and toxicological research should be done.

2.3. Chemistry of *Salsola*

Phytochemical composition and biological consequences of the genus have gotten very little attention. Only a few species from the genus *Salsola* have been examined chemically and biologically. The secondary metabolites in *Salsola* (**Figure 2.3**) include flavonoids (**1–53**), phenolic compounds (**54–70**), phenolic acids (**71–95**), nitrogenous compounds (**96–126**), saponins (**127–137**), triterpenes (**138–144**), sterols (**145–151**), fatty acids (**152–186**), volatile constituents (**187–195**), lignans (**196–200**), magastigmane (**201–207**), coumarins (**208–219**), cardiac glycosides (**220–224**), alcohols (**225–228**), cyanogenic, isoprenoid, and sulphur containing compounds (**229–231**). The structures of different secondary metabolites are presented in **Figures (2.4–2.14).**

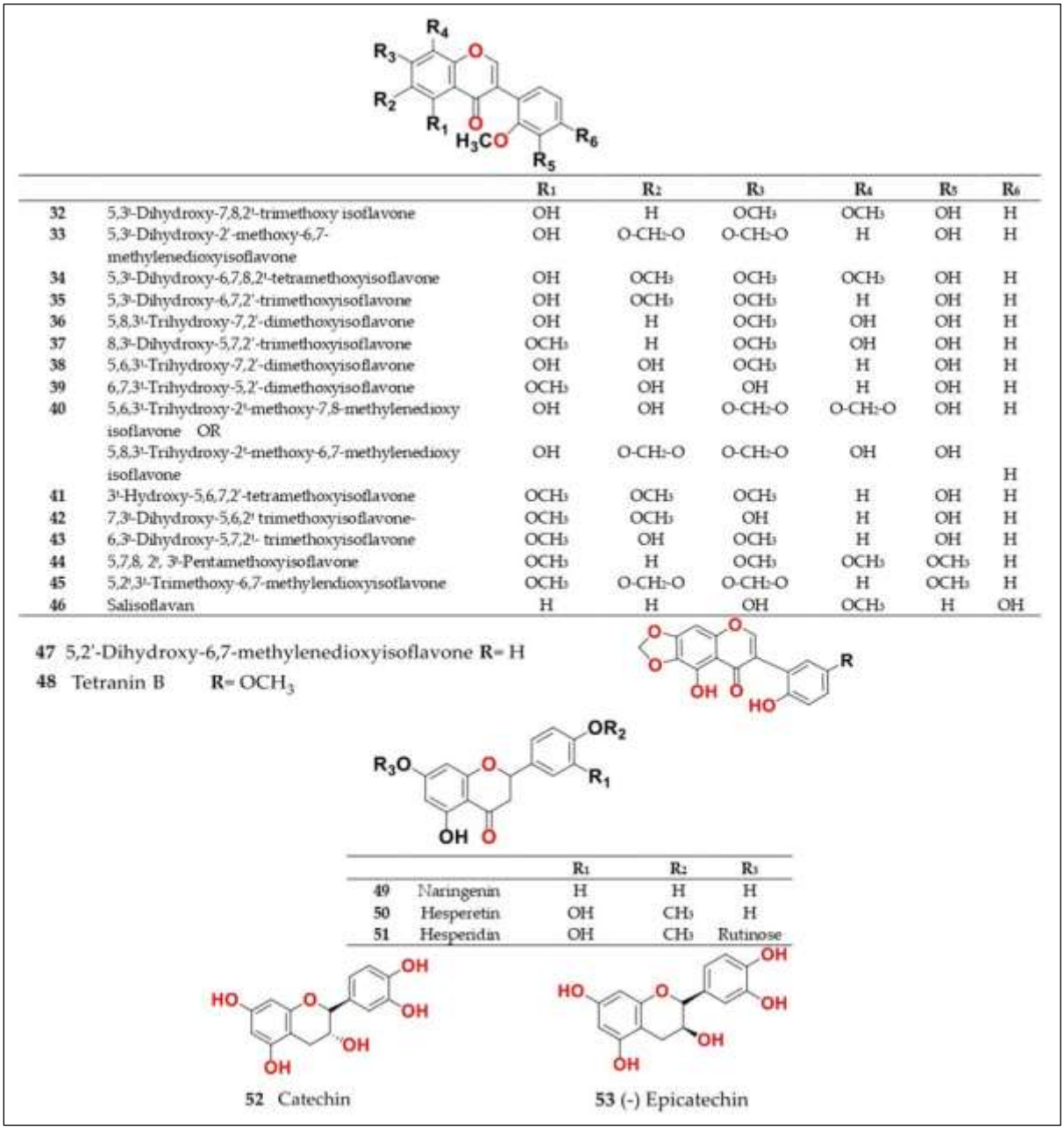
Figure 2.3: Different chemical constituents in genus *Salsola* (Murshid et al., 2022).



	Compound	R1	R2	R3
1	Quercetin	OH	H	H
2	Quercetin-3-methyl ether	OH	CH_3	H
3	Quercetin-3-glucopyranoside (Isoquercitrin)	OH	Glc	H
4	Quercetin-3-galactopyranoside	OH	Gal	H
5	Quercetin-3-rhamnopyranoside (Quercitrin)	OH	Rh	H
6	Quercetin-3-rutinoside	OH	Rutinose	H
7	Rhamnetin	OH	H	CH_3
8	Isorhamnetin	OCH_3	H	H
9	Isorhamnetin-3-O- glucopyranoside	OCH_3	Glc	H
10	Isorhamnetin-3-O-glucouronide	OCH_3	Glucuronic acid	H
11	Isorhamnetin-3-O-galactopyranoside	OCH_3	Gal	H
12	Isorhamnetin-7-O- glucopyranoside	OCH_3	H	Glc
13	Isorhamnetin-3-O- rutinoside (Narcissin)	OCH_3	Rutinose	H
14	Isorhamnetin-3-O-β-D-glucuronyl (1‴-4″) β-D-glucuronide	OCH_3	1-4 Diglucuronic acid	H
15	Isorhamnetin-3-O-β-D- glucuronyl (1‴-4″) β-D-glucuronide-6″,6‴- dimethyl ester	OCH_3	1,4 Diglucuronic dimethyl ester	H
16	Isorhamnetin-3-O-α-L-arabinopyranosyl (1-6)-β-D-glucopyranoside	OCH_3	α-L- arabinopyranosyl (1-6)-β-D-glucopyranoside	H
17	Manghaslin	OH	2,6-di-O-α-L-rhamnopyranosyl-β-D-glucopyranose	H
18	Kaempferol	H	H	H
19	Kaempferol-3-methyl ether	H	CH_3	H
20	Kaempferol-3-O- glucopyranoside (astragalin)	H	Glc	H
21	Kaempferol-3-O-rutinoside	H	Rutinose	H
22	Tiliroside	H	Coumaroyl glucopyranoside	H

		R1	R2	R3	R4
25	Apigenin	H	OH	H	H
26	Tricin	OCH_3	OH	OCH_3	H
27	Selagin	OCH_3	OH	OH	H
28	Tricin-7-O-glucopyranoside	OCH_3	OH	OCH_3	Glc
29	Tricin-4′-O-β-D-apiopyranoside	OCH_3	O-Apioside	OCH_3	H
30	Chrysoeriol-7-O- β-D-glucopyranoside	OCH_3	OH	H	Glc
31	Chrysin	H	H	H	H

Figure 2.4: Chemical structure of flavonoids isolated from genus *Salsola* (Murshid et al., 2022).



		R1	R2	R3	R4	R5	R6
32	5,3'-Dihydroxy-7,8,2'-trimethoxy isoflavone	OH	H	OCH_3	OCH_3	OH	H
33	5,3'-Dihydroxy-2'-methoxy-6,7-methylenedioxyisoflavone	OH	O-CH_2-O	O-CH_2-O	H	OH	H
34	5,3'-Dihydroxy-6,7,8,2'-tetramethoxyisoflavone	OH	OCH_3	OCH_3	OCH_3	OH	H
35	5,3'-Dihydroxy-6,7,2'-trimethoxyisoflavone	OH	OCH_3	OCH_3	H	OH	H
36	5,8,3'-Trihydroxy-7,2'-dimethoxyisoflavone	OH	H	OCH_3	OH	OH	H
37	8,3'-Dihydroxy-5,7,2'-trimethoxyisoflavone	OCH_3	H	OCH_3	OH	OH	H
38	5,6,3'-Trihydroxy-7,2'-dimethoxyisoflavone	OH	OH	OCH_3	H	OH	H
39	6,7,3'-Trihydroxy-5,2'-dimethoxyisoflavone	OCH_3	OH	OH	H	OH	H
40	5,6,3'-Trihydroxy-2'-methoxy-7,8-methylenedioxy isoflavone OR	OH	OH	O-CH_2-O	O-CH_2-O	OH	H
	5,8,3'-Trihydroxy-2'-methoxy-6,7-methylenedioxy isoflavone	OH	O-CH_2-O	O-CH_2-O	OH	OH	H
41	3'-Hydroxy-5,6,7,2'-tetramethoxyisoflavone	OCH_3	OCH_3	OCH_3	H	OH	H
42	7,3'-Dihydroxy-5,6,2' trimethoxyisoflavone-	OCH_3	OCH_3	OH	H	OH	H
43	6,3'-Dihydroxy-5,7,2'- trimethoxyisoflavone	OCH_3	OH	OCH_3	H	OH	H
44	5,7,8, 2', 3'-Pentamethoxyisoflavone	OCH_3	H	OCH_3	OCH_3	OCH_3	H
45	5,2',3'-Trimethoxy-6,7-methylendioxyisoflavone	OCH_3	O-CH_2-O	O-CH_2-O	H	OCH_3	H
46	Salisoflavan	H	H	OH	OCH_3	H	OH

47 5,2'-Dihydroxy-6,7-methylenedioxyisoflavone **R**= H

48 Tetranin B **R**= OCH_3

		R1	R2	R3
49	Naringenin	H	H	H
50	Hesperetin	OH	CH_3	H
51	Hesperidin	OH	CH_3	Rutinose

52 Catechin

53 (-) Epicatechin

Figure 2.4 (Cont.): Chemical structure of flavonoids isolated from genus *Salsola* (Murshid et al., 2022).

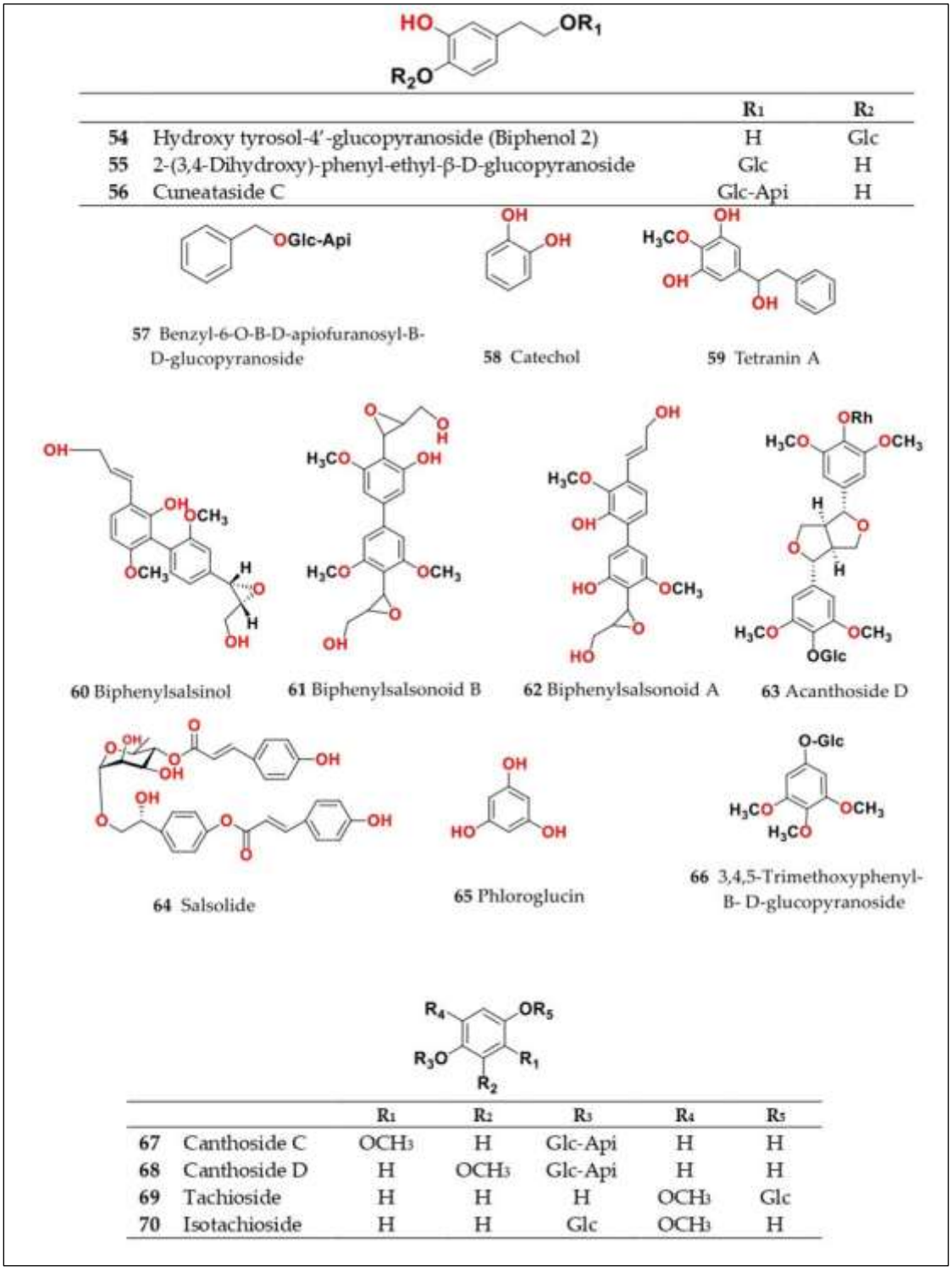
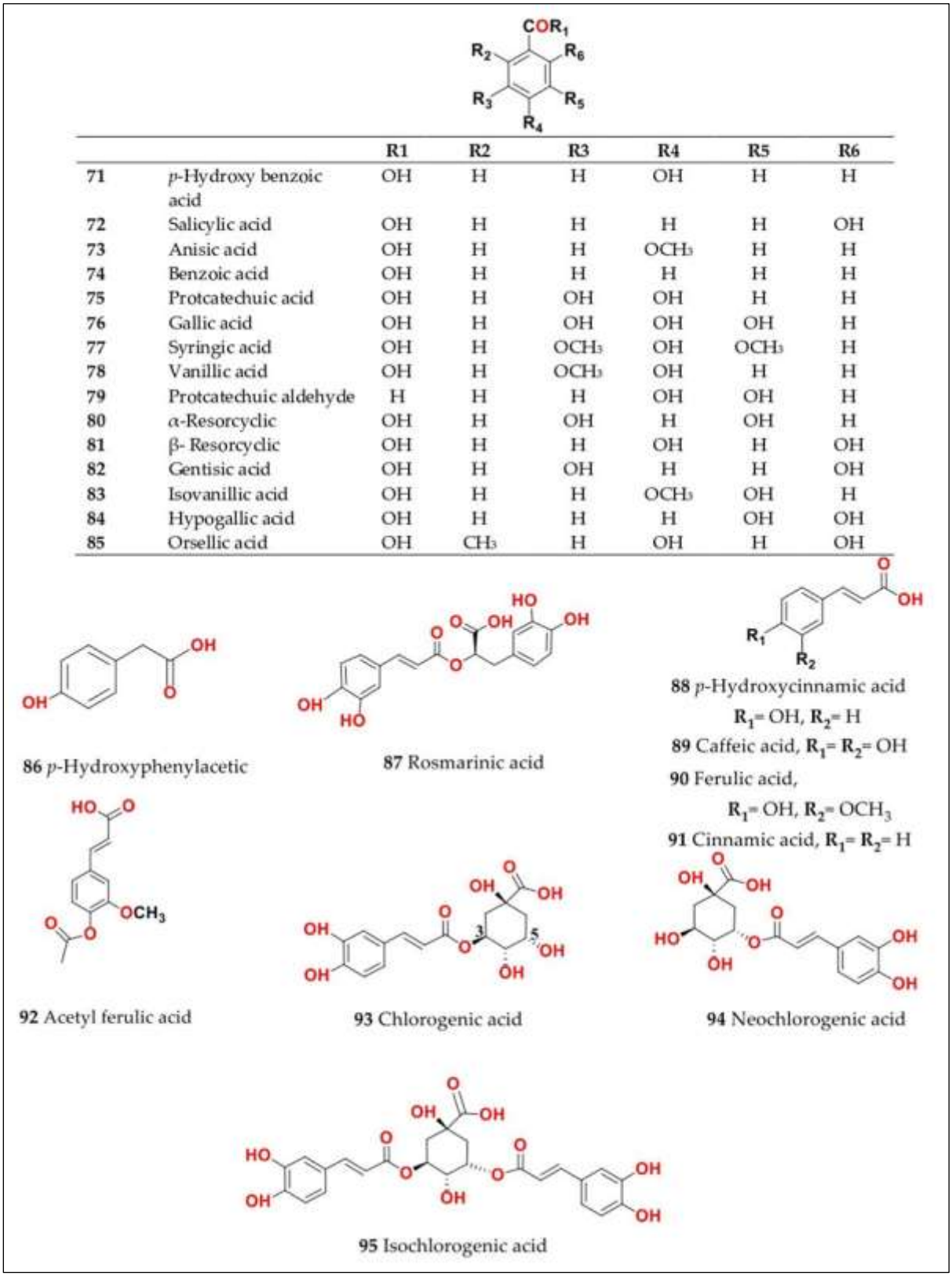


Figure 2.5: Chemical structure of phenolic compounds isolated from genus *Salsola* (Murshid et al., 2022).



		R1	R2	R3	R4	R5	R6
71	*p*-Hydroxy benzoic acid	OH	H	H	OH	H	H
72	Salicylic acid	OH	H	H	H	H	OH
73	Anisic acid	OH	H	H	OCH_3	H	H
74	Benzoic acid	OH	H	H	H	H	H
75	Protcatechuic acid	OH	H	OH	OH	H	H
76	Gallic acid	OH	H	OH	OH	OH	H
77	Syringic acid	OH	H	OCH_3	OH	OCH_3	H
78	Vanillic acid	OH	H	OCH_3	OH	H	H
79	Protcatechuic aldehyde	H	H	H	OH	OH	H
80	α-Resorcyclic	OH	H	OH	H	OH	H
81	β- Resorcyclic	OH	H	H	OH	H	OH
82	Gentisic acid	OH	H	OH	H	H	OH
83	Isovanillic acid	OH	H	H	OCH_3	OH	H
84	Hypogallic acid	OH	H	H	H	OH	OH
85	Orsellic acid	OH	CH_3	H	OH	H	OH

86 *p*-Hydroxyphenylacetic

87 Rosmarinic acid

88 *p*-Hydroxycinnamic acid

R_1= OH, R_2= H

89 Caffeic acid, R_1= R_2= OH

90 Ferulic acid,

R_1= OH, R_2= OCH_3

91 Cinnamic acid, R_1= R_2= H

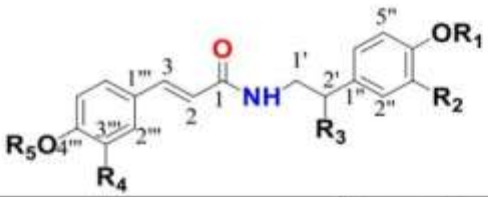
92 Acetyl ferulic acid

93 Chlorogenic acid

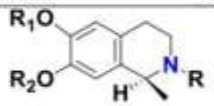
94 Neochlorogenic acid

95 Isochlorogenic acid

Figure 2.6: Chemical structure phenolic acids isolated from genus *Salsola* (Murshid et al., 2022).

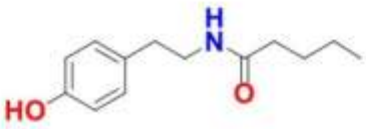


	Compound	R1	R2	R3	R4	R5
96	Moupinamide	H	H	H	OCH_3	H
97	2′-Hydroxymoupinamide	H	H	OH	OCH_3	H
98	2′-Hydroxy- 3″- methoxylmoupinamide	H	OCH_3	OH	OCH_3	H
99	*N*-[2′-(3″,4″-dihydroxyphenyl)-2′-hydroxyethyl]-3-(4‴-methoxyphenyl)prop-2-enamide	H	OH	OH	H	CH_3
100	*N*-[2′-(3″,4″-dihydroxyphenyl)-2′-hydroxyethyl]-3-(3‴,4‴-dimethoxyphenyl)prop-2-enamide	H	OH	OH	OCH_3	CH_3
101	*N*-[2′-(3″-hydroxy-4″-methoxyphenyl)-2′-hydroxyethyl]3-(4‴-methoxyphenyl)-prop-2-enamide	CH_3	OH	OH	H	CH_3
102	*N-trans*-Feruloyl 3-O-methyldopamine	H	OCH_3	H	OCH_3	H
103	*N-trans*-Feruloyl tyramine	H	H	H	OCH_3	H
104	*trans-N*-Feruloyltyramine-4‴-O-β-D-glucopyranoside	Glc	H	H	OCH_3	H
105	S(-)*trans-N*-Feruloyloctopamine	H	H	OH	OCH_3	H
106	*N*-Caffeoyltyramine	H	H	H	OH	H
107	*N*-Feruloyl- 3‴-methoxytyramine	H	OCH_3	H	OCH_3	H
108	*N*-(3',4'-dimethoxy-cinnamoyl)-norepinephrine	H	OH	OH	OCH_3	CH_3
109	*N*-(4'-Methoxy-cinnamoyl)-norepinephrine	H	OH	OH	H	CH_3



		R	R1	R2
110	Salsoline	H	H	CH_3
111	*N*-Methylisosalsolidine	CH_3	CH_3	H
112	Salsolidine	H	CH_3	CH_3
113	Carnegine	CH_3	CH_3	CH_3

114 Salsoline A R_1= H, R_2= OH
115 Salsoline B R_1= OH, R_2= H



116 *N*-(4-Methylpentanoyl) tyramine

Figure 2.7: Chemical structure of nitrogenous compounds isolated from genus *Salsola* (Murshid et al., 2022).

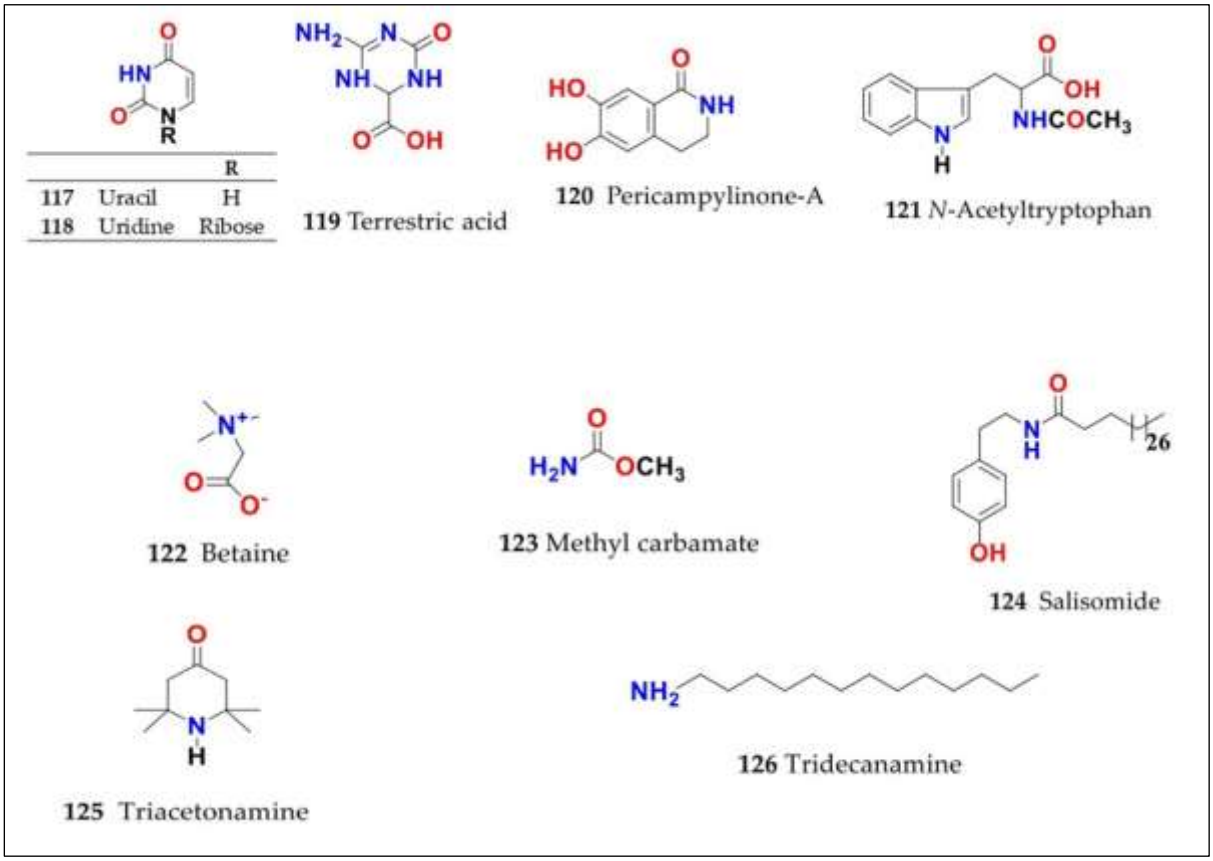


Figure 2.7 (Cont.): Chemical structure of nitrogenous compounds isolated from genus *Salsola* (Murshid et al., 2022).

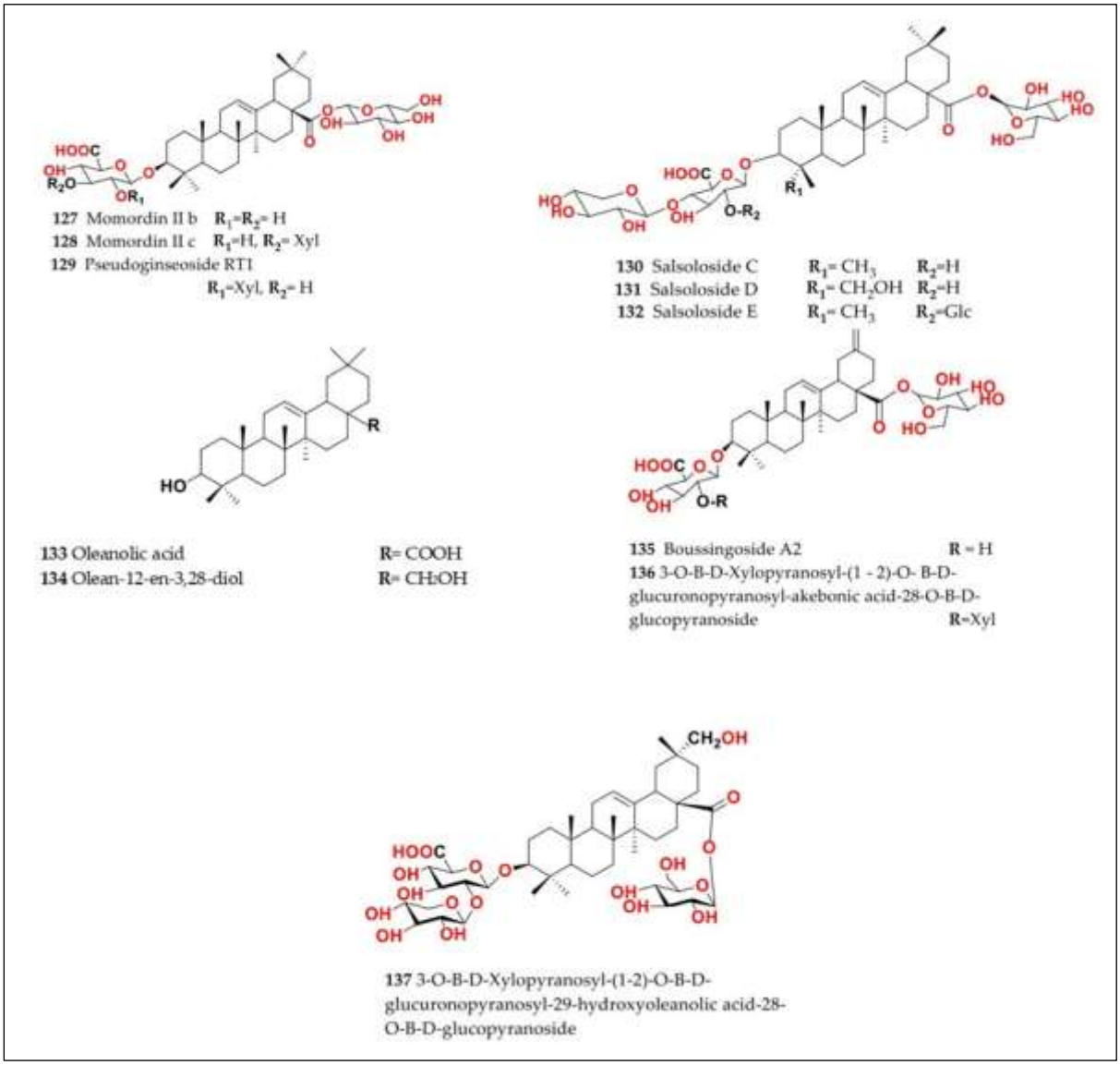
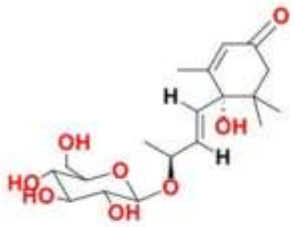
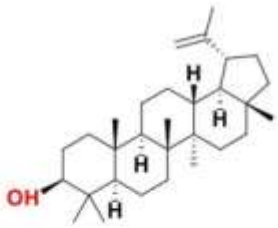


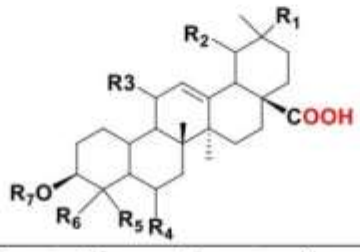
Figure 2.8: Chemical structure of saponins isolated from genus *Salsola* (Murshid et al., 2022).



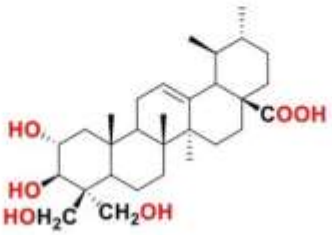
138 Corchoionoside C



139 Lupeol

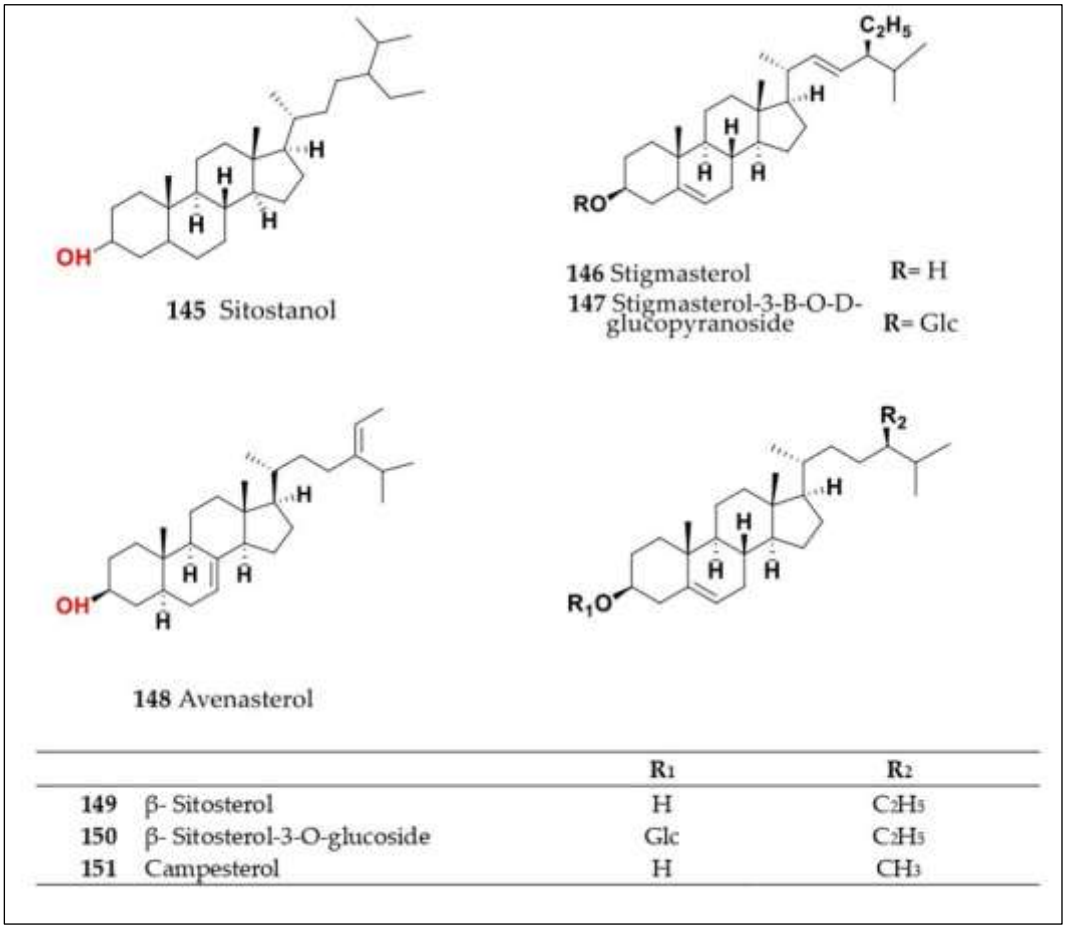


		R_1	R_2	R_3	R_4	R_5	R_6	R_7
140	Salsolic acid	CH_3	H	H	OH	CH_2OH	CH_3	H
141	Ursolic acid	H	CH_3	H	H	CH_3	CH_3	H
142	Salsolin A	CH_2OH	H	OH	H	CH_2OH	CH_3	H
143	Salsolin B	CH_3	H	OH	OH	CH_2OH	CH_2OH	Glc



144 2-α, 3β, 23, 24-Tetrahydroxyurs-12-en-28-oic acid

Figure 2.9: Chemical structure of terpenoid compounds isolated from genus *Salsola* (Murshid et al., 2022).



		R1	R2
149	β- Sitosterol	H	C_2H_5
150	β- Sitosterol-3-O-glucoside	Glc	C_2H_5
151	Campesterol	H	CH_3

Figure 2.10: Chemical structure of sterols isolated from genus *Salsola* (Murshid et al., 2022).

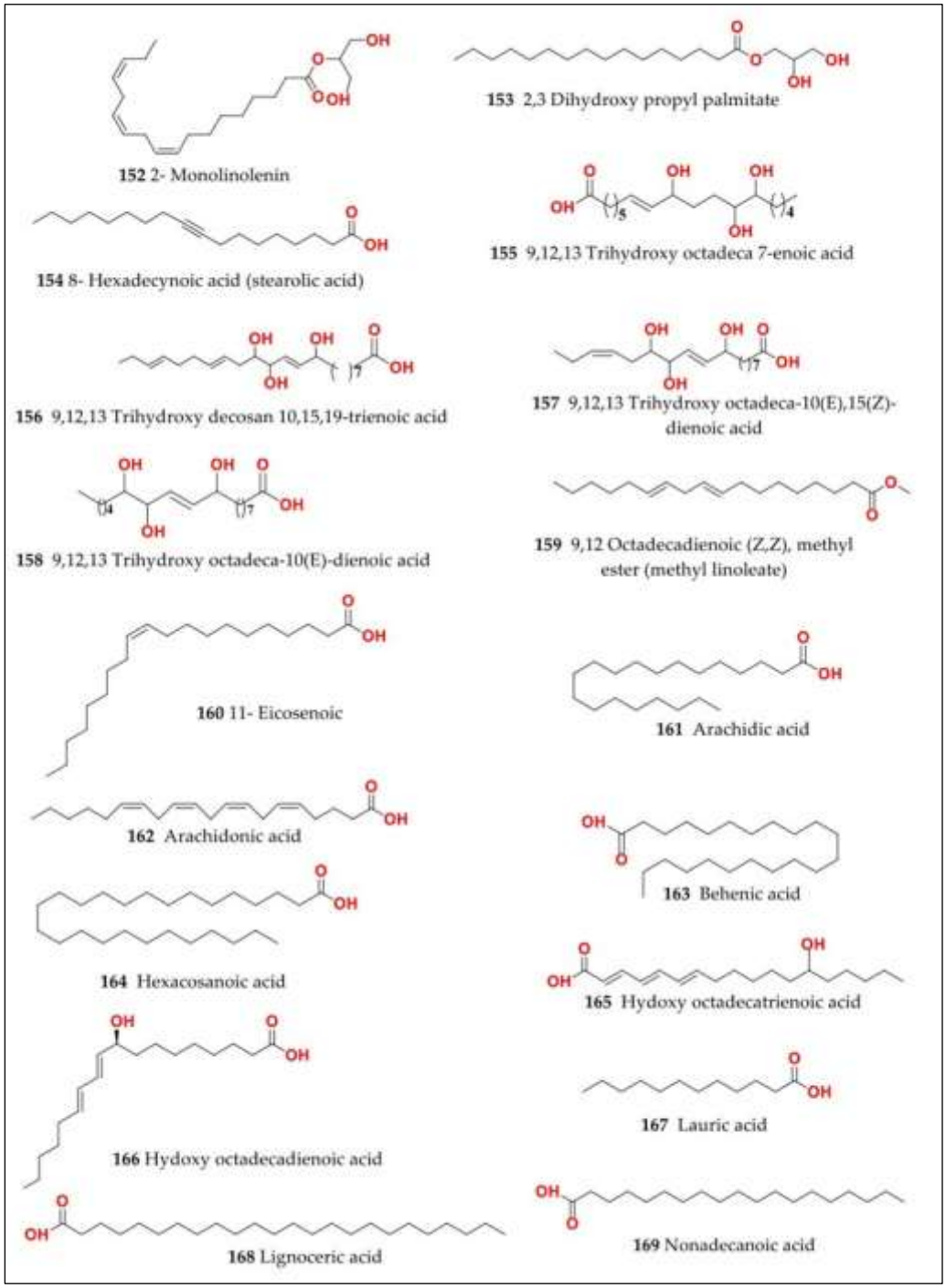


Figure 2.11: Chemical structure of fatty acids isolated from genus *Salsola* (Murshid et al., 2022).

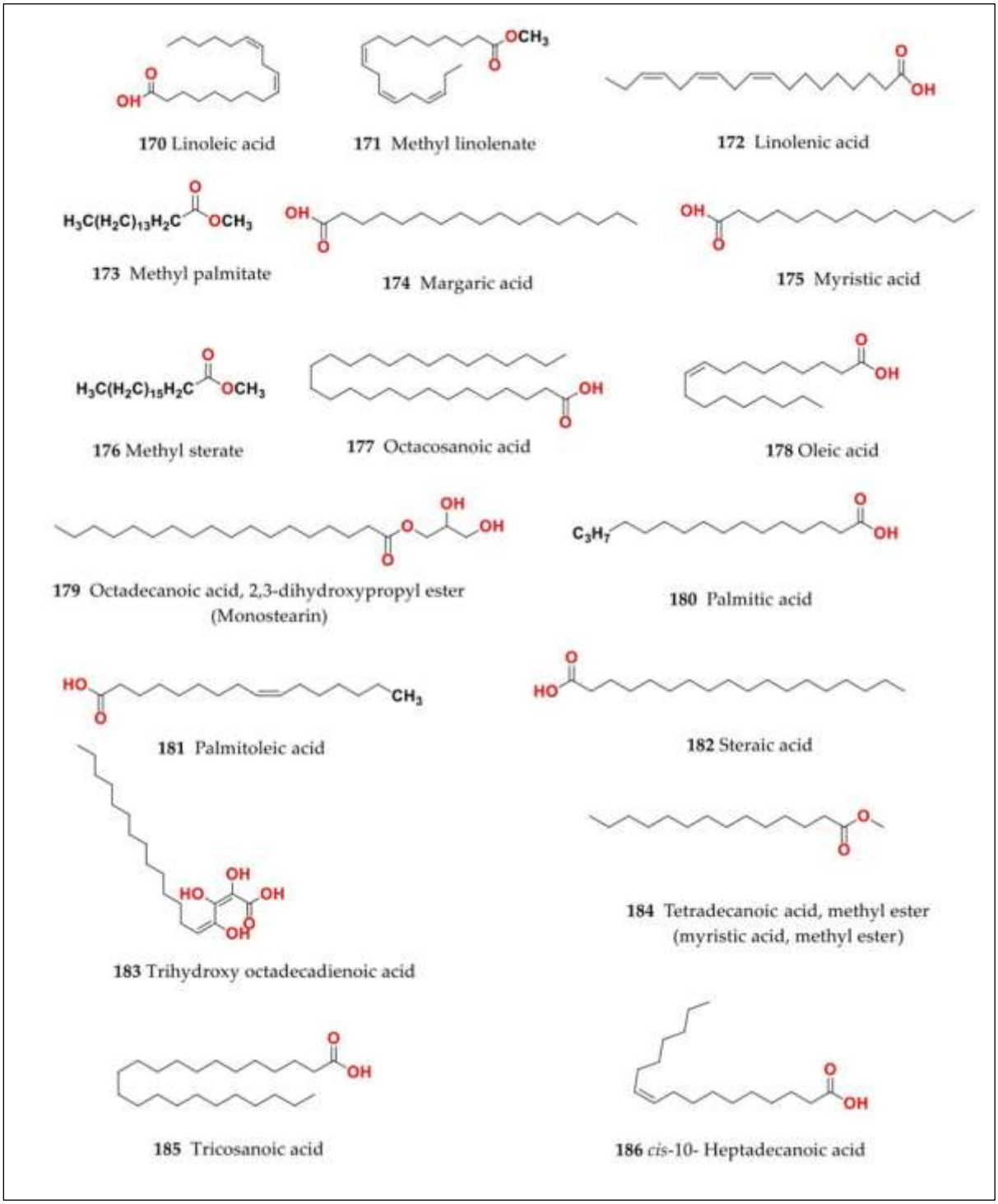


Figure 2.11 (Cont.): Chemical structure of fatty acids isolated from genus *Salsola* (Murshid et al., 2022).

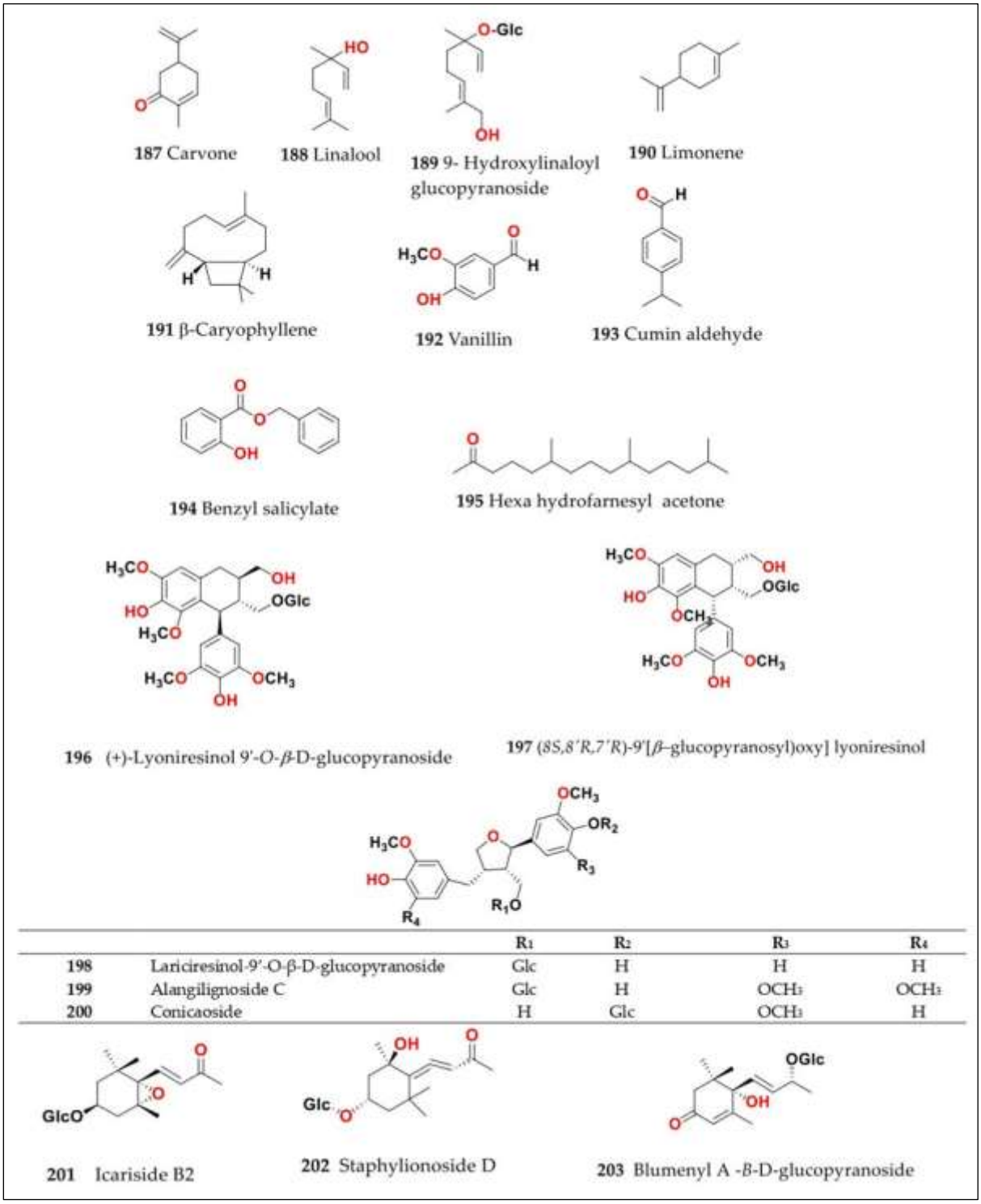


Figure 2.12: Chemical structure of volatile constituents, their glycosides, lignans and megastegmanes isolated from genus *Salsola* (Murshid et al., 2022).

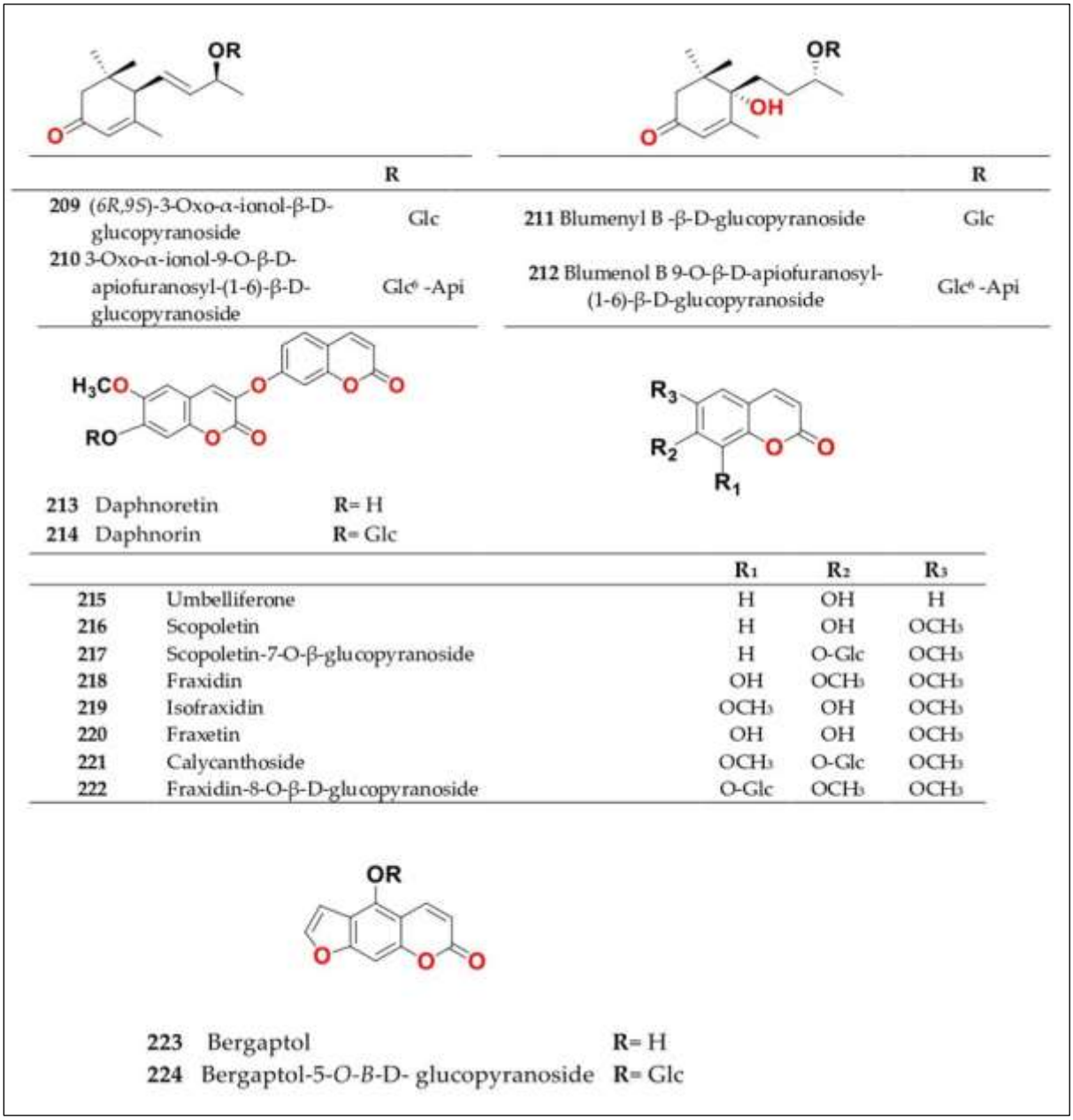


Figure 2.13: Chemical structure of megastegmanes (cont.) and coumarins isolated from genus *Salsola* (Murshid et al., 2022).

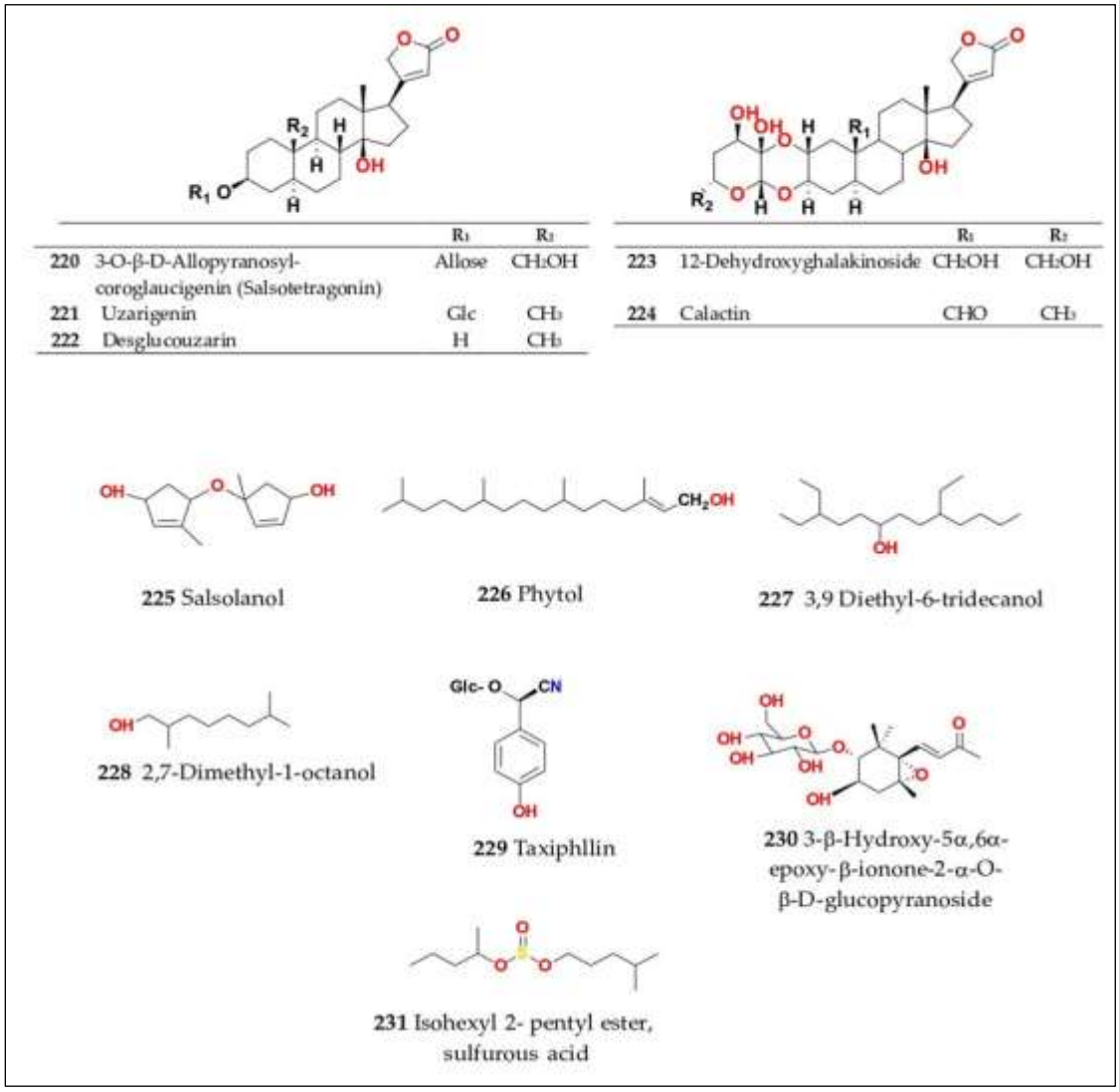


Figure 2.14: Chemical structure of cardiac glycoside, alcohols, cyanogenic, isoprenoid, and Sulphur containing compounds isolated from genus *Salsola* (Murshid et al., 2022).

Flavonoids, phenolic compounds, and phenolic acids predominate in most of the species in this genus. Volatile constituents were examined only in *S. vermiculata* and *S. cyclophylla*. Meanwhile, lignans and magastigmanes were isolated only from *S. komarovii*. On the other hand, cardiac glycosides were isolated only from *S. tetragona*. The naming of many active compounds in *Salsola* was very confusing. Some active compounds were given a name derived from the genus like salcolin A (**23**) and B (**24**) (flavonoid nucleus), Salsoline A (**114**) and B (**115**) (nitrogenous compound) and Salsolin A (**142**) and B (**143**) (triterpene nucleus). Some isolated compounds were given confusing common names, for example, Biphenol 2 (**54**) was given to hydroxy tyrosol-4`-glucopyranoside (Cho et al., 2014). Moreover, tetranin A (**59**) was given to a phenolic compound while tetranine B (**48**) (Beyaoui et al., 2012) was given to isoflavonoid, although they were isolated by the same authors. Therefore, naming of new isolated compounds

from this genus in future need careful revision of the previously isolated compounds to avoid any confusion.

The following section will outline the important isolated and identified compounds in different *Salsola* species as well as general procedures of their isolation.

2.3.1. General Procedures for Isolation of Bioactive Compounds from the Genus

Genus *Salsola* is rich in different types of phytoconstituents that need different techniques in isolation of their active compounds. Generally, dried plant material is extracted with suitable organic solvents such as methanol or aqueous ethanol. Total extract is fractionated with different solvents *viz*. hexane, chloroform, ethyl acetate and butanol. Hexane fraction is rich in nonpolar constituents including sterols and triterpenes which is separated on silica gel columns using eluting system formed from Hexane:Ethyl acetate with gradual increasing of polarity (Ahmad et al., 2007; Ahmad et al., 2008b; Alturkistani et al., 2017). Meanwhile, chloroform fraction is rich in coumarins, phenolic compounds and flavonoid aglycones. The separation of these compounds is also performed on silica gel columns using chloroform-methanol mixtures with gradual increasing of polarity (Ahmad et al., 2006). Sephadex may be used in purification of the isolated compounds using methanol as an eluting agent (Ahmad et al., 2006; Jin et al., 2011). The flavonoid glycosides as well as saponins could be detected in ethyl acetate or butanol fractions. These fractions could be treated on Diaion or polyamide columns to remove sugars and get flavonoids and their glycosides in less contaminated form (Küçükboyacı et al., 2016; Orhan et al., 2017). Flavonoid glycosides could be then isolated on normal silica gel using mixtures of chlo-roform:methanol with gradual increasing of polarity or by reversed phase silica (RP-18) using water:methanol mixtures in isolation (Küçükboyacı et al., 2016; Orhan et al., 2017). Saponins need different treatment as they detected in butanol fraction and could be purified using silica gel columns and chloroform:methanol with gradual increasing of polarity (Hamed et al., 2011). Alkaloids usually detected in chloroform or ethyl acetate fractions and separated on silica gel columns using mixtures of chloroform:methanol with gradual increasing of polarity (Zhao and Ding, 2004; Xiang et al., 2007b; Jin et al., 2011). Cardinolides are usually detected in chloroform (aglycones) or in butanol (glycosides). Aglycones are separated on silica gel columns using mixtures of chloroform:methanol with gradual increasing of polarity meanwhile, their glycosides are isolated on RP-18 eluted with H_2O-MeOH (Ghorab et al., 2017).

2.3.2. *S. arabica*

Using GC-MS analysis, eight compounds were identified from the *n*-hexane extract. Oleic acid (**178**) was the most common component (75.57%) among the components found (Elwekeel et al., 2023).

2.3.3. *S. baryosma* (Schult.) Dandy (*Caroxylon imbricata* var. *imbricatum*)

S. baryosma has tested positively for alkaloids (Khan et al., 2003), flavonoids, coumarins and sterols (Ahmad et al., 2006). Phytochemical investigation of chloroform soluble fraction of *S. baryosma* resulted in the isolation of polyoxygenated triterpenes named as salsolin A (**142**) and salsolin B (**143**) along with 2*α*,3*β*,23,24-tetrahydroxyurs-12-en-28-oic acid (**144**) (Ahmad et al., 2008b). In addition, salsolide (**64**), *p*-hydroxyphenylglycol derivative, coumarins as daphnoretin (**208**), daphnorin (**209**), scopoletin (**211**), bergaptol (**218**), bergaptol-5-*O*-*β*-D-glucopyranoside (**219**) and a flavonoid; chrysoeriol-7-*O*-*β*-D-glucopyranoside (**30**) have been isolated from the ethyl acetate soluble fraction of the whole plant (Ahmad et al., 2006; Ahmad et al., 2008a). Meanwhile, salsolic acid (**140**), an oleane-type triterpene, was isolated from the chloroform fraction of *S. baryosma* (Ahmad et al., 2008b). Quercetin (**1**) and kaempferol (**18**) have been isolated from root, shoot and fruit of *S. baryosma*. Among plant parts, maximum content of total flavonoids [quercetin (**1**) and kaempferol (**18**)] was observed in fruits, followed by shoot and roots (Kaur and Bains, 2012).

2.3.4. *S. collina* Pall.

The herb *S. collina* is widespread in northeast, north, northwest, and southwest of China (Li et al., 2021). It contains various amino acids, flavonoids, phenolic acids, glycosides, steroids, glycoalkaloids and vitamins (Khurelbat et al., 2014; Glushchenko et al., 2015). A previous investigation of the aerial part showed the presence of alkaloids that were isolated and identified as moupinamide (**96**), 2'-hydroxymoupinamide (**97**), 2'-hydroxy-3"-methylmoupinamide (**98**), *N*-*trans*-feruloyl-3-*O*-methyldopamine (**102**), salsoline A (**114**), salsoline B (**115**), uracil (**117**), uridine (**118**), pericampylinone-A (**120**), and *N*-acetyltryptophan (**121**). Glycoalkaloids, salsoline (**110**) and salsolidine (**112**) were also isolated from the aerial parts and extracted with aqueous or aqueous alcohol with the alcohol concentration of 30%, 50% and 70%. Identification of acyl transferases mediating for production of these amino acid phenolic conjugates has yet to be determined considering their abundance among most listed *Salsola* species. It has been found that the largest content of alkaloids is extracted with a 70% alcohol (Glushchenko et al., 2015). It also contained quercetin (**1**), quercetin-3-*O*-*β*-D-glucopyranoside (**3**), quercetin-3-*O*-rutinoside (rutin) (**6**), isorhamnetin (**8**), isorhamnetin-3-*O*- *β*-D-glucopyranoside (**9**), isorhamnetin-7-*O*- *β*-D-glucopyranoside (**12**), narcissin (**13**), isorhamnetin-3-*O*-*α*-L-arabinopyranosyl(1→6)- *β*-D-

glucopyranoside (**16**), kaempferol (**18**), tricin (**26**), selagin (**27**), tricin-7-*O*-*β*-D-glucopyranoside (**28**), tricin-4'-*O*-*β*-D-apioside (**29**), 5,2'-dihydroxy-6,7- methylenedioxyisoflavone (**47**), acanthoside D (**63**), *p*-hydroxybenzoic acid (**71**), salicylic acid (**72**), anisic acid (**73**), protocatechuic aldehyde (**79**), *p*-hydroxycinnamic acid (**88**), ferulic acid (**90**), acetyl ferulic acid (**92**), terrestric acid (**119**), corchoionoside C (**138**), and vanillin (**192**) (Syrchina et al., 1989; Zhao and Ding, 2004; Xiang et al., 2007a; 2007b; Jin et al., 2011).

Butanol fraction of *S. collina* aerial parts afforded tricin derivatives that were identified as Salcolin A (**23**) **[**tricin 4'-*O*-(erythro-*β*-guaiacylglyceryl) ether**]** while, Salcolin B (**24**) was identified as tricin 4'-*O*-(threo-*β*-guaiacylglyceryl) ether (Syrchina et al., 1992). Hexane and chloroform fractions of the aqueous ethanolic extract of epigeal part of *S. collina* afforded sterols as sitostanol (**145**), stigmasterol (**146**), *β*-sitosterol (**149**), campesterol (**151**) and their glycoside together with fatty acids as linoleic acid (**170**), linolenic acid (**172**), oleic acid (**178**), and palmitic acid (**180)** (Syrchina et al., 1989; Zaikov et al., 1992). The major components in the ethyl acetate fraction of *S. collina* were identified using HPLC and LC/MS analysis. Nine compounds were assigned as 4-hydroxybenzoic acid (**71**), salicylic acid (**72**), protocatechuic acid (**75**), syringic acid (**77**), vanillic acid (**78**), orsellic acid (**85**), 4-hydroxycinnamic acid (**88**), caffeic acid (**89**), and ferulic acid (**90**) (Zhao et al., 2020). Meanwhile, butanol fraction of seeds of *S. collina* which were exhaustively extracted with ethyl alcohol afforded glycine betaine (**122**) and flavonoids as quercetin (**1**), quercetin-3-*O*-*β*-glucopyranoside (**3**), quercetin-3-*O*-rutinoside (rutin) (**6**) isorhamnetin (**8**), isorhamnetin-3-*O*-*β*-D glucopyranoside (**9**), and kaempferol (**18**) (Zaikov et al., 1992). Moreover, different carbohydrates, such as D-glucose and D-fructose, carbohydrate ethers, such as ethyl-*β*-D-glucopyranoside and ethyl-*β*-D-fructopyranoside, and polyhydric alcohols, such as myo-inositol and D-mannitol, were also extracted from the butanol soluble fraction of an ethanolic extract of *S. collina* (Syrchina et al., 1991). Li *et al*., employed ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS), a widely targeted metabolomics method, to analyze the primary and secondary metabolites of *S. collina* shoots and roots, involving over ten different types of substances (Li et al., 2021). The main metabolites found in both the shoots and roots were flavonoids, phenolic acids, lipids, and amino acids according to the results. The shoots had significantly lower levels of most of the differential primary metabolites. On the other hand, the levels of the primary differential bioactive secondary metabolites, such as lignans, coumarins, flavonoids, alkaloids, and terpenes, were greater in the shoots than in the roots. More than half of alkaloids and terpenoids showed higher relative concentrations in the roots. It's interesting to note that all of the coumarins, most flavonoids, lignans, and the alkaloid key, salsoline A (**114**), showed higher

relative concentrations in the shoots. As a result, shoots with higher medicinal value were reported.

2.3.5. *S. cyclophylla* (Baker) (Synonyme of *Caroxylon cyclophyllum* (Baker) Akhani & Roalson)

Volatile constituents from *S. cyclophylla* herb were identified by GC and GC/MS and showed thirty-two volatile compounds (98.16%). Of which 34.59% belong to ketones, aldehydes and ester and 27.97% accounted for benzoic acid ester derivatives including mainly benzyl salicylate (**194**) (9.07%). Furthermore, the ketone hexahydrofarnesyl acetone (**195**) made up 27.14 % of the constituents of *S. cyclophylla* volatile oils. In addition, saturated, and unsaturated hydrocarbons were also detected in the volatile constituents. Therfore, benzoic acid ester derivatives as well as saturated hydrocarbons are the major constituents of essential oil from *S. cyclophylla* (Mohammed et al., 2019). Benzoate esters have been found in *S. cyclophylla*, although cinnmate esters have been found in other species. It is necessary to identify the biochemical pathways involved in the formation of benzoates versus cinammates.

2.3.6. *S. foetida* Vest ex Schult. (Synonyme of *Suaeda foetida* (Vest ex Schult.) Moq.)

A phytochemical study on the whole plant of *S. foetida* yielded the isolation of three nitrogenous compounds; *N*-[2'-(3",4"-dihydroxyphenyl)-2'-hydroxyethyl]-3-(4"'-methoxyphenyl)prop-2-enamide (**99**), *N*-[2'-(3",4"-dihydroxyphenyl)-2'-hydroxyethyl]-3-(3"',4"'-dimethoxyphenyl)prop-2-enamide (**100**) and *N*-[2'-(3"-hydroxy-4"-methoxyphenyl)-2'-hydroxyethyl]3-(4"'-methoxyphenyl)-prop-2-enamide (**101**) (Khan et al., 2003).

2.3.7. *S. grandis* Freitag, Vural & Adigüzel

Ethanolic extract of *S. grandis* aerial parts afforded ten flavonoids, quercetin (**1**), quercetin-3-*O*-methyl ether (**2**), quercetin-3-*O*-galactoside (**4**), quercetin-3-*O*-rhamnoside (**5**), quercetin-3-*O*-rutinoside (**6**), isorhamnetin-3-*O*-glucoside (**9**), isorhamnetin-3-*O*-glucuronide (**10**), isorhamnetin-3-*O*-rutinoside (**13**), manghaslin (**17**) and tiliroside (**22**). One amino acid derivative, *N*-acetyltryptophan (**121**), and two oleanane-type saponins, momordin II b (**127**) and momordin II c (**128**) (Küçükboyacı et al., 2016; Orhan et al., 2017).

2.3.8. *S. imbricata* Forssk. Moq. (Synonyme of *Caroxylon imbricatum* (Forssk.) Moq.

It is a tiny shrub that grows to a height of 0.3-1.2 m and is found across Egypt and native to Saudi Arabia. The other names of *S. imbricata* is "harm" , Lani and Lana, which is used as a source of camel food (Ajaib et al., 2019). Chemical investigation of different parts of *S. imbricata* revealed isolation of steroids, triterpenoids, triterpene glycoside, isoflavonoids, flavonoids, anthraquinones, tannins, coumarins, alkaloids, phenolics and sterols (Munir et al., 2014; Shehab and Abu-Gharbieh, 2014). The chloroform residue of the alcoholic extract of the whole plant grown in the eastern region of Saudi Arabia resulted in the isolation of 7 compounds as *β*-sitosterol-3-*O*-*β*-D-glucoside-6`-palmitate which is reported for the first time from the Amaranthaceae family and *β*-sitosterol-3-*O*-*β*-D-glucoside (daucosterol) (**150**) which is reported for the first time from *S. imbricata*. In addition, nitrogenous compounds as momor-cerebroside I, phytolacca cerebroside, glyceride 1,2-di-*O*-palmitoyl-3-*O*-(6-sulfoquinovopyranosyl)-glycerol, isorhamnetin-3-robino-bioside flavonoid as well as isorhamnetin-3-*O*-rutinoside (**13**) were reported for the first time from the genus *Salsola* (Suleiman et al., 2022).

Phytosterols were the primary metabolites, mostly found in the petroleum ether and methylene chloride fractions, according to a comprehensive chemical profile of the aerial parts carried out using UHPLC-QqQ-MS. The most abundant class were phenylalkylamines, which were primarily concentrated in the butanol extract, butanol, and methylene chloride fractions. Biphenylpropanoids, on the other hand, were mostly concentrated in the ethyl acetate and methylene chloride fractions. Oleanane saponins and sesquiterpene lactones were mainly found in the ethyl acetate and butanol fractions, isoquinoline alkaloids were primarily found in the methylene chloride and ethyl acetate fractions, while phenolic acids were only found in the latter. The OPLS-DA model coefficient plot revealed a positive correlation between *S. imbricata* and tetradecanoic acid (**184**), and *β*-sitosterol (**149**) (Selim et al., 2023).

The phenolic and flavonoids compounds were highly concentrated in the leaf part of the plant (Saleem et al., 2009; Hamed et al., 2011). HPLC analysis of the ethanol leaf extract showed the presence of phenol and flavonoid contents as catechin (**52**), catechol (**58**), *p*-hydroxy benzoic acid (**71**), and vanillic acid (**78**) as major contents. Other contents were detected as quercetin (**1**), rutin (**6**), kaempferol (**18**), syringic acid (**77**), caffeic acid (**89**), and ferulic acid (**90**) (Soliman et al., 2022).

HPLC analysis of the ethanolic crude extract of the aerial parts revealed the presence of quercetin (**1**), benzoic acid (**74**), gallic acid (**76**), syringic acid (**77**), and chlorogenic acid (**93**) (Javed and Jabeen, 2021).

Methanol extract of its root afforded momordin II b (**127**), pseudoginsenoside RT1 (**129**), 3-*O*-*β*-D-xylopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranosyl-akebonic acid-28-*O*-*β*-D-glucopyranoside (**136**), and 3-*O*-*β*-D-xylopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranosyl-29-hydroxyoleanolic acid-28-*O*-*β*-D-glucopyranoside (**137**) (Tanaka et al., 1985); in addition to nor-triterpene glycoside boussingoside A2 (**135**) (Espada et al., 1990; Oueslati et al., 2006; Hamed et al., 2011). Ethyl acetate soluble fraction of the alcoholic extract from their roots afforded *p*-hydroxy benzoic acid (**71**), isovanillic acid (**83**), and ferulic acid (**90**), in addition to an alkaloidal phenolic; *N-trans*-feruloyltyramine (**103**) (Osman et al., 2016). Moreover, Bi-phenylpropanoids named biphenylsalsonoid B (**61**) and biphenylsalsonoid A (**62**) were also isolated (Oueslati et al., 2017).

The flavonol; quercitrin (**5**) and the phenolic acid; rosmarinic acid (**87**) were isolated from the whole plant of *S. imbricata*. Methanolic extract of its leaves afforded nine phenolic compounds; among them two compounds were isolated from butanol fraction, isorhamnetin-3-*O*-*β*-D-glucuronyl(1'''→4'')-*β*-D-glucuronic acid (**14**) and isorhmnetin-3-*O*-*β*-D-diglucuronate dimethyl ester (**15**). Meanwhile, ethyl acetate fraction afforded seven compounds from which three were identified as isorhamnetin (**8**), isorhamnetin-3-*O*-*β*-D-glucopyranoside (**9**), and isorhamnetin-3-*O*-*β*-D-galactopyranoside (**11**).

Furthermore, analysis of the hydrolysed-methanol extract by RP-HPLC resulted in the identification and quantification of polyphenols, namely, phenolic acids and flavonoids using two different wavelengths. At short wavelength (λ = 280 nm), salicylic acid (**72**), protocatechuic acid (**75**), gallic acid (**76**), vanillic acid (**78**), caffeic acid (**89**), ferulic acid (**90**), cinnamic acid (**91**), and chlorogenic acid (**93**) were the major identified phenolic acids with predominancy of *p*-hydroxy cinnamic acid (**88**). Apart from catechol (**58**), two flavonoids, chrysin (**31**) and catechin (**52**), were found; nevertheless, only one non-phenolic compound was identified as benzoic acid (**74**) (Shehab and Abu-Gharbieh, 2014).

On the other hand, at longer wavelength (λ = 330 nm), eight components were identified, among which seven were of flavonoids nature as quercetin (**1**), rutin (**6**), apigenin (**25**), naringenin (**49**), hesperetin (**50**), and hesperidin (**51**) with major quercitrin (**5**); besides, rosmarinic acid (**87**) was the only detected phenolic acid (Shehab and Abu-Gharbieh, 2014). Meanwhile, alcoholic extract from aerial parts of *S. imbricata* yielded two secondary metabolites, salisoflavan (**46**) and salisomide (**124**) (Saleem et al., 2009). Investigation of the role of rosmarinic acid in that species

and involved biosynthetic pathways can help further agronomic and molecular approaches to improve its yield.

2.3.9. *S. inermis* Forssk. (Synonyme of *Caroxylon inerme* (Forssk.) Akhani & Roalson.

Alcoholic extract from *S. inermis* aerial parts afforded quercetin-3-rutinoside (**6**), isorhamnetin-3-*O*-*β*-glucopyranoside (**9**), kempherol (**18**), kaempferol 3-methyl ether (**19**), kaempferol-3-*O*-*β*-glucopyranoside (**20**), (-) epicatechin (**53**), hypogallic acid (**84**), *trans*-*N*-feruloyl tyramine-4'''-*O*-*β*-D-glucopyranoside (**104**), olean-12-en-28-oic acid (**133**), olean-12-en-3,28- diol (**134**), stigmastanol (sitostanol) (**145**), stigmasterol (**146**), stigmasterol-3-*β*-*O*-D-glucopyranoside (**147**), *β*-sitosterol (**149**), 9,12,13-trihydroxydecosan-10,15,19-trienoic acid (**156**), umbelliferone (**210**), and scopoletin (**211**) (Taia et al., 2018).

2.3.10. *S. jordanicola* Eig. (Synonym *Caroxylon jordanicola* Eig.)

The plant is listed in literature as one of the widely distributed plants found in the Arabian Peninsula and Iraq's deserts. A complete chemical profile of the aerial parts was conducted using UHPLC-QqQ-MS. The results showed that the main components of the ethyl acetate fraction were phenylalkylamines, followed by flavonoids, fatty acids that were detected in the petroleum ether fraction and cardenolides were concentrated in the petroleum ether and methylene chloride fractions. The OPLS-DA model coefficient plot revealed a positive correlation between *S. jordanicola* and ferulic acid (**90**), *N*-feruloyloctopamine (**105**), and *N*-feruloyltyramine (**103**) (Selim et al., 2023).

2.3.11. *S. kali* L. (*S. spinosa* Lam.)

Aerial parts of *Salsola kali* L. contains tetrahydroisoquinoline alkaloids; salsoline (**110**), *N*-methylisosalsoline (**111**), salsolidine (**112**), and carnegine (**113**) which were also separated from the aerial parts of *S. soda* L., *S. oppositifolia* and *S. ruthenica* methanol extract (Loizzo et al., 2007).

Its aerial parts contained some fatty acids like arachidonic (**162**), linolenic (**172**), oleic (**178**), palmitic (**180**), and stearic (**182**). Moreover, its aerial parts afforded sterols like sitostanol (**145**), stigmasterol (**146**), avenasterol (**148**), *β*-sitosterol (**149**), and *β*-sitosterol-3-*O*-glucoside (**150**) which were also found in *S. tetrandra*, *S. rigida* and *S. longifolia* (Karawya et al., 1972; Salt and Adler, 1985). Also, triterpene like lupeol (**139**) and ursolic acid (**141**) were found in the whole plant (Alturkistani et al., 2017).

Moreover, quercetin (**1**), quercetin-3-glucoside (**3**), quercetin-3-rhamnoside (**5**), quercetin-3-rutinoside (rutin) (**6**), rhamnetin (**7**), isorhamnetin-3-*O*-glucoside (**9**), isorhamnetin-3-*O*-rutinoside (narcissin) (**13**), and kempferol (**18**) were also identified in *S. kali* (Tundis et al., 2007; Taia et al., 2018). In addition, caffeic (**89**), ferulic (**90**), chlorogenic (**93**), neo-chlorogenic (**94**) and isochlorogenic (**95**) were the major phenolic acids identified in leaves of *S. kali* L. (Lodhi, 1979; Tundis et al., 2007).

Moreover, aerial parts and roots of *S. kali* afforded phenolic acids that were present free or liberated from their sugar after hydrolysis. The phenolic acids were identified as *p*-hydroxybenzoic (**71**), protocatechuic (**75**), syringic (**77**), vanillic (**78**), α and *β*-resorcylic (**80, 81**), gentisic (**82**), *p*-hydroxyphenylacetic (**86**), *p*-hydroxy cinnamic (**88**), caffeic (**89**) and ferulic (**90**) (Sokolowska-Krzaczek et al., 2009).

The presence of certain phytochemicals was shown by HPLC testing on methanolic extract of leaves and stems as; quercetin (**1**), hyperoside (**4**), kaempferol (**18**), catechol (**58**), syringic acid (**77**), salsoline (**110**) and fraxidin (**213**) (Boulaaba et al., 2019).

Detailed phytochemical profiling in parallel to gene expression can help establish different biosynthetic pathways in different organs. Moreover, gallic acid (**76**) and hypogallic acid (**84**), the precursor of hydrolysable tannins were found in their aerial parts. (-)Epicatchin (**53**) which is the condensed tannins precursor was found in the most of the *Salsola* species except *S. kali* and *S. tetragona* (Taia et al., 2018).

2.3.12. *S. komarovii* Iljin

Methanol extract of *S. komarovii* aerial parts afforded five lignan glycosides as (+)-lyoniresinol 9'-*O*-*β*-D-glucopyranoside (**196**), (8*S*,8'*R*,7'*R*)-9'-[(*β*-glucopyranosyl)oxy]lyoniresinol (**197**), lariciresinol-9'-*O*-*β*-D-glucopyranoside (**198**), alangilignoside C (**199**) and conicaoside (**200**); seven mega-stigmane glycosides identified as icariside B2 (**201**), staphylionoside D (**202**), blumenyl A *β*-D-glucopyranoside (**203**), (6*R*,9*S*)-3-oxo-α-ionol *β*-D-glucopyranoside (**204**), 3-oxo-α-ionol 9-*O*-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside (**205**), blumenyl B *β*-D-glucopyranoside (**206**) and blumenol B 9-*O*-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside (**207**) and seven phenolic compounds determined as biophenol 2 (**54**), 2-(3,4-dihydroxy)-phenyl-ethyl-*β*-D-glucopyranoside (**55**), cuneataside C (**56**), benzyl 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside (**57**), canthoside C (**67**), tachioside (**69**) and isotachioside (**70**) (Cho et al., 2014). Moreover, seven flavonoids as isoquercitrin (**3**), rutin (**6**), isorhamnetin (**8**), isorhamnetin-3-*O*-glucoside (**9**), isorahmnetin-3-*O*-rutinoside (**13**), kaempferol 3-*O*-glucoside (astragalin) (**20**), kaempferol-3-*O*-rutinoside (**21**), and two phenolic amides as *N-trans*-feruloyl-3-*O*-

methyldopamine (**102**) and *N-trans*-feruloyl tyramine (**103**) and were identified from the aerial parts of the ethyl acetate fraction of *S. komarovii* (Lee et al., 2012).

2.3.13. *S. laricifolia* Litv. ex Drobow

Ethanol extract of *S. laricifolia* epigeal parts which is collected in the fruit-bearing period from South-Gobi arimak, Mongolia contained coumarins identified as fraxidin (**213**), isofraxidin (**214**), fraxetin (**215**), isofraxidin-7-*O*-*β*-D- glucopyranoside (calycanthoside) (**216**), and fraxidin-8-*O*-*β*-D-glucopyranoside (**217**) from the $CHCl_3$ fraction and scopoletin-7-*O*-*β*-glucopyranoside (**212**) from EtOAc and BuOH fractions (Narantuyaa et al., 1986). Moreover, two unusual coumarin lignans were isolated from the aerial parts and identified as cleomiscosin B and cleomiscosin D which were formed by the association with another cinnamic acid moiety (Proksa et al., 1990).

2.3.14. *S. longifolia* Forssk.

S. longifolia stem was reported to contain quercetin (**1**), quercetin-3-rhamnoside (**5**), kempferol (**18**), (-) epicatechin (**53**) protocatchuic acid (**75**) and gentisic acid (**82**) (Taia et al., 2018).

2.3.15. *S. micranthera* Botsch. (Synonym of *Caroxylon micrantherum* (Botsch.) Sukhor.)

Salsolosides C (**130**), D (**131**), and E (**132**) are triterpene glycosides isolated from the aerial part of *S. micranthera* (Annaev et al., 1983; 1984).

2.3.16. *S. oppositifolia* Pall.

Isorhamnetin-3-*O*-glucoside (**9**) and isorhamnetin-3-*O*-rutinoside (**13**) flavonols were isolated from ethyl acetate fraction of aerial parts of *S. oppositifolia*. Meanwhile, *β*-sitosterol (**149**), 2-monolinolenin (**152**), methyl linolenate (**171**), methyl palmitate (**173**), methyl stearate (**176**), palmitic acid (**180**) and phytol (**226**) were the major constituents isolated from *n*-hexane fraction while 2-monolinolenin (**152**), methyl linoleate (**159**), linoleic acid (**170**), methyl linolenate (**171**) and palmitic acid (**180**) were identified from CH_2Cl_2 fraction by GC-MS. In addition, GC-MS analysis of the diethyl ether fraction revealed the presence of salsoline (**110**) and salsolidine (**112**) alkaloids (Tundis et al., 2008).

2.3.17. *S. passerine* (Bunge) Botschantz

S. passerine is among the most prevalent species in Mongolia. The aerial parts were found to contain approximately 13% (w/w) of pectic polysaccharides that were extractable with water and aqueous solutions of ammonium oxalate and sodium carbonate.

The highest yield was found in the fractions extracted using aqueous sodium carbonate solutions, while the lowest yield was found in those extracted using cold water. Rhamnogalacturonan-I (RG-I), homoga-lacturonan (HG), and arabinan with regions formed by 3,5-substituted and by 1,5-linked arabinose residues were identified as the structural units of the obtained polysaccharides (Golovchenko et al., 2022).

2.3.18. *S. soda* L. (Synonym of *Soda inermis Fourr.*)

Chemical investigations of wild and cultivated *S. soda* revealed the presence of four flavonoids, quercetin-3-*O*-glucouronopyranoside (**3**), rutin (**6**), isorhamnetin-3-*O*- glucuronopyranoside (**10**), and isorhamnetin-3-*O*-rutinoside (**13**). Furthermore, a saponin; momordin II c (**128**) was identified. Even at the young twigs stage, when it is used as food, cultivated *S. soda* produced a significant number of secondary metabolites. Both flavonoids and saponins were found in varying amounts in the two types, according to the LC-MS quantitative analysis (Iannuzzi et al., 2020).

2.3.19. *S. somalensis* N.E.Br.

Roots of *S. somalensis* afforded twelve isoflavones, 5,3'-dihydroxy-7,8,2'-trimethoxyisoflavone (**32**), 5,3'-dihydroxy-2'-methoxy-6,7-methylenedioxyisoflavone (**33**), 5,3'-dihydroxy-6,7,8,2'-tetramethoxyisoflavone (**34**), 5,3'-dihydroxy-6,7,2'-trimethoxyisoflavone (**35**), 5,8,3'-trihydroxy-7,2'-dimethoxyisoflavone (**36**), 8,3'-dihydroxy-5,7,2'-trimethoxyisoflavone (**37**), 5,6,3'-trihydroxy-7,2'dimethoxyisoflavone (**38**), 6,7,3'-trihydroxy5,2'- dimethoxyisoflavone (**39**), 5,8,3'-trihydroxy-2'-methoxy-6,7-methylendioxyisoflavone [5,6,3'-trihydroxy-2'-methoxy-7,8-methylenedioxy isoflavone] (**40**), 3'-hydroxy-5,6,7,2'-tetramethoxyisoflavone (**41**), 7,3'-dihydroxy-5,6,2'-trimethoxyisoflavone (**42**) and 6,3'-dihydroxy-5,7,2'-trimethoxyisoflavone (**43**) besides two more compounds named as 5,7,8,2',3'-pentamethoxyisoflavone (**44**) and 5,2',3'-trimethoxy-6,7-methylendioxyisoflavone (**45**) (Woldu and Abegaz, 1990; Abegaz and Woldu, 1991). While isoflavones are restricted in a few plant families mostly Fabaceae, their ecological role in *Salsola* has yet to be determined.

2.3.20. *S. tetragona* Delile (Synonym of *Caroxylon tetragonum* (*Delile*) *Moq.*)

It is native to the arid regions of Mauritania, Algeria, Tunisia, Morocco, and Libya (Ozenda, 1977). This species is relatively widespread in Algeria's Northern and Western Sahara regions.

The phenolic and flavonoid components of the hydromethanolic extract of the aerial parts were examined using LC-HR/MS. Sixteen phenolic compounds were identified by the analysis; five of these, quercetin (**1**), hyperoside (**4**), quercitrin (**5**), naringenin (**49**), and salicylic acid (**72**) were

found in *S. kali*, *S. grandis*, and *S. cyclophylla*. Additionally, *S. collina* Pall, *S. imbricate*, *S. vermiculata*, *S. tetrandra*, and *S. grandis* were found to contain quercetin (**1**) (ElNaggar et al., 2022). Ascorbic acid (159.60 mg/kg extract) and salicylic acid (**72**) were the two main compounds in the extract (274 mg/kg extract) (Cherrada et al., 2023b).Therefore, the presence and concentration of these compounds may have an impact on the crude extract's and its fractions' effectiveness (Chemsa et al., 2018).

The aerial parts of *S. tetragona* afforded five cardenolides: salsotetragonin (**220**), uzarigenin (**221**), desglucouzarin (**222**), 12-dehydroxy-ghalakinoside (**223**) and calactin (**224**); three flavonoids: quercetin-3-*O*-*β*-D-glucopyranoside (**3**), quercetin-3-rutinoside (**6**) and kaempferol-3-*O*-*β*-D-glucopyranoside (**20**); four phenolic compounds: canthoside C (**67**), canthoside D (**68**), protocatchuic acid (**75**) and vanillic acid (**78**). In addition to two fatty acids: 2,3-dihydroxypropylpalmitate (**153**) and oleic acid (**178**) (Ghorab et al., 2017; Taia et al., 2018). Whether cardenolides exist in other species has yet to be confirmed by profiling of many other species for comparison.

2.3.21. *S. tetrandra* Forssk. (Synonym of *Caroxylon tetrandrum (Forssk.) Akhani & Roalson*)

Coumarins, saponins, alkaloids, terpenes, and steroids were detected in aqueous ethanol extract from the aerial portions of *S. tetrandra* (Rasheed et al., 2013). The metabolite profile of the methanol extract of aerial parts and root of *S. tetrandra* revealed detection of a total of 29 metabolites, from which only 24 were identified using ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS) and nuclear magnetic resonance (NMR). Classification of detected metabolites was assessed using principal component analysis (PCA). Under optimized conditions, the metabolites discovered belonged to distinct classes including five hydroxycinnamic acid conjugates of norepinephrine and tyramine as *N*-*trans*-feruloyl tyramine (**103**), *N*-caffeoyl tyramine (**106**), *N*-feruloyl-3′′′-methoxy tyramine (**107**), *N*-(3′,4′-dimethoxy-cinnamoyl)-norepinephrine (**108**) and *N*-(4′-methoxy-cinnamoyl)-norepinephrine (**109**); six flavonoids with high abundance of kaempferol derivatives as kaempferol trihexoside, kaempferol pentosyl dihexoside, kaempferol-*O*-rhamnosyl dihexoside, rutin (**6**), isorhamnetin-3-*O*-glucopyranoside (**9**) and isorhamnetin-3-*O*-rutinoside (**13**); eight fatty acid derivatives as 9,12,13-trihydroxy octadeca-7-enoic acid (**155**), hydroxy octadecatrienoic acid (**165**), hydroxy octadecadienoic acid (**166**), octadecadienoic acid (linoleic acid) (**170**), octadecatrienoic acid (linolenic acid) (**172**), octadecenoic acid (oleic acid) (**178**), palmitic acid (**180**) trihydroxy octadecadienoic acid (**183**), and nitrogenous compounds as salsoline A (**114**). The aerial parts were higher in flavonoids whereas the roots were higher in hydroxycinnamic acid conjugates

(Rasheed et al., 2013). Few studies have reported on the application of chemometrics for classification and or differentiation between the different *Salsola* species and should be considered in the future and from large more specimens to help identifying which species presents best source of certain class and or identification of markers.

On the other hand, the presence of different compounds in the unsaponifiable matter from petroleum ether extract include tridecanamine (**126**), 8-hexadecynoic acid (stearolic acid) (**154**), 9,12-octadecadienoic(*Z,Z*), methyl ester (methyl linoleate) (**159**), 11- eicosenoic acid (**160**), icosanoic (arachidic) acid (**161**), docosanoic (behenic) acid (**163**), hexacosanoic acid (**164**), lauric acid (**167**), tetracosanoic (lignoceric) acid (**168**), nonadecanoic acid (**169**), linoleic acid (**170**), linolenic acid (**172**), methyl palmitate (**173**), heptadecanoic (margaric) acid (**174**), myristic acid (**175**), octacosanoic acid (**177**), oleic acid (**178**), octadecanoic acid, 2,3-dihydroxypropyl ester (monostearin) (**179**), palmitic acid (**180**), palmitoleic acid (**181**), stearic acid (**182**), myristic acid methyl ester (**184**), tricosanoic acid (**185**), *cis*-10-heptadecanoic acid (**186**), 3,9-diethyl-6-tridecanol (**227**), 2,7-dimethyl-1-octanol (**228**) and isohexyl-2-pentylester sulfurous acid (**231**). Saturated fatty acids reached up to 43.16% while, unsaturated fatty acids were 56.84% (with predominancy of polyunsaturated FA 48.59% while monounsaturated was 8.25%) (Elsharabasy et al., 2015). Furthermore, given the limited phytochemical research for this species that has been published, a coumarinolignan, estrone, cholesterol and three bases as betaine (**122**), triacetonamine (**125**) (Karawya et al., 1971) and methyl carbamate (**123**) (Karawya et al., 1972), have been identified and were detected also in *S. kali*, *S. longifolia* and *S. rigida* (Karawya et al., 1971).

Aerial parts of *S. tetrandra* afforded D-glucopyranoside (**66**), *N-trans* feruloyltyramine (**103**), *S*-(-)-*trans*-*N*-feruloyloctopamine (**105**) in addition to long-chain hydroxyl fatty acids: 3,4,5-trimethoxyphenyl-*β*- 9,12,13-trihydroxyoctadeca-10(*E*),15(*Z*)-dienoic acid (**157**) and 9,12,13-trihydroxyoctadeca-10(*E*)-dienoic acid (**158**), 9-hydroxylinaloyl glucoside (**189**), taxiphyllin (**229**) and norisoprenoid; 3-*β*-hydroxy-5*α*,6*α*-epoxy-*β*-ionone-2-*α*-*O*-*β*-D-glucopyranoside (**230**) (Oueslati et al., 2006).

Isoflavonoid; tetranin B (**48**) and tetranin A (**59**) (bibenzyl derivative) were isolated from the roots of *S. tetrandra* (Beyaoui et al., 2012). Flavonoids as quercetin (**1**), rutin (**6**), kempherol (**18**) and other phenolic compounds as (-) epicatechin (**53**), phloroglucin (**65**) and hypogallic acid (**84**) were isolated from *S. tetrandra* stem (Taia et al., 2018).

2.3.22. *S. tomentosa* (Moq.) Spach

Phenolic components (tannins, flavonoids, and total phenols), and saponins were detected as major constituents in aerial parts of *S. tomentosa* collected from Qum province from Iran. Methanol extraction either by soxhelt or maceration give the highest concentration of total phenolic and flavonoid (Mohammadi et al., 2016).

2.3.23. *S. vermiculata* L. (Synonym of *Caroxylon vermiculatum* (L.) Akhani & Roalson

This is an annual plant with a wide range of distribution in Southwest Asia (Cheng et al., 2009). High concentration of terpenoids were found in the roots, terpenoids and oils in the seeds, flavonoids and terpenoids in the leaves, and phenols and flavonoids in the flowers, according to phytochemical screening of the methanol crude extract. There are significant amounts of oils in the roots and seeds; terpenoids in the leaves, according to the analysis of fixed oil fractions. High concentrations of phenols and flavonoids were found in the leaves and flowers of the EtOAc fraction. Conversely, the roots' aqueous acid fractions revealed elevated levels of alkaloids (Mollaei et al., 2021a). Furthermore, employing Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC-ESI-MS), GC, and GC-MS, metabolite profiling of crude methanolic extracts and their fractions were detected in different parts of *S. vermiculata*. The detected metabolites belonged to phenolic compounds, alkaloids, fatty acid derivatives, and volatile oil compounds, among other types. LC-ESI-MS was used to isolate and identify alkaloids and phenolic compounds, while GC and GC-MS were used to identify fatty acids and volatile oil components (Mollaei et al., 2021b).

Ethyl acetate fractions contained phenolic compounds. Three phenolic acids, protocatechuic acid (**75**), ferulic acid (**90**), and cinnamic acid (**91**) were found exclusively in the ethyl acetate fraction of flowers; one phenolic acid, caffeic acid (**89**), was found only in the leaves extract; salicylic acid (**72**) and gallic acid (**76**) were found in both leaves and flowers extracts. Furthermore, vanillic acid (**78**) was found in all extracts. In the leaves and flowers, three flavonoids quercetin (**1**), rutin (**6**), and kaempferol (**18**) were found, followed by two flavonoids rutin (**6**) and kaempferol (**18**) in the seeds. The aerial part of *S. vermiculata* was found to be higher in rutin (**6**) and kaempferol derivatives than root samples in another research (Rasheed et al., 2013). As a result, phenolic compound-rich ethyl acetate fractions of leaves and flowers can be considered suitable candidates for various biological activities. According to LC-ESI-MS analysis of the aqueous-acid fractions, three alkaloids were found in the roots extract, including salsoline (**110**), salsolidine (**112**), and salsoline A (**114**), whereas just one alkaloid, salsoline A (**114**), was found in the seeds extract. The dominance identified fatty acids in roots samples were linoleic acid

(**170**), linolenic acid (**172**), oleic acid (**178**), palmitic acid (**180**), and stearic acid (**182**). In the fixed oil fraction of seeds, linoleic acid (**170**), oleic acid (**178**), and palmitic acid (**180**) predominated. Arachidic acid (**161**) was found only in the seeds. Lignoceric acid (**168**), on the other hand, was exclusively found in roots. Furthermore, the fixed oil fraction of leaves included just three fatty acids: linolenic acid (**172**), oleic acid (**178**), and stearic acid (**182**), according to the data (Salt and Adler, 1985; Mollaei et al., 2021b). In the flowers fraction, just two fatty acids, linolenic acid (**172**), oleic acid (**178**), were found. As a result, it may be stated that fatty acid composition is highly dependent on plant parts. Because of the unique properties of fatty acids, parts containing these chemicals could be used as a viable source of biological agents. The types and quantities of volatile chemicals found in different parts *of S. vermiculata* differed significantly. At the roots, the main components of the volatile compounds were carvone (**187**), linalool (**188**), *β*-caryophyllene (**191**), and cumin aldehyde (**193**). The main constituents of the volatile compounds in the seeds were carvone (**187**), linalool (**188**), limonene (**190**), *β*-caryophyllene (**191**), and cumin aldehyde (**193**). Meanwhile the constituents in the leaves were as follows: carvone (**187**), limonene (**190**), linalool (**188**), cumin aldehyde (**193**), *β*-caryophyllene (**191**). Finally, carvone (**187**), linalool (**188**), limonene (**190**), and cumin aldehyde (**193**) were shown to be the most abundant volatile elements in the flowers. According to their chemical formula, the volatile compounds were divided into five groups and according to the findings, oxygenated monoterpenes accounted for the majority of the volatile compounds (Mollaei et al., 2021b).

The metabolite profile of *S. vermiculata* detected a total of 28 metabolites, where only 24 of them were identified in the methanol extract of aerial portions and root using (UPLC-MS) and (NMR). Classification of detected constituents was performed using (PCA). Under optimized conditions, the identified metabolites belonged to various classes including 5 hydroxycinnamic acid conjugates of norepinephrine and tyramine as *N-trans*-feruloyl tyramine (**103**), *N*-caffeoyl tyramine (**106**), *N*-feruloyl-3′′′-methoxy tyramine (**107)**, *N*-(3′,4′-dimethoxy-cinnamoyl)-norepinephrine (**108**) and *N*-(4′-methoxy-cinnamoyl)-norepinephrine (**109**); six flavonoids as kaempferol trihexoside, kaempferol pentosyl dihexoside, kaempferol-*O*-rhamnosyl dihexoside, rutin (**6**), isorhamnetin-3-*O*-glucopyranoside (**9)** and isorhamnetin-3-*O*-rutinoside (**13**); eight fatty acid derivatives as 9,12,13-trihydroxy octadeca-7-enoic acid (**155**), hydroxy octadecatrienoic acid (**165**), hydroxy octadecadienoic acid (**166**), linoleic acid (**170**), linolenic acid (**172**), oleic acid (**178**), palmitic acid (**180**) and trihydroxy octadecadienoic acid (**183**), two nitrogenous compounds as salsoline A (**114**) and *N*-(4-methylpentanoyl) tyramine (**116**) (Rasheed et al., 2013). Hydroxycinnamic acid conjugates were plentiful in the roots, whereas areal parts

were rich in flavonoids with quercetin derivatives being the most common flavonoids (Rasheed et al., 2013).

The volatile fractions produced by hydrodistillation of *S. vermiculata* leaves, stems, and roots were analyzed chemically, and forty-four compounds belonging to several chemical classes were identified. Twenty-eight constituents make up 95.9% of the total constituents in the volatile fraction of leaves. The major compounds of this fraction are carvone (**187**), linalool (**188**), *β*-caryophllene (**191**) and cumin aldehyde (**193**). Meanwhile sixteen compounds representing 98% of volatile fractions from stem were identified. The major identified compounds were carvone (**187**), linalool (**188**), limonene (**190**) and *β*-caryophllene (**191**). On the other hand, thirty-three constituents amounted to 94% were identified from volatile constituents of root. The majority compounds are carvone (**187**), linalool (**188**), *β*-caryophllene (**191**) and cumin aldehyde (**193**). Oxygenated monoterpenes are the dominant class of volatile fractions present in *S. vermiculate*. Carvone (**187**) is the main major component of this class (Gannoun et al., 2016). Few studies have presented on the volatile's composition in *Salsola* species and should be compared to that reported in *S. vermiculate* in the future.

2.3.24. *S. villosa* Schult. (Synonym of *Caroxylon villosum* (Schult.) Akhani & Roalson)

The phytochemical screening of the 95% ethanol extract of the whole plant of *S. villosa* revealed the presence of alkaloids, saponins, tannins, flavonoids, sterols/terpenes and coumarins (Al-Saleh et al., 1997). Previous work led to isolation of biphenylsalsinol (**60**) and secondary cyclic alcohol, salsolanol (**225**) from the chloroform fraction of the aerial parts of *S. villosa* (Oueslati et al., 2015). In addition, GC-MS analysis of the *n*-hexane extract resulted in detection of twenty-two compounds. Hexadecanoic acid methyl ester (26.15%) (methyl palmitate) (**173**) was the most abundant component among the constituents that were found (Elwekeel et al., 2023).

2.3.25. *S. volkensii* Schweinf. & Asch.

Quercetin (**1**), quercetin-3-glucoside (**3**), quercetin-3-rutinoside (**6**), (-) epicatechin (**53**), phloroglucin (**65**) and hypogallic acid (**84**) were isolated from the stem of *S. volkensii* (Taia et al., 2018).

2.4. Overview of the Benefits, Uses and Medicinal Properties of *Salsola* Genus

There have only been a few chemical and biological studies of *Salsola* genus. Halophytic plants have been used for medicinal purposes because of the presence of health promoting bioactive compounds (Ksouri et al., 2012). In this regard, species of *Salsola* genus have a significant therapeutic value (**Figures 2.15 and 2.16**). *Salsola* species have a variety of constituents that give a wide range of biological activities and have been reported to be utilized in folk medicine all throughout the world, according to the literature. In the following sections, different medicinal uses, and benefits of different species in genus *Salsola* were outlined.

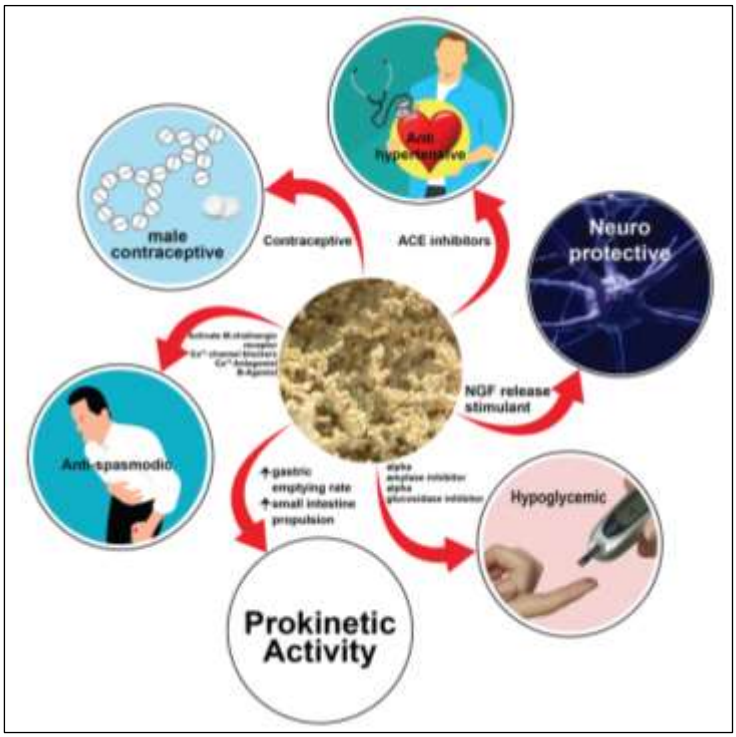


Figure 2.15: Some important biological activities of genus *Salsola* and their mechanism (Murshid et al., 2022).

2.4.1. Anti-inflammatory, Analgesic and Anti-nociceptive Activity

Pain, fever, and inflammation are immune reactions that are thought to be the body's natural defense mechanism and are vital to good health (Taylor et al., 2002; C Recio et al., 2012). Yet, under certain conditions, such as rheumatoid arthritis and asthma, these reactions injure the body and cause the onset of certain illnesses. Prostaglandins are significant inflammatory mediators that are created from certain fatty acids found in cell membranes through processes that are catalyzed by the cyclooxygenase enzyme (Ricciotti and FitzGerald, 2011). There are two isoforms of this enzyme, called COX-1 and COX-2 (Taylor et al., 2002). Normal conditions

cause cells to constitutively express COX-1, which results in the synthesis of compounds that protect the kidney and stomach. Consequently, there are several negative effects associated with COX-1 suppression. Conversely, in pathological circumstances, pro-inflammatory agents like cytokines induce COX-2. Therefore, when there is inflammation or tissue damage, COX-2 levels rise. The COX-2 produced prostanoid causes fever, edema, and pain (Taylor et al., 2002). The incidence of inflammatory diseases is becoming common in almost all the countries around the world. Despite of their well-known side effects, non-steroidal anti-inflammatory drugs remain the most commonly used to relieve inflammatory pain (Maroon et al., 2010) by blocking the COX enzyme. These medications' lack of selectivity for the COX-2 isoform results in a number of adverse effects, including gastrointestinal issues (Taylor et al., 2002).

Natural products and traditional medicines as alternatives to these drugs offer a great hope in the development of efficient agents for treatment of inflammatory diseases (Reynolds et al., 1995; Gautam and Jachak, 2009). In this regard, total methanol extract together with petroleum ether, chloroform and ethyl acetate fractions of *S. kali* was investigated for anti-inflammatory activity using rat paw edema test. Petroleum ether fraction demonstrated the highest activity (60%). Meanwhile, the chloroform, ethyl acetate fractions and methanol extract displayed 35.0 %, 20% and 40% reduction in rat-paw, respectively, relative to indomethacin (Alturkistani et al., 2017). The significant anti-inflammatory activity produced by the petroleum ether fraction was attributed to its sterols′ contents lupeol (**139**), ursolic acid (**141**), *β*-sitosterol (**149**) and *β*-sitosterol-3-*O*-glucoside (**150**) that were detected in petroleum ether extract of *S. kali*. Moreover, these compounds were proved as an anti-inflammatory by different mechanisms (Garcia et al., 1999; Saleem et al., 2009; Shehata et al., 2018). Moreover, phenolic acid; ferulic (**90**) that was also identified in *S. kali* is known for its strong anti-inflammatory activity (Ou and Kwok, 2004; Sokolowska-Krzaczek et al., 2009).

The traditional use of *S. imbricata* in inflammatory conditions was evaluated previously (Janbaz et al., 2021) through testing of the aqueous-ethanol extract of the aerial parts. The results confirmed the anti-inflammatory activity of *S. imbricate* as it significantly inhibited carrageenan-induced paw edema in rats. The same research team also used rat models of formalin-induced paw licking and NaCl-induced writhing to assess the analgesic efficacy of *S. imbricata* extract. The findings they obtained demonstrated that, at all tested doses (100, 300, and 500 mg/kg), *S. imbricata* exhibited a dose-dependent analgesic activity by reducing the number of abdominal writhing mediated by 4% NaCl intraperitoneal injection. However, it only reduced rats' paw-licking duration when given at a dose of 500 mg/kg. Furthermore, in the rat model of pyrexia caused by brewer's yeast, *S. imbricate* demonstrated strong antipyretic activity. The total aqueous

methanol extract of *S. imbricata* leaves and the six isolated phenolic compounds; isorhamnetin (**8**), isorhamnetin-3-*O*-*β*-D- glucopyranoside (**9**), isorhamnetin-3-*O*-*β*-D-galactopyranoside (**11**), isorhamnetin-3- *O*-*β*-D-glucuronyl (1'''→4'') -*β*-D-glucuronic acid (**14**), isorhamnetin-3-*O*-*β*-D-diglucuronate dimethyl ester (**15**) and *N*-*trans*-feruloyltyramine (**103**), showed distinctly *in vitro* anti-inflammatory activities with no toxicity on Raw murine macrophages cells (RAW 264.7) using nitric oxide assay at concentration level of 100 µg/mL for all samples. It is noteworthy that isorhamnetin-3-*O*-*β*-D-glucopyranoside (**9**) showed the highest anti-inflammatory activity than its corresponding galactopyranoside aglycone (**8**) and glycoside (**11**) (Osman et al., 2016).

The *ex-vivo* anti-inflammatory activity of *S. imbricata* and *S. jordanicola's* aerial parts was studied. Reduced TNF-α 5 gene upregulation induced by LPS to levels less than those generated by piroxicam was observed upon fractionation of *S. imbricata*. Furthermore, compared to piroxicam, the petroleum ether extracts of *S. jordanicola*, *S. imbricata*, and *S. jordanicola's* butanol fraction were more successful in lowering the expression of the IL-6 gene. The coefficients plot showed that the pro-inflammatory markers TNF-*α*, IL-1*β*, and IFN-γ were downregulated in strong positive correlation with aegicin, cleomiscoside, sitostanol (**145**), *β*-sitosterol (**149**) and methyl palmitate (**173**). The only major metabolites that demonstrated a strong positive correlation with the inhibition of the pro-inflammatory marker IL-6 were norepinephrine derivatives (**108 and 109**) (Selim et al., 2023). Inclusion of an *in vivo* model should now be followed to be more conclusive.

COX and other mediators implicated in the pathophysiology of pain alleviation, as well as anti-nociceptive activity, are inhibited by a hydroalcoholic extract from the aerial portions of *S. inermis* (Elsharabasy and Hosney, 2013).

Using carrageenan-induced paw edema and *p*-benzoquinone-induced nociception models, the anti-inflammatory and anti-nociceptive effects of the ethanol extract and BuOH fraction of *S. grandis*, as well as their major constituents, were examined *in vivo* on male swiss albino mice. The inhibitory effect of BuOH fraction on carrageenan-induced paw edema was 27.8–32.9%. On the other hand, 37.6% inhibition was detected in *p*-benzoquinone-induced nociception model. Quercetin-3-*O*-galactoside (**4**) and tiliroside (**22**) were shown to have the most powerful inhibitory effects in the employed models, according to the findings (Küçükboyacı et al., 2016).

S. komarovii ethanol extract exerted anti-inflammatory effects by significant decreasing lipopolysaccharide (LPS)-induced interleukin IL-6 production like hydrocortisone. It worked by another mechanism rather glucocorticoids induction which is the main side effect of gluococorticoids (Seo et al., 2020).

In addition, aqueous-ethanolic extract of the aerial parts of *S. cyclophylla* exhibited strong analgesic activity in mice in hot plate model of pain induction as well as carrageenan-induced paw edema model. The activity was attributed to its high phenolic contents of the plant (Mohammed et al., 2021).

In the UVB *in-vitro* model for inflammation, it has been reported that the synthetic analogue of *S. tuberculata's* active principle, 2-(4-acetoxyphenyl)2-chloro-*N*-methylethylammonium-chloride, inhibits UVB-induced intracellular interleukin-1alpha (icIL-1α). On the other hand, *S. tuberculata* methanol extract increased cytotoxicity and UVB-induced IL-1α production, which in turn produced a pro-inflammatory effect. However, the dichloromethane extract had no apparent effect on the inflammation of skin cells (Magcwebeba et al., 2014). It was also proposed that the synthetic analogue under investigation would increase *in vivo* free corticosterone by competitively inhibiting glucocorticoid binding to corticosteroid-binding globulin (CBG), thereby exhibiting its anti-inflammatory and contraceptive properties (Louw and Swart, 1999; De Bosscher et al., 2005).

Using a protein denaturation assay at various concentrations, the anti-inflammatory properties of the hydromethanolic extract of *S. tetragona* aerial parts and its fractions (*n*-hexane, DCM, EtOAc, *n*-BuOH, and aqueous) were identified. The ethyl acetate fraction and the aqueous fraction showed significantly higher activity with an IC_{50} of 13 ±5 µg/mL and 40 ±7 µg/mL for inflammation, respectively, exceeding aspirin as positive control (Cherrada et al., 2023b). The strong anti-inflammatory properties can be attributed to the existence of bioflavonoids like quercetin (**1**) and naringenin (**49**) (Oluwole et al., 2022).

Salsola arabica, *S. cyclophylla*, *S. imbricata*, and *S. villosa* are the four *Salsola* species whose *n*-hexane extracts were tested for their ability to inhibit the COX-1 and COX-2 enzymes, a measure of their anti-inflammatory potential. When the COX-2 inhibitory activity of the tested extract was evaluated, *S. villosa* was found to have the highest level (IC_{50} 4.6 ±0.13 µg/mL), followed by *S. arabica* (IC_{50} 13.1 ±0.37 µg/mL) and *S. cyclophylla* (IC_{50} 20.1 ±0.57 µg/mL). With a selectivity index (SI) of 10, the *S. villosa* extract exhibited selective inhibition of COX-2, which is interestingly similar to the levels of the standard drugs celecoxib (SI = 16.6) and indomethacin (SI = 0.18). However, the most notable inhibitory activity against COX-1 was shown by the *S. imbricate* extract (IC_{50} 10.2 ±0.52 µg/mL), which did not significantly differ from the standard medication celecoxib (IC_{50} 13.9 ±0.70 µg/mL). The phytoconstituents found in the extracts of *S. villosa* and *S. imbricata* were examined for their potential to interact with the active sites of the two isoforms of the cyclooxygenase enzyme using molecular docking, based on the results of bioactivity; 12,15-Octadecadiynoic acid, methylester, 10,13-octadecadiynoic acid, methylester,

methyl octadec-6,9-dien-12-ynoate, 9,12-octadecadienoic acid (*Z,Z*) -,methyl ester (Methyl linoleate), 9-octadecenoic acid (*Z*)- methyl ester (Methyl oleate) and 13-docosenoic acid; Erucic acid exhibited the highest binding affinities to COX-2 with free binding energies from -8.966 to -8.166 kcal/mol compared to that of the co-crystallized ligand flufenamic acid (-6.9 kcal/mol), while (2-phenyl-1,3-dioxolan-4-yl) methyl(9*E*)-9-octadecenoate, 12,15-Octadecadiynoic acid, methyl ester and methyl 9,12,15-octadecatrienoate exhibited the highest binding scores with COX-1 (-8.817, -8.658, and -8.160 kcal/mol, respectively) compared to the co-crystallized ligand mofezolac (-8.662 kcal/mol). Findings suggested that those compounds could be integrated into the enzymes' active sites, where some of them bind with their polar ends into cavities beyond Arg120 and their aliphatic chains oriented towards the catalytically significant Tyr385 like arachidonic acid, the natural substrate. This suggests that these compounds could be good candidates for the development of selective COX-2 inhibitors in the future (Elwekeel et al., 2023). Oleic acid (**178**) is a non-essential monounsaturated fatty acid that was reported to have a beneficial role as anti-inflammatory agent in autoimmune diseases and protective effects in breast cancer (Sales-Campos et al., 2013). It was the most common compound reported of the GC-MS profiling of the *n*-hexane extract (lipophilic constituents) of *S. arabica* (Elwekeel et al., 2023).

2.4.2. Antibacterial Activity

Ferulic acid (**90**), a phenolic acid identified in *S. kali* as well as salsoline A (**114**), an alkaloid isolated from *S. collina* showed an appreciable antibacterial activity (Ou and Kwok, 2004; Wang et al., 2004; Wang et al., 2020b). The antibacterial activity of a methanol extract of *S. kali* aerial parts was evaluated using the agar well diffusion method against seven pathogenic bacterial strains at a concentration of 0.5µg/mL. The highest activity was against *Staphylococcus aureus*, *Streptococcus mutans*, *Bacillus subtilis* and *Streptococcus pneumoniae*, while it showed moderate bactericidal activity against *Pseudomonas aeruginosa*. It inhibits the growth of *Escherichia coli* and *Sarcina lutae*. Pure methanol was used as negative control while ampicillin, amoxicillin, levofloxin, tetracycline, vancomycin, ciprofloxacin, and penicillin were positive control (Mughal et al., 2010).

The antimicrobial properties of the *S. kali* leaves and stems methanol extract were investigated (Boulaaba et al., 2019). Since the stem and leaf extracts contained significantly more phenolic compounds than the root, the stem extract exhibited higher activity. It demonstrated an inhibition zone diameter (IZD) of 10 mm against *P. aeruginosa* and *M. luteus*, demonstrating good antibacterial activity. It exhibited negligible or minimal efficacy against *Candida* sp. and other bacterial pathogens.

The *in vitro* antibacterial activity of the ethyl acetate extract from the roots of *S. imbricata* and the two biphenylpropanoids A (**62**) and B (**61**) was evaluated by minimum inhibitory concentration (MIC) method against three Gram-positive bacteria; *S. aureus*, *S. epidermidis* and *M. luteus* and three Gram negative bacteria; *E. coli*, *P*. aeruginosa and *Salmonella typhimurium*. The two compounds have similar effectiveness against the bacteria tested, with MIC values ranging from 16 to 64 µg/mL. On the other hand, biphenylsalsonoid B (**61**) showed higher potency than biphenylsalsonoid A (**62**) against *M. luteus* (Oueslati et al., 2017).

S-(−)-*trans*-*N*-feruloyloctopamine (**105**) and taxiphyllin (**229**) isolated from *S. tetrandra* displayed mild antibacterial activity against *S. aureus* at concentration of 200 µg/mL, with minimal bactericidal concentration (500 and 600 µg/mL, respectively) (Oueslati et al., 2006).

It was found that 95% ethanol extract of the whole plant of *S. villosa*; which contain a high concentration of alkaloids and flavonoids, showed a wide spectrum of antimicrobial activity at different concentrations against *S. aureus* and *P. aeruginosa* using the agar diffusion method and antibiotics discs of streptomycin and chloramphenicol as positive controls (Al-Saleh et al., 1997). Different fractions of *S. villosa* revealed different degrees of antimicrobial activity against gram positive and negative micro-organisms (Mahasneh et al., 1996). Meanwhile, Oueslati and Al-Ghamdi *et al*., 2015 stated that biphenylsalsinol (**60**) and salsolanol (**225**) from *S. villosa* exhibited antibacterial activities. The highest antimicrobial effect was observed for bi-phenylsalsinol (**60**) (Oueslati et al., 2015).

The antibacterial activity of the fixed oil fraction of *S. vermiculate* roots showed the highest activity against *S. aureus*, with inhibition zone 18.37 ± 0.31 mm. This was followed by the fixed oil fraction of seeds and the volatile fraction of roots, both of which had MIC 7.5 mg/mL. The crude extract showed moderate to high inhibitory activity. Thus, the presence of linoleic (**170**), oleic (**178**), and palmitic acids (**180**) may be connected to the fixed oil fraction's strong antibacterial activity (Mollaei et al., 2021a). Moreover, the antimicrobial activity of extracts prepared from different organs of *S. vermiculate* (10 mg/mL) was evaluated using the microdilution technique for determination of the (MIC). *E. faecalis* and *S. aureus* were the most affected by *S. vermiculate* extracts (MICs 0.28 to 4.16 mg/mL). Ethanol extract of the root was the most effective on *S. aureus* meanwhile, *E. coli* and *P. aeruginosa* were the most resistant bacteria. The antibacterial activity was referred to carvone (**187**) (Znini et al., 2011). It has the ability to destabilize the phospholipid bilayer, interact with enzymes and proteins of the membrane, and reduce pH gradient across the membrane (Aggarwal et al., 2002).

The antibacterial activity of *S. tetragona* extracts against *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213) was examined. The findings demonstrated that the crude extract of the aerial parts, with MIC values of 125 µg/mL and 250 µg/mL for the fractions (*n*-hexane, DCM, EtOAc, *n*-BuOH, and aqueous), had a moderate effect on inhibiting the growth of *E. coli* (ATCC 25922). Conversely, the crude extract's and its fractions' MICs (250 µg/mL) against *S. aureus* (ATCC 29213) were similar (Cherrada et al., 2023b). Therefore, the presence of secondary metabolites associated with polyphenols was identified as the main cause of the antibacterial activity observed in *S. tetragona* (Bouarab-Chibane et al., 2019) followed by flavonoids (quercetin (**1**) and naringenin (**49**)) in these fractions (Oluwole et al., 2022).

The agar diffusion method was used to perform the antimicrobial assay of *S. cyclophylla*. Positive control drug disc 10 µg/mL amoxicillin and gentamycin; inhibition zone diameter (IZD) and a broth microdilution test were chosen to determine the MIC for selected microorganisms. It had no effect on *Staphylococcus epidermidis* but was effective against *S. aureus* and *Streptococcus pyogenes* with 16 and 11 mm IZD, and MIC equal to 45 and 72 mg/mL, respectively. Furthermore, it showed activity against *P. aeruginosa* gram-negative strain having 11 mm IZD and 75 mg/mL MIC, respectively. In contrast, it showed 10 mm IZD with MIC equal to 79 mg/mL against *E. coli*. As a result, potent antimicrobial activity was proven which is remarkable from the viewpoint of this herb being a common camel feed (Mohammed et al., 2019). It should be noted that most results for extracts and/or compounds assessed for antimicrobial assays were based on *in vitro* or agar diffusion assays with no animal models tested to confirm efficacy and should now follow.

2.4.3. Antiviral Activity

Salsoline A (**114**), an alkaloid in *S. collina* showed moderate antiviral activity against influenza virus A and B (Wang et al., 2004). The activity was assessed by infection of Madin-Darby canine kidney (MDCK) cell monolayers with influenza virus A or B using ribavirin as standard antiviral agent. Salsoline A (**114**), showed antiviral activity against influenza virus A with IC_{50} 56.8 µg/mL (Wang et al., 2004).

Therefore, chalcone and flavones in the dichloromethane (STD) and ethyl acetate (STE) aerial part extracts of *S. tetragona*, can hinder the binding of the viral spike protein to its angiotensin converting enzyme-2 (ACE2 receptor). In tests of inhibition, STD extract was 20% less effective at 50 µg/mL and demonstrated better activity as Anti-SARS-CoV-2. Nevertheless, additional investigation is required to assess their effectiveness and safety *in vivo* (Cherrada et al., 2023b).

2.4.4. Antifungal Activity

The petroleum ether fraction of the whole plant of *S. kali* exhibited a significant *in vitro* antifungal activity against *Rhizoctonea solani* and *Nattrassi mangifera* (21.1mm and 25.3 mm, respectively) using the agar disc diffusion assay (Boughalleb et al., 2009). Moreover, the methanolic extract of *S. kali* showed a promising activity against *A. flavus* (Mughal et al., 2010). Mahasneh *et al.* (1996) studied the antifungal activity of the whole aerial parts of the butanol extract of *S. villosa* which showed significant antifungal activity (13-14 mm inhibition zones) against *Candida albicans* and *Fusarium oxysporum* comparable to the antifungal miconazole nitrate (Mahasneh et al., 1996).

The antifungal activity of *S. vermiculate* leaves, roots, and stems extracts (100 mg/mL) was tested against three pathogenic *Candida* species; *C. glabrata*, *C. krusei* and *C. parapsilosis* by the diffusion method in a solid medium (Sabouraud Chloramphenicol). The results showed that the activity varied according to the pathogen and the plant extract. It also appears that these activities were weak with inhibition zone diameters ranging from 6.5 to 9.5 mm. The butanol fraction of roots methanol extract was the most active on *C. parapsilosis* (ϕIZ = 9.5 mm). However, it has been reported that an aqueous extract of *S. vermiculata's* aerial parts is a potent antifungal that can be used as a preservative when storing grains. The reduction in fungal growth on wheat samples coated with *S. vermiculata* aqueous extract, dried, and stored for a year was used to measure this activity (Moghtet et al., 2018). The richness of *S. vermiculate* leaves, stems and roots volatile fractions in carvone (**187**) (52.2%, 53% and 49.9%, respectively) could explain its antifungal activity (Gannoun et al., 2016).

S. cyclophylla volatile oil demonstrated a powerful effect against *C. albicans* compared with clotrimazole with inhibition zone 16 mm IZD and 14.5 mg/mL MIC, respectively (Mohammed et al., 2019). Terrestric acid (**119**) from *S. collina* showed positive antifungal activity evaluated by the standard broth microdilution method of the NCCLS (Jin et al., 2011).

2.4.5. Antioxidant, Iron Chelation, Hepato-protective and Cardio-protective Activity

Active polymers such as free radicals (reactive oxygen species or reactive nitrogen species) are overproduced or eliminated too slowly under oxidative stress. A variety of chronic disorders, such as diabetes mellitus (DM) and Alzheimer's disease, are linked to an oxidation-antioxidation imbalance (Reddy et al., 2009; Umeno et al., 2017; Zhao et al., 2017). As long as the body maintains a dynamic balance between oxidation and anti-oxidation, excess ROS and RNS can be rapidly removed from the body. A cellular damage occurs as a result of overproduction of RNS

and ROS, resulting in damage to all cellular components, including DNA, proteins, and lipids (Fu et al., 2012), which causes disordered cell function and metabolism. Excess ROS and RNS have been reported to be eliminated by natural antioxidants, as well as preventing free radicals from oxidizing and harming cells.

Salsola is an important halophytic genera of the family Amaranthaceae and is considered genera of plants containing antioxidants compounds with low caloric composition (Chauhan et al., 2018). *Salsola* species have been studied for their antioxidant potential in greater detail than any other. The results that have been published suggest that the antioxidant activity may be significantly impacted by the plant parts that were used as well as the extraction solvent. Most of the compounds responsible for antioxidant activities are flavonoids and their glucosidal derivatives. However, other substances with only moderate activities included as biphenylpropanoids, alkaloids, and components of essential oils (ElNaggar et al., 2022).

The primary potential antioxidant component was flavonoids, which were found in higher relative concentrations in the shoots of *S. collina* than in the roots (Li et al., 2021). It has been reported that the ethanol extract of *S. collina* has antioxidant activity through DPPH radical scavenging capacity (Oh et al., 2014). Ethyl acetate extract of *S. collina* alleviates diabetic gastroparesis (DGP), possibly through promoting gastric emptying in DGP Male Sprague-Dawley rats due to its oxidative stress inhibition ability and increase the number of gastric neurons, combined with its hypoglycemic and lipid lowering effects (Zhao et al., 2020). Patients with chronic hepatitis have reportedly shown a significant hepato-protective effect when using lochein, a liquid extract of the Russian thistle *S. collina* Pall (Beloborodova et al., 2000). Additionally, the Russian Federation's Ministry of Health has authorized it as an active food supplement (Vengerovskii et al., 2010). Rats with paracetamol-induced liver damage showed fewer symptoms when exposed to a 25% ethanol extract of the aerial parts of *S. collina*, and this extract was shown to have superior hepatoprotective properties than silymarin, the reference medication. It has also been shown to improve bilirubin and ammonia detoxification while lowering liver enzyme and lipid peroxidation product levels (Vengerovskii et al., 2010).

Polyoxygenated triterpenes are named as salsolin A (**142**) and B (**143**) together with 2*α*,3*β*,23,24-tetrahydroxyurs-12-en-28-oic acid (**144**) have been reported to possess significant antioxidant activity in the chloroform soluble subfraction of *S. baryosma* (Ahmad et al., 2008b). Moreover, EtOAc fraction of the whole plant gave 73% anti-oxidant activity whilst other fractions (ethanol 80%, *n*-hexane and *n*-BuOH) had antioxidant activity below 57% which was determined by DPPH radical scavenging method (Ahmed et al., 2006). Rats exposed to paracetamol-induced hepatorenal toxicity demonstrated both preventative and therapeutic hepatoprotective effects

from 70% of the ethanol extracts of *S. tetrandra* and *S. baryosma*. The findings demonstrated that *S. tetrandra* was more active and had a greater capacity to reduce the levels of inflammatory markers, including tumor necrosis factor alpha (TNF-α) and interleukin-1*β* (IL-1*β*) (Mahmoud et al., 2016).

Using the DPPH method, the antioxidant potential *n*-hexane extracts of four *Salsola* species: *S. arabica*, *S. cyclophylla*, *S. imbricata*, and *S. villosa* was evaluated. In comparison to ascorbic acid, which had an IC_{50} of 0.16 ±0.01 mg/mL as a positive control, *S. villosa* had the highest antioxidant activity (IC_{50} 0.99 ±0.05 mg/mL), closely followed by *S. cyclophylla* (IC_{50} 1.36 ± 0.06 mg/mL), *S. imbricata* (IC_{50} 2.01 ±0.05 mg/mL), and *S. arabica* (IC_{50} 3.70 ±0.08) (Elwekeel et al., 2023).

When CCl_4 was used to induce hepatotoxicity in Sprague Dewaly rats, the alcoholic extracts of *S. volkensii* and *S. villosa* demonstrated hepatoprotective effects with a wide safety margin, suggesting their potential application in the management of liver damage (Nofal et al., 2002).

Aqueous-methanol extracts (80% v/v) of *S. vermiculata* and *S. baryosma*, along with other Algerian herbs, were tested for their antioxidant activity using the Fe^{3+}–TPTZ complex reductive power assay, hydroxyl (OH), nitroxide (NO), and ($ABTS^{+}$) radicals scavenging assays. Despite its low phenolic content, the results demonstrated that *S. baryosma* had the highest antioxidant activity in the OH radical assay, with an EC_{50} of 0.26 ppm (Djeridane et al., 2015). Furthermore, using the DDPH assay, it was found that the crude extracts of *S. vermiculata's* leaves and flowers had high levels of phenolic content and antioxidant activity (IC_{50} values of 36.41 ±1.44 and 37.29 ±1.61 µg/mL), and that their EtOAc fractions had higher activities 12.8, 9.4 (mg GAE/g), respectively, than other fractions. A correlation exists between the presence of phenolic compounds and antioxidant activity (Mollaei et al., 2021a).

Salsola imbricate leaf ethanolic extract has been shown to inhibit and regulate acrylamide-induced liver side effects by controlling oxidative stress, inflammation, and genes linked to apoptosis and antiapoptosis at the biochemical, molecular, and cellular levels. As a result, in high altitude locations, it is advised as an oxidative stress reliever against environmental toxicity (Soliman et al., 2022).

Biphenylsalsonoids A (**62**) and B (**61**) which were isolated from the roots of *S. imbricata* showed a moderate activity towards DPPH with IC_{50} values of 86.5 ±1.3 and 122.3 ±1.4 µg/mL, respectively and ABTS with IC_{50} value of 95.1 ±1.5 and 137.7 ±1.2 µg/mL, respectively. Biphenylsalsonoid A (**62**) has a relatively higher activity due to the presence of two phenol groups (Oueslati et al., 2017). Quercitrin (**5**) and rosmarinic acid (**87**), both isolated from *S.*

imbricata, have been shown to protect against CCl_4-induced hepatotoxicity and have a high antioxidant potential (Shehab et al., 2015).

In vitro DPPH radical scavenging activity of the methanol extract of the aerial parts of *S. tetrandra* exhibited a strong antioxidant activity with an IC_{50} of 24.98 µg/mL comparable with ascorbic acid standard (24.7 µg/mL). This finding agrees with the enrichment of the extract with polyphenols, particularly flavonoids (Rasheed et al., 2013). Tetranins A (**59**) (bibenzyl derivative) and B (**48**) (isoflavonoid) were isolated from the EtOAc extract of *S. tetrandra* roots. They demonstrated a significant antioxidant effect in DPPH free radical scavenging activity and ABTS assays. In DPPH assay, tetranin A (**59**) possessed a higher antioxidative capability than tetranin B (**48**) with IC_{50} of 0.17 mM and 1.09 mM, respectively. In the ABTS assay, tetranin A (**59**) was lightly less antioxidant than tetranin B (**48**) with trolox equivalent antioxidant capacity (TEAC) of 2.39 mM and 2.06 mM, respectively (Beyaoui et al., 2012).

The hydroalcoholic extract from the aerial parts of *S. inermis* exhibited antioxidant activity (Elsharabasy and Hosney, 2013). Methanol and acetone extracts of the aerial parts of *S. tomentosa* have good *in vitro* antioxidant activity by DPPH and *β*-carotene bleaching methods (Mohammadi et al., 2016).

Using (DPPH) colorimetric assay method, the antioxidant activity of *S. cyclophylla* extracts was evaluated. The aqueous-ethanolic extract exhibited the highest potential for scavenging DPPH-free radicals, with activity comparable with quercetin. Using a ferrozine-based assay, the ethyl acetate extract exhibited the highest ferrous ions (Fe^{2+}) chelating activity. The same group reported the antioxidant activity of *S. cyclophylla* essential oils obtained by steam water distillation. The scavenging effect of essential oils was 32% compared with the standard quercetin and trolox. The antioxidant activity may be attributed to the presence of a noticeable proportion of benzoic acid ester derivatives (27.97%) and the ketone hexahydrofarnesyl acetone (27.14%) (Mohammed et al., 2019).

Ferulic acid (**90**) identified in *S. kali* is known for its strong antioxidant activity (Sokolowska-Krzaczek et al., 2009). It decreases the synthesis of cholesterol and lipids levels and protects against coronary disease (Ou and Kwok, 2004). Pretreatment with aqueous extract of *S. kali* (200 mg/kg orally) had a potential antioxidant activity which ameliorated adriamycin (ADR)-induced cardiotoxicity in male Swiss albino mice. The protective mechanisms may be caused by inhibiting lipid peroxidation (LPO) and enhancing antioxidant status in the heart (Aniss et al., 2014). The methanolic extract of different plant parts of *S. kali* was examined for its antioxidant activity using DDPH test, Fe-reduced, and iron chelation. The highest iron chelation activity was

demonstrated by leaf and root extracts (EC_{50} values ranged from 5.85 mg/mL to 6.15 mg/mL) compared to EDTA, while the highest antioxidant activity was demonstrated by leaf and stem extracts (IC_{50} values were 10.67 µg/mL and 11.67 µg/mL, respectively) compared to BHT. The nature and the synergism of the bioactive substances in the extract appear to account for this biological effect (Boulaaba et al., 2019).

Phenolic compounds isolated from *S. foetida; N*-[2'-(3",4"-dihydroxyphenyl)-2'-hy-droxyethyl]-3-(4'"-methoxyphenyl)prop-2 enamide (**99**), *N*-[2'-(3",4"-dihydroxyphenyl)-2'-hydroxyethyl]-3-(3'",4'"-dimethoxyphenyl)prop-2-enamide (**100**) and *N*-[2'-(3"-hydroxy-4"-methoxyphenyl)-2'-hydroxyethyl]3-(4'"-methoxyphenyl)-prop-2- enamide (**101**) exhibited moderate antioxidant activity using DPPH radical scavenging assay with IC_{50} 383, 427 and 378 µM, respectively, compared with 3-(*tert-butyl*)-4-hydroxyanisol and propylgallate as a positive control (Khan et al., 2003).

The phenolic antioxidant constituents in aerial parts of *S. komarovii* extract were determined using the online HPLC coupled $ABTS^+$ based assay (HPLC)-$ABTS^+$ and (HPLC-ESI/MS). Isoquercitrin (**3**), rutin (**6**), isorhamnetin (**8**), and astragalin (**20**) were determined as major anti-oxidant compounds (Lee et al., 2012).

The *in vitro* antioxidant activity of alkaloids extracts of *S. oppositofolia*, *S. soda* and *S. tragus* was determined by the DPPH method using ascorbic acid (IC_{50} 2 µg/mL) as a positive control. The results revealed a significant antioxidant with an IC_{50} value of 16.3 µg/mL for *S. oppositifolia*. In comparison, *S. soda* and *S. tragus* extracts exhibited an IC_{50} value of 24.3 µg/mL and 26.2 µg/mL, respectively (Tundis et al., 2009).

Significant antioxidant activity of the aqueous ethanolic extract of *S. cyclophylla* aerial parts is expressed as DPPH free radicals scavenging reactivity at IC_{50} 0.615 ±0.06 mg/mL (Mohammed et al., 2021).

Polysaccharide fractions that were isolated from the aerial parts of *S. passerine* showed moderate antioxidant activity. In comparison to commercial apple pectin, fractions isolated with cold water and sodium carbonate were significantly more effectively scavenged the DPPH radical at 0.2-1.8 mg/mL. Anionic exchange chromatography's correlation analysis revealed a relationship between the amount of phenolic compounds present in polysaccharide fractions and their ability to scavenge DPPH (Golovchenko et al., 2022).

Antioxidant activity of aerial parts of *S. tetragona* fractions (*n*-hexane, DCM, EtOAc, and *n*-BuOH) was assessed using DPPH, anti-Hemolysis, and ferric reducing capacity (FRAP) assays. In the DPPH test, the DCM fraction exhibited the highest level of significant antioxidant activity

with IC_{50} 62 ±4.10 µg/mL, while the *n*-hexane fraction performed better in the antihemolysis test, with an IC_{50} of 200 ±2.10 µg/mL. The EtOAc fraction demonstrated the highest level of antioxidant activity in the FRAP test, with an IC_{50} of 910 ±30 µg/mL (Cherrada et al., 2023a). Furthermore, using various tests such as DPPH, $ABTS^{+}$, Cupric Reducing Antioxidant Activity (CUPRAC), and metal chelating assays, the crude hydromethanolic extract and the five fractions (*n*-hexane, DCM, EtOAc, *n*-BuOH, and aqueous) demonstrated significant antioxidant activity, which was expressed by IC_{50} values. In the DPPH and $ABTS^{+}$ tests, the DCM fraction demonstrated the highest level of activity, with IC_{50} values of 62.54 ±0.48 µg/mL and 19.11 ±0.70 µg/mL, respectively. With an IC_{50} of 116.74 ±2.39 µg/mL, the EtOAc fraction was superior to the *n*-hexane fraction in the metal chelating activity test, while the latter had the best IC_{50} of 95.13 ±0.69 µg/mL. For the metal chelation test, the outcomes were compared with those of α-tocopherol, BHA, and EDTA. With higher IC_{50} values, the antioxidant activity is less powerful (Cherrada et al., 2023b). As a result, the type and concentration of phenolic and flavonoid biocompounds, such as ascorbic acid, quercetin (**1**), and other antioxidant molecules, may be responsible for the fractions' antioxidant capacity.

S. soda afforded quercetin-3-*O*-glucuronopyranoside (**3**), rutin (**6**), isorhamnetin-3-*O*-glucuronopyranoside (**10**), and isorhamnetin-3-*O*-rutinoside (**13**) as major constituents. These compounds proved to be helpful in the management of diabetic problems, inflammatory diseases, and medication resistance to anthracycline-based anti-cancer therapy (Iannuzzi et al., 2020).

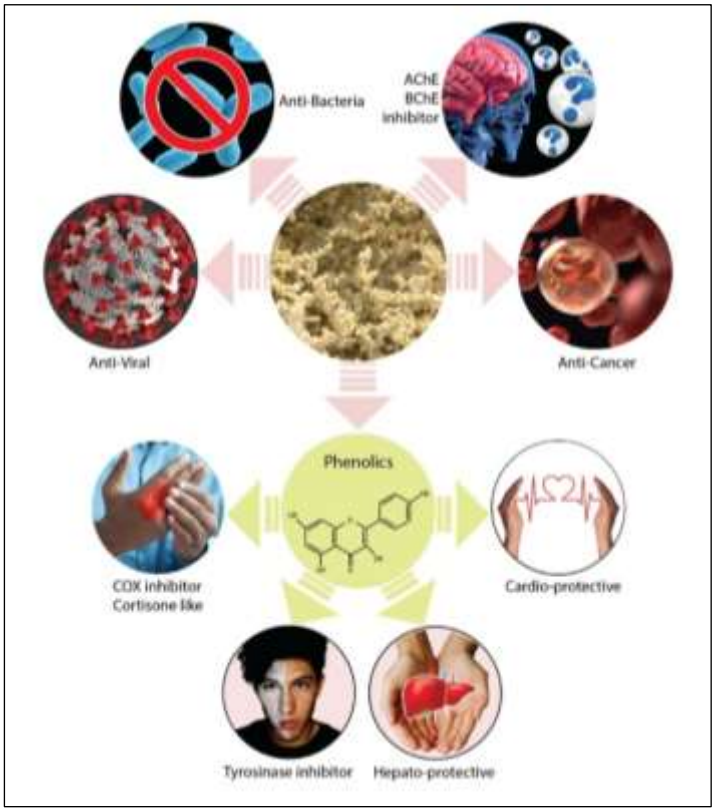


Figure 2.16: Important biological activities of genus *Salsola* (Murshid et al., 2022).

2.4.6. Contraceptive Effect

It is usually possible to classify contraceptive methods as either traditional or modern. Herbal medicine has always supported the potential health benefits of plants. Today, they are highly regarded as a source of safe phyto-pharmaceuticals (Shehab and Abu-Gharbieh, 2014).

The contraceptive activity of *Salsola* plants was first described by Ploss in 1960. He reported the use of the aqueous extract of an undefined *Salsola* sp. as an oral contraceptive in Algeria (Swart et al., 2003). It was reported that the compound responsible for this activity was reported to be a labile synephrine analogue with a reactive aziridine group. Therefore, they synthesized the compound, 2–(4-acetoxyphenyl) 2-chloro-*N*-methylethylammonium-chloride, as a stable analogue for the active principle of *S. tuberculatiformis*. This compound was found to disturb the mammalian steroid hormones homeostasis and to inhibit adrenal steroid-genesis (Swart et al., 2003).

Oral administration of the ethanolic extract (cold maceration in 70% ethanol) of the whole plant of *S. imbricata* at two doses (250 and 500 mg/kg b. wt) over a 65-day period was used to examine the contraceptive effect in male albino rats. It was reported to cause a slight decrease in the testis weight and to cause a significant decline in the sperm count suggesting its potential use

as a reversible male contraceptive. Prior to biological evaluation, an acute toxicity study was conducted to ensure its safety. It was found to be safe up to dose 5g/kg. The male contraceptive activity was related to its phenolic contents, especially quercitrin (**5**) (Shehab and Abu-Gharbieh, 2014).

2.4.7. Antispasmodic and Bronchodilator Activity

Constipation and indigestion are two of the most frequent ailments. Constipation affects up to 27 % of the population, while indigestion affects 11%–29.2% of the population (Mahadeva and Goh, 2006; Cheng et al., 2009). There is growing evidence that several compounds present in medicinal plants could treat gastrointestinal diseases like indigestion and constipation in a synergistic manner (Sangiovanni et al., 2015; Rtibi et al., 2016). Furthermore, medicinal plants are thought to be generally safe and beneficial when used for a long time, particularly in individuals with chronic gut motility issues.

Ethyl acetate extract of *S. collina* has significant prokinetic activity. It was effective *in vivo*, in promoting gastric emptying and small intestinal propulsion in normal Male Sprague Dawley rats, showing a dose-dependent manner *via* a mechanism that mainly involves in modulating plasma ghrelin and gastrin, expressions of vasoactive intestinal peptide receptor 2 in the duodenum. *In vitro*, atropine promoted the contraction of both normal and relaxed gastric antrum strips; thus, activating M-cholinergic receptor. This establishes a pharmacological foundation for treating gastrointestinal motility problems with *S. collina* extract (Wang et al., 2020b).

Total extract as well as EtOAc and aqueous fractions of *S. imbricata* caused relaxation effect on gut and tracheal tissues through $Ca^{\gamma+}$ antagonist as well as *β*-adrenergic receptor agonist effects. This explains its medicinal value in gastrointestinal and respiratory problems like stomach colic, diarrhea, cough, and asthma (Aslam and Janbaz, 2017). The ethyl acetate fraction was found to be more effective in relaxing smooth muscle spasms than the original extract and its aqueous fraction.

The 80% ethanol extract of the whole plant of *S. baryosma* growing in Cholistan desert demonstrated antispasmodic activity in isolated rabbit jejunum preparations. When compared to the control verapamil, it likewise suppressed K^+-induced contractions by 70% at 1–5 mg/mL, implying a calcium channel blocking activity (Ahmed et al., 2006).

2.4.8. Antiulcer Activity

GIT disorders, which are among the leading causes of human illness, are widespread public health issues worldwide (Mehmood et al., 2011). Numerous effects on the gastrointestinal tract have been reported for various plants in the *Salsola* genus, including anthelmintic, antispasmodic, and gastroprotective activity against ulcer. *S. imbricata* has a legendary reputation for treating a variety of gastrointestinal problems (Aslam and Janbaz, 2017). In Swiss albino mice, ethanol crude extract of *S. imbricata* aerial parts was found to alleviate acetic acid-induced inflammatory bowel disease (IBD) as ulcerative colitis (UC) by modulating dysregulated antioxidant enzyme system (supporting antioxidant enzymes as SOD and GPX-1, decreasing inflammatory cytokines mediators as (IL)-Iβ, IL-6, TNF-α signalling pathways and promoting the repair of epithelial layers by normalizing the structure integrity of cells and reducing the damage of the epithelial mucosa. It was determined that the maximum dosage of 5000 mg/kg, p.o. was safe. The presence of quercetin (**1**), benzoic acid (**74**), gallic acid (**76**), syringic acid (**77**), and chlorogenic acid (**93**) is linked to this gastroprotective and anti-inflammatory action (Javed and Jabeen, 2021).

The alcoholic extract (70% alcohol in H_2O) of the aerial parts of *S. tetrandra* showed an ulcer protective effect like that of ranitidine against aspirin-induced gastric ulceration in rats in a dose-dependent manner. The ulcer index decreased significantly ($p < 0.05$) in the *Salsola*-treated rats, according to histopathological and histochemical data. In contrast, stomach mucus production increased while mucosa erosion decreased (Elsharabasy et al., 2015).

The ameliorating effect of 500 mg/kg of 50% alcohol extract of *S. komarovii* against gastritis and gastric ulcers induced by HCl-ethanol- gastritis model was studied. It has inhibitory effects against gastritis and gastric ulcers that were more potent than 300 mg/kg of ranitidine and could be used to develop a novel antigastric ulcer medication (Hong et al., 2014).

2.4.9. Anthelmintic Activity

The isoflavonoids 5,3'-dihydroxy-7,8,2'-trimethoxyisoflavone (**32**), 5,3'-dihydroxy-2'-methoxy-6,7-methylenedioxyisoflavone (**33**), and 5,3'-dihydroxy-6,7,8,2'-tetramethoxyisoflavone (**34**) were isolated from the *S. somalensis* roots and showed modest anthelmintic effect in earthworms (Abegaz and Dagne, 1978; Woldu and Abegaz, 1990). The anthelmintic activity of *S. imbricata* bark extract was observed against *Haemonchus contortus* worms (Ajaib et al., 2019).

2.4.10. Cytotoxic Activity

A few studies have been conducted to examine the cytotoxic activity of *Salsola* spp., such as *S. cyclophylla, S. oppositifolia, S. collina*, and *S. baryosma*. The MTT assay was used to examine the cytotoxic activity of a 95% aqueous-ethanolic extract of *S. cyclophylla's* aerial parts against M14 melanoma derived epithelial breast cancer (MDA cells), human pancreatic cancer (PANC-1), breast cancer cells (MCF-7) and normal human fibroblast cells. Only at high concentrations (50–400 mg/mL) of the aqueous ethanolic extract of *S. cyclophylla* exhibited low to moderate cytotoxic activity against the tested cell lines; at low concentrations (< 50 mg/mL), no discernible cytotoxic effect was seen (Mohammed et al., 2021).The ethanol extract of *S. collina* has been shown to have anti-cancer properties on human colon carcinoma HT29 cells in a dose-dependent manner by cell cycle regulation (Oh et al., 2014) and caused cell cycle arrest in the G2/M phase. Different fractions (*n*-hexane, CH_2Cl_2, EtOAc and diethyl ether) and isolated flavonols (from EtOAc fraction) from *S. oppositifolia* aerial parts were evaluated for their cytotoxic activity against five human tumor cell lines, renal adenocarcinoma ACHN, hormone-dependent prostate carcinoma LNCaP, human breast adenocarcinoma MCF-7, amelanotic melanoma C32 and large cell lung carcinoma COR-L23. *n*-Hexane fraction was more selective against lung carcinoma compared with amelanotic melanoma cell lines with IC_{50} values of 19.1 µg/mL and 24.4 µg/mL, respectively. Lower activity was found against renal adenocarcinoma and hormone-dependent prostate carcinoma cells (IC_{50} values of 43.4 µg/mL and 45.1 µg/mL, respectively). Also, the dichloromethane fraction showed the most interesting biological activity on large cell lung carcinoma (IC_{50} 30.4 µg/mL) and amelanotic melanoma cells (IC_{50} 33.2 µg/mL). Against renal adenocarcinoma and hormone-dependent prostate cancer cells, comparable results to the *n*-hexane fraction were found (IC_{50} values of 40.4 µg/mL and 41.9 µg/mL, respectively). Meanwhile, the EtOAc fraction exhibited a cytotoxic activity with IC_{50} values ranging from 56.4 µg/mL against amelanotic melanoma to 88.6 µg/mL against renal adenocarcinoma cells. Interestingly, it demonstrated a selective cytotoxic activity against human breast adenocarcinoma cells (IC_{50} 67.9 µg/mL) compared to other fractions. The major active constituents of this fraction were isorhamnetin-3-*O*-glucoside (**9**) and isorhamnetin-3-*O*-rutinoside (**13**) which showed an interesting activity against human breast adenocarcinoma cell line with IC_{50} values of 18.2 and 25.2 µg/mL, respectively. Moreover, isorhamnetin-3-*O*-glucoside (**9**) showed good cytotoxic activity against the renal adenocarcinoma and the hormone-dependent prostate carcinoma cells with IC_{50} values of 26.1 and 28.5 µg/mL, respectively. Isorhamnetin-3-*O*-rutinoside (**13**) exhibited potent activity against the hormone-dependent prostate carcinoma cell line with an IC_{50} of 20.5 µg/mL. Diethyl ether fraction was selective against the renal adenocarcinoma cell line (IC_{50} values of 46.8 µg/mL). The remarkable cytotoxic

effect of the two non-polar fractions (*n*-hexane and diethyl ether), specifically against COR-L23 and C32 cells, may be attributed to the presence of fatty acids and methyl esters, based on their chemical makeup (Tundis et al., 2008).

The IC_{50} of the ethyl acetate fraction of the whole plant of *S. baryosma* was determined using a brine shrimp assay and the number of larvae survived after the addition of various amounts of test sample using permethrin (236 g/cm^3) as a standard was calculated to be 1mg/mL. On the other hand, all fractions of *S. baryosma* (ethanol 80%, *n*-hexane, EtOAc and *n*-BuOH) were found phytotoxic to a varying degree from 52% to 100% which was assessed by inhibition of growth of Lemna minor plant in a dose-dependent manner using paraquat as standard drug (0.9025 µg/mL) (Ahmed et al., 2006).

With IC_{50} values of 33.88 µg/mL and 42.92 µg/mL, respectively, the crude extracts of *S. vermiculata's* seeds and roots, respectively showed significant cytotoxic activity against the A549 cell line in the MTT assay. Only the fixed oil fractions demonstrated cytotoxic activity, which may have been related to the presence of linoleic (**170**) oleic (**178**), and palmitic acids (**180**) (Mollaei et al., 2021a).

The human skin fibroblast cells CCD-1079Sk and the breast cancer cell line MCF-7 were used in the cytotoxicity test of the crude extract and the five fractions (*n*-hexane, DCM, EtOAc, *n*-BuOH, and aqueous) of *S. tetragona* aerial parts. The DCM fraction exhibited remarkable efficacy and outperformed the other fractions in a concentration-dependent manner, with IC_{50} values of 64 µg/mL and 98 µg/mL, respectively. The effects on cell migration and viability were assessed using the MTT test and the *in vitro* scratch assay. To determine the precise cytotoxic effects of these substances, more research on different cell lines is necessary (Cherrada et al., 2023b).

The inhibitory effect of *S. komarovii* crude extracts (CH_2Cl_2 and MeOH) (SKI) and four fractions (*n*-hexane, 85% aq. MeOH, *n*-BuOH, and H_2O) on matrix metalloproteinases (MMPs), such as MMP-2 (gelatinase A) and MMP-9 (gelatinase B), was examined by (Kil et al., 2020). According to the findings, SKI included MMP inhibitory compounds with potentially distinct action mechanisms and distinct molecular structures. The gelatin zymography assay revealed that 85% aq. MeOH fraction was highly effective in suppressing the enzymatic activity of MMP-2 and MMP-9. Additionally, the *n*-hexane fraction, which was marginally more effective than 85% aq. MeOH, downregulated the expression of mRNA and protein levels. Overall, the *n*-BuOH fraction was the most effective at suppressing the MMP-2 and MMP-9 levels, with *n*-hexane coming in second. The aqueous fraction performed the lowest out of all the fractions tested in terms of

suppressing MMP-2 and MMP-9 expression, release, and activity. Taking into account the chemical structures of the previously identified components from *S. komarovii* extracts (Cho et al., 2014), it was suggested that polyphenolic and flavonoid ingredients of SKI, which were suggested to be abundant in 85% aq. MeOH fraction of halophyte extracts (Trabelsi et al., 2010; Faustino et al., 2019), were responsible for direct enzymatic inhibition of MMP-2 and MMP-9. However, it was suggested that the *n*-hexane fraction contained derivatives of coumarin and quercetin, which were isolated from both methanol and *n*-hexane extracts (Vilegas et al., 1997). These derivatives were responsible for the downregulation of intracellular MMP expression and release. The results of this study indicate that *S. komarovii* may be a source of inhibitors against MMP-2 and MMP-9, two important enzymes involved in chronic inflammation and cancer metastasis. However, more research is required to clarify the bioactive components of active extracts and describe their mechanisms of action. This will help to improve the use of *S. komarovii* as a natural product.

Finally, taxiphyllin (**229**) from *S. tetrandra* showed high cytotoxic activity in the *Artemia salina* lethality bioassay with ED_{50} value of 0.96 µM (Oueslati et al., 2006). Likewise, most cytotoxic results are based on cell-based inhibition with no tumor xenografted animal model to prove efficacy and should be considered as the next step.

2.4.11. Vasoactivity Effect

Salsola sp. has been known to exhibit antihypertensive properties since its early pharmacological studies. Alkaloids salsoline (**110**) and salsolidine (**112**) have been isolated from *S. kali* and used in the treatment of hypertonia, hypertension and headache (as hydrochloride) by stimulating the activity of sleep and as a nervous system tonifier (Sokolowska-Krzaczek et al., 2009). Also Ammon *et al*. attributed the antihypertensive activity of *S. kali* and *S. longifolia* Forsk to salsoline (**110**) and salsolidine (**112**) due to their ability to stimulate respiration and to decrease blood pressure (Ammon et al., 1987).

Captopril was used as a reference ACE inhibitor to examine ethyl acetate extracts of the aerial parts of *S. oppositifolia*, *S. soda*, and *S. tragus* for their hypotensive activities. With IC_{50} values of 181.04 and 284.27 g/mL, *S. oppositifolia* and *S. soda* showed an interesting suppression of ACE activity, respectively. *S. tragus*, on the other hand, showed minimal action, with an inhibition percentage of 36.21± 0.4 %. Furthermore, using water as a negative control, a gelatin salt block test was used to reduce the false positive effect caused by tannins. Thus, tannins are not the only factor in the efficacy of *S. oppositifolia* and *S. soda* EtOAc extracts to inhibit ACE (Loizzo et al., 2007).

2.4.12. Hypoglycemic Effect

A popular therapeutic strategy for the treatment of diabetes is to inhibit digestive enzymes that are involved in the hydrolysis of carbohydrates, such as α-amylase and α-glucosidase, in order to reduce post-prandial hyperglycemia. Consequently, an extensive amount of research was done on the inhibitory effects of α-amylase and α-glucosidase on various *Salsola* species (Tundis et al., 2007; Djeridane et al., 2015).

The hypoglycemic effects of methanol extract of the aerial parts of *S. kali*, *S. soda*, and *S. oppositifolia* were evaluated using an *in vitro* assay based on suppression of the α-amylase digesting enzyme. Ethyl acetate fraction of the extract was the most active with IC_{50} value of 0.022 mg/mL. The bioassay-guided chromatographic separation of this most active fraction resulted in the isolation of two flavonol glycosides, of which isorhamnetin-3-*O*-rutinoside (**13**) displayed significant α-amylase inhibitory activity with an IC_{50} value of 0.129 mM (Tundis et al., 2007).

α-Glucosidase inhibitory activity of *S. vermiculata* crude extracts was examined *in vitro* (Mollaei et al., 2021b). The ethyl acetate fraction of leaves showed the strongest inhibitory activity (IC_{50}: 62.37 µg/mL) followed by the flowers (IC_{50}: 101.61 mg/mL) duo to its richness of flavonoids as quercetin (**1**) and rutin (**6**). There was no inhibitory activity observed in the root or seed crude extracts.

Aquous-methanol extracts of *S. vermiculata* and *S. baryosma* was tested for its ability to inhibit the activities of the enzymes α-amylase and α-glucosidase (Djeridane et al., 2015). *S. baryosma* showed the highest level of competitive inhibitory activity against α-amylase and α-glucosidase, with inhibition constants (Ki) of 7 and 16 mM, respectively. These values suggested that *S. baryosma* may be useful in the management of type 2 diabetes.

In addition, *N*-acetyltryptophan (**121**), which is a derivative of amino acid and was isolated from *S. collina*, showed moderate inhibition of α-amylase activity using the Caraway iodine/potassium iodide (IKI) method (Jin et al., 2011).

The chemical profile of *S. soda's* cultivated buds was examined and compared with the plant's natural profile (Iannuzzi et al., 2020). Additionally, the compounds isolated from their *n*-BuOH fraction were evaluated for inhibitory activity against three aldo/keto reductase superfamily enzymes: carbonyl reductase 1 (*h*CBR1), aldose reductase (*h*AKR1B1), and aldose-reductase-like protein (*h*AKR1B10). The only flavonoid found in both plant types, quercetin-3-*O*-glucuronopyranoside (**3**), was shown to be the most effective inhibitor of the enzymes tested, and its use as a functional nutraceutical to prevent diabetic complications was recommended.

The fruit, roots, bark, and leaves of *S. tetragona* are usually used in powder or decoction form in management of diabetes (Ghourri et al., 2013). Aerial parts of *S. tetragona* was investigated for its ability to prevent diabetes, through studying its effect on α-amylase and α-glucosidase (Cherrada et al., 2023a). The *n*-butanol and ethyl acetate fractions showed the highest degree of inhibition on α-amylase, according to the results ($p < .05$), with an estimated IC_{50} value of 70 ± 1.80 µg/mL and 70 ±1.30 µg/mL, respectively. This inhibitory capacity was comparable to that of the acarbose control, which had an activity level of 62 ±2.80 µg/mL. At the same time, dichloromethane and hexane fractions displayed lower inhibitory activity ($p < .05$), with IC_{50} values of 124 ±5.10 µg/mL and 130 ±0.40 µg/mL, respectively. The ethyl acetate and butanol fractions showed similar inhibitory activity ($p <.05$) in relation to the α-glucosidase enzyme's inhibitory properties, with an IC_{50} value of 70 ±1.80 µg/mL. Compared to the acarbose control, which had an IC_{50} value of 60 ±1 µg/mL. The *n*-hexane and dichloromethane fractions' inhibitory activity were similar ($p < .05$), with IC_{50} values of 130 ±1.20 µg/mL and 132 ±3.20 µg/mL, respectively. Therefore, the type of solvent used to extract the compounds as well as the quantitative and qualitative amounts of active natural compounds (polyphenol and flavonoids) are mainly responsible for the inhibitory effect of *S. tetragona* fractions. This discovery opens the door to the development of medications for diabetes and related disorders and supports its long-standing use in the treatment of the disease.

2.4.13. Anti-acetylcholinesterase and Anti-butyrylcholinesterase Activity

Triterpene salsolic acid (**140**) isolated from the chloroform fraction of *S. baryosma* showed inhibitory activity against the enzyme butyrylcholinesterase (BChE) ((Ahmad et al., 2007; Ahmad et al., 2008b). Moreover, amino acid derivative, *N*-acetyltryptophan (**121**), which was isolated from the ethanolic extract of the aerial parts of *S. grandis*, displayed a marked inhibitory activity against acetylcholinesterase (AChE) (64.90 ± 1.61%) at a dose of 50 µg/mL using a microtiter assay. Moreover, molecular modelling experiments have been performed for the interactions of *N*-acetyltryptophan (**121**) at atomic level with AChE using *in silico* experiments. Thus, *N*-acetyltryptophan (**121**) could be a valuable preclinical molecule for AChE inhibitors with neuroprotective potential, especially in the treatment of Alzheimer's disease (AD) (Orhan et al., 2017).

In addition, *S. vermiculata's* acetylcholinesterase inhibiting activity was measured using a microplate colorimetric technique. According to the findings, the EtOAc fractions of leaves and the aqueous-acid fraction of roots had the strongest inhibitory activity (IC_{50}: 17.24 µg/mL) due to their high concentration of phenolic and alkaloids components (Mollaei et al., 2021b). Moreover, due to high catecholamines in their roots, it could also inhibit AChE-with an IC_{50}

value of 0.45 ± 0.17 mg/mL comparable with that of eserine (physostigmine) (Rasheed et al., 2013).

Moreover, alkaloid fractions prepared from *S. oppositofolia*, *S. soda*, *S. tragus* aerial parts showed promising activity against AChE and BChE enzymes. The activity of *S. tragus* was the highest against AChE and BChE (with IC_{50} of 30.2 g/mL and IC_{50} of 26.5 g/mL, respectively). Meanwhile, with IC_{50} values of 34.3 g/mL and 32.7 g/mL, respectively, *S. soda* and *S. oppositifolia* alkaloid fraction had a specific inhibitory action against BChE. The high activity of *S. tragus* against AChE and BChE enzymes could be referred to its high contents of alkaloids salsoline (**110**) (36.5%) and salsolidine (**112**) (17.7%) (Tundis et al., 2009).

The AChE enzyme inhibition was investigated by Cherrada *et al*. on various fractions derived from the aerial parts of *S. tetragona*. The findings showed that the fractions had a strong inhibitory capacity when compared to galantamine as a positive control. The ethyl acetate fraction showed the most significant levels of inhibition with an IC_{50} value of 30 ±0.30 µg/mL, which was comparable to and slightly less than the inhibitory potency of galantamine, 28 ±0.30 µg/mL. The *n*-butanol fraction exhibited a somewhat lower inhibitory capacity with an IC_{50} value of 32 ±0.60 µg/mL. The dichloromethane and hexane fractions also displayed inhibitory capacity, with IC_{50} values of 60 ±0.10 µg/mL and 63 ±0.60 µg/mL, respectively. Plant fractions of *S. tetragona* are thought to exhibit inhibitory effects due to the presence of active compounds, specifically flavonoids and polyphenols. Unlike the well-known inhibitor galantamine, these substances had been demonstrated to inhibit AChE through a variety of different mechanisms (Cherrada et al., 2023a).

Other components in the *Salsola* matrix with higher specific activity may, however, perform additively or synergistically, and may eventually be relevant for the anti-acetylcholinesterase effect (Rasheed et al., 2013). However, more *in vivo* studies are needed to completely understand the mechanism of enzyme inhibition, the extent to which the body absorbs and uses the substances, and their metabolic pathways (Cherrada et al., 2023a).

2.4.14. Neuroprotective Activity

Exogenous nerve growth factor (NGF) improves the cholinergic neuron system and offers therapeutic potential for neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, and diabetic polyneuropathy (Aloe et al., 2012). Nineteen isolated compounds from the MeOH extract of the aerial parts of *S. komarovii* were tested on C6 glial cells to see how they affected NGF induction. Cell viability was determined by MTT assay, and 6-shogoal was used as a positive control. (*8S,8′R,7′R*)-9'-[(*β*-glucopyranosyl)oxy] lyoniresinol (**197**) was stimulant for

NGF secretion in C6 cells (127.3 ±10.3%) but was cytotoxic at low concertation. Also, alangilignoside C (**199**), conicaoside (**200**) and blumenyl B *β*-D-glucopyranoside (**206**) were found to upregulate (NGF) secretion without significant cell toxicity. The most effective stimulator of NGF release, conicaoside (**200**) may have neuroprotective properties through stimulating NGF secretion (Cho et al., 2014).

2.4.15. Tyrosinase Inhibitory Activity

The three isolated phenolic compounds, *N*-[2'-(3",4"-dihydroxyphenyl)-2'-hydroxyethyl]-3-(4"'-methoxyphenyl)prop-2-enamide (**99**), *N*-[2'-(3",4"-dihydroxyphenyl)-2'-hydroxyethyl]-3-(3"',4"'-dimethoxyphenyl)prop-2-enamide (**100**) and *N*-[2'-(3"-hydroxy-4"-methoxyphenyl)-2'-hydroxyethyl]3-(4"'-methoxyphenyl)-prop-2-enamide (**101**) from the whole plant of *S. foetida* were studied on mushroom tyrosinase inhibition. They exhibited pronounced tyrosinase inhibition activity with IC_{50} 2.61, 1.85, and 0.40 µM, respectively. As a result, *S. foetida* can be utilized to treat disorders like hyperpigmentation caused by excessive melanocyte production (Khan et al., 2003).

2.4.16. Other Activities

Many species of this genus can act as an allergenic substance (Assarehzadegan et al., 2009). *S. baryosma* is used as a diuretic agent, vermifuge, and its ash is applied to itches (Al-Saleh et al., 1997). In comparison to the standard insecticidal compound, cypermethrin (which causes 37.64% mortality), the ethanol extract of *S. baryosma* was reported to cause moderate insecticidal activity (22.08% mortality) against *Trogoderma granarium* insects (Everts) (Mansoor-ul-Hassan et al., 2005). Furthermore, an aqueous extract of *S. collina* is an effective means of cholelithiasis prophylaxis by (i) inhibiting the development of inflammation in the mucous membrane of the gallbladder against the background of an aggressive atherogenic diet; (ii) favoring cholesterol absorption by the mucous membrane of the gallbladder; (iii) stimulating the absorption of water, thus maintaining a high concentration of bile acids in the gallbladder bile; (iv) preventing the precipitation of calcium allodeoxycholate crystals and the formation of a biliary slough (Nikiforov et al., 2002). Remarkably, *S. laricifolia* Turcz is said to be one of the medications that strengthens the immune system, and a pharmaceutical product that comes from it called "Salimon" is among the most popular immunostimulant medications in the Mongolian pharmaceutical market (Cooper and Deakin, 2020).

2.4.17. As a Fodder

Given the potential effects of climate change, global conditions are strongly pressuring us to alter our dietary habits. Conventional edible plants are struggling due to a variety of issues, including

salinization leading to soil degradation, a shortage of high-quality irrigation water, rising temperatures, and many others. In the arid and semi-arid regions, the situation is considerably more concerning, with issues growing more urgently (Slama et al., 2015). *Salsola* species, especially in the autumn and winter in deserts, can be utilized as a partial substitute for feed concentrates. The aerial parts of *S. cyclophylla*, which grow in marshy areas of central Saudi Arabia, are used for both medicinal and feeding purposes frequently (Mohammed et al., 2019) as a potential alternative food supply during food shortages and drought times (Mohammed et al., 2021) and as nutraceuticals which was corroborated with the richness of phytoconstituents such as flavonoids and phenols (Mohammed et al., 2019). *S. arbusculiformis* has comparatively high crude protein content, digestibility, and metabolic energy during vegetative growth, therefore it might make a good fodder resource for sheep and goats in arid environment (Asaadi et al., 2014). Moreover, *S. arbuscula* might make the best feed due to the large amount of crude protein and water-soluble carbohydrates (Abtahi and Zandi Esfahan, 2017).

Moreover, *Salasola* species are a promising camel feed in Pakistan's Cholistan desert (Ali et al., 2009). Its development as a viable fodder species in arid regions was aided by a number of characteristics such as excellent nutritional qualities, prolific seed production, resistance to high temperatures, and long-term drought tolerance (Fowler et al., 1992).

2.5. The Choice of *Salsola kali* for this Study

Salsola kali L. (**Figures 2.17-2.19**) belongs to the Chenopodiaceae family showing xerophytic habits. In comparison to the other species, *S. kali* has entirely distinct anatomical features. Argentina, Chile, Canada, Mexico, South Africa, Indonesia, Australia, New Zealand, and the United States have all reported *S. kali* as an alien weed (Crompton and Bassett, 1985; Beckie and Francis, 2009). Because of its value as fodder, the US Department of Agriculture introduced it as cattle food (Khan and Qaiser, 2006). It can be found in inland sandy-soil habitats, as well as in deserts and waste areas. Beyond its ecological significance, *Salsola kali* holds medicinal and nutritional value. Traditional medicine systems have utilized various parts of the plant for their therapeutic properties. It is believed that *S. kali* possesses diuretic, anti-inflammatory, and antiseptic properties, making it useful in treating conditions such as urinary tract infections, rheumatism, and skin ailments. Additionally, some communities have incorporated *S. kali* into their diets, as it is a source of dietary fiber, vitamins, and minerals. Its consumption can contribute to a balanced diet, particularly in regions where food resources are limited.

Even though this plant contains the different valuable phytoconstituents (**Table 2.2**), *S. kali* growing in Saudi Arabia has not received much attention in the literature. Taking this into

consideration, along with the numerous chemical classes that have been previously documented from this plant, I carried out a phytochemical evaluation of the aerial parts of *S. kali* plant from Saudi Arabia.

Figure 2.17: General view of *Salsola kali* used in this study.

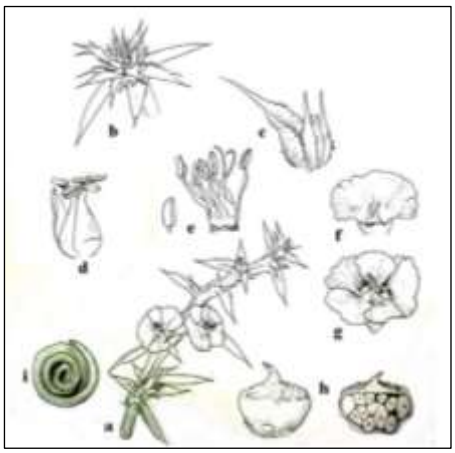


Figure 2.18: *Salsola kali* flower morphology (**a**) branch, (**b**) terminal glomerule, (**c**) bud with bract and bracteoles, (**d**) flower with and without tepals, (**e**) anthers, (**f**) tepal at fruiting stage, (**g**) perianth at fruiting stage, (**h**) entire and longitudinal section of the fruit, (**i**) seed (embryo) (Arafeh, 2005).

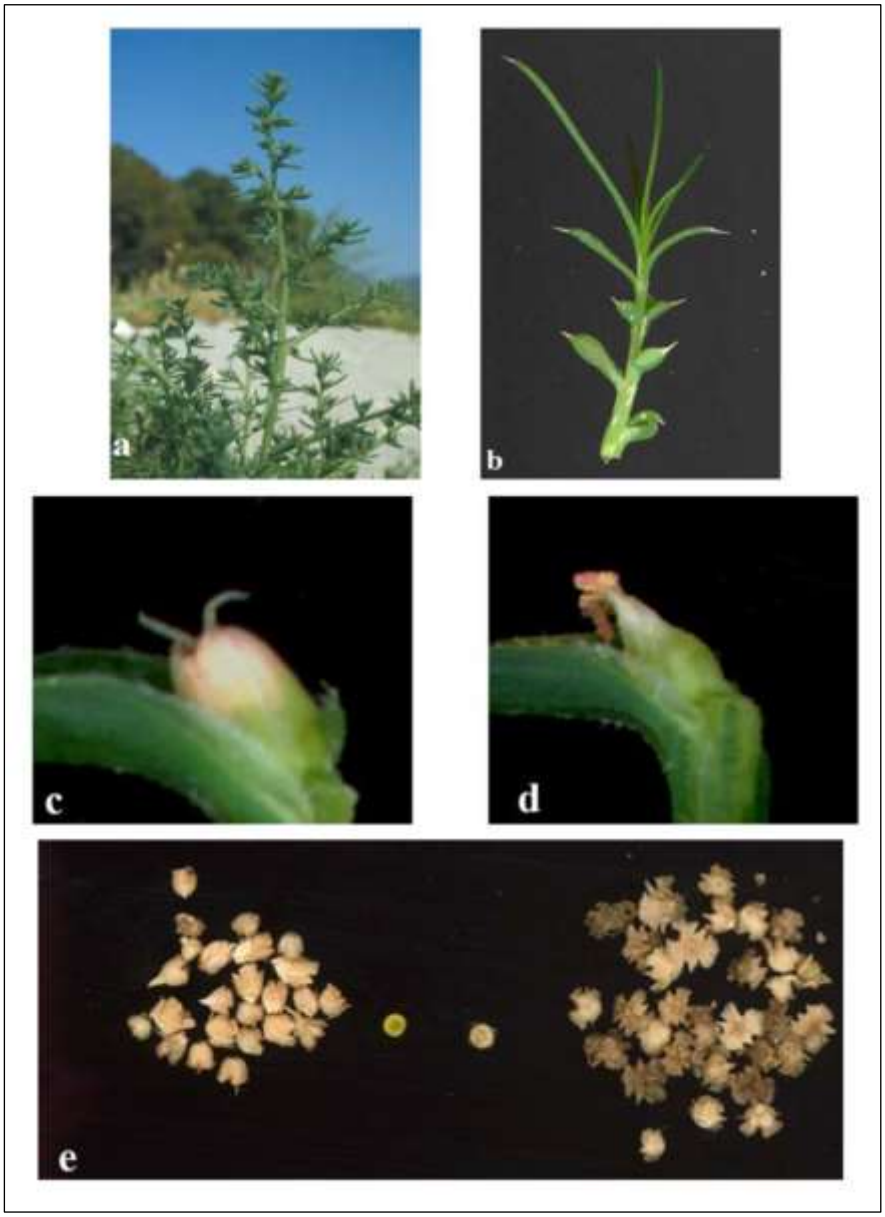


Figure 2.19: *Salsola kali* morphology (**a**) intact plant, (**b**) morphological plasticity of *S. kali*. (**c-d**) flowering phenology of *S. kali* where stigma lopes appear first then anthers, (**e**) un-winged fruits (left), seeds (middle), and winged fruits (right) collected from the same plant (Arafeh, 2005).

Table 2.2: A summary of compounds isolated previously from *Salsola kali*

Isolated compounds	Chemical class	Reference
ferulic acid protocatechuic acid caffeic acid gentisic acid *p*-hydroxycinnamic acid *p*-hydroxybenzoic acid *p*-hydroxyphenylacetic syringic acid vanillic acid α and β-resorcylic hypogallic acid gallic acid chlorogenic acid isochlorogenic acid neochlorogenic acid	Phenolic acid compounds	(Lodhi, 1979; Tundis et al., 2007; Xiang et al., 2007b; Sokolowska-Krzaczek et al., 2009; Jin et al., 2011; Shehab and Abu-Gharbieh, 2014; Osman et al., 2016; Ghorab et al., 2017; Taia et al., 2018; Boulaaba et al., 2019; Zhao et al., 2020)
lupeol ursolic acid	Triterpene	(Alturkistani et al., 2017)
sitostanol, stigmasterol avenasterol β-sitosterol β-sitosterol-3-*O*-glucoside	Sterols	(Karawya et al., 1972; Salt and Adler, 1985; Syrchina et al., 1989; Zaikov et al., 1992; Elsharabasy and Hosney, 2013; Aniss et al., 2014; Alturkistani et al., 2017)
salsoline salsolidine *N*-methylisosalsoline carnegine	Alkaloids	(Loizzo et al., 2007; Tundis et al., 2008; Tundis et al., 2009; Glushchenko et al., 2015; Boulaaba et al., 2019)
quercetin kaempferol quercetin-3-glucoside quercetin-3-rhamnoside quercetin-3-rutinoside quercetin-3-glactoside isorhamnetin-3-*O*-glucoside isorhamnetin -3-*O*-rutinoside rhamnetin luteolin-7-*O*-glucoside	Flavonoids and their glycoside	(Lodhi, 1979; Syrchina et al., 1989; Zaikov et al., 1992; Tundis et al., 2007; Xiang et al., 2007b; Jin et al., 2011; Kaur and Bains, 2012; Lee et al., 2012; Rasheed et al., 2013; Shehab and Abu-Gharbieh, 2014; Shehab et al., 2015; Küçükboyacı et al., 2016; Ghorab et al., 2017; Orhan et al., 2017; Taia et al., 2018; Boulaaba et al., 2019; Iannuzzi et al., 2020)
linolenic acid oleic acid arachidonic acid palmitic acid stearic acid	Fatty acid	(Karawya et al., 1972; Zaikov et al., 1992; Tundis et al., 2008; Rasheed et al., 2013; Elsharabasy et al., 2015; Ghorab et al., 2017)
triacetonamine betaine methyl carbamate catechol fraxidin	Nitrogenous compounds Phenolic compound coumarins	(Karawya et al., 1971; Karawya et al., 1972; Narantuyaa et al., 1986; Shehab and Abu-Gharbieh, 2014; Boulaaba et al., 2019)

CHAPTER III: GENERAL EXPERIMENTAL PROCEDURES

The work presented in this thesis was carried out in different well-reputed institutions, such as the Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia; the Institute of Pharmacy and Biomedical Sciences (SIPBS); and the Department of Pure and Applied Chemistry, Strathclyde University, Glassgow.

3. Research Materials and Methodology

3.1. Introduction

The current chapter discusses the materials and methods used in the sampling of *S. kali* plant materials, as well as performance of phytochemical activity screening of the respective extracts of the plants, isolation of bioactive compounds responsible for such activity.

3.2. Health and Safety

All the procedures and experiments in this study were performed in accordance with the laboratory health and safety risk assessments of the University of Strathclyde, Institute of Pharmacy and Biomedical Sciences (SIPBS). A risk assessment was conducted, for which Control of Substances Hazardous to Health Regulation (CoSHH) forms were completed; the outcome was favorable, allowing to work in reasonably practicable and safe working environment conditions.

3.3. Materials

3.3.1. Solvents

Different solvents were used in this work for plant extraction, chromatographic purposes such as, column chromatography, TLC analysis, LC-HRMS and MPLC. HPLC reagent grade including acetonitrile (ACN) and formic acid (FA) both were obtained from Sigma-Aldrich, Poznań, Poland. HPLC grade acetone, methanol (MeOH), isopropanol, dichloromethane (DCM), *n*-hexane, *n*-BuOH were purchased from Fisher Scientific (Loughborough, UK) and from Sigma-Aldrich, Poznań, Poland. Ethyl acetate (EtOAc) which was used as extraction solvent was purchased from Fisher Scientific (Loughborough, UK) and from Sigma-Aldrich, Poznań, Poland. While analytical- grade acetone, DCM from VWR, Fontenay-sous-Bois Cede, France were used for multi-purpose. Analytical- grade methanol was purchased from Fisher Scientific (Loughborough, UK). Dimethyl sulphoxide (DMSO) and deuterated (DMSO-d_6) were purchased

from Sigma Aldrich (Poznań, Poland). Ultrapure water for solvent was obtained using a Direct Q-3® water purifier system (Merck Millipore, Massachusetts, USA).

3.3.2. Glassware

Such as flasks, funnels, separating funnels, beakers (25 mL, 50 mL, 100 mL, 150 mL, 500 mL, 1000 mL, 5000 mL), vials, graduated cylinders, pasture pipetts, volumetric pipetts (1 mL, 5 mL, 10 mL, 25 mL), test tubes and stirring glass rods.

3.3.3. Chromatography

3.3.3.1. Stationary Phases

Celite ® S was purchased from Sigma Aldrich (Dorset, UK) and Celite® 545AW-Reagent Grade was obtained from Supelco, USA. Sephadex® LH-20 (Pharmacia) and Silica gel 60, (230-40 µm mesh) (E. Merck) were used for conventional column chromatography. The normal phase thin layer chromatography plates (TLC silica gel 60 F_{254}) and preparative TLC plates (TLC silica gel 60 F_{254} on 20 x 20 cm aluminium sheets) were from Merck KGaA, Germany.

3.3.3.2. Spray Reagent

The reagents were stored in amber-colored bottles and kept refrigerated until use. TLC was used to monitor the identity of each of the fractions and the qualitative purity of the isolated compounds. It was also utilized to optimize the solvent system that would be applied for column chromatography.

Table 3.1: The constituents of anisaldehyde/H_2SO_4 spray reagent

Methanol	85 mL
Glacial acetic acid	10 mL
Conc H_2SO_4	5 mL (added slowely)
p-Anisaldehyde	0.5 mL

Sulfuric acid was obtained from Fisher Scientific, UK. Glacial acetic acid was purchased from Sigma-Aldrich, US. *p*-Anisaldehyde was obtained from Acros Organics, Belgium.

3.3.3.3. Chromatographic Materials

Glass columns of different sizes and dimensions, column chromatography (CC) was performed using normal phase silica gel 60, particle size 0.04 - 0.063 mm, glass jars fitted with covers were used for TLC, capillary tubes for application of the test solution to TLC plates were obtained from Hirschmann, Germany, spray bottles, 100 mL capacity. NMR tubes obtained from Norell, New York, USA. HPLC-MS vials (sourced from Theromoscientific, C4015-88).

3.3.4. Equipment

3.3.4.1. General Equipment

The rotary evaporator model number R-110 and R-3 were from BÜCHI, Flawil, Switzerland was used for sample concentration and drying under vacuo. The Ultrawave sonicator from Scientific Laboratory Supplies, Nottingham, UK was used during the extraction process to enhance solubilization. The UV lamp (UVGL-55 Handheld UV Lamp) was from UVP, Cambridge, UK. Magnetic mixer from Stuart, Stone, UK and Vortex Genie 2 from Scientific Industries Inc, London, UK were used for multi-purposes including mixing or solubilization either during plant extraction or sample preparation and fractions produced by preparaitive thin layear chromatography extraction. The heat gun HL 2010 E Type 3482 was from Steinel, USA. The concenterated extracts which were reconstituted in EtOAc and dried under nitrogen in a Stuart® block heater SBH130D/3 was from Bibby Scientific Ltd., Staffordshire, UK. Sample concentrator SBHCONC/1 from Stuart, Stone, UK. The freeze dryer, model Christ Alpha 2-4 was from Martin Christ Gefriertrocknunsanlagen GmbH, Germany. Hot plate, Laminar Air Flow (BioMAT^2) was obtained from Medical Air Technology, Manchester, UK. Electric Balance (ae ADAM) and Fume hood (Thermo Scintific, Hamilton SAFEAIRE II). The automatic pipette (Eppendorf PhysioCare) was used for all measurements.

3.3.4.2. High Resolution Liquid Chromatography- Mass Spectrometry Instrument (HR-LCMS)

For extract and fractions, samples were run on an (Accela HPLC) High Pressure Liquid Chromatography (UltiMate-3000) from (Thermo Scientific, Germany) coupled to Mass Spectrometry (Exactive) (Orbitrap, Germany). Electron spray isolation (ESI) was used so that the HPLC could be interfaced with the mass spectrometer, meaning that less fragmentation data was produced for structure elucidation.

3.3.4.3. Nuclear Magnetic Resonance Spectroscopy Instrument

The Nuclear Magnetic Resonance spectroscopy instrument used was a 400 MHz Jeol-LA400 FT-NMR spectrometer system equipped with a 40TH5AT/FG probe (**Figure 3.1**). In addition, an AVANCE-III 600 instrument with a 14.1 T Bruker UltraShield magnet from the Department of Pure and Applied Chemistry was used as well. It has a 24 position autosampler, 3 channel console, is DQD and Waveform-equipped and used a BBO-z-ATMA-[^{31}P-183W/^{1}H] probe from Bruker, Rheinstetten, Germany.

Figure 3.1: Jeol-LA400 FT-NMR spectrometer system equipped with a 40TH5AT/FG probe at SIPBS.

3.3.4.4. Flash Chromatography Equipment

The BÜCHI MPLC instrument Sepacore® Purification System (**Figure 3.2**), consisting of two C-601 pump modules and the C-615 pump manager (BÜCHI, Switzerland). This allowed binary solvent gradients with flow rates of 2.5 to 250 mL/min to be run. The columns and the column stand were purchased from VersaFlash/Supelco, Sigma-Aldrich, Germany. The fraction collector (CF2) was from Spectrum Labs.

Another system, the Biotage® Isolera™ One 2.0.4 Spektra Flash Purification System ISO-1SV (**Figure 3.3**), a product of Biotage, Uppsala, Sweden, was also used. This has a photodiode array (PDA) detector covering an UV wavelength range of 200-400 nm but did not have an evaporative light scattering detector (ELSD). The Biotage had four solvent channels allowing four solvents to be used in a binary gradient during a single run. Biotage® SNAP loading cartridges were used. The sample extract was dissolved in a suitable solvent prior to loading it to a samplet which permitted the loading of samples onto frits before being placed into the column. The samplet was then dried to get rid of the carrier solvent and was placed over the column. This minimised problems with the solubility of the samples.

The appropriate size of the column and the flow rate, which ranges from 4 to 200 mL were set according to the amount and complexity of the respective samples in terms of the number of compounds to be separated. The pressure was automatically adjusted according to the size of column used. The collector was built into the system and trays recognised by the software. At the end of the run the chromatograms could be saved and printed.

Figure 3.2: MPLC system and flasks used to collect fractions.

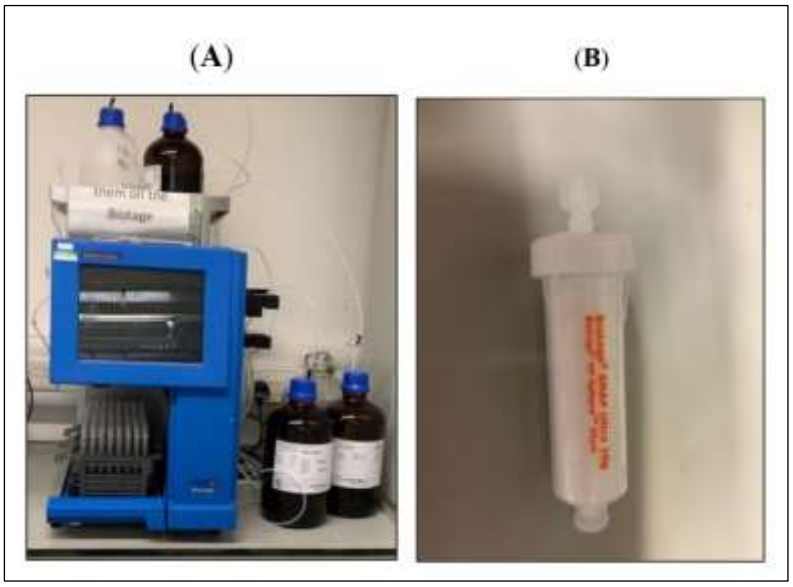


Figure 3.3: (**A**) Isolera from Biotage® used for fractionation and the (**B**) prepacked column used as stationary phase (Biotage® SNAP Ultra 10 g, (21 x 55 mm), (Biotage® HP-Sphere™ 25 um)).

3.3.5. Software

Thermo "Xcalibur 2.2" (Thermo Scientific, Heidelberg, Germany) was used to monitor HR-LCMS raw data. The MassConvert file converter from ProteoWizard (pwiz) (https://sourceforge.net/projects/proteowizard/), USA was used for data splitting to convert and separate the raw data into positive and negative ionisation mode files (Kessner et al., 2008). While splitted data analysis was done with MZmine 2.53 to process the HR-LCMS database. MZmine is a freeware and can be downloaded through http://mzmine.sourceforge.net/. An In-house EXCEL-MACRO was customised to couple the analysis of the mass spectral data with the Dictionary of Natural Products database (DNP version 2021) published by CRC Press, BocaRaton, USA (Pluskal et al., 2010; Macintyre et al., 2014) for dereplication work. The

MestReNova (MNova) 14.2 by Mestrelab Research, S.L, (Santiago de Compostela, Spain) was used to process all the NMR data. The SIMCA 17 (Umetrics AB, Umeå, Sweden) was used for multivariate data analysis.

3.4. Methods

3.4.1. Plant Collection and Identification

Plants can produce various compounds at different concentrations, depending on the season and the stresses that surround the plants (Figueiredo et al., 2008). Therefore, it is sometimes challenging to get the same activity from the plants that have been collected in different seasons. *Salsola kali* was selected based on the ethno-medicinal usage in local medicinal system and wide distribution/occurrence in Saudi Arabia. The aerial parts of *S. kali* (**Figure 3.4**) were collected from Al-Bahah, southwestern part of the Hejazi region, Saudi Arabia (**Figure 3.5**) by taxonomist Dr. Dhafer Ahmed Al-Zahrani, Faculty of Science, Department of Biology, King Abdulaziz University, Jeddah, Saudi Arabia during the flowering stage in December 2017. The plant was thereafter authenticated, and a Voucher Specimen (SK-3-017) was issued and deposited at the Herbarium of Natural Products Department, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia. Plant identity was confirmed by Dr. Dhafer as well. The plant materials were air-dried to minimize degradation of materials and to prevent mould contamination.

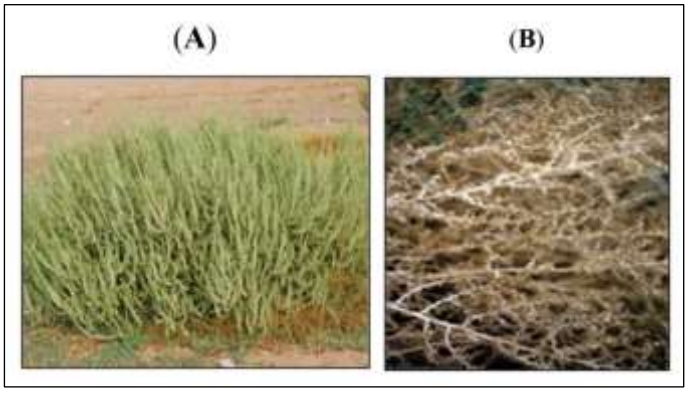


Figure 3.4: *Salsola* kali (**A**) before sampling (**B**) after air drying.

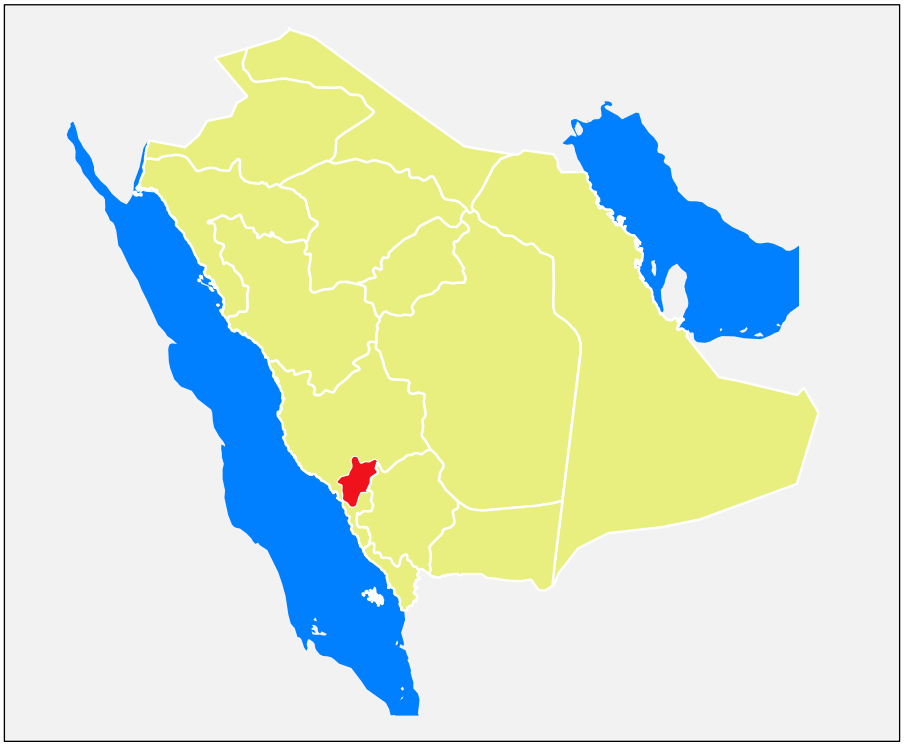


Figure 3.5: Map of Saudi Arabia with Al-Bahah highlighted.

3.4.2. Plant Grinding

Air-dried aerial parts of *S. kali* were cut into smaller pieces and coarsely ground in a grinder using a commercial blender to produce powder weighed 1.5 kg for easier extraction so that the extraction time could be expedited because of the increased surface area.

3.4.3. Plant Extraction, Solvent Partitioning-Preparation of Samples for Analysis and Biological Assay

The qualitative and quantitative studies of bioactive compounds from plant materials are mostly reliant on the choice of proper method of extraction (Smith, 2003; Sasidharan et al., 2011). Extraction is the preliminary step for any medicinal plant study as it plays a crucial role in the result and outcome of compounds obtained. The most common factors affecting extraction processes are matrix properties of the plant part, solvent, temperature, pressure and time (Hernández et al., 2009).

Maceration is the most common methods of extracting plant materials (Azmir et al., 2013). Maceration is the process in which plant extracts are yielded by soaking the plant materials in a solvent (Jones and Kinghorn, 2005). Herein, cold, and continual maceration, with the use of an overhead and/or magnetic stirrer, was selected and subsequently employed throughout the entire experimental project. This mixture of solvent and sample was filtered using filter paper. Because

of the uninterrupted stirring of the ground material within the solvent, the chemical constitute could be extracted on an ongoing basis from the original plant material using this approach. According to some researchers (Harborne, 1998; Barnes and Heinrich, 2004; Waksmundzka-Hajnos et al., 2008), this approach is efficacious due to its comprehensiveness, particularly when organic solvents are used, with methanol, acetone and ethylacetate being the most common, which the active constituents are extracted, and is a good all-purpose solvent for preliminary extractions.

3.4.3.1. Plant Extraction for Chemical and Biological Studies

In the present study, dried crude substances were employed in the experimental procedure; the experiment was undertaken on a large scale using these dried-out crude materials.

The extraction method was done using two solvents (acetone and methanol) to extract phytochemical compounds.

1) 250 g coarsely ground air-dried aerial parts of *S. kali* were properly immersed in 2.5 L analytical grade acetone.

2) The extraction was soaked overnight at room temperature. The solvent was filtered (**Figure 3.6**) the next day and collected into a round bottom flask. A rotary evaporator was subsequently used, R-110 and R-3 (BUCHI Labortechnik AG, Switzerland) (**Figure 3.7**) to concentrate the extract by evaporating the solvent at a standardized temperature of 40°C under reduced pressure along side with pooling the organic layer, which appeared in yellow colour, and the procedure was repeated until the yellow colour of the organic layer had finally diminished and become transparent.

3) The extraction process was repeated three times (2.5 L x 3) on the residual plant material using fresh acetone each time. The concentrated acetone extract afforded a dark greenish brown residue was reconstituted with minimum amount of acetone and pooled together in one round bottom flask.

4) Maceration step was repeated on the residual plant material using analytical grade methanol (2.5 L).

5) The extraction was soaked overnight at room temperature. The solvent was filtered the next day and collected into a round bottom flask then concentrated using rotary evaporator at a standardized temperature of 40°C under reduced pressure.

6) The extraction process was repeated three times (2.5 L x 3) on the residual plant material using fresh methanol each time.

7) The acetone and methanol supernatants were pooled together in a round bottom flask and concentrated using the rotary evaporator.

8) Once dry, all the samples were transferred to accurately labelled 5 mL small, tared vials by reconstituting the samples in the appropriate solvent (acetone, methanol, or a combination of those solvents). Extracts that were hard to redissolve, were sonicated (Scientific Laboratory Supplies, Cardiff, United Kingdom).

9) The samples were then further concentrated on heating blocks (STUART Bibby Scientific Limited Stone, Staffordshire, United Kingdom) at 40°C under a stream of nitrogen gas under the fume hood. The dried total crude extract was weighed to determine the final yield (30 g).

Figure 3.6: Filtration process of aerial parts of S. *kali*.

Figure 3.7: Rotary evaporator used for the concentration of crude extracts and fractions.

Prepartion of KM Extract for Bioassay Screening:

1200 g was immersed properly in methanol at room temperature for a period of 4 days with stirring followed by filtration. The filtrate was concentrated with a rotary evaporator at 40ºC to obtain about 150 g of total methanol extract (**KM**) which was subjected to bioassay screening for antioxidant and cytotoxic activities as shown on **Figure 3.8**.

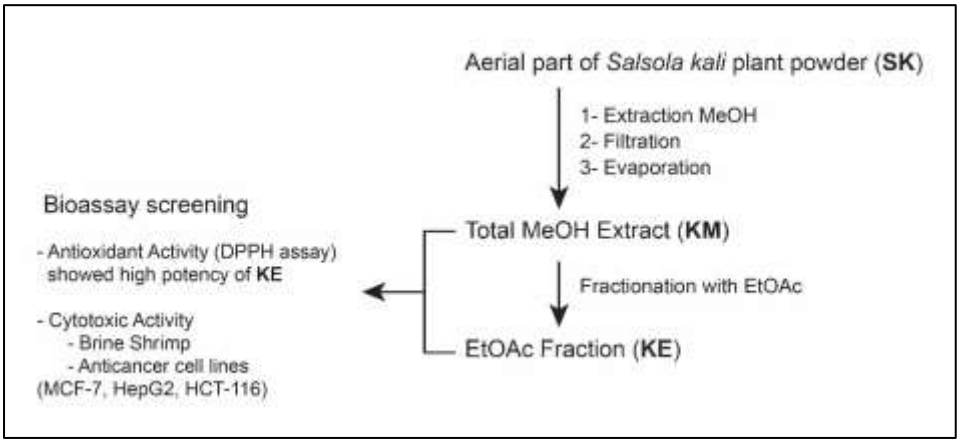


Figure 3.8: Schematic diagram for extraction of plant material for biological assay

3.4.3.2. Preparation of KE Extract by Solvent Partitioning

Two phase partitioning was done to separate each extract into aqueous and semi-polar fractions.

1) The dried total crude extract (30 g) was reconstituted with 100 mL distilled water and transferred to a 1 L separating funnel.

2) Ethyl acetate 100 mL was added. The funnel was closed. This was followed by liquid-liquid extraction and separation, which involved initially separating the aqueous and ethyl acetate (organic) phases by shaking gently in an eight-motion rotation, with air being released following every motion rotation until reaching equilibrium (**Figure 3.9**).

3) The organic layer was collected, and the aqueous phase was partitioned twice with ethyl acetate in the separating funnel.

4) The remaining aqueous layers were lyophilized in the freeze dryer (CHRIST, Germany) at -80°C while the organic layer was evaporated at 40°C under reduced pressure using a rotary evaporator.

5) The residue was dissolved in a small volume of ethyl acetate then dried over nitrogen and transferred to a tared vial that was again weighed after being dried to determine the weight of the extract.

6) The extract (**KE**) 4 g was then concentrated and stored in a closed vial at 4 °C prior to analysis.

Figure 3.9: Separation of aqueous layer (lower) and organic layer (upper) (ethyl acetate) during extraction process.

3.4.3.3. Prepartion of Samples for Analysis and Biological Assay

The crude extracts (**KM**, **KE**) and EtOAc fractions were then reconstituted with Acetone:MeOH (50:50) and subjected to three screening tests namely for NMR, LC-HRMS, and bioassay. Samples were prepared as summarized in **Table 3.2.**

Table 3.2: Required sample concentration for screening.

Screening test	Concentration	Solvent used
LC-HRMS	1 mg/mL	MeOH
NMR	5 mg/600 µL	DMSO-d_6
DPPH assay	10 mg/mL	DMSO

3.4.4. LC-HRMS Analysis of Crude Extract (KE) and Fractions

The crude extract (**KE**) along with its 15 fractions, that were obtained after fractionation using MPLC, were prepared to a concentration of 1 mg/mL in HPLC-grade methanol. A blank solvent was also included, which was HPLC-grade MeOH. The experiment was carried out according to an established standard operating procedure (Macintyre et al., 2014) using the Thermo Finnigan Exactive Orbitrap Mass Spectrometry (Thermofisher Corporation, Hemel Hempstead, United Kingdom) in both positive and negative ionization modes in a mass range ranging from *m/z* 150 to 1500. The spray voltage was 4.5 kV at the time of the experiment. The temperature of the capillary was 320°C. The mobile phase consisted of two solvent components: HPLC-grade acetonitrile (Solvent A) and ultrapure water (Solvent B), both of which contained 0.1% formic acid. A silica C-18 HPLC column (ACE 5 C18) with the size of 75.0 x 3.0 mm, particle size of 5

µm and pore size of 100 °A (Hichrom Limited, United Kingdom) was used. The injection volume was at 10 µL. Each of the samples were eluted at a flow rate of 300 µL/min using a linear gradient of 10% B to 100% B for 30 min, followed by isocratic elution at 100% B for 5 min and a linear gradient of 100% B to 10% B for 1 min, after which the column was further re-equilibrated with the same solvent system for another 9 min. The standard gradient is shown in **Table 3.3**.

The pressure and temperature were monitored to be within the normal range of 37-70 bars and 22°C, respectively, for the instrument to operate smoothly. LC-HRMS chromatograms and spectra were viewed using Thermo Xcalibur version 2.2 (Thermo Scientific, Bremen, Germany). Then, the data was analysed using MZmine 2.53, Excel-MACRO, and SIMCA-P V 17.0 software.

Table 3.3: Elution gradient used for LC-HRMS. Acetonitrile (solvent A) and water (solvent B)

Time (minutes)	% A	% B
0	90	10
30	0	100
35	0	100
36	90	10
45	90	10

3.4.5. NMR Analysis of Crude Extract (KE) and Fractions

The crude extract (**KE**) and its 15 fractions, that were obtained after fractionation using MPLC, were prepared at a concentration of 5 mg/mL. The samples were dissolved in 600 µL deuterated NMR solvent dimethyl sulfoxide (DMSO-d_6) (Sigma-Aldrich, Dorset, UK) and placed in 5 mm NMR tubes (Wilmad, Sigma-Aldrich, Dorset, UK) to a depth of 4 cm. For fractions with limited sample weights (<5 mg), the total fraction was dissolved in 200 µL DMSO-d_6 and transferred to a 3 mm NMR tubes (GPE scientific, Bedfordshire, UK) (**Figure 3.10**). The ^{1}H NMR was carried out on a 400 Hz instrument and, for selected samples, 500 and 600 Hz instruments. ^{1}H proton and presaturation experiments were performed for all samples with a sufficient extract weight. 2D and 1D carbon NMR analyses in JEOL-LA400 FT-NMR instrument equipped with a 40TH5AT/FG probe (JEOL, Tokyo, Japan) were carried out for several samples deemed interesting from results of ^{1}H proton experiments. Chemical shifts were given in ppm and coupling constants in Hz. Spectra were processed using MestReNova (Mnova 14.2) software (Mestrelab Research, Santiago de Compostela, Spain) to confirm chemical structures. The spectrum was processed under certain conditions, which included normalization, Whittaker Smoother for baseline correction and Gaussian was set to 1 for apodization. Structures of

identified compounds were then drawn using ChemDraw Professional software, Version 17.1.0.105 (19) (PerkinElmer, Yokohama, Japan).

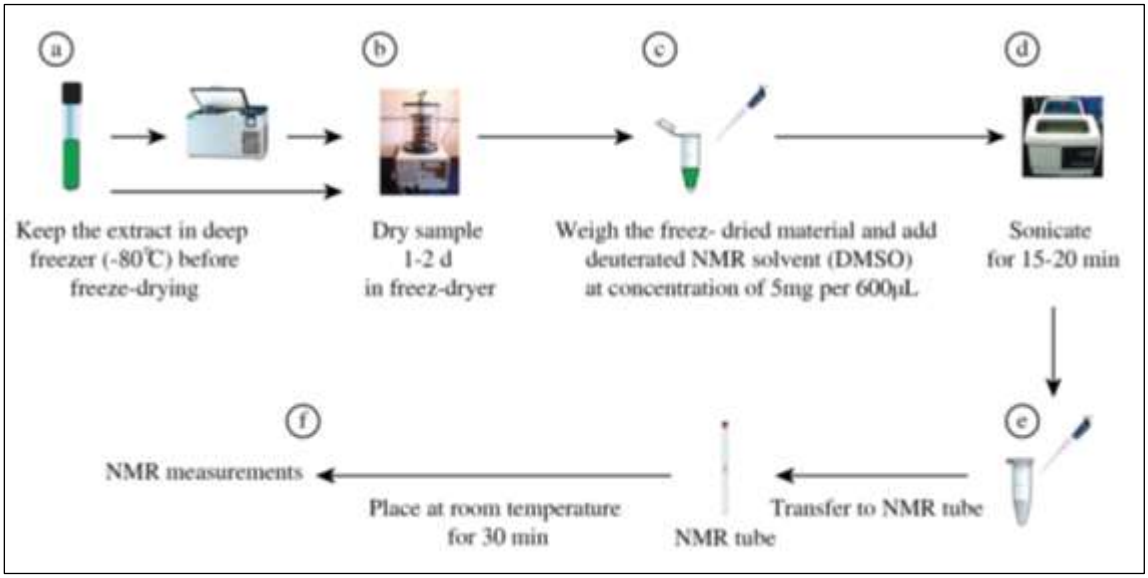


Figure 3.10: Schematic diagram for sample preparation for NMR based metabolomics analysis

For structure elucidation, all compounds were submitted to Applied Chemistry department at Strathclyde University to use their 500 MHz Bruker NMR instruments. This was done to perform ^{1}H, HMBC, HSQC and COSY especially for compounds with low yields (less than 5 mg).

3.4.6. Bioassay Screening of Crude Extract (KE) and Fractions

Bioassays are required to select crude materials and isolate potential new drug agents from natural sources. The assay must be reliable, reproducible, sensitive and predictive (Dey and Harborne, 1991). To determine the true efficacy of potential drug agents, it is important to evaluate their potency in more advanced testing systems followed by pre-clinical trials. For early-stage and for the purpose of screening for bioactive fractions, bioassay-guided fractionation was applied for further separation and purification.

The crude extract (**KE**) and 15 fractions, that were obtained after fractionation using MPLC, were prepared at a concentration of 10 mg/mL. The samples were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Dorset, UK). The samples were prepared as summarized previously in **Table 3.2**. SIMCA-P V 17.0 software was used to analyse spectral data and perform multivariate analysis.

3.4.6.1. Antioxidant Assay

Medicinal plants have great antioxidant potential which is due to their contents of variable phytochemical compounds. The most important free radical in biological systems is radical derivatives of oxygen that is why phenolic compounds possess the largest group among other phytochemical groups in such activity (Al-Jaber et al., 2011).

The screening studies for antioxidant properties of medicinal and food plants have been performed increasingly for the last few decades in hope of finding an efficient remedy for several present-day diseases and means to delay aging symptoms (Halliwell, 2008; Pilipović et al., 2023). There is also a huge demand for natural antioxidants in the food industry, for replacing the synthetic preservatives used to prevent fat rancidity or color loss. Oxidizing agents may damage several biological molecules such as nucleic acids, membrane lipids, enzymes, or synovial fluid polysaccharides.

In this thesis, the crude extracts of *Salsola kali* (**KM, KE**) and 15 KE fractions obtained by MPLC were tested for antioxidant activity using DPPH assay.

3.4.6.1.1. DPPH Assay

The model system of scavenging 2,2-diphenyl-1- picrylhydrazyl (DPPH) free radical, which is stable at room temperature, is a simple method for evaluating the antioxidant activity of compounds. Due to its operational simplicity, this assay has been used in many research laboratories to study antioxidant capacity (Huang et al., 2005). DPPH assay is based on the reduction of DPPH in presence of ethanol due to the hydrogen–donating antioxidant leading to the formation of the non-radical form of DPPH. This transformation results in a colour change from purple to yellow, which is measured spectro-photometrically at 512 nm. DPPH radicals react with suitable reducing agent, the electrons become paired off and the solution loses colour stoichiometrically depending on the number of electrons taken up. Decrease in the absorbance of the DPPH solution is an indication of an increase in the DPPH radical-scavenging activity. DPPH free radical-scavenging activity is considered as *in vitro* screening for possible *in vivo* antioxidant potentialities. The result of the different tests carried on the plant materials are compared with standard result of ascorbic acid (Robak and Gryglewski, 1988; Olalye and Rocha, 2007). Stock solution of 1 mg/mL of semi-polar plant extract was prepared and dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, UK).

DPPH (Sigma Aldrich, Poznań, Poland) was stored at 4°C until used. A stock concentration of 10 mM was made by dissolving 7.98 mg of DPPH in 2 mL absolute ethanol (Sigma Aldrich, UK). The working DPPH solution concentration was 200 µM and therefore the solution was prepared

at 400 µM as it was diluted twice in the assay plate. For an assay plate, 60 µl of the stock was added to 3 mL absolute ethanol. Samples (plant extract and fractions) were prepared at 30 µg/mL. Ascorbic acid (Sigma Aldrich, Poznań, Poland) was used as a positive control as it is a known anti-oxidant (Baba and Malik, 2015). The displacement curve of ascorbic acid was obtained by using a range of ascorbic acid concentrations from 100 µM to 30 nM.

The assay was performed in a 96-well half-area clear flat bottom plate (Grenier Bio-One, UK). Twenty µl of ascorbic acid or 20 µL of sample were added to wells. Then, 20 µL of DPPH was added to all wells then shaked vigorously. The plate was covered with foil and incubated for 20 min at room temperature in dark place. Optical density (OD) was read at a wavelength of 512 nm on a M5 Spectramax Plate Reader using Softmax Pro software. The percent inhibition of DPPH was calculated as follows:

%DPPH inhibition = (OD control - OD sample)/(OD control) X 100

Bar charts were plotted by Microsoft Excel 2016 wherease the dose-response curves between % of antioxidant activity *vs* concentration was performed by non-linear regression analysis using GraphPad Prism for Windows V 6.0 (GraphPad software, San Diego, CA, USA). The mean inhibitory concentration (IC_{50}) values indicate the concentration of sample (Mm) required to scavenge 50% of the DPPH radical were determined *via* cell viability data where the measurements were done in triplicate independent bioassay (n=3).

3.4.6.2. Cytotoxic Activity

The crude extracts of *Salsola kali* (**KM, KE**) were tested for cytotoxic activity using Brine Shrimp Lethality Bioassay and MTT assay using 3 human cell lines; human breast cancer cell line (MCF-7), human hepatocellular carcinoma cell line (HepG2) and human colorectal carcinoma cell line (HCT-116), respectively.

3.4.6.2.1. Brine Shrimp Lethality Bioassay

Now a days, brine shrimp lethality bioassay is commonly used. It is an important tool for the preliminary cytotoxicity assay of plant extract and others based on the ability to kill a laboratory cultured larvae (nauplii) (Ghosh et al., 2015; Kibiti and Afolayan, 2016). The nauplii were exposed to different concentrations of plant extract for 24 hours. The number of motile nauplii was calculated for the effectiveness of the extract. It is a simple, rapid, comprehensive, and cost-effective test that requires a small amount of test material and no special equipments.

Artemia salina encysted eggs were incubated in 100 mL of sea water under artificial light at 28°C, pH 7-8. Nauplii were collected after incubation for 24 h then kept under the same

conditions for another 24 h. **KM** and **KE** crude extract was dissolved in DMSO (500 µg/mL), then diluted serially to 250 µg/mL, 125 µg/mL, 62.5 µg/mL and the volume was adjusted to 5 mL with sea water. The concentration was prepared in triplicate. Nauplii (10-20) were added to each set of vials containing the sample. A control with 50 µL of DMSO in sea water was included. The survivors and dead nauplii were counted after 24 h and LC_{50} value was determined (Meyer et al., 1982).

$$\%\boldsymbol{Death} = \frac{Number\ of\ dead\ nauplii\ \times 100}{Number\ of\ dead\ nauplii + Number\ of\ live\ nauplii}$$

3.4.6.2.2. Anticancer Cell Lines and Culture Medium

The human breast cancer cell line (MCF-7), the human hepatocellular carcinoma cell line (Hep G2) and the human colorectal carcinoma cell line (HCT-116) which were used in the present study were obtained from American Type Culture Collection (ATCC). The MCF-7 and HepG2 cells were cultured in Eagle's Minimum Essential Medium (EMEM). HCT-116 were cultured in McCoy's 5a Medium Modified. All media were supplemented with 10% (v/v) fetal bovine serum (FBS), and 10,000 units/mL penicillin/streptomycin, at 37 ºC in humidified 5% CO_2 incubator.

Principle

Assessment of cytotoxicity using MTT assay (Van Meerloo et al., 2011). The MTT assay is a colorimetric assay for measuring cell metabolic activity. It is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye MTT to its insoluble formazan, which has a purple color (**Figure 3.11**).

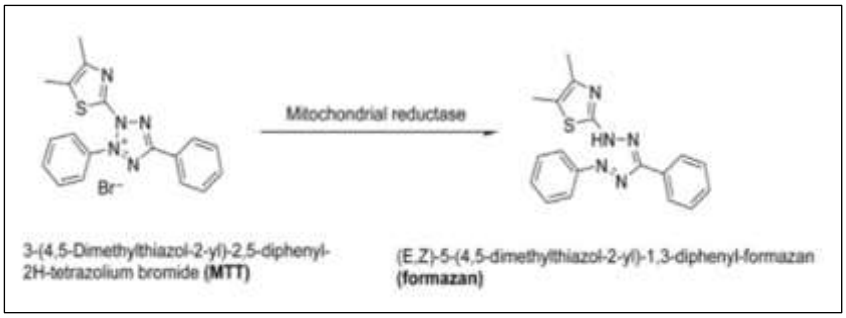


Figure 3.11: Reduction of MTT by mitochondrial reductase.

Procedure

Breast MCF-7, hepatic HepG2 and colorectal HCT-116 cancer cells were added at 3 X 103 cells/mL into a 96-well plate with three replicates and incubated overnight at 37 ºC in a 5% CO_2 humidified atmosphere. Drug concentrations at six serial dilutions (100.0, 50.0, 10.0, 1.0, 0.5, and 0.1 µM/mL) were added in triplicate and incubated at 37 ºC and 5% CO_2 for 72 h. Drugs

were dissolved in 0.1% DMSO as a vehicle. Untreated cells were used as control. Thereafter, each well for each time point was removed and replaced with 100 µM of full medium containing 10% 3-(4,5- dimethylthiaxolyl-2)-2,5-diphenyltetrazoliumbromide (MTT) (10 mg/mL). Then, the media was removed and 100 µL of DMSO was added, and cells were incubated for a further 5 min at 37 ºC and 5% CO_2. Plates were quantified using the SpectraMax M3 plate reader at 570 nm. The percentage inhibition was calculated as

100 - (mean OD of treated cell X 100)/Mean OD of vehicle treated cells (DMSO).

All the experiments were repeated in at least three independent experiments.

3.4.7. Chromatographic Techniques

Since plant extracts usually contain mixtures of various type of bioactive compounds or phytochemicals with different polarities, their separation remains a big challenge for the process of identification and characterization of bioactive compounds and therefore different separation techniques are required to isolate and purify the compounds present in them (Harborne, 1998; Romanik et al., 2007). Chromatography is efficacious because of the various affinities the many constituents of a mixture have toward their mobile and stationary phases. The insoluble matrix is called the stationary phase, while the solution which passes through it is called the mobile phase (Luo et al., 2009). There are different types of chromatographic techniques which can be utilised to separate the compounds. In the present study, several techniques employed as part of normal phase chromatography, three chromatographic techniques will be discussed.

3.4.7.1. Column Chromatography (CC)

This popular type of chromatography technique is used to isolate and fractionate bioactive natural compounds both polar and non-polar from mixtures (Hostettmann and Marston, 2001). The separation takes place through selective distribution of the components between a mobile phase and a stationary phase. Compounds move down the column at different rates and therefore, CC separates compounds based on differential adsorption to the adsorbent to yield fractions and pure compounds. This technique is commonly applied to plant mixtures because of several reasons. It is a simple and inexpensive technique which allows for the disposal of the stationary phase; thus, cross-contamination is prevented. CC has a variety of column sizes allowing the procedure to be carried out on both small- and large-scale materials (from milligrams to kilograms). There are eluting techniques which can be used which are either isocratic elution and or gradient elution. Isocratic elution employs only one mobile phase while gradient elution employs a sequence of mobile phases usually in order of increasing polarity. Gradient elution is normally employed when isolating and/or fractionating natural bioactive compounds from crude

samples (Gurib-Fakim, 2006). Column chromatography was performed using two types of stationary phase, Sephadex® LH-20 and silica gel.

3.4.7.1.1. Sephadex® LH-20 Chromatography

Sephadex® LH-20 is a hydroxypropylated dextran that has both hydrophilic and lipophilic properties. It is prepared by crosslinking water-soluble dextran with epichlorohydrin to be size-exclusion stationary phase. It makes use of molecular sieving to separate the analytes. The separation is on the basis of molecular size and shape of the analyte molecules; accordingly, molecules elute in order of decreasing size. It is particularly ideal for labile natural products and for removal of chlorophyll from plant extracts. It is useful in separating lipids, steroids, and low molecular weight peptides. Sephadex® LH-20 usually does not adsorb compounds irreversibly and can be used for several experiments without the need for regeneration.

3.4.7.1.2. Adsorption Chromatography Using Silica Gel

Silica gel is regarded as a typical polar sorbent and has a weakly acidic surface. Polar compounds containing carboxylic, or hydroxyl group are strongly absorbed in silica gel. It was only used for a few lipophilic fractions. It is a common stationary phase for adsorption chromatography. The surface of the silica gel has silanol groups which interact with the compounds. The less polar compounds have fewer interactions with these hydroxyl groups and thus elute much faster from the column (Braithwaite, 1996).

3.4.7.2. Medium Pressure Liquid Chromatography (MPLC)

Flash chromatography or medium pressure liquid chromatography (MPLC) is a separation technique that is similar in principle to conventional column chromatography. Flash chromatography utilises air pumps and compressed air; the mobile phase of the chromatography is pushed through the column by the pressure of the compressed air, and thus a higher flow-rate in the mobile phase is achieved by a vacuum line and to obtain higher resolution (Still et al., 1978).

The Biotage® Isolera™ Spektra flash chromatography system is among the most contemporaneous and most reliable automated systems. Normal phase columns were employed in Buchi, and the Biotage® Isolera™ Spektra, and they were conditioned with the beginning solvent solution before loading dry samples that were combined with celite. Flash setup equipped with prepacked cartridges for 250 mg to 1 g fractions. Separation was carried out by an isocratic or gradient solvent system that is either MeOH/H_2O elution system to fractionate the polar fractions on a reversed phase silica or EtOAc/Hexan or EtOAc/DCM system for the non-polar

fractions on normal phase silica. The conditions, parameters and mobile phases used are mentioned in the result section.

3.4.7.3. Thin Layer Chromatography (TLC)

TLC is one of the fastest and most widely used chromatographic techniques in the separation of natural products. It provided not only simple, inexpensive and direct analytical technique but also highly sensitive to achieve a good resolution for partition of respective compounds (Wall, 2005; Wall, 2007). TLC is employed to identify compounds based on their R_f value and colour under UV light or after reaction with various spraying reagents. In addition it is a fundamental tool for separating compounds in mixtures (Stahl and Mangold, 1975). It is used to determine the purity of a sample and to estimate the number of compounds present in an extract and it is helpful in decision making when it comes to choosing mobile phases for other separation methods such as column chromatography (CC), flash chromatography (FC) and vacuum liquid chromatography (VLC). TLC has the advantage of being a highly cost-effective qualitative technique since many samples can be analysed simultaneously. TLC separation is achieved by applying a spot of samples (which have already been dissolved in a suitable non-polar solvent) by capillary tube above the bottom edge (1 cm) of a TLC-grade silica gel-coated sheet (Marston, 2011). The sample volume applied on the starting point should be small to obtain a small spot and also to avoid tailing or double spot formation after the development of the chromatogram (Svendsen and Verpoorte, 2011). The TLC plate that is already spotted is inserted inside the TLC tank. The mobile phase is allowed to move until it reaches 1 cm from the top of the TLC plate. The TLC plate can be visualised under UV light at short and long wavelengths UVGL-55 Handheld UV light (UVP, Cambridge, UK) (λ 254 nm and 366 nm), respectively. The TLC plates were coated with anisaldehyde/ H_2SO_4 reagent followed by heating using a heat gun HL 2010 E Type 3482 (Steinel, USA) at 200°C to visualise bands of organic compounds with no UV absorbance for a total of roughly two minutes. Despite the fact that the mechanism by which the visible colour of the stain remained is not known, according to (Fried and Sherma, 1996), the likely detection is explained as shown in **Figure 3.12**

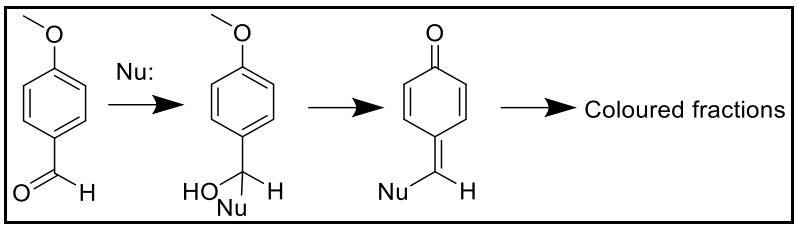


Figure 3.12: The mechanism by which the coloured fractions can be observed when *p*-Anisaldehyde/sulfuric acid reagent is sprayed on TLC plates.

Steroids, terpenes, sugars, phenolic acids, sapogenins and essential oil are examples of compounds that are not UV-active but will react with the spray reagent to be visualized (Wall, 2005). The distance travelled by each spot (sample) is then divided by the total distance travelled by the mobile phase to give R_f values for the samples using the following equation (Cheng et al., 2011):

$$\boldsymbol{R_f value} = \frac{distance\ of\ analyte\ migration}{distance\ of\ mobile\ phase\ migration}$$

The R_f value of the spots should be between 0.3 and 0.5 to prevent a fast rate of elution of the individual components in the sample. The mobile phases that were used are described in the result chapters.

3.4.7.4. Preparative Thin Layer Chromatography (PTLC)

Meanwhile, preparative TLC was done to recover compounds from the small volume of fraction and analytical TLC have been performed in advance to obtain the suitable solvent system for good chromatogram separation. 10 mg of sample was dissolved in 150 µL of acetone and MeOH (70:30) and applied to each TLC plate. The samples were spotted as band along a line 2 cm above the bottom edge of the TLC plate. The TLC chamber that contained 400 mL mobile phase was allowed to equilibrate for 10 min by placing filter paper inside the chamber. This step will make sure that the chamber was saturated or properly equilibrated with the solvent system to develop a good chromatogram separation. In addition, the plates can be developed several times to improve resolution (Wall, 2005). The eluted bands were observed under UV light and were marked using a carbon pencil. The respective compound from each eluted band was recovered by cutting the marked bands into several pieces and macerated in 100 mL acetone overnight with stirring for 2 hours. The stirring step was repeated twice, and the supernatant was filtered and collected in a tared round bottom flask to dry using the rotary evaporator. The dried residue was weighed and subjected to further analytical work.

3.4.8. LC-HRMS Data Analysis Using MZmine 2.53 Adapted from Macintyre et al., (2014)

The LC-HRMS chromatograms and spectra were viewed in Thermo Xcalibur 2.2. The raw LC-MS data from both positive and negative ionization modes were sliced using the ProteoWizard's MassConvert file slicer to separate both positive and negative masses. The mass files were uploaded separately to import and process the sliced MS data using MZmine 2.53 software (Pluskal et al., 2010). The chromatograms were first cropped to 0.0-45.0 minutes. The raw data methods were processed using peak detection, started with mass detection by mass detector was set as centroid, noise level at 1000 and MS level as 1. Followed by chromatogram builder was set at 0.2 min for minimum time span, minimum height at 10000 and *m/z* tolerance of 0.001 *m/z* or 5 ppm. The analysis continued with peak detection from peak list methods, for the chromatogram deconvolution, where the algorithm was set as local minimum search. The chromatographic threshold was set to 90%, search minimum retention time (RT) at 0.4 min, minimum relative height at 5%, minimum absolute height at 10000, minimum ratio of peak top/edge as 2 and the peak duration range within 0.2 – 5.0 min. Isotopes were detected using the isotopic peaks grouper. The *m/z* tolerance was set to 0.001 *m/z* or 5 ppm, retention time (RT) tolerance at 0.2 min absolute, the maximum charge as 2, and the most intense representative isotope utilised. RT normalization was performed using the RT normalizer with *m/z* tolerance of 0.001 *m/z* or 5.0 ppm while the RT tolerance and the minimum standard intensity were set to 5% (relative) and 5.0E4 respectively. The peak lists were aligned uniformly using the join aligner (*m/z* tolerance 0.001 *m/z* or 5.0 ppm, weight for *m/z*: 20, RT tolerance 5.0 percent relative, weight for RT: 20). The aligned peak list was gap-filled using the peak finder function (tolerances for intensity: 30%, *m/z*: 0.001 *m/z* or 5.0 ppm, and RT: 0.5 min). The RT tolerance was set to 0.2 absolute (min), the *m/z* tolerance to 0.001 *m/z* or 5.0 ppm, and the maximum relative adduct peak height to 30%. The adducts Na, K, and NH_4 were used in the positive mode, formate in the negative mode, and ACN+H in both modes. Additionally, a sophisticated search was conducted utilising $[M+H]^+$ for positive ionisation and $[M-H]^-$ for negative ionisation. RT Tolerances was set to 0.2 absolute (min), *m/z* to 0.001 or 5.0 ppm, and the maximum complex peak height was set to 50%. The data was exported as CSV file until further clean-up process. All the MZmine processing steps were then repeated with negative mode with some modification such as an adduct search was set as formate and ACN+H, complex search ionization was set as M-H and formula prediction ionization was set as $[M-H]^-$.

An In-house EXCEL Macro was developed by Dr Tong Zhang and was adapted in (Macintyre et al., 2014) for both dereplication of the LCMS data and metabolomics study. The EXCEL Macro

file was coupled with Dictionary of Natural Product (DNP) database for peak identification and dereplication. The positive and negative ionization modes were combined, and any blank peaks were removed from the background of individual peak, so that remaining peaks from both ionization modes were overlaid for further statistical analysis. The generated database of known and unknown metabolites from the Macro was utilised to pinpoint the putative discriminating metabolites from the sample extracts, which provided information of individual peaks including by assigning a feature ID number along with the respective ionization mode, *m/z*, retention time, molecular formula, molecular weight, biological sources and peak intensity. The data was then converted into CSV file and was exported into SIMCA V 17.0 (Umetrics, Umeå, Sweden) for multivariate analysis.

3.4.9. Multivariate Analysis Using SIMCA V 17.0

The processed data generated from the EXCEL Macro was further analysed using SIMICA-P V 17.0 for multivariate analysis. For the mass spectral data, the MZmine feature ID number was merged with the ionization mode to generate a unique primary ID in SIMICA while the other variables like retention time, *m/z*, and molecular weight were considered secondary IDs. While for the NMR data, the chemical shift in ppm is used to generate the unique primary ID and there are no secondary IDs considered for variables. The data were preliminarily analysed with an unsupervised statistical method by using principal component analysis (PCA). Following that, a supervised statistical analysis method was done with orthogonal partial least squares discriminant analysis (OPLS-DA) when comparing groups and discriminating metabolites according to the known variables between groupings. PCA and OPLS-DA analysis were done using Pareto scaling and the models were validated based on multiple correlation coefficients (R^2) and cross-validation (Q^2) coefficients, as well as permutation tests for the supervised method. The above-mentioned in-house EXCEL macro was then used to determine the top twenty features (ordered by peak intensity) and their related putative identities in each sample by producing unique CSV files for each extract. ChemBioFinder version 13 was used to get database hits (PerkinElmer Informatics, Cambridge, UK).

Both liquid chromatography-high resolution mass spectroscopy (LC-HRMS) and nuclear magnetic resonance (NMR) spectroscopy are commonly used in metabolomics. LC-HRMS has the advantage of being more sensitive than the NMR, detecting compounds that are present as femtograms in the extracts. Moreover, it can identify compounds based on their exact mass and fragmentation pattern and the addition of other limiters such as retention time could enhance its identification. However, it is limited by the ionisation capability of the metabolites (Griffiths et al., 2010; Tawfike et al., 2013; Krug and Müller, 2014). On the other hand, NMR is better for

structure elucidation. Nevertheless, it is not sensitive enough to detect minor metabolites that present in lower concentrations. Dereplication is defined as the process of implementing spectroscopy in the identification of known metabolites in the early stages of isolation (Krug and Müller, 2014; Harvey et al., 2015). This is achieved by using LC-HRMS, where hits with certain *m/z* values are compared to available databases like Dictionary of Natural Products (DNP). Along with multivariate analysis that pinpoints the active compounds, this allows prioritising fractions for further purification and helps save time and money in the process of isolating novel bioactive compounds. Combining data given by LC-HRMS or NMR to a multivariate data analysis tool allows scientists to compare and detect differential metabolites in biological samples.

Multivariate data analysis is a chemometrics tool that is applied to extract relevant information from measured data. Thus, visualising this data and enabling the prediction of its outcomes. The use of common instrumental analysers produces multivariate collinear data. Measured variables, which describe the system, provide similar information content. These collinear variables and thus, the structure of data, could be combined and described by fewer factors, called latent variables or principal components (Rajalahti and Kvalheim, 2011).

Different approaches in multivariate analysis include principal component analysis (PCA), soft independent modelling by class analogy (SIMICA), partial least squares, or projections to latent structures, discriminant analysis (PLS-DA), orthogonal partial least squares discriminant analysis (OPLS-DA) and modified orthogonal projections to latent structures (O2PLS) were use (Wiklund, 2008).

CHAPTER IV: FRACTIONATION, ISOLATION, BIOLOGICAL EVALUATION AND MULTIVARIATE ANALYSIS OF *SALSOLA* EXTRACT

4. Fractionation, Isolation, Biological Evaluation and Multivariate Analysis of *Salsola kail* Etheyl acetate Extract

4.1. Fractionation of Bioactive Secondary Metabolites from *Salsola kali* Ethyl acetate Extract (KE) Using MPLC from BŪCHI

Performing TLC experiments on the crude extract predetermined an appropriate gradient system to use for fractionation. The *S. kali* ethyl acetate crude (**KE**) extract (4 g) was loaded into a column and fractionated with the two-pump BÜCHI Sepacore® semi-automated flash chromatography system (BÜCHI, Switzerland) using the following method:

Column: VersaPak Silica (Spherical) 20-45 µm, 40 x 75 mm (48 g) Supleco, USA

Mobile Phase: EtOAc (**A**), MeOH (**B**)

Flow rate: 15 mL/min.

Gradient: 0-5 min	0% B	equilibration
5-180 min	0-30 % B	
180-190 min	30% B	

Flash liquid chromatography set-up from BŪCHI was used to isolate the bioactive compounds from the **KE** extract. The crude extract (4 g) was reconstituted with 6 mL of EtOAc or with the minimum possible amount and added to 9 g of celite. The concentrated extract solution was added to the celite drop-wise to obtain homogeneous mixing then dried overnight under the fume hood. A prepacked column of VersaPak Silica (Spherical) 20-45 µm was used as a stationary phase with 40 x 75 mm dimension. Two main solvents were used in this system, Ethyl acetate and Methanol for Solvents A and B, respectively. The percentage of MeOH in the mobile phase must not exceed 80%, to avoid the solvation of silica from the pre- packed column. Prior to chromatographic separation, the column was equilibrated by eluting with 100% of A in 250 mL conical flask for 5 min at a flow rate of 15 mL/min. The chromatography continued with a linear gradient elution from 0% to 30% B for 180 min, the fraction collection volume was set at 35 mL/flask. Followed by a washing step using a 70:30 ratio of EtOAc and MeOH for 10 min. All

fractions were manually collected in Erlenmeyer flasks at volumes 100 mL per fraction every 15 seconds, which gave a total of 76 fractions (**Table 4.1)**, (**Figure 4.1**), while the waste was collected in a 1 L conical flask. The column was inverted and washed with 250 mL of 70:30 EtOAc:MeOH. For monitoring purposes, each fraction was spotted on a TLC plate along with the EtOAc extract sample as reference. The TLC was performed on normal-phased aluminium-backed plates (Merck, UK). The 76 fractions collected were analysed by TLC using mobile phase combinations of ethyl acetate: MeOH and dichloromethane:isopropanol to perform the normal-phase TLC. The chromatograms were observed under short and long wavelength UVGL-55 Handheld UV light (UVP, Cambridge, UK) at 254 and 365 nm, respectively. Anisaldehyde/H_2SO_4 reagent was used to spray TLC plates followed by heating using a heat gun at 170°C to visualize bands of organic compounds and those with the same chromatographic profiles were pooled together yielding 15 major fractions (**Figure 4.1**). The total percentage recovery of the extract fractions was 68%. The respective fractions were concentrated and dried under *vacuo* then weighed as listed under **Table 4.2**. The resulting fractions were analyzed using ^{1}H NMR and HRESI-MS for metabolomic profiling studies and tested for antioxidant activity.

Table 4.1: Mobile phase used for the BŪCHI Sepacore® fractionation of the EtOAc extract of *Salsola kali*

	Fractions	Time (Min.)	MeOH%	EtOAc%
1	F1-2	0	0	100
2	F3	7	2	98
3	F4	9	4	96
4	F5	11	6	94
5	F6	14	8	92
6	F7-9	21	10	90
7	F10-22	51	12	88
8	F23-32	75	14	86
9	F33-42	98	16	84
10	F43-44	102	18	82
11	F45-46	107	20	80
12	F47-51	119	23	77
13	F52-55	128	26	74
14	F56-60	140	27	73
15	F61-76	180	30	70

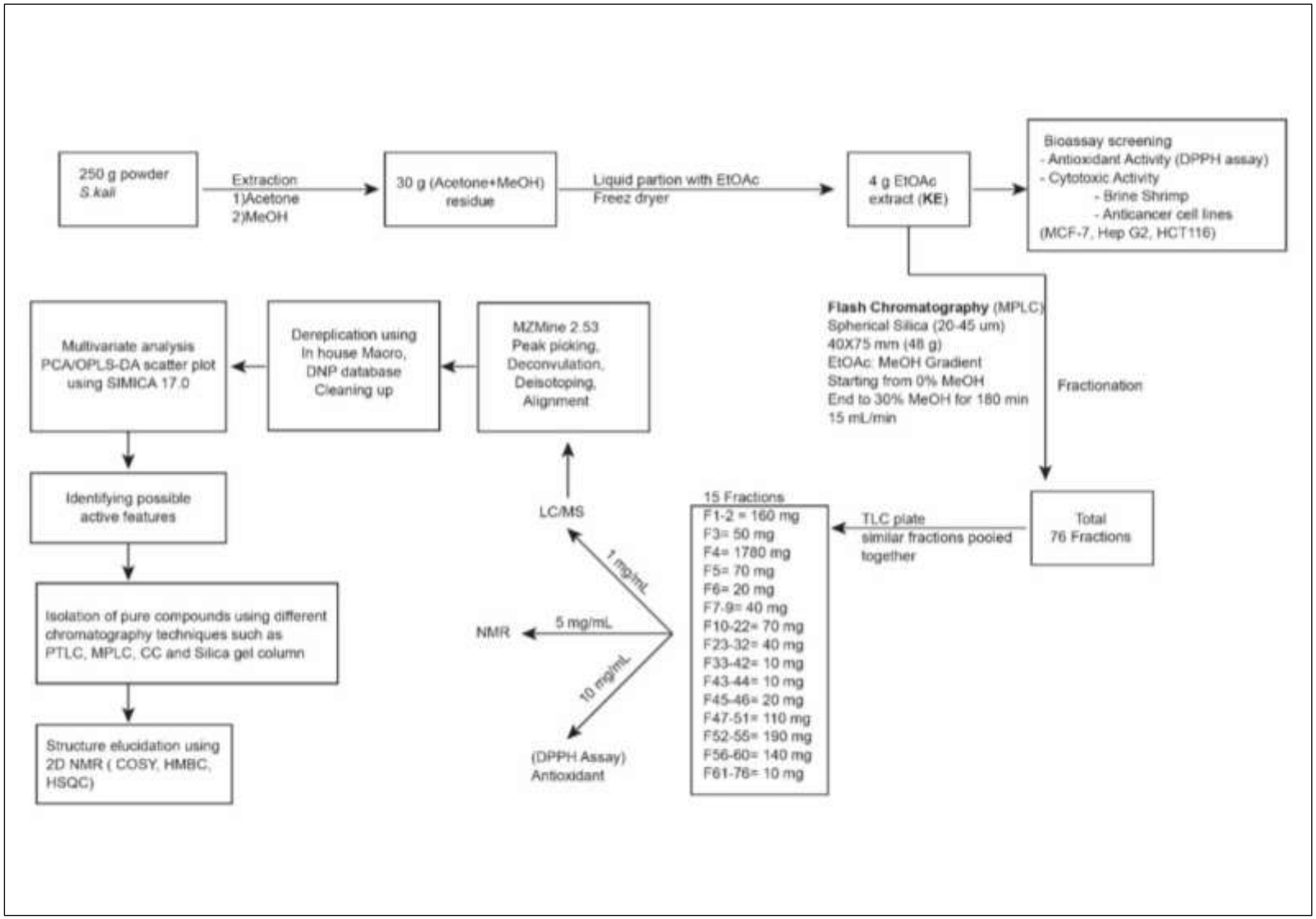


Figure 4.1: Schematic diagram of *Salsola kali* extraction, solvent partitioning-preparation for metabolomic study and isolation of major constituents.

Table 4.2: Weights of fractions obtained from the BŪCHI Sepacore® fractionation of the EtOAc extract of *Salsola kali*

Fraction No.	Fraction Code	Weight (mg)	Fraction No.	Fraction Code	Weight (mg)
1	F1-2	160	9	F33-42	10
2	F3	50	10	F43-44	10
3	F4	1780	11	F45-46	20
4	F5	70	12	F47-51	110
5	F6	20	13	F52-55	190
6	F7-9	40	14	F56-60	140
7	F10-22	70	15	F61-76	10
8	F23-32	40		**Total amount**	2720
				Recovery%	68%

4.2. Phytochemical Methods for Isolation of Compounds

For the extraction and purification procedures, various phytochemicals were used on the extract. Spectroscopic and chromatographic techniques were used more than any other technique. The chemical profiling of LC-HRMS, TLC and ^{1}H-NMR was undertaken in addition to the previously mentioned procedures. A phytochemistry profile of the aerial parts of *S. kali* was the outcome of the chemical profiling and biological assay.

Fractions F4, F5, F6-9, F52-55, and F56-60 had the potential for further fractionation and purification work. Fractions were subjected for extended isolation work using (CC) either Silica gel or Sephadex® LH-20, (PTLC) and (MPLC). The fractions were prioritised for further isolation work not only depending on the non-complexity but also the novelty of their chemical profile, fraction yield, and potency of their antioxidant activity. The schematic diagram for the preparation of plant extract and its fractionation for compounds separation is illustrated in **Figure 4.2**.

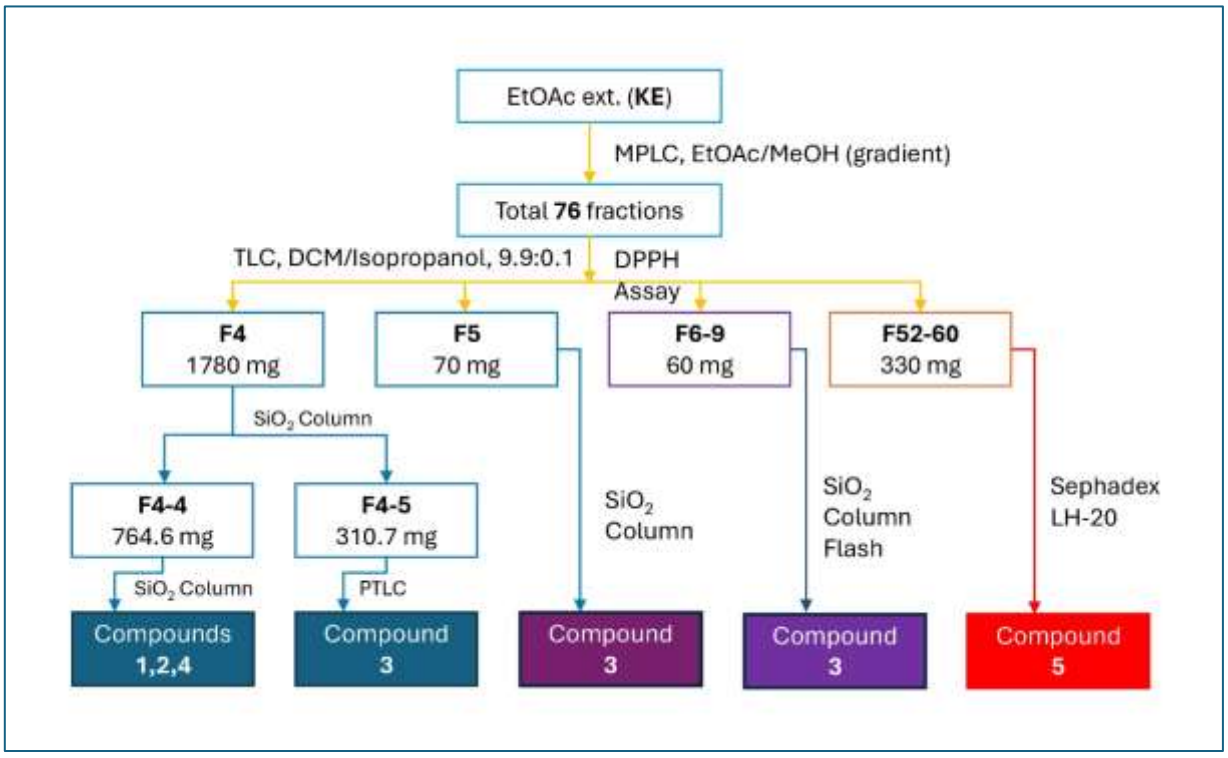


Figure 4.2: Summary of schematic isolation of the bioactive compounds**.**

4.2.1. Purification of Subfraction F4.4.3.3 from F4 led to Isolation of Compound **1**

Fraction 4 (1780 mg) was dissolved in MeOH and transferred to the top of glass column (41 x 5.5 cm) packed with Sephadex LH-20. The column was previousely equilibrated with 100% MeOH and was eluted with the same solvent to obtain 185 subfractions. All subfractions were checked by TLC using DCM:isopropanol (9.9:0.1) as a developing system. Spots were revealed by UV and anisaldehyde/H_2SO_4 spray reagent. Similar fractions were grouped together to yield 5 subfractions shown in **Table 4.3** and evaporated under reduced pressure.

The residue of Subfraction 4 (794.6 mg) was subjected to further purification using a conventional column (25 x 5 cm) in which silica was used as the stationary phase. It was dissolved in Hex/DCM (50:50) and adsorbed on a small amount of silica gel then left to dry. The slurry was loaded on an open normal-phase silica column equilibrated with Hex/DCM (50:50). A gradient elution was performed commencing with 50% Hex and culminating with 80% MeOH as shown in **Table 4.4.** Fractions of 50 mL were collected and examined using the same conditions under F4. Similar fractions were combined to yield 11 subfractions which was shown in **Table 4.5** and evaporated under reduced pressure.

Subfraction 3 (22.2 mg) was subjected to further purification and dissolved in Hex/DCM (20:80) and adsorbed on a small amount of silica gel then left to dry. The slurry was loaded on an open normal-phase silica column (15 x 0.7 cm) equilibrated with Hex/DCM (20:80). A gradient elution was performed commencing with 20% hexane and culminating with 95% DCM as shown in **Table 4.6.** Fractions of 10 mL were collected and examined using the same conditions under F4. Similar fractions were combined to yield 4 subfractions was shown in **Table 4.7** and evaporated under reduced pressure.

Subfraction 3 (5 mg) was added to subfraction 4 (3.6 mg) and subjected to further purification then dissolved in Hex/DCM (50:50) and adsorbed on a small amount of silica gel then left to dry. The slurry was loaded on an open normal-phase silica column (11.5 x 1 cm) equilibrated with Hex/DCM (50:50). An Isocratic elution was performed with Hex/DCM (50:50). Fractions of 10 mL were collected and examined using the same conditions under F4. Similar fractions were combined to yield 6 subfractions and evaporated under reduced pressure. **Subfraction 2** shows a distinct spot and is highlighted in **Table 4.8** (**Compound 1 = 2.8 mg**). While subfraction 3 (0.4 mg) from the same column was pooled together with fraction F4.5.3 (7 mg) then dissolved in Hex/DCM (50:50) and adsorbed on a small amount of silica gel then left to dry. The slurry was loaded on an open normal-phase silica column (11.5 x 1 cm) equilibrated with Hex/DCM

(50:50). An Isocratic elution was performed with Hex/DCM (50:50). Fractions of 10 mL were collected and examined using the same conditions under F4. Similar fractions were pooled together to yield three subfractions and evaporated under reduced pressure. **Subfraction 1** shows distinct spot (**Compound 1 = 2.4 mg**) and is highlighted in **Table 4.9.** The method is outlined below in **Figure 4.3.**

Table 4.3: Yields of fractions from fractionation of F4 using Sephadex column

Fraction code	Yield (mg)
F4.1	160.6
F4.2	139.3
F4.3	326.4
F4.4	794.6
F4.5	310.7

Table 4.4: Elution gradient used for fractionation of F4.4 using silica gel column

Eluting System							
Hex	**DCM**	**MeOH**	**Total volume collected (mL)**	**Hex**	**DCM**	**MeOH**	**Total volume collected (mL)**
50	50	0	100	0	93	7	300
40	60	0	100	0	92	8	300
30	70	0	1000	0	91	9	400
20	80	0	300	0	90	10	200
10	90	0	300	0	87.5	12.5	200
5	95	0	300	0	85	15	200
2	98	0	300	0	82.5	17.5	200
0	100	0	1000	0	80	20	200
0	99	1	300	0	70	30	200
0	98	2	300	0	60	40	200
0	97	3	300	0	50	50	200
0	96	4	300	0	30	70	200
0	95	5	300	0	20	80	200
0	94	6	300				

Table 4.5: Yields of fractions from fractionation of F4.4 using silica gel column

Fraction code	Yield (mg)	Fraction code	Yield (mg)
F4.4.1	42.6	F4.4.7	42.1
F4.4.2	30.9	F4.4.8	40.3
F4.4.3	22.2	F4.4.9	114.2
F4.4.4	42.7	F4.4.10	44.8
F4.4.5	41.3	F4.4.11	129.4
F4.4.6	202.6		

Table 4.6: Elution gradient used for fractionation of F4.4.3 using silica gel column

Eluting System					
Hex	**DCM**	**Total volume collected (mL)**	**Hex**	**DCM**	**Total volume collected (mL)**
20	80	100	12	88	100
19	81	100	11	89	100
18	82	100	10	90	100
17	83	100	9	91	100
16	84	100	8	92	100
15	85	100	7	93	100
14	86	100	6	94	100
13	87	100	5	95	100

Table 4.7: Yields of fractions from fractionation of F4.4.3 using silica gel column

Fraction code	Yield (mg)
F4.4.3.1	8.2
F4.4.3.2	5.0
F4.4.3.3	5.0
F4.4.3.4	3.6

Table 4.8: Yields of fractions from fractionation of F4.4.3.3+F4.4.3.4 using silica gel column

Fraction code	Yield (mg)
F4.4.3.3.1	2.1
*F4.4.3.3.2	2.8
F4.4.3.3.3	0.4
F4.4.3.3.4	1.4
F4.4.3.3.5	0.9
F4.4.3.3.6	0.7

**The highlighted raw represent the compound that was semi-purified in this work and are further presented in Chapter 5*

Table 4.9: Yields of fractions from fractionation of F4.4.3.3.3+F4.5.3 using silica gel column

Fraction code	Yield (mg)
*F4.4.3.3.3.1	2.4
F4.4.3.3.3.2	1.4
F4.4.3.3.3.3	2.8

**The highlighted raw represent the compound that was semi-purified in this work and are further presented in Chapter 5*

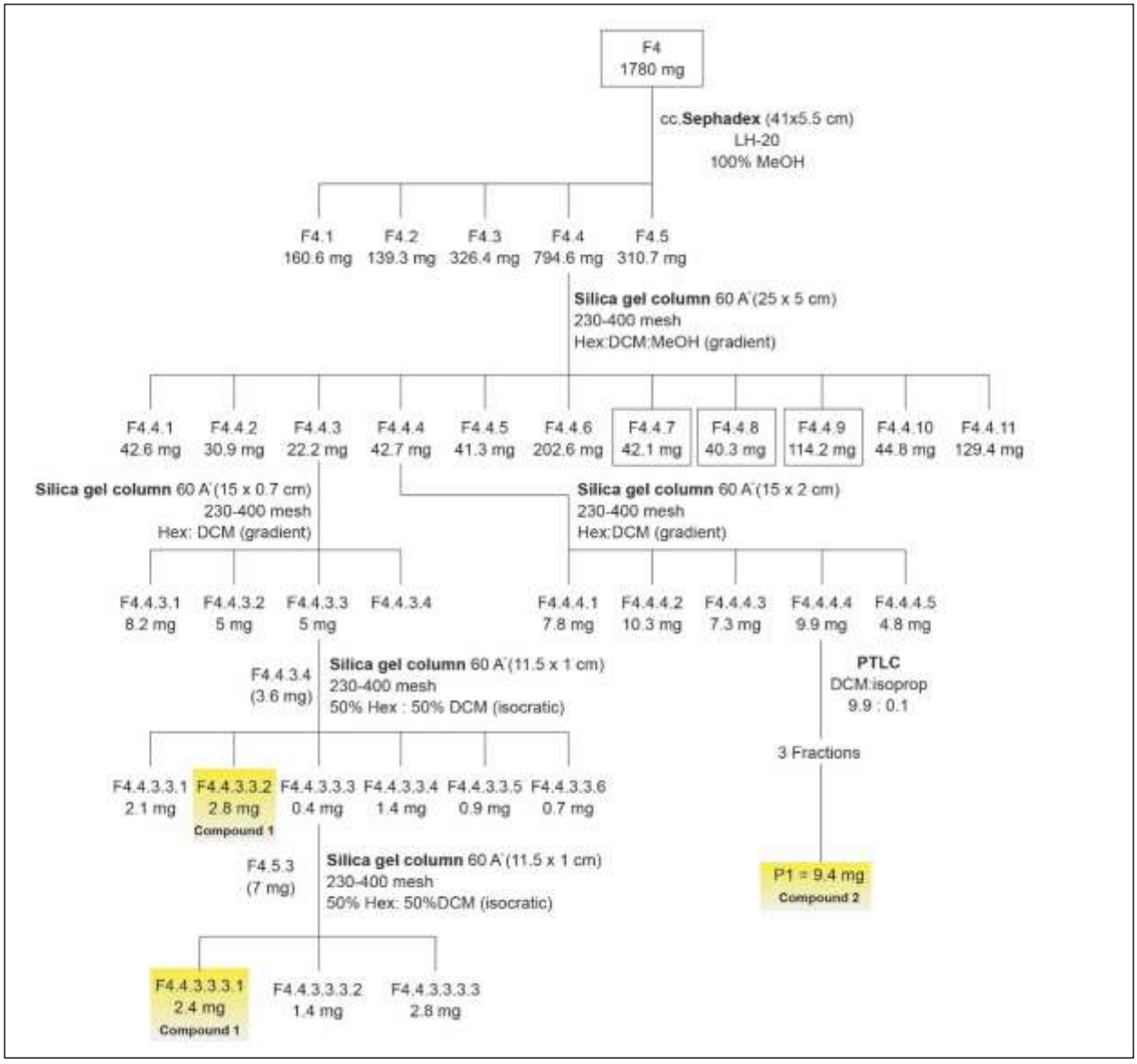


Figure 4.3: Schematic diagram represent the isolation of compounds **1**&**2** using silica gel column and PTLC chromatography.

4.2.2. Purification of Subfraction F4.4.4.4 from Fraction F4 led to Isolation of Compound **2**

The residue of F4.4.4 (42.7 mg) dissolved in Hex/DCM (10:90) and adsorbed on a small amount of silica gel then left to dry. The slurry was loaded on an open normal-phase silica column (15 x 2 cm) equilibrated with Hex/DCM (10:90). Gradient elution was performed with Hex/DCM commencing with 10% hexane and culminating with 100% DCM as shown in **Table 4.10.** Fractions of 15 mL were collected, examined by TLC and developed with DCM:isopropanol (9.9:0.1). Spots were revealed by UV and anisaldehyde/H_2SO_4 spray reagent. Similar fractions were combined to yield 5 subfractions as shown in **Table 4.11** and evaporated under reduced pressure. Subfraction 4 (F4.4.4.4) exhibited a distinct spot on the TLC.

Preparative TLC was chosen for the further purification of F4.4.4 of fraction 4 (F4.4.4.4) due to its low yield of (9.9 mg). The fraction was dissolved in 148 µl of acetone and MeOH (70:30). A volume of 150 µl was loaded as a band on every 20 × 20 cm silica TLC using one TLC plate. DCM and isopropanol (99:1) were used as mobile phase. TLC plates were developed three times to ensure the maximum separation between bands. The plates were viewed under short wavelength and long wavelength UV. Three major bands were observed under UV light (**Figure 4.3**)**.** The bands were cut, placed into 100 mL conical flasks, and extracted with acetone 50 mL (HPLC grade) three times. These were stirred for an hour, after which the acetone was filtered, and fresh acetone was placed into the flasks. The pooled filtrate was then evaporated under vacuum and **compound 2 yielding 9.4 mg** was elucidated using 1D and 2D NMR. The method is outlined below in **Figure 4.3.**

Table 4.10: Elution gradient used for fractionation of F4.4.4 using silica gel column

Eluting System					
Hex	**DCM**	**Total volume collected (mL)**	**Hex**	**DCM**	**Total volume collected (mL)**
10	90	100	4	96	100
9	91	100	3	97	100
8	92	100	2	98	100
7	93	100	1	99	100
6	94	100	0	100	100
5	95	100			

Table 4.11: Yields of fractions from fractionation of F4.4.4 using silica gel column

Fraction code	Yield (mg)
F4.4.4.1	7.8
F4.4.4.2	10.3
F4.4.4.3	7.3
F4.4.4.4	9.9
F4.4.4.5	4.8

Figure 4.4: Preparative TLC of MPLC F4 from crude extract

4.2.3. Purification of Subfraction F4.5.4 from Fraction F4.5 led to Isolation of Compound **3**

Subfraction F4.5 (310.7 mg) was dissolved in $CHCl_3$ and adsorbed on a small amount of silica gel then left to dry. The slurry was loaded on an open normal-phase silica column (30 x 3 cm) equilibrated with 100% $CHCl_3$. Gradient elution was performed with $CHCl_3$/MeOH commencing with 100% $CHCl_3$ and culminating with 100% MeOH. Fractions of 15 mL were collected, examined by TLC and the developing system DCM:MeOH (9.5:0.5). Spots were revealed by UV and anisaldehyde/H_2SO_4 spray reagent. Similar fractions were combined to yield 16 subfractions as shown in **Table 4.12** and evaporated under reduced pressure. Subfraction 4 (F4.5.4) showed a distinct spot on the TLC.

F4.5.4 was selected for further investigation as TLC-screening showed one major spot while all other fractions showed a small amount with a series of compounds. **Preparative TLC** was chosen for the sub fractionation 4 of fraction 4.5 (F4.5.4) (44.7 mg). The fraction was dissolved in 670 µL of acetone and MeOH (70:30). A volume of 150 µL was loaded as a band on every 20 × 20 cm silica TLC using four TLC plates in total. 100% DCM was used as mobile phase. TLC plates were developed three times to ensure the maximum separation between bands. The plates were viewed under short wavelength and long wavelength UV. Ten major bands were observed

under UV light. The bands were cut, placed into 100 mL conical flasks and extracted with acetone 50 mL (HPLC grade) three times. These were stirred for an hour, after which the acetone was filtered, and fresh acetone was placed into the flasks. The pooled filtrate was then evaporated under vacuum and **compound 3 yielding 4.2 mg** was analysed using 1D and 2D NMR. The method is outlined below in **Figure 4.5**.

Table 4.12: Yields of fractions from fractionation of F4.5 using silica gel column

Fraction code	Yield (mg)	Fraction code	Yield (mg)
F4.5.1	9.2	F4.5.9	20.6
F4.5.2	8.3	F4.5.10	13.1
F4.5.3	7.0	F4.5.11	17.9
F4.5.4	44.7	F4.5.12	16.5
F4.5.5	14.6	F4.5.13	17.2
F4.5.6	16.3	F4.5.14	17.8
F4.5.7	15.0	F4.5.15	41.5
F4.5.8	14.5	F4.5.16	23.6

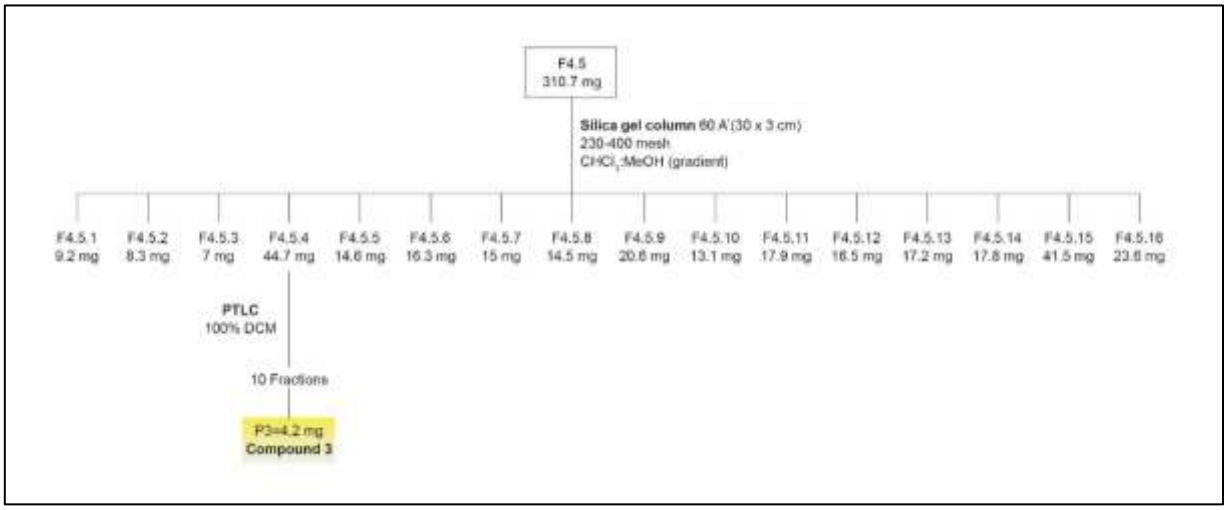


Figure 4.5: Schematic diagram represent the isolation of compound **3** using PTLC chromatography

4.2.3.1. Purification of Fraction F5 led to Isolation of Compound **3**

Fraction F5 (70 mg) was further fractionated using the Biotage® MPLC Isolera™ One 2.0.4 Spektra flash purification system (ISO-1SV). The sample was loaded on a normal phase Biotage®SNAP Ultra 10 g, (21 x 55 mm), (Biotage® HP-Sphere™ 25 um). A 40-minute isocratic elution method with 90% Hex (A) and 10% Acetone (B) was used, then increased to 30% acetone for 15 min prior to washing. The flow rate was at 36 mL/min and maximum collection volume was set at 18 mL. The system marked the respective test tubes based on the eluting peaks

at real time as determined by UV from 210 to 350 nm. The column was then flushed with 100 mL of DCM:Isopropanol (70:30) for 15 min and this was collected as the wash. Fractions were then pooled using UV read-outs from the Isolera™ One 2.0.4 Spektra flash purification system and similarities on Si60 normal phase TLC plates using Hex and Acetone (5:5) as solvent system. Similar fractions were pooled together yielding 17 fractions. Fractions exhibited as distinct spots on the TLC plates (**compound 3** = **3 mg**) were highlighted in **Table 4.13**. The method is outlined below in **Figure 4.6.**

Table 4.13: Yields of fractions from fractionation of F5 using Biotage®

Fraction code	Yield (mg)
F5(1-2)	2.0
F5(3)	1.3
F5(4-5)	0.8
F5(6-13)	1.1
F5(14-29)	3.2
F5(30-61)	5.0
F5(62-74)	1.6
F5(75-80)	0.5
F5(81-82)	3.5
F5(83-86)	5.5
F5(87-91)	2.3
F5 (92-110)	5.0
*F5(111-120)	3.0
F5(121-132)	1.1
F5(133-138)	9.5
F5(139-140)	4.5
WASH	6.9

**The highlighted raw represent the compound that was semi-purified in this work and are further presented in Chapter 5*

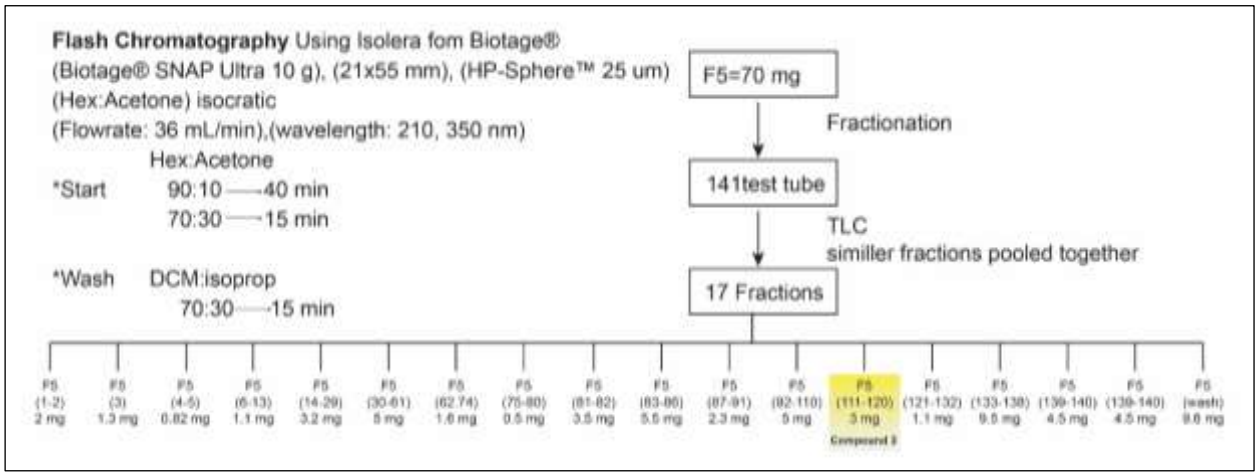


Figure 4.6: Schematic diagram represent the isolation of compound **3** using Biotage®-1

4.2.3.2. Purification of Fraction F6-9 led to Isolation of Compound **3**

Fraction F6 (20 mg) and F7-9 (40 mg) were pooled together (60 mg) then further fractionated using the Biotage® MPLC Isolera™ One 2.0.4 Spektra flash purification system (ISO-1SV). The sample was loaded on a normal phase Biotage®SNAP Ultra 10 g, (21 x 55 mm), (Biotage® HP-Sphere™ 25 um). The Isocratic elution method for 5 min was used with 100% EtOAc and the flow rate was at 36 mL/min and maximum collection volume was set at 18 mL. The system marked the respective test tubes based on the eluting peaks at real time as determined by UV from 230 to 280 nm. Fractions were then pooled using UV read-outs from the Isolera™ One 2.0.4 Spektra flash purification system and similarities in TLC on Si60 normal phase plates using EtOAc and Isopropanol (9.7:0.3) as solvent system. Similar fractions pooled together. 5 fractions were obtained. **Fraction 6-9E(5-10) 3 mg** (**compound 3**) showed a distinct spot on the TLC and is highlighted in **Table 4.14.** The method is outlined below **Figure 4.7.**

Table 4.14: Yields of fractions from fractionation of F6-9E using Biotage®

Fraction code	Yield (mg)
F6-9E(1-4)	1.9
*F6-9E(5-10)	3.0
F6-9E(11-20)	5.9
F6-9E(21-28)	1.7
F6-9E(WASH)	2.4

**The highlighted raw represent the compound that was semi-purified in this work and are further presented in Chapter 5*

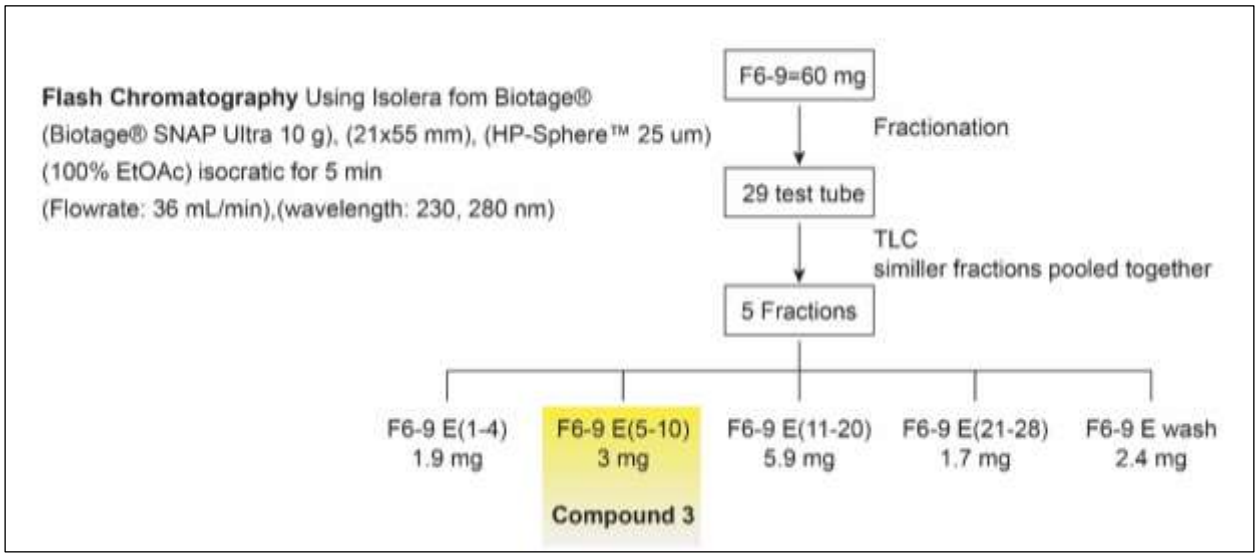


Figure 4.7: Schematic diagram represent the isolation of compound **3** using Biotage®-2

4.2.4. Purification of Subfraction F4.4.7- F4.4.9 led to Isolation of Compound **4**

Fraction F4.4.7-F4.4.9 (196.6 mg) was further fractionated using the Biotage® MPLC Isolera™ One 2.0.4 Spektra flash purification system (ISO-1SV).

The sample was loaded on a normal phase Biotage®SNAP Ultra 10 g, (21 x 55 mm), (Biotage® HP-Sphere™ 25 um). The gradient elution method was performed using DCM (A) and Acetone (B) as solvent system. The gradient was carried out for 60 min. The run started with 1% B at 0 min to 5% B in 20 min and increased to 10% B at 30 min then for every 5 min, the % of solvent B was stepwise increased by 10% until it reaches 70% as listed in **Table 4.15**. The flow rate was at 30 mL/min and maximum collection volume was set at 15 mL. The system marked the respective test tubes based on the eluting peaks at real time as determined by UV from 230 to 280 nm. The column was then flushed with 100 mL of 100% Acetone for 10 min and this was collected as the wash. Fractions were then pooled using UV read-outs from the Isolera™ One 2.0.4 Spektra flash purification system and their similarities on Si60 normal phase TLC plates using DCM and Acetone (9:1) as solvent system. The MPLC purification procedure yielded 10 fractions. **Fraction D(1-7) (6.3 mg)** yielded **compound 4** that exhibited a distinct spots on the TLC as outlined in **Table 4.16** and **Figure 4.8.**

Table 4.15: Elution gradient used for fractionation of F4.4.7-F4.4.9 using Biotage®

Time (min)	% Mobile phase		
	DCM	Acetone	Flow rate mL/min
0	99	1	30
10	99	1	30
20	95	5	30
30	90	10	30
35	80	20	30
40	70	30	30
45	60	40	30
50	50	50	30
55	40	60	30
60	30	70	30
70	0	100	30

Table 4.16: Yields of fractions from fractionation of F4.4.7-F4.4.9 using Biotage®

Fraction code	Yield (mg)
*D(1-7)	6.3
D(8)	1.2
D(9-30)	3.3
D(31)	1.7
D(32-34)	0.7
D(35-40)	7.0
D(41-61)	23.7
D(62)	6.1
D(63-100)	39.0
D(101-154)	8.2

**The highlighted raw represent the compound that was semi-purified in this work and are further presented in Chapter 5*

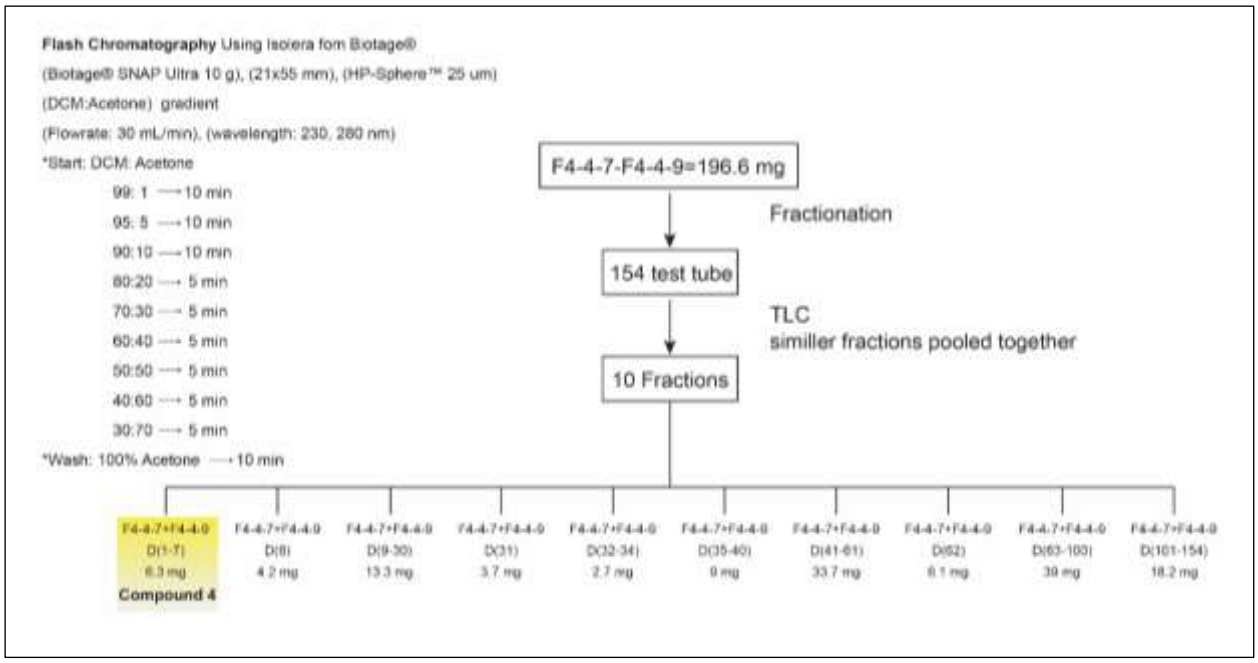


Figure 4.8: Schematic diagram represent the isolation of compound **4** using Biotage®

4.2.5. Purification of Fraction F52-60 led to Isolation of Compound **5**

F52-60 (330 mg) was dissolved in MeOH and chromatographed on a Sephadex LH-20 column (41 x 5.5 cm). The column was equilibrated with 100% MeOH using the same solvent as eluting agent. All subfractions were monitored by TLC using DCM:isopropanol (8:2) as a developing system. The eluted similar fractions were pooled together based on monitoring by UV and anisaldehyde /H_2SO_4 spray reagent. The purified subfractions were further treated on series of columns of Sephadex LH-20 to give **compound 5** = **8.8 mg**.

4.3. Biological Assay Results of the Bioactive Fractions Obtained from Buchi

Chromatographic fractionation is a purification technique applied to isolate and purify the targeted bioactive metabolites from the crude extract. The crude extract itself holds unique chemical properties that is responsible for the bioactivity. The extract comprised of a diverse metabolite profile, which can act synergistically by the interaction of the metabolites producing a combined bioactive effect greater than the sum of their separate effects. Nevertheless, when the crude extract is subjected to fractionation and purification, it can miss reported bioactivity. Loss or change in bioactivity can also be due to variations in concentrations of the bioactive metabolites in the different fractions. The concentration of the bioactive metabolite can decrease after fractionation rendering the fraction inactive when bioactivity is concentration-dependent and not only due to the mere presence or absence of the metabolite.

4.3.1. DPPH Antioxidant Assay

The obtained *S. kali*-derived crude extracts and EtOAc fractions were assessed for their differential antioxidant activities through easy and rapid *in vitro* testing. The free-radical α,α-diphenyl-*β*-picrylhydrazyl (DPPH) scavenging assay was adopted where the EtOAc fractions served as hydrogen providers (anti-oxidants) for the purple free-radical DPPH to generate its lower absorbance state, decolorized DPPH-H (Baliyan et al., 2022).

Measured at 517 nm, differential antioxidant activities were depicted for the crude EtOAc and methanolic extracts (KE and KM, respectively) as well as EtOAc fractions (F*x*) as compared to ascorbic acid serving as positive reference control (**Figure 4.9**). Screening testing at single concentrations (30 µg/mL) showed higher antioxidant activities for the EtOAc crude extract as compared to its methanolic congruent. However, neither of the two crude extracts depicted higher antioxidant activities than that of the ascorbic acid control (40 %) where the latter served as a threshold for significant antioxidant activity. On the other hand, several EtOAc fractions including F4, F5, F6, F7-9, F52-55, F56-60, and F61-76 showed significant antioxidant activities within the range from 41 % and up to almost 65 %.

Thrilled by the obtained antioxidant findings, the top active EtOAc fractions were proceeded through multi-dose response DPPH assay for estimating their corresponding concentrations for furnishing half the corresponding maximum response (EC_{50}) (**Figure 4.10** and **Table 4.17**). Interestingly, all tested EtOAc fractions showed relevant antioxidant activities with EC_{50} at two-digit microgram concentrations. The fractions F6 and F56-60 were of superior antioxidant activities over those of the other tested fractions with EC_{50} values at 17.92 and 17.02,

respectively. On the contrary, F4 was assigned with the modest antioxidant EC_{50} with values up to 49 µg/mL. It is worth mentioning that the EC_{50} of all EtOAc fractions were at higher values as compared to that of the positive control (ascorbic acid; EC_{50} = 5.09 µg/mL). The latter would confer higher concentrations of these fractions to be needed for achieving half their respective maximum antioxidant responses. Nevertheless, several top-active fractions can achieve higher antioxidant potencies (higher % levels) than that of the ascorbic acid when raising up to their higher concentrations as being depicted in **Figure 4.9**. This can be considered beneficial for these fractions as long as safety profiles can be assured at these higher concentrations.

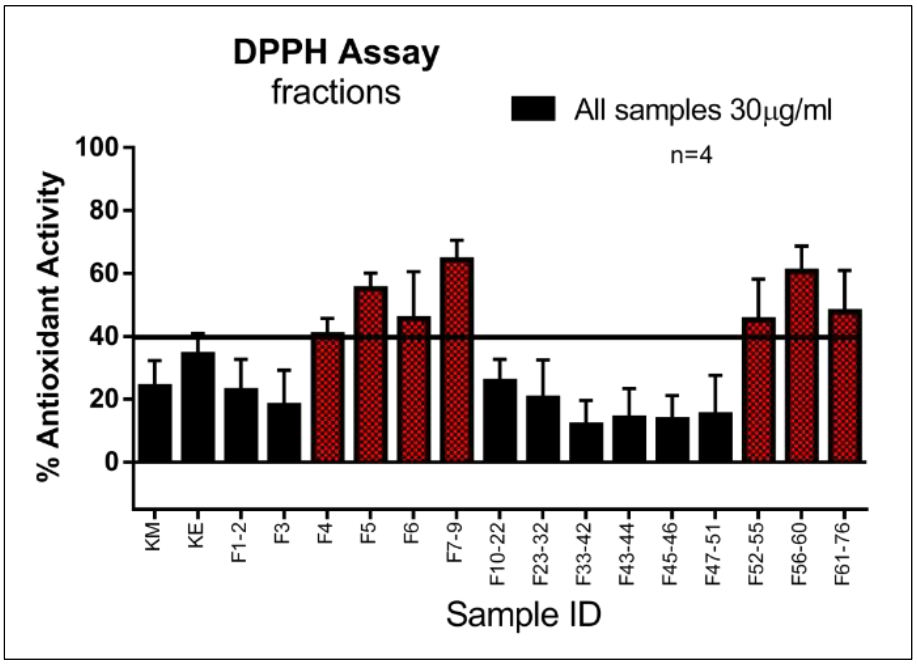


Figure 4.9: Single-dose of the *in vitro* free-radical DPPH scavenger assay for *S. kali*-derived crude extract and EtOAc 15 MPLC fractions obtained from Buchi. Percentage antioxidant activities are represented for corresponding crude extract and EtOAc fractions at single concentration (30 µg/mL) of sample. Ascorbic acid was used as the positive control with significant antioxidant threshold (i.e. 40 %).

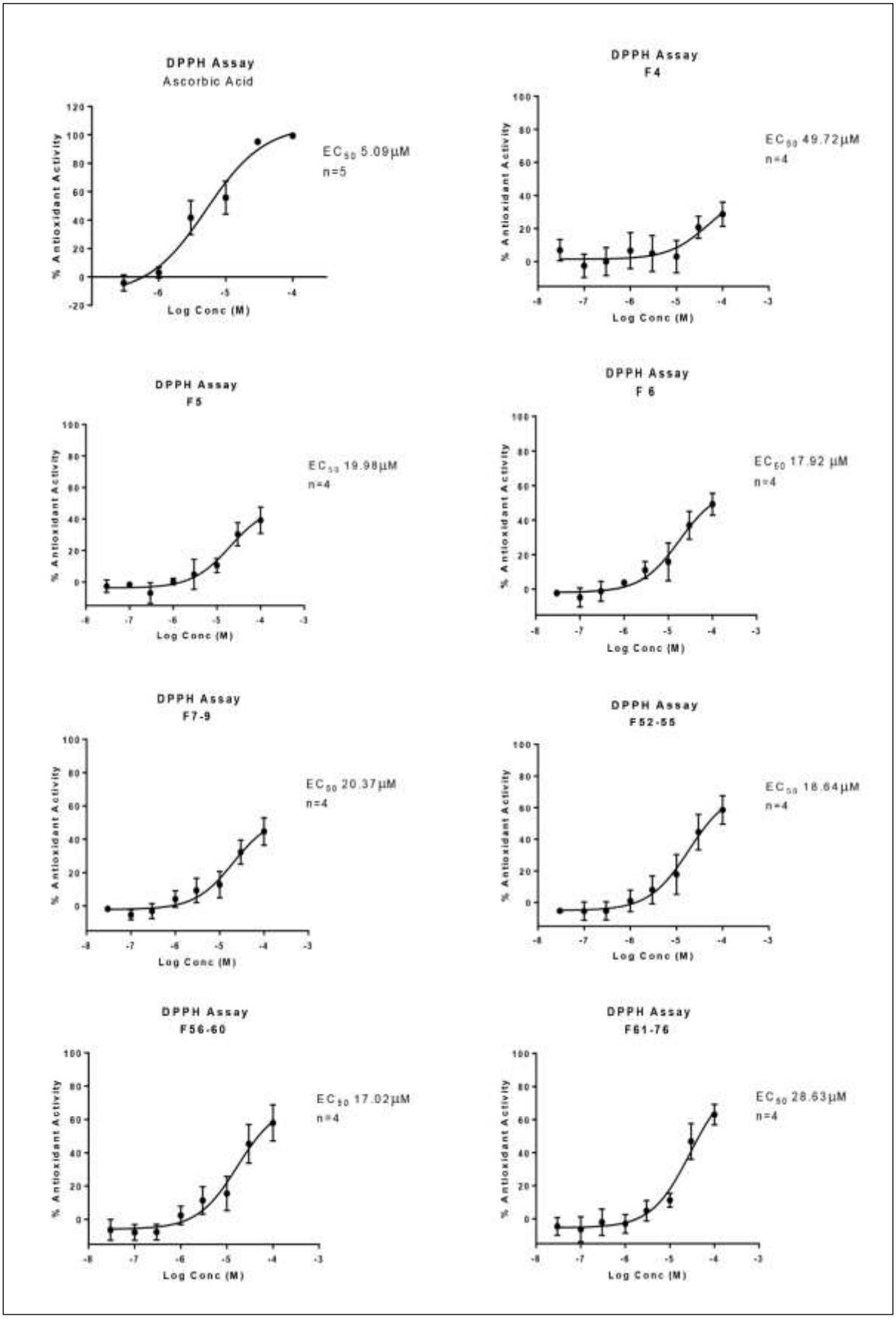


Figure 4.10: Multidose-response curve of the *in vitro* free-radical DPPH scavenger assay for *S. kali*-derived top-bioactive EtOAc fractionated samples and positive control. The concentrations of fractions that achieved half the respective maximal responses were represented as EC_{50}.

Table 4.17: The *in vitro* EC_{50} values for the top active EtOAc fractions possessing relevant antioxidant activities at higher % levels than the positive control ascorbic acid

№.	Fraction code	EC_{50} (µg/mL)	*n**
1	F1-2	N/D	–
2	F3	N/D	–
3	F4*	49.72	4
4	F5*	19.98	4
5	F6*	17.92	4
6	F7-9*	20.37	4
7	F10-22	N/D	–
8	F23-32	N/D	–
9	F33-42	N/D	–
10	F43-44	N/D	–
11	F45-46	N/D	–
12	F47-51	N/D	–
13	F52-55*	18.64	4
14	F56-60*	17.02	4
15	F61-76*	28.63	4
Ascorbic acid		5.09	5

**n = number of repeated sample measurements; N/D = not determined*

** The highlighted raw represent the fractions that show promising antioxidant activity*

4.3.2. Cytotoxic Assay

Cytotoxic activities of the *S. kali*-derived crude extracts and their impacts on cellular survival were evaluated through *in vitro* cell-proliferation MTT assay and brine shrimp lethality bioassays. The adopted cell-proliferation MTT bioassay was performed to estimate the IC_{50} values of the *S. kali*-derived crude extracts against three human cancerous cell lines; breast cancer (MCF-7), hepatocellular carcinoma (HepG2), and colorectal carcinoma (HCT-116). Generally, cytotoxic activity has been correlated to the reduced cellular activity of NADPH-dependent cellular oxido-reductases on the MTT tetrazolium dye [bromide salt of 2,5-diphenyl-3-(4,5-dimethylthiazol-2-yl)tetrazoline] for producing the sparingly water-soluble formazan chromone (Berridge et al., 2005).

Interestingly, the *S. kali* methanolic crude extract showed higher cytotoxicity values (Lower-value IC_{50}) across all tested cancerous cell lines as compared to those of EtOAc crude extract (**Table 4.18** and **Figure 4.11**). The highest cellular sensitivity patterns for both EtOAc and methanolic crude extracts were against the HepG2 cell lines being the most sensitive (104.5 and 34.5 µg/mL, respectively). On the contrarily, the breast MCF-7 cell line was of the least

sensitivity towards the *S. kali* EtOAc with IC_{50} up to 326.3 µg/mL. Concerning the colorectal carcinoma, both crude extracts showed almost comparable cytotoxicity patterns. It is worth noting that both *S. kali* crude extracts showed lower comparative cytotoxicity relative to the positive control, doxorubicin, over the three cancerous cell lines. Based on the above cytotoxicity findings, both *S. kail* crude extracts (methanolic and EtOAc) showed week cytotoxicity activites against the cancerous cell lines under investigation.

Table 4.18: *In-vitro* experimental data of MTT cytotoxicity bioassay for *S. kali* crude extracts exhibiting moderate-to-relatively high activity against three tested human cancerous cell lines

	MCF-7 **IC_{50}* (µg/mL)**	**HepG2** **IC_{50} (µg/mL)**	**HCT-116** **IC_{50} (µg/mL)**
$K_{methanol}$ (**KM**)	167.3 µg/mL	34.5 µg/mL	234 µg/mL
K_{EtOAc} (**KE**)	326.3 µg/mL	104.5 µg/mL	246 µg/mL
DOX	0.41 µg/mL	0.85 µg/mL	0.11 µg/mL

**IC_{50} values confer effective concentrations of tested crude extracts capable of inducing 50% inhibitory response of their own maximum responses following 48 h of exposure*

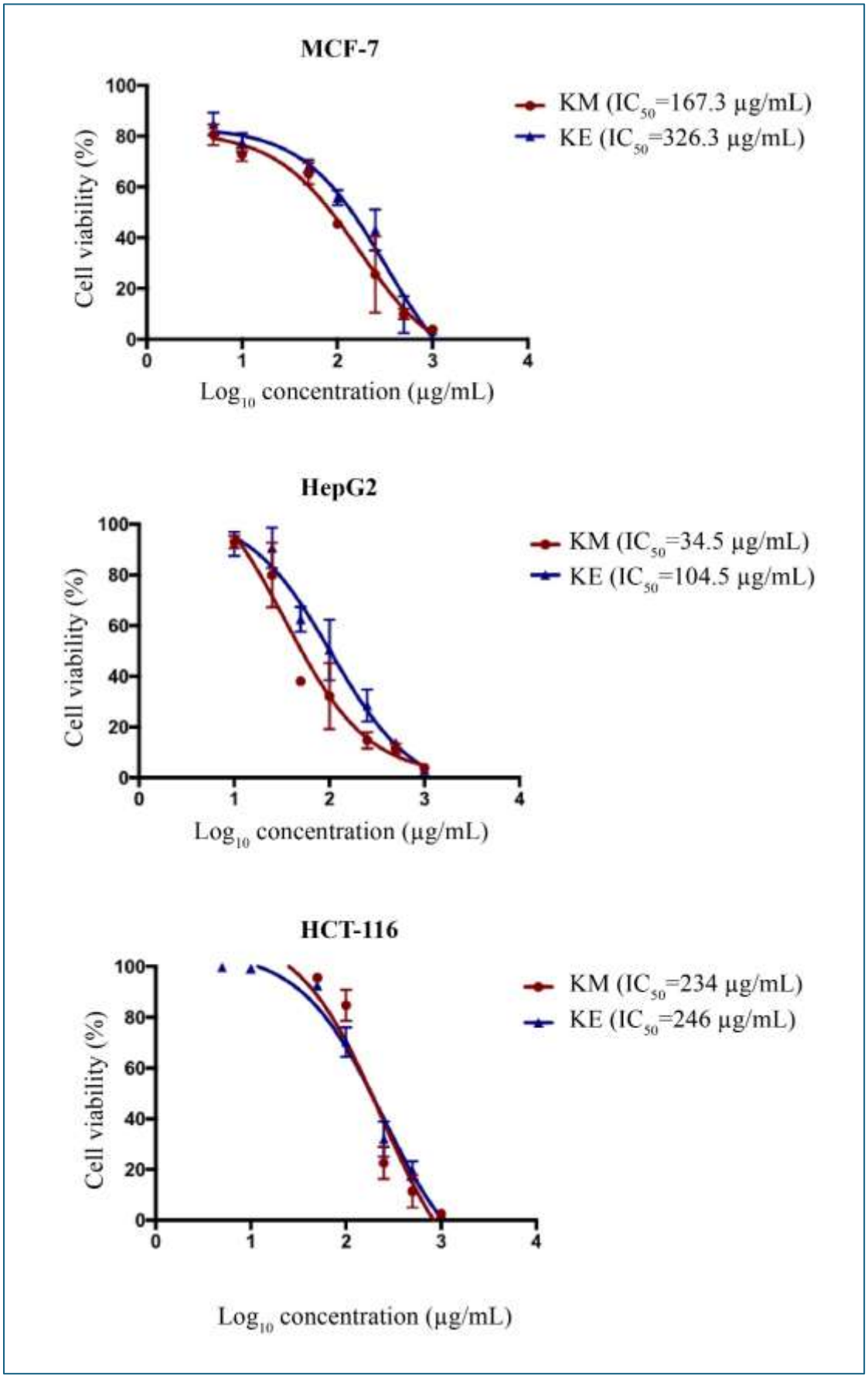


Figure 4.11: Multidose-response curve of the MTT cell-proliferation inhibitory bioassay of the bioactive *S. kali* fractionated crude extract samples against the three cancerous cell lines.

Cancerous cytotoxic activities of *S. kali* crude extracts have been recapitulated through the estimated brine shrimp lethality at four various escalating concentrations. The adopted cytotoxicity bioassay relied on simple estimation of the extracts' lethal concentrations being capable of the killing *Artemia salina*, the zoological organism-brine shrimps (Wu, 2014).

Notably, both *S. kali* crude extracts depicted organism killing starting at 250 µg/mL concentrations with higher lethality pattern being assigned for the methanolic crude extract (**Table 4.19**). This experimental observation was consistent with the superior cancerous cytotoxicity of the methanolic extract as compared to its EtOAc congruent. At the highest applied extract concentrations (500 µg/mL), almost half the treated organisms were killed in case of the methanolic crude extract. Finally, cytotoxicity of *S. kali* crude extracts against brine shrimps was demonstrated at a dose-dependent manner following the extract concentration threshold of 250 µg/mL.

Table 4.19: *In-vitro* experimental data of brine shrimp cytotoxicity bioassay for *S. kali* crude extracts exhibiting dose-dependent manner following a concentration threshold

Conc. (µg/mL)	K $_{methanol}$ (KM)		K $_{EtOAc}$ (KE)	
	Dead number	*Dead number %	Dead number	*Dead number %
62.5	0	0 %	0	0 %
125	0	0 %	0	0 %
250	2	20 %	1	10 %
500	4	40 %	3	30 %

** Total number of tested brine shrimp per sample in n= 10; Samples were done in quadruplicates (n = 4).*

4.4. Multivariate Analysis of the Fractions

To investigate the chemical characteristics, all fractions were subjected to ^{1}H NMR and HRESI-MS. The ^{1}H NMR spectra were stacked and presented in **Figure 4.12**. The biological activity was done by testing the antioxidant activity using DDPH assay. A high ratio of peaks at the aliphatic region between 0.80 and 3.0 ppm was afforded by all fractions. Resonating peaks at a wide range of chemical shifts from the aliphatic to aromatic region with a higher ratio of glycosylated compounds (combination of glycone and aglycone) as indicated by the occurrence of anomeric proton peaks between 4.7 and 5.5 ppm and aromatic compounds (e.g. phenolics) that were accounted for peaks found between 6.0 and 9.0 ppm. Furthermore, signals corresponding to fatty acids were detected (1-1.50 ppm) with characteristic long chain methylene units that were the most relevant structural units.

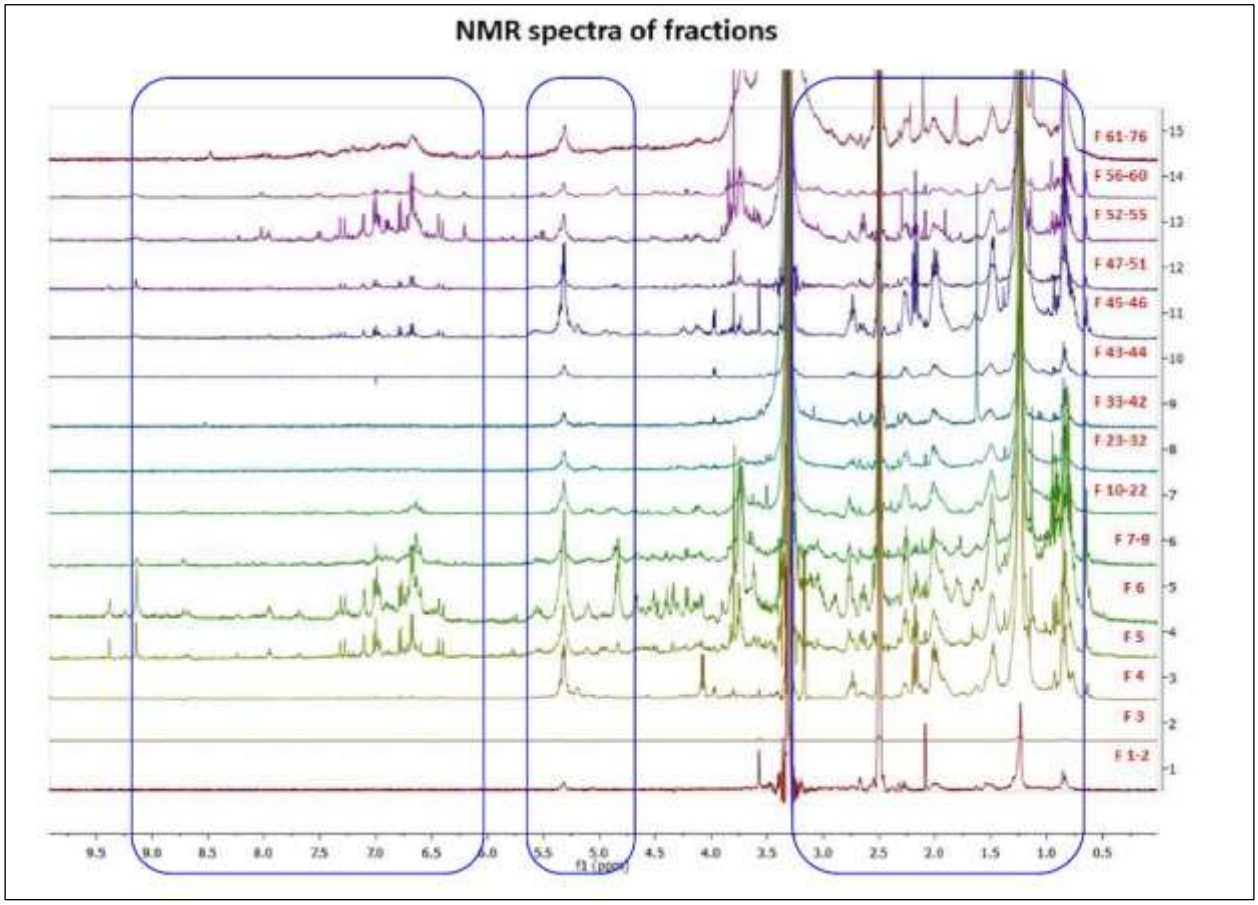


Figure 4.12: Stacked ^{1}H NMR spectra of 15 MPLC fractions. Sample concentration is 5 mg/600µL of DMSO-d_6 measured at 400 MHz

4.4.1. Multivariate Analysis of NMR Spectral Data of *Salsola kali* Fractions

The DPPH assay results were incorporated with the spectral data set for multivariate analysis. The PCA scores scatter plot of the NMR spectral data (NMR fractions M1) of the fractions naturally grouped the bioactive extracts as shown in **Figure 4.13**. It was observed that active and inactive fractions were not completely separated. However, most of the inactive ones were displayed on the upper quadrants, with only three fractions from the active ones, while the active fractions were mostly found in the lower quadrants. The variation $R^2X_0[1]$ between groups was 32.4% while within groups was 30.8%. The large variation both between the groups and within it indicated significant variation in their chemical profiles.

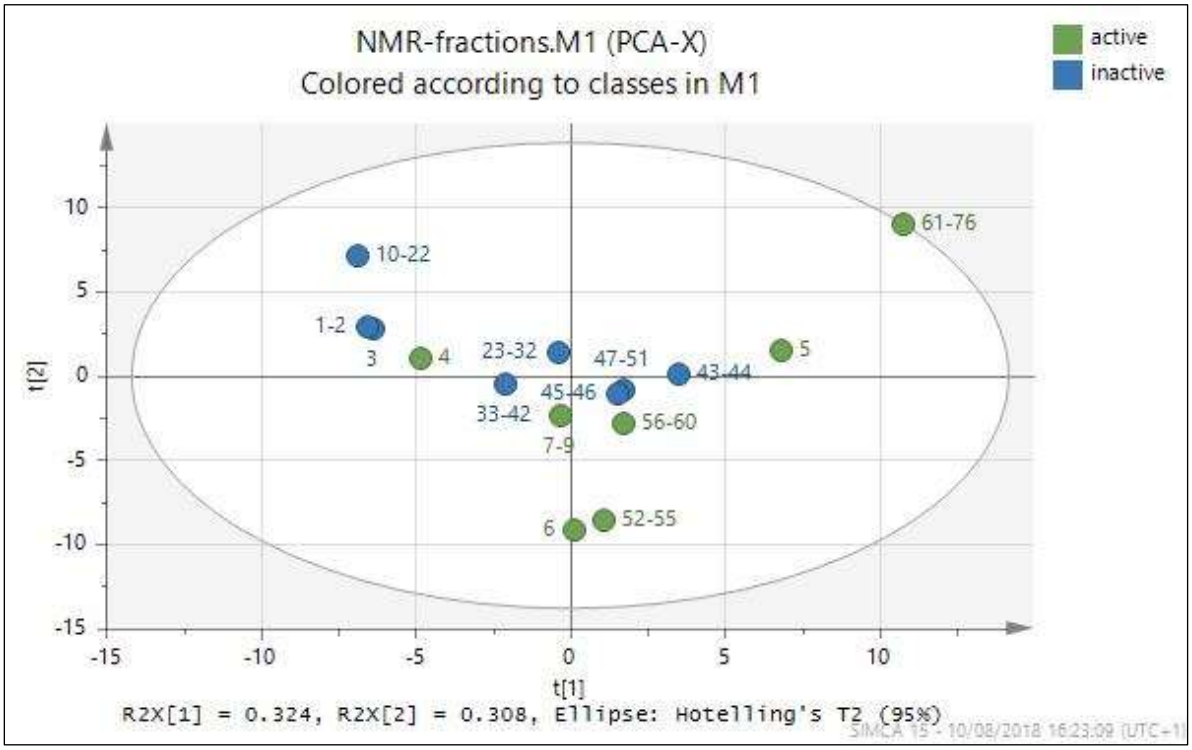


Figure 4.13: PCA scores plot of the NMR spectral data of the 15 MPLC fractions

The PCA-loadings plot in **Figure 4.14** showed the chemical shifts or functional group unique to certain segregated fractions on the same quadrent. These included the active fractions 61-67 with chemical shifts at 2.497 and 3.337 ppm representing acetylated and hydroxylated components; and active fractions on the lower quadrants with resonances at 1.257 and 1.217 ppm belonging to aliphatic alkyl units as lipids, steroids, and triterpenes; while the inactive fractions on the upper left quadrant were presented by signals at 3.297 and 3.377 ppm observed aliphatic hydroxylated groups as sugar and glycosides.

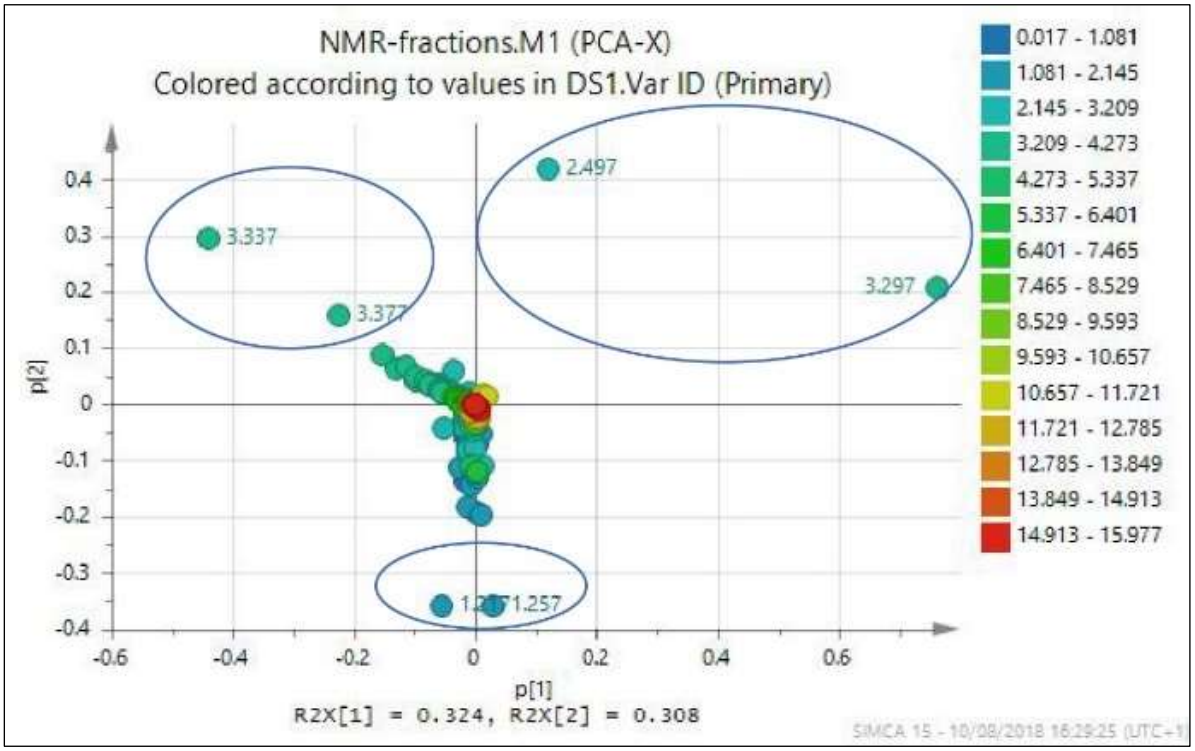


Figure 4.14: PCA loadings plot of the NMR spectral data of the 15 MPLC fractions

By applying OPLS-DA to the NMR fractions, the scores plot is shown in **Figure 4.15.** The fractions were pre-classified according to their bioactivity result in the DPPH assay and were absolutely segregated from each other. The bioactive fractions were dispersed on the left quadrant. This could be explained by the different chemical profiles for the respective fractions. On the other hand, the inactive fractions were in the right quadrant of the scores plot. Overlapping fractions that were clustered together indicated high similarity with no huge difference in their chemical profiles. The variation $R^2X_0[1]$ between groups was 11.9% while within groups was 22.7%. The large variation within the group suggests a significant chemical profile differences among the individuals. This may be attributed to the present fractions present in OPLS-DA loadings plot in **Figure 4.16**.

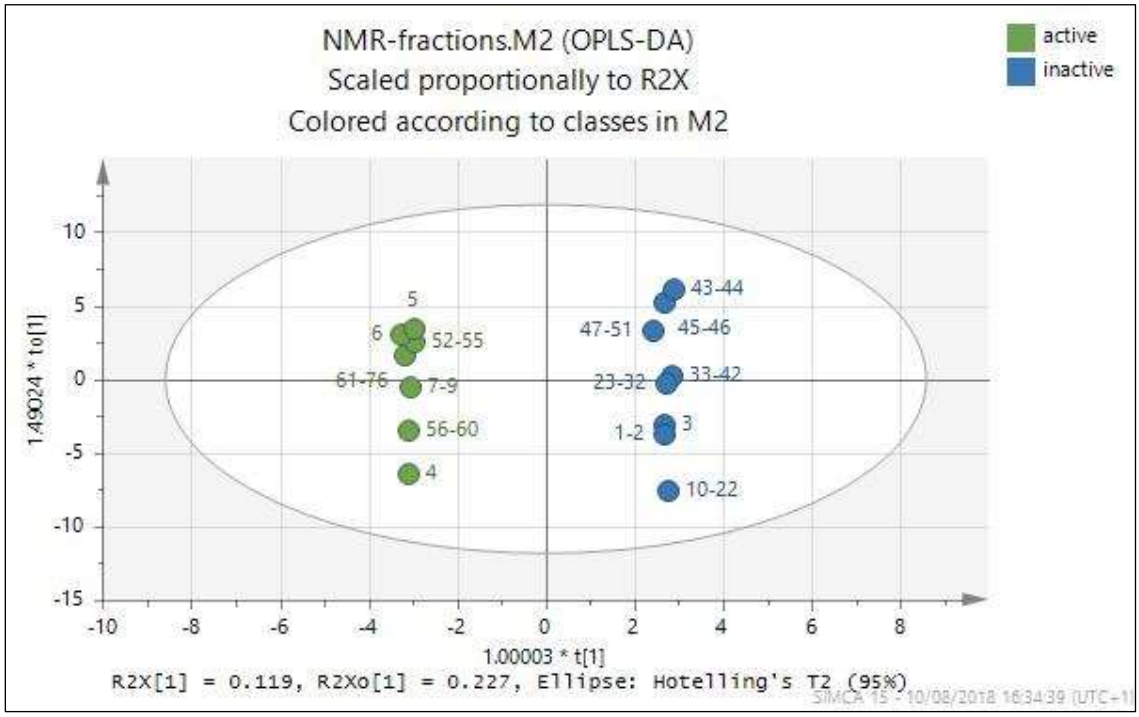


Figure 4.15: OPLS-DA scores plot of the NMR spectral data of the 15 MPLC fractions according to their bioactivity in DPPH assay result.

Figure 4.16 displayed the discriminating chemical shifts for the active fractions that were found at 1.257 and 3.297 ppm for aliphatic hydroxylated moieties. These results were similar to some extent to those obtained in **Figure 4.14.**

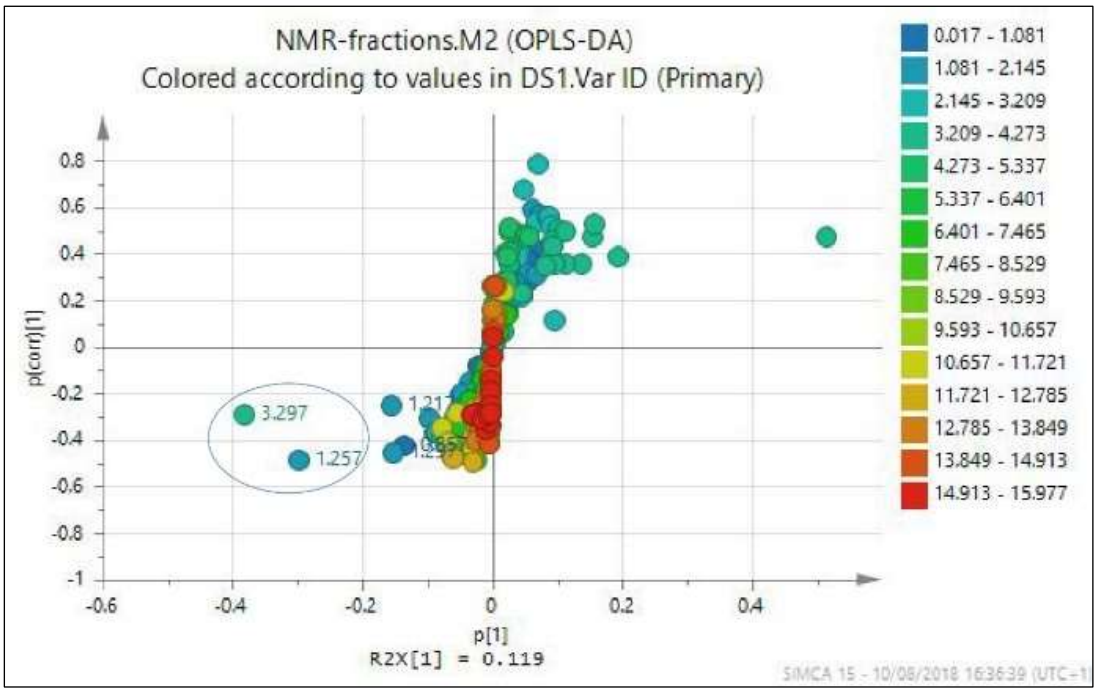


Figure 4.16: OPLS-DA loadings plot of the NMR spectral data of the 15 MPLC fractions according to their bioactivity in DPPH assay result.

4.4.2. Multivariate Analysis of LC-HRMS Data of *Salsola kali* Fractions

The crude extract of *S. kali* was investigated by high resolution LC-ESI-MS analytical method to put an emphasis on its phytochemical profile. Tentative chemical identification of *S. kali* metabolites was achieved by comparing the *m/z* values of the molecular ions, MS transitions and retention times with those in the literature and mass spectral records and databases. In the current investigation, 43 hits were detected in *S. kali* extract.

OPLS modelling was applied on the mass spectral data of the fractions to assess the variation between different fractions according to their bioactivity result in the DPPH assay. As shown in **Figure 4.17**, it was observed that the bioactive fractions were displayed on the right side, dispersed on both upper and lower quadrants, completely segregated from the inactive fractions. Each component represents one metabolite. This could be explained by the different chemical profiles for the respective fractions. The variation $R^2X_0[1]$ between groups was 21.5% while $R^2X[1]$ within groups was 17.6%.

In addition, Hotelling's T2 analysis for identified sample outliers display that the observations almost were distributed within the 95% confidence regions, reflecting the observations were available and the PCA model presented in this study was stable and reliable. OPLS-DA loadings plot offered the metabolites that discriminate between the active and inactive fractions.

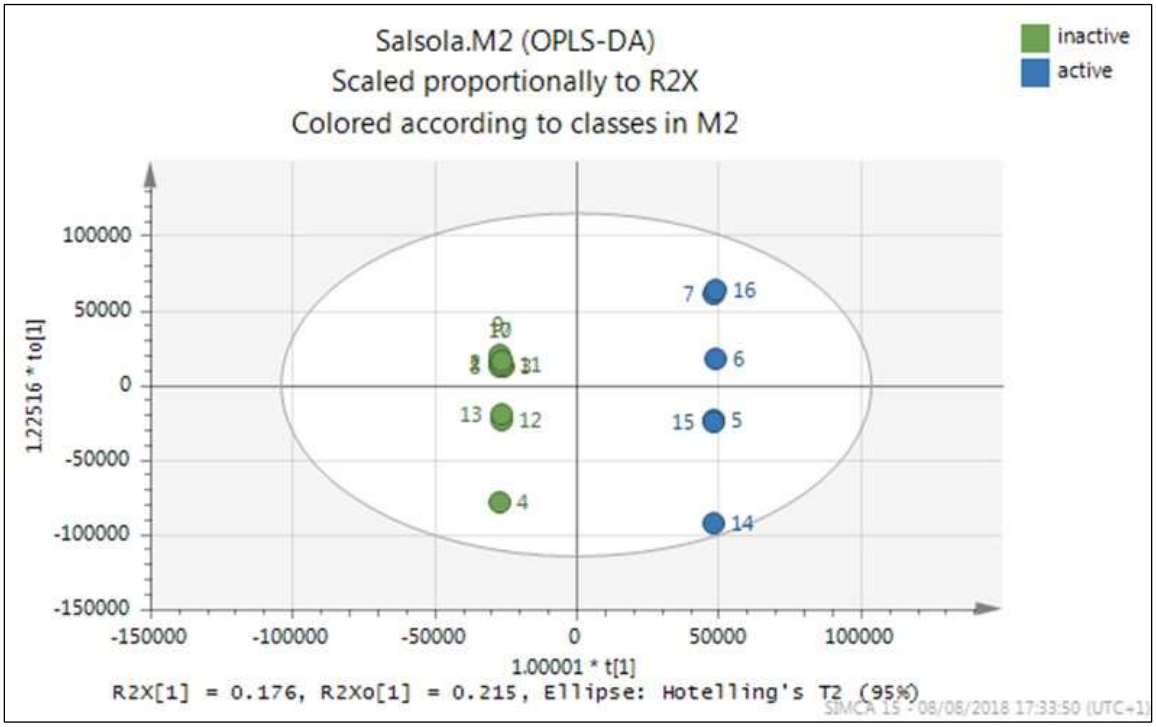


Figure 4.17: OPLS-DA scores plot of the mass spectral data of the 15 MPLC fractions according to their bioactivity in DPPH assay result.

By applying OPLS-DA to different fractions to investigate their relation to each other's. As shown in **Figure 4.18**. It was observed that all the fractions were closely related to each other as most of them were superimposed, except for a few fractions. The active metabolites appear on the right quadrant with molecular weight between 400-800 daltons and *p* value between 0.06 to 0.2. One fraction was detected as an outlier on the upper right quadrant.

The compounds listed in **Table 4.20 and Figure 4.19** are the discriminating metabolites listed on the active quadrant. Most of these compounds fall under the chemical class of phenolics, more especially flavonoids and their derivatives. In addition to organic acids, nitrogenous compounds and sugar derivatives were recorded.

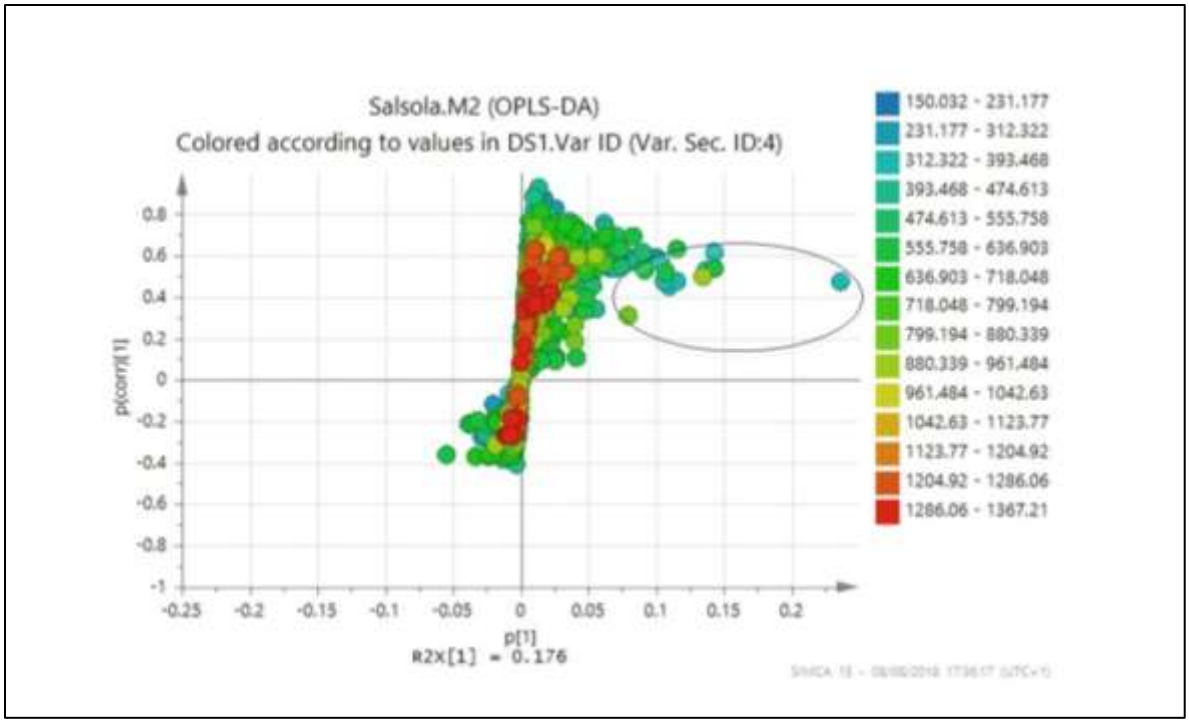


Figure 4.18: OPLS-DA loadings plot of the mass spectral data of the 15 MPLC fractions according to their bioactivity in DPPH assay result. Encircled features on the loadings plot indicate the discriminating *m/z* ion peaks for the biologically active fractions listed under **Table 4.20**

Table 4.20: Dereplication identified in *S. kali* crude extract using LC-ESI-MS

MZmine ID	*m/z*	Rt (min)	MWt g/mol	Precursor type	Chemical formula prediction	Fraction number	Compound name and Ref
P2033	169.0495	5.76	168.0423	$[M+H]^+$	$C_8H_8O_4$	F4	vanillic acid (Sokolowska-Krzaczek et al., 2009; Shehab and Abu-Gharbieh, 2014; Ghorab et al., 2017; Zhao et al., 2020)
P2150	199.0601	5.81	198.0529	$[M+H]^+$	$C_9H_{10}O_5$	F4	syringic acid (Sokolowska-Krzaczek et al., 2009; Zhao et al., 2020)
P8017	465.1028	7.06	464.0955	$[M+H]^+$	$C_{21}H_{20}O_{12}$	F61-76	quercetin-3-*O*-glucoside (isoquercitrin) (Syrchina et al., 1989; Lee et al., 2012; Ghorab et al., 2017; Taia et al., 2018; Iannuzzi et al., 2020)
P6536	611.1606	7.70	610.1533	$[M+H]^+$	$C_{27}H_{30}O_{16}$	F52-55	rutin (Zaikov et al., 1992; Jin et al., 2011; Lee et al., 2012; Rasheed et al., 2013; Shehab and Abu-Gharbieh, 2014; Küçükboyacı et al., 2016; Orhan et al., 2017; Taia et al., 2018; Iannuzzi et al., 2020)
P7873	344.14927	7.72	343.142	$[M+H]^+$	$C_{19}H_{21}NO_5$	F61-76	*N*-[2'-(3"-hydroxy-4"-methoxyphenyl)-2'-hydroxyethyl]3-(4'"-methoxyphenyl)-prop-2-enamide (Khan et al., 2003) OR *N*-(3',4'-dimethoxy-cinnamoyl)-norepinephrine (Rasheed et al., 2013)
P2735	330.13353	7.82	329.12626	$[M+H]^+$	$C_{18}H_{19}NO_5$	F5	*N*-[2'-(3",4"-dihydroxyphenyl)-2'-hydroxyethyl]-3-(4'"-methoxyphenyl)prop-2-enamide

Table 4.20: Dereplication identified in *S. kali* crude extract using LC-ESI-MS

MZmine ID	*m/z*	Rt (min)	MWt g/mol	Precursor type	Chemical formula prediction	Fraction number	Compound name and Ref
							(Khan et al., 2003) OR *N*-(4'-methoxy-cinnamoyl)-norepinephrine (Rasheed et al., 2013)
P2637	312.123 312.1229	7.83	311.116 311.1157	$[M-H_2O]^+$	$C_{18}H_{17}NO_4$	F52-55	*N*-[2'-(3",4"-dihydroxyphenyl)-2'-hydroxyethyl]-3-(4'"-methoxyphenyl)prop-2-enamide. (Khan et al., 2003)
P7264	449.1078	8.19	448.1006	$[M+H]^+$	$C_{21}H_{20}O_{11}$	F56-60	kaempferol-3-*O*- glucoside (astragalin) (Lee et al., 2012; Elsharabasy and Hosney, 2013; Ghorab et al., 2017)
P5986	479.1183	8.42	478.1111	$[M+H]^+$	$C_{22}H_{22}O_{12}$	F52-55	isorhmnetin-3-*O*-glucoside (Syrchina et al., 1989; Zaikov et al., 1992; Tundis et al., 2007; Xiang et al., 2007b; Tundis et al., 2008; Lee et al., 2012; Elsharabasy and Hosney, 2013; Rasheed et al., 2013; Küçükboyacı et al., 2016; Osman et al., 2016; Orhan et al., 2017) OR *isorhmnetin-3-*O*-galactopyranoside (Osman et al., 2016) OR isorhmnetin-7-*O*-glucoside (Xiang et al., 2007b)
P5821	317.0655	8.43	316.0582	$[M+H]^+$	$C_{16}H_{12}O_7$	F56-60	isorhamnetin (Syrchina et al., 1989; Zaikov et al., 1992; Jin et al., 2011; Lee et al., 2012; Osman et al., 2016) OR

Table 4.20: Dereplication identified in *S. kali* crude extract using LC-ESI-MS

MZmine ID	*m/z*	Rt (min)	MWt g/mol	Precursor type	Chemical formula prediction	Fraction number	Compound name and Ref
							rhamnetin (Tundis et al., 2007; Taia et al., 2018)
P7905	361.0918	8.72	360.0845	$[M+H]^+$	$C_{18}H_{16}O_8$	F61-76	rosmarinic acid (Shehab and Abu-Gharbieh, 2014; Shehab et al., 2015)
P7770	271.0600	8.97	270.0528	$[M+H]^+$	$C_{15}H_{10}O_5$	F61-76	apigenin (Shehab and Abu-Gharbieh, 2014)
N1363	329.0667	9.22	330.074	$[M-H]^-$	$C_{17}H_{14}O_7$	F56-60	6,7,3'-trihydroxy5,2'-dimethoxyisoflavone (Abegaz and Woldu, 1991)
P7119	291.0863	9.23	290.079	$[M+H]^+$	$C_{15}H_{14}O_6$	F56-60	(-) epicatechin (Taia et al., 2018)
P5816	314.1385	9.50	313.1313	$[M+H]^+$	$C_{18}H_{19}NO_4$	F6	moupinamide (Jin et al., 2011) *N-trans*-feruloyltyramine (Oueslati et al., 2006; Lee et al., 2012; Rasheed et al., 2013; Osman et al., 2016)
N796	315.0510	9.63	316.0583	$[M-H]^-$	$C_{16}H_{12}O_7$	F56-60	Isorhamnetin (Syrchina et al., 1989; Zaikov et al., 1992; Jin et al., 2011; Lee et al., 2012; Osman et al., 2016) OR rhamnetin (Tundis et al., 2007; Taia et al., 2018)
N862	609.1462	9.66	610.1535	$[M-H]^-$	$C_{27}H_{30}O_{16}$	F56-60	rutin (Zaikov et al., 1992; Jin et al., 2011; Lee et al., 2012; Rasheed et al., 2013; Shehab and Abu-Gharbieh, 2014; Küçükboyacı et al., 2016; Orhan et al., 2017; Taia et al., 2018; Iannuzzi et al., 2020)

Table 4.20: Dereplication identified in *S. kali* crude extract using LC-ESI-MS

MZmine ID	*m/z*	Rt (min)	MWt g/mol	Precursor type	Chemical formula prediction	Fraction number	Compound name and Ref
P2656	314.1385	9.69	313.1313	$[M+H]^+$	$C_{18}H_{19}NO_4$	F6	moupinamide (Jin et al., 2011) *N-trans*-feruloyltyramine (Oueslati et al., 2006; Lee et al., 2012; Rasheed et al., 2013; Osman et al., 2016)
N883	285.0405	9.87	286.0477	$[M-H]^-$	$C_{15}H_{10}O_6$	F56-60	kaempferol (Zaikov et al., 1992; Kaur and Bains, 2012; Taia et al., 2018; Iannuzzi et al., 2020)
P2657	314.1385	10.19	313.1313	$[M+H]^+$	$C_{18}H_{19}NO_4$	F5	moupinamide (Jin et al., 2011) *N-trans*-feruloyltyramine (Oueslati et al., 2006; Lee et al., 2012; Rasheed et al., 2013; Osman et al., 2016)
N859	463.0882	10.38	464.0955	$[M-H]^-$	$C_{21}H_{20}O_{12}$	F61-76	quercetin-3-*O*-glucoside (isoquercitrin) (Syrchina et al., 1989; Lee et al., 2012; Ghorab et al., 2017; Taia et al., 2018; Iannuzzi et al., 2020)
P7890	355.1024	10.74	354.0951	$[M+H]^+$	$C_{16}H_{18}O_9$	F61-76	neochlorogenic acid (Lodhi, 1979)
P7705	195.0653	10.74	194.0580	$[M+H]^+$	$C_{10}H_{10}O_4$	F4	*ferulic acid (Lodhi, 1979; Xiang et al., 2007b; Sokolowska-Krzaczek et al., 2009; Shehab and Abu-Gharbieh, 2014; Osman et al., 2016; Zhao et al., 2020)
N1406	193.0498	11.01	194.0571	$[M-H]^-$	$C_{10}H_{10}O_4$	F4	*ferulic acid (Lodhi, 1979; Xiang et al., 2007b; Sokolowska-Krzaczek et al., 2009; Shehab and Abu-Gharbieh, 2014; Osman et al., 2016; Zhao et al., 2020)
P3880	625.2546	11.27	624.2473	$[M+H]^+$	$C_{28}H_{32}O_{16}$	F52-55	isorhmnetin-3-*O*-rutinoside (narcissin)

Table 4.20: Dereplication identified in *S. kali* crude extract using LC-ESI-MS

MZmine ID	*m/z*	Rt (min)	MWt g/mol	Precursor type	Chemical formula prediction	Fraction number	Compound name and Ref
							(Syrchina et al., 1989; Tundis et al., 2007; Tundis et al., 2008; Lee et al., 2012; Rasheed et al., 2013; Küçükboyacı et al., 2016; Orhan et al., 2017; Iannuzzi et al., 2020)
N834	269.0455	11.55	270.0528	$[M-H]^-$	$C_{15}H_{10}O_5$	F52-55	apigenin (Shehab and Abu-Gharbieh, 2014)
N923	301.0355	12.29	302.0428	$[M-H]^-$	$C_{15}H_{10}O_7$	F56-60	quercetin (Lodhi, 1979; Zaikov et al., 1992; Tundis et al., 2007; Jin et al., 2011; Kaur and Bains, 2012; Rasheed et al., 2013; Shehab and Abu-Gharbieh, 2014; Shehab et al., 2015; Küçükboyacı et al., 2016; Orhan et al., 2017; Taia et al., 2018)
P2586	303.0863	12.60	302.0791	$[M+H]^+$	$C_{16}H_{14}O_6$	F4	hesperetin (Shehab and Abu-Gharbieh, 2014)
P2501	287.0549	12.92	286.0477	$[M+H]^+$	$C_{15}H_{10}O_6$	F4	kaempferol (Zaikov et al., 1992; Kaur and Bains, 2012; Taia et al., 2018; Iannuzzi et al., 2020)
N66	623.2551	13.61	624.2624	$[M-H]^-$	$C_{28}H_{32}O_{16}$	F5	isorhmnetin-3-*O*-rutinoside (narcissin) (Syrchina et al., 1989; Tundis et al., 2007; Tundis et al., 2008; Lee et al., 2012; Rasheed et al., 2013; Küçükboyacı et al., 2016; Orhan et al., 2017; Iannuzzi et al., 2020)
P3881	625.2544	13.63	624.2472	$[M+H]^+$	$C_{28}H_{32}O_{16}$	F52-55	isorhmnetin-3-*O*-rutinoside (narcissin) (Syrchina et al., 1989; Tundis et al., 2007; Tundis et al., 2008; Lee et al.,

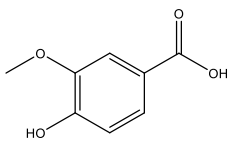
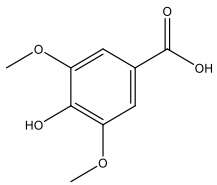
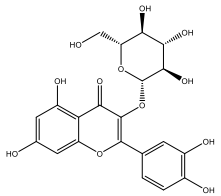
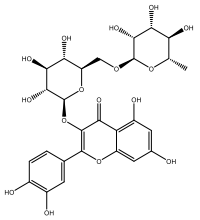
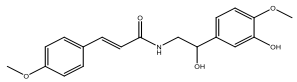
Table 4.20: Dereplication identified in *S. kali* crude extract using LC-ESI-MS

MZmine ID	*m/z*	Rt (min)	MWt g/mol	Precursor type	Chemical formula prediction	Fraction number	Compound name and Ref
							2012; Rasheed et al., 2013; Küçükboyacı et al., 2016; Orhan et al., 2017; Iannuzzi et al., 2020)
P2578	301.0706	13.75	300.0634	$[M+H]^+$	$C_{16}H_{12}O_6$	F4	kaempferol-3-methyl ether (Elsharabasy and Hosney, 2013)
P2014	163.039	16.72	162.0317	$[M+H]^+$	$C_9H_6O_3$	F4	umbelliferone (Elsharabasy and Hosney, 2013)
P2083	181.0496	16.76	180.0423	$[M+H]^+$	$C_9H_8O_4$	F4	caffeic acid (Lodhi, 1979; Sokolowska-Krzaczek et al., 2009; Shehab and Abu-Gharbieh, 2014; Zhao et al., 2020)
P5929	415.3941	24.41	414.3868	$[M+H]^+$	$C_{29}H_{50}O$	F7-9	*B*- sitosterol (Zaikov et al., 1992; Tundis et al., 2008; Elsharabasy and Hosney, 2013; Alturkistani et al., 2017)
P3375	457.3685	26.73	456.3613	$[M+H]^+$	$C_{30}H_{48}O_3$	F4	ursolic acid (Alturkistani et al., 2017)
N665	281.2485	26.89	282.2558	$[M-H]^-$	$C_{18}H_{34}O_2$	F52-55	oleic acid (Karawya et al., 1972; Zaikov et al., 1992; Rasheed et al., 2013; Elsharabasy et al., 2015; Ghorab et al., 2017)
P7634	625.1763	8.04	624.169	$[M+H]^+$	$C_{28}H_{32}O_{16}$	F56-60	isorhmnetin-3-*O*-rutinoside (narcissin) (Syrchina et al., 1989; Tundis et al., 2007; Tundis et al., 2008; Lee et al., 2012; Rasheed et al., 2013; Küçükboyacı et al., 2016; Orhan et al., 2017; Iannuzzi et al., 2020)
P1863	413.3791	29.46	412.3719	$[M+H]^+$	$C_{29}H_{48}O$	F23-32	stigmasterol (Karawya et al., 1972; Salt and Adler,

Table 4.20: Dereplication identified in *S. kali* crude extract using LC-ESI-MS

MZmine ID	*m/z*	Rt (min)	MWt g/mol	Precursor type	Chemical formula prediction	Fraction number	Compound name and Ref
							1985; Syrchina et al., 1989; Zaikov et al., 1992; Elsharabasy and Hosney, 2013)
N525	277.2171	31.25	278.2244	$[M-H]^-$	$C_{18}H_{30}O_2$	F7-9	linolenic acid (Karawya et al., 1972; Zaikov et al., 1992; Rasheed et al., 2013; Elsharabasy et al., 2015)
N512	353.3425	31.80	354.3498	$[M-H]^-$	$C_{23}H_{46}O_2$	F7-9	tricosanoic acid (Elsharabasy et al., 2015)
P1873	427.3979	36.57	426.3906	$[M+H]^+$	$C_{30}H_{50}O$	F4	*lupeol (Alturkistani et al., 2017)
P7154	179.0703	5.47	178.0631	$[M+H]^+$	$C_{10}H_{10}O_3$	F4	**trans*-4-methoxy cinnamic acid (Kim et al., 2012b; Świsłocka et al., 2012)

***** *Highlighted in dark blue were the isolated compounds in this study*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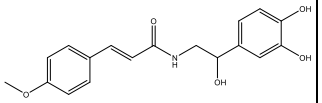	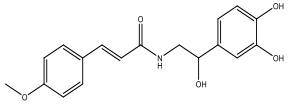	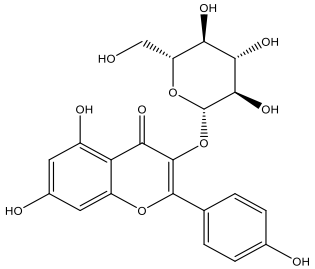	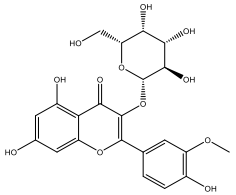	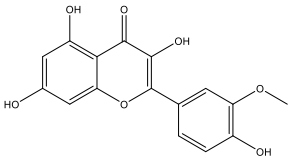
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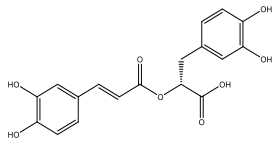	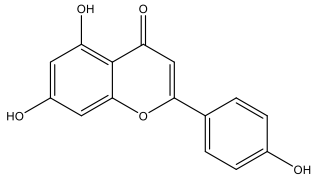	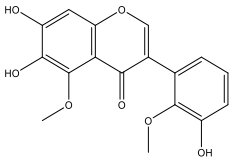	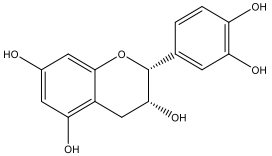	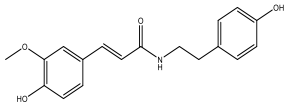
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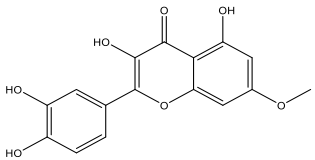	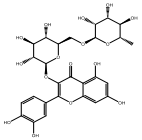	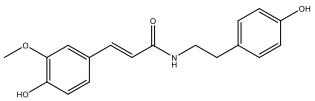	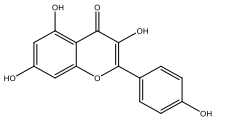	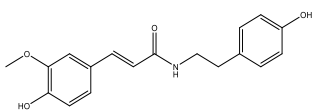
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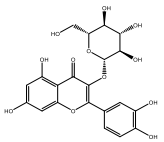
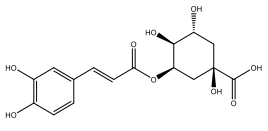
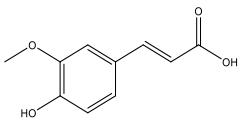
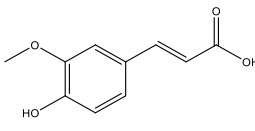
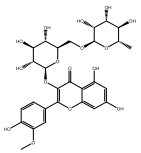
				
N859	**P7890**	**P7705**	**N1406**	**P3880**

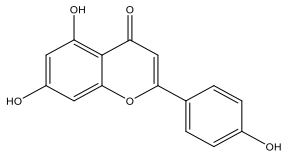
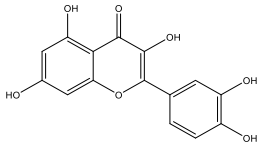
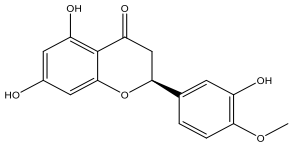
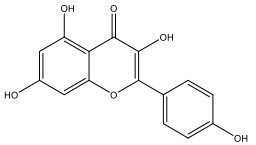
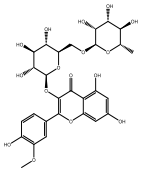
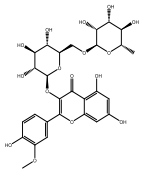
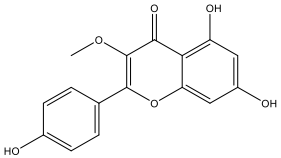
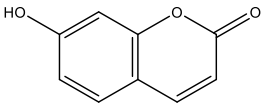
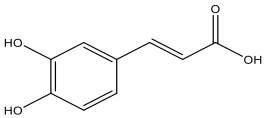
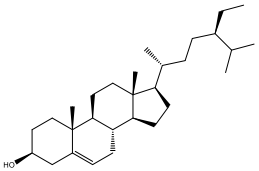
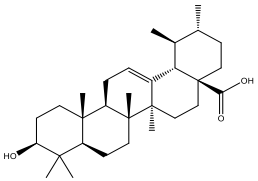
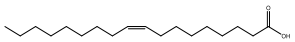
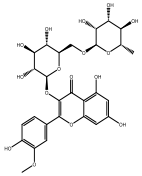
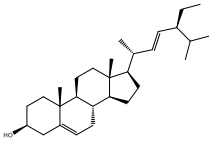
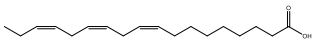
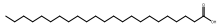
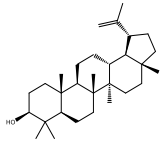
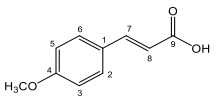
 **N834**	 **N923**	 **P2586**	 **P2501**	 **N66**
 **P3881**	 **P2578**	 **P2014**	 **P2083**	 **P5929**
 **P3375**	 **N665**	 **P7634**	 **P1863**	 **N525**
 **N512**	 **P1873**	 **P7154**		

Figure 4.19: Chemical structures of the identified compounds.

CHAPTER V: STRUCTURE ELUCIDATION OF ISOLATED COMPOUNDS

5. Structure Elucidation of Isolated Compounds

5.1. Structure Elucidation of Bioactive Compounds

The bioactive compounds were submitted for ^{1}H NMR, 2D NMR and LC-HRMS. The dereplication study was performed by using the HPLC-MS data, which help to find major metabolites present in the active fractions that were unidentified using the DNP database. Dereplication can be performed by analysis of exact mass and fragmentation patterns, as well as using retention time, which can aid in identification of compounds.

5.1.1. Structure Elucidation of Compound **1**

Compound **1** was isolated from fraction F4.4.3.3.2 and F4.4.3.3.3.1 as white crystal needles soluble in chloroform with a yield of 2.8 mg and 2.4 mg, respectively, exhibited typical colour reactions for triterpenes. Molecular ion peak at *m/z* 427.3979 $[M+H]^{+}$, indicating the molecular weight as 426.3906 g/mol, corresponding to the molecular formula $C_{30}H_{50}O$. ^{13}C NMR revealed 30 carbons including seven methyls, eleven methylenes, six methines and six quaternary carbons. The ^{1}H NMR spectrum in **Figures 5.2, 5.3 and 5.4** showed seven singlets at δ_H 0.66 (3H, s), 0.77 (3H, s), 0.78 (3H, s), 0.88 (3H, s), 0.92 (3H, s), 0.99 (3H, s) and 1.65 (3H, s) assigned for tertiary methyl groups 24, 28, 25, 23, 27, 26, and 30, respectively. Moreover, one secondary hydroxyl group as broad multiplet at δ_H 3.42 typical for an oxymethine proton at C-3 of a triterpene. The splitting pattern of this proton confirmed the *β* orientation of the C-3 oxygenated substituent. It also showed a pair of germinal coupled olefinic protons at δ_H 4.55 (1H, m, H-29b) and δ_H 4.68 (1H, m, H-29a) representing the exocyclic double bond.

The above spectral data suggested that compounds **1** are identical and were elucidated as a lupane triterpene having a secondary hydroxyl group at C-3. In comparison with the ^{1}H and ^{13}C NMR data of the literature, the structure of compound **1** was assigned as **lupeol** (Burns et al., 2000; Prakash and Prakash, 2012). The ^{1}H and ^{13}C NMR assignments are presented in **Table 5.1.**

Table 5.1: ^{1}H-NMR and ^{13}C-NMR of compound **1** (DMSO, 400, 100 MHz)

Position	δ_H (mult., *J* (Hz)	δ_C
1	0.91, 1.64 (1H, m)	38.4
2	1.61 (1H, m)	21.3
3	3.42 (1H, m H-3)	76.1
4	-	38.0
5	0.67 (1H, m)	55.3
6	1.39 1.52 (1H, m)	18.2
7	1.36 (1H, m)	34.2
8	-	42.8
9	1.33 (1H, m)	50.3
10	-	38.8
11	1.28, 1.43 (1H, m)	20.9
12	1.08, 1.70 (1H, m)	23.7
13	1.62 (1H, m)	37.1
14	-	43.0
15	0.99, 1.61 (1H, m)	25.1
16	1.48 (1H, m)	35.6
17	-	40.0
18	1.39 (1H, m)	48.3
19	2.35 (1H, m)	48.8
20	-	151.3
21	1,25 (1H, m)	29.8
22	1.19 (1H, m)	40.0
23	0.88 (3H, s),	27.4
24	0.66 (3H, s)	16.5
25	0.78 (3H, s)	16.2
26	0.99 (3H, s)	16.0
27	0.92 (3H, s)	14.5
28	0.77 (3H, s)	18.0
29	4.55 (1H, m, H-29b) 4.68 (1H, m, H-29a)	110.7
30	1.65 (3H, s)	19.3

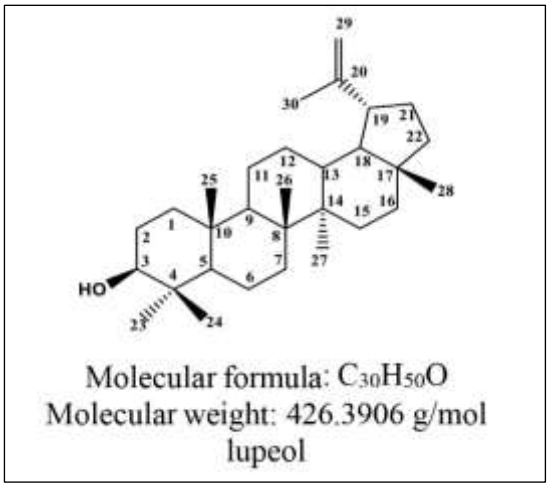


Figure 5.1: Chemical structure of compound **1**

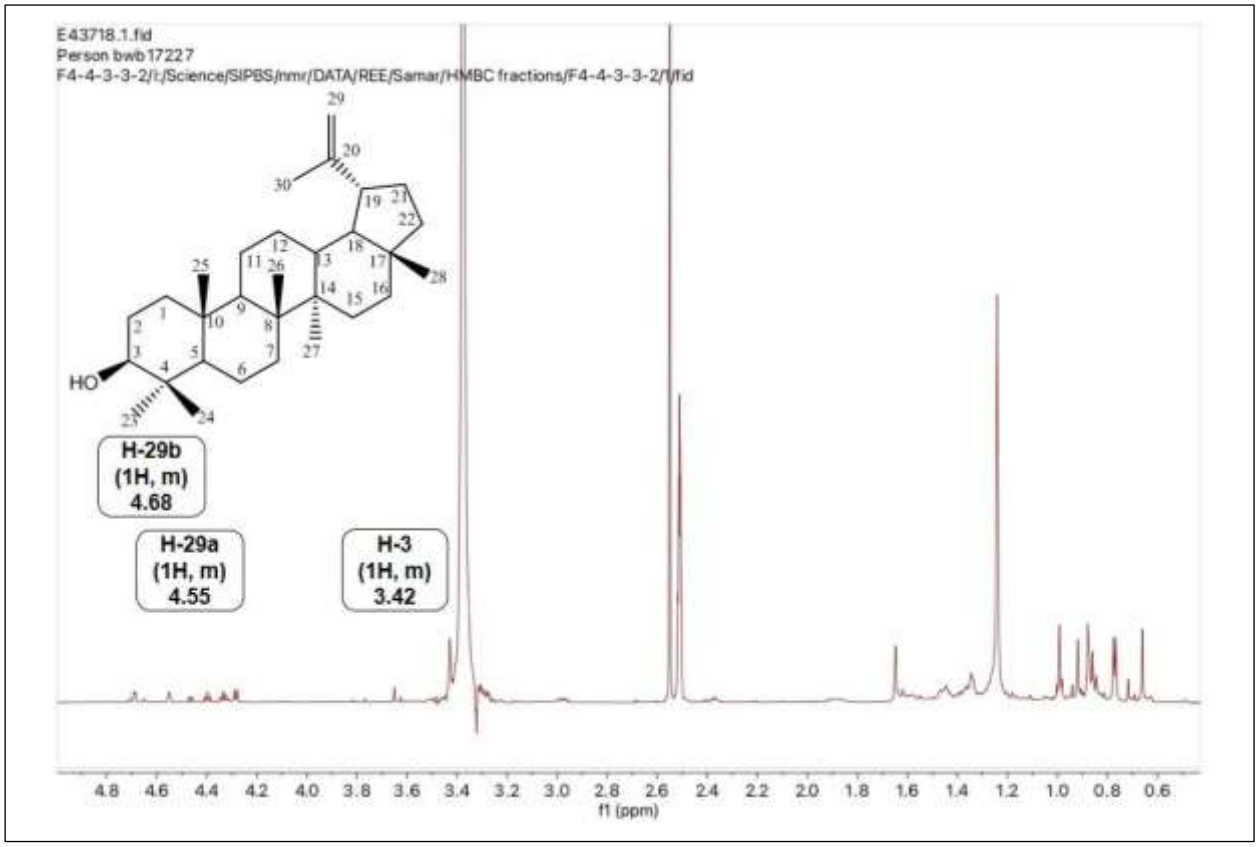


Figure 5.2:[1]H-NMR spectrum of compound **1** in DMSO measured at 400 MHz

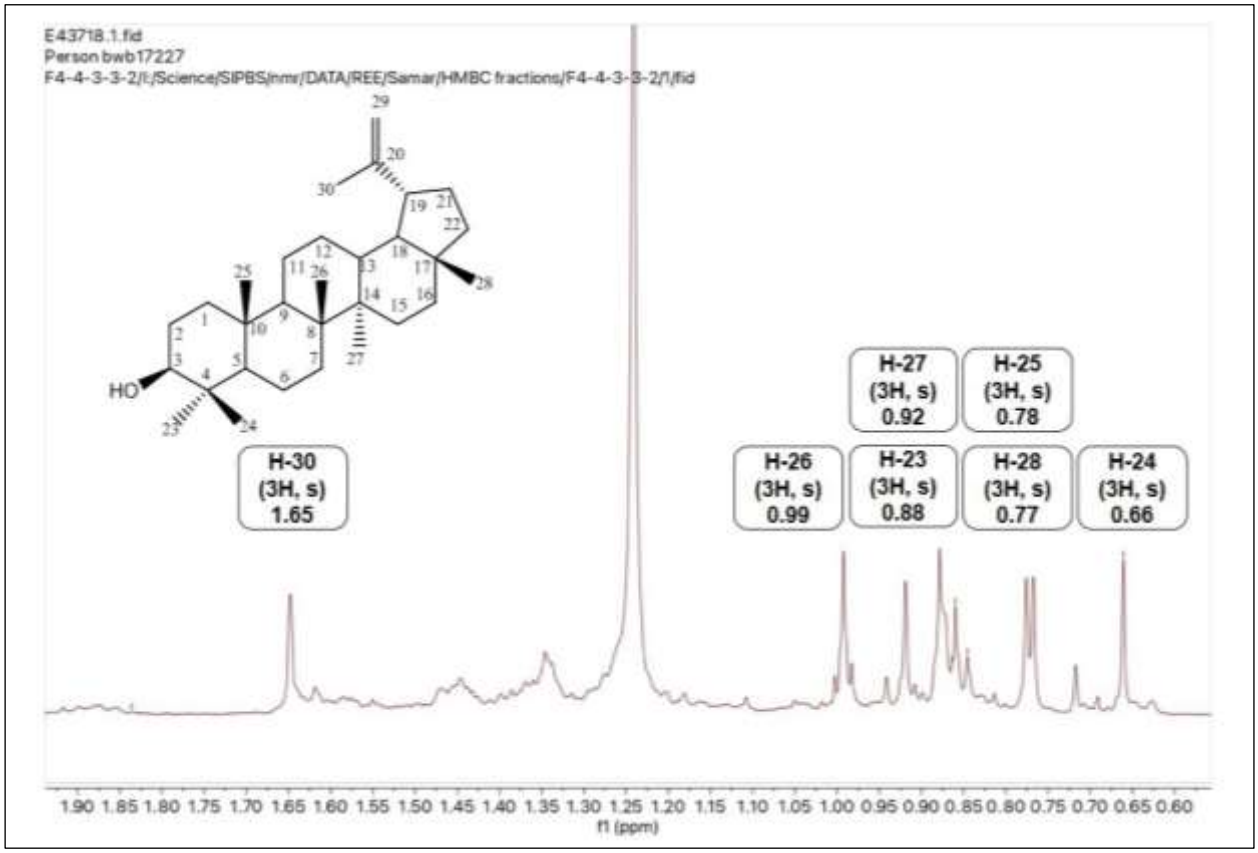


Figure 5.3:[1]H-NMR spectrum expansion of compound **1** in DMSO measured at 400 MHz.

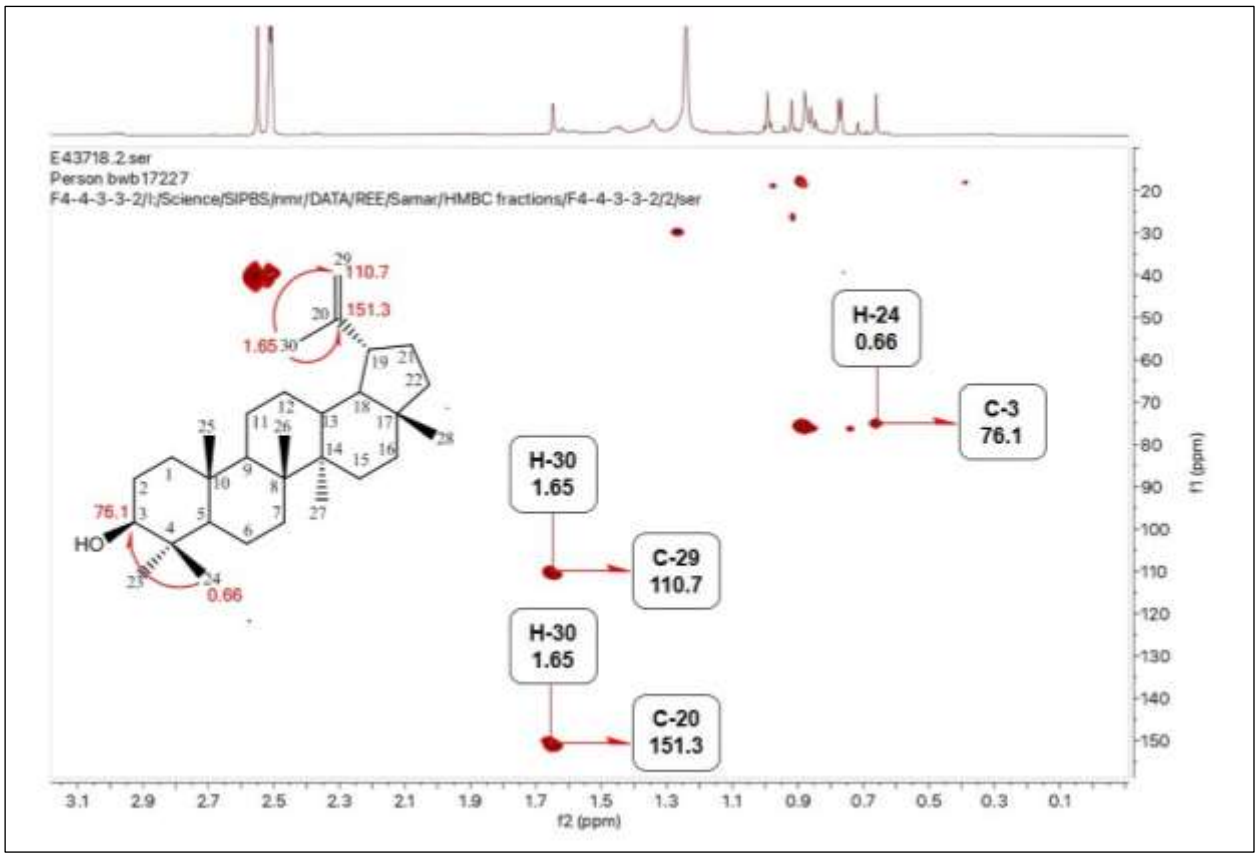


Figure 5.4: HMBC spectrum of compound **1** in DMSO measured at 400 MHz

5.1.2. Structure Elucidation of Compound **2**

Compound **2** was isolated as white amorphous powder (9.4 mg). It is soluble in MeOH and insoluble in $CHCl_3$ and *n*-Hexane. It showed a violet color single spot on a pre-coated silica gel plate after spraying with *p*-anisaldehyde/H_2SO_4 spray then heating at 110 ºC for 2 min. Molecular ion peak was shown at *m/z* 179.0703 $[M+H]^+$, indicating the molecular weight as 178.0631g/mol, corresponding to the molecular formula $C_{10}H_{10}O_3$. ^{1}H-NMR spectrum in **Table 5.2** and **Figure 5.6** and **5.7** exhibited doublet signals at δ_H 7.31 (d, J = 8.8 Hz) for H-2,6 and at 6.90 (d, J = 8.5 Hz) for H-3,5 each integrated for two protons that indicated chemical and magnetical equivalence. On the other hand, the NMR spectrum exhibited one proton appear as doublet at δ_H 6.58 (d, J = 16.0 Hz) characteristic for H-8, in addition downfield doublet proton for H-7 appeared at δ_H 7.48 (d, J = 16.0 Hz) indicating presence of *trans* double bond configuration. In addition, there is a singlet signal at δ_H 3.72 (s) belonging to the methoxy at C-4. The ^{13}C-NMR of compound **2** (**Table 5.2**) showed signals for nine carbon atoms. Special signals were detected at δ_C 129.2 for (C-1), at δ_C 115.2 ppm (C-3), C-4 was assigned at δ_C 159.5 and 130.8 for C-2 and C-6, respectively. A downfield signal appeared at δ_C 168.5 and was assigned for C-9. Due to the double bond, there are signals at δ_C 144.5 (C-7) and 125.8 (C-8). It was identified as ***trans* 4-methoxy cinnamic acid** by comparing with the literature (Świsłocka et al., 2012).

Table 5.2: NMR spectral data of compound **2** (DMSO, 400, 100 MHz)

Position	δ_H (mult., J (Hz)	δ_C
1	-	129.2
2	7.31 d (8.8)	130.8
3	6.90 d (8.5)	115.2
4	-	159.5
5	6.90 d (8.5)	115.2
6	7.31 d (8.8)	130.8
7	7.48 d (16.0)	144.5
8	6.58 d (16.0)	125.8
9	-	168.5
4-OCH_3	3.72 (s)	56.5

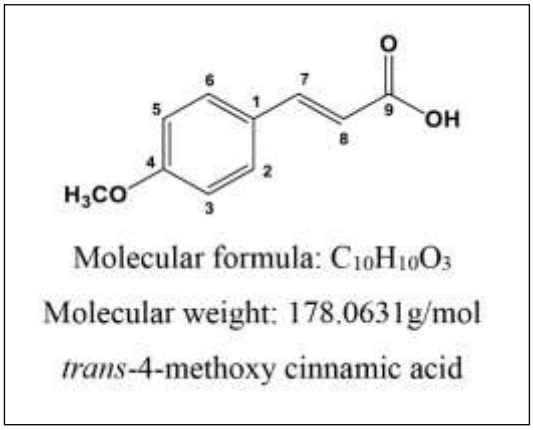


Figure 5.5: Chemical structure of compound **2**

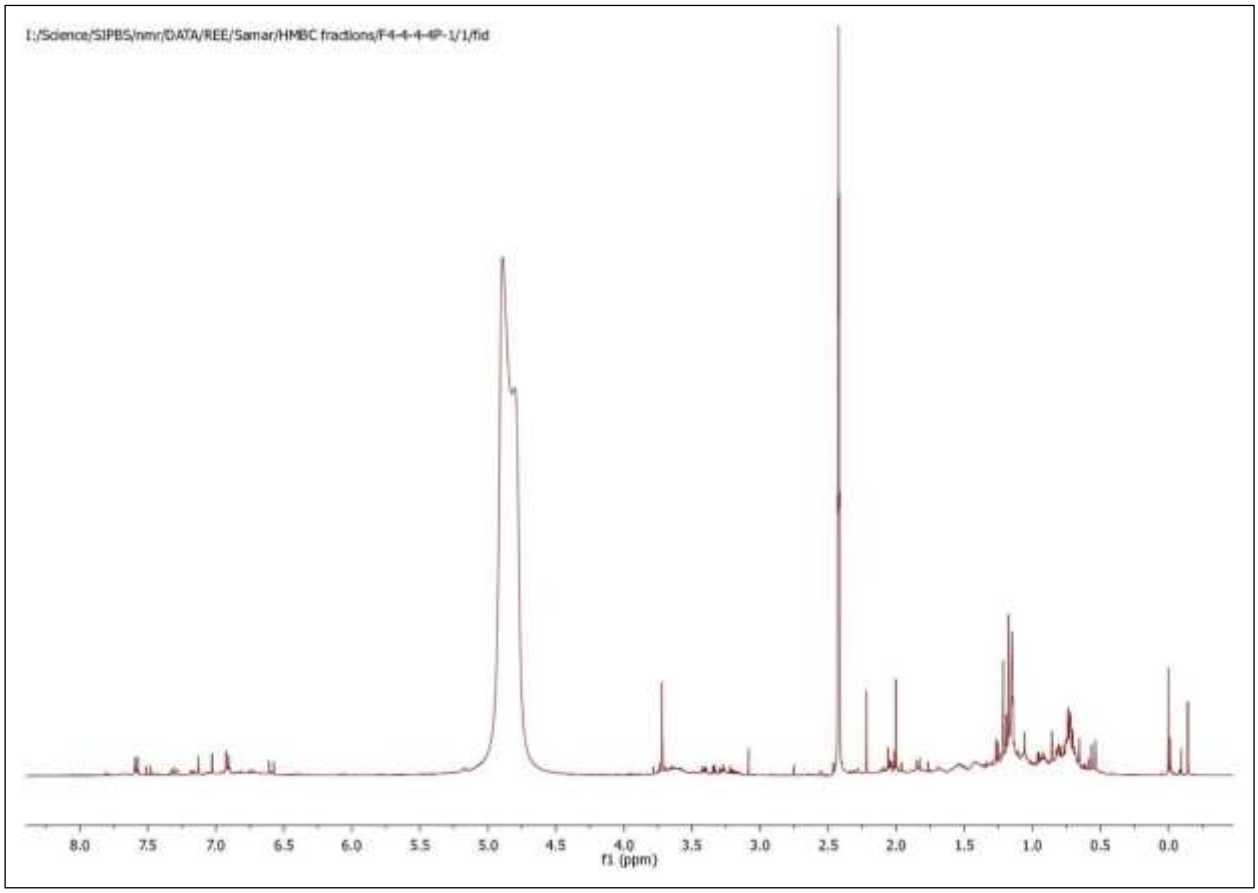


Figure 5.6:[1]H-NMR spectrum of compound **2** in DMSO measured at 400 MHz

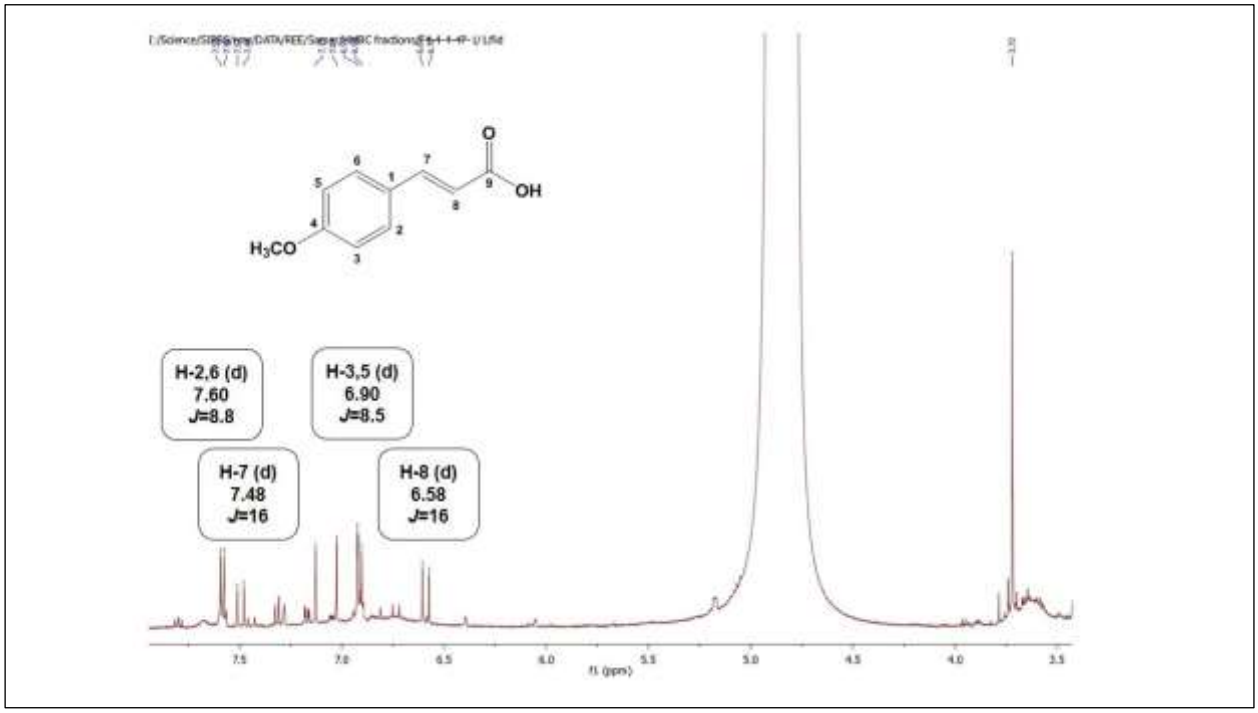


Figure 5.7:[1]H-NMR spectrum expansion of compound **2** in DMSO measured at 400 MHz

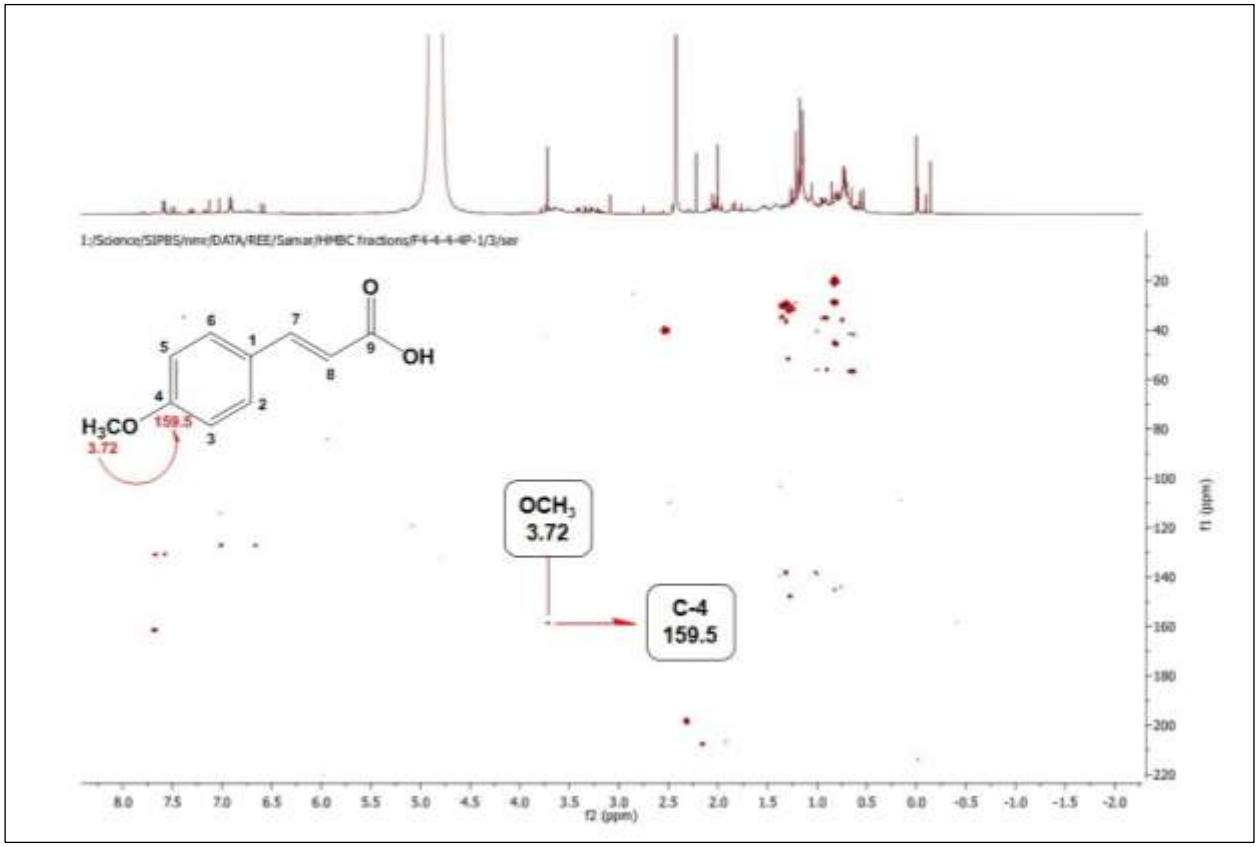


Figure 5.8: HMBC spectrum of compound **2** in DMSO measured at 400 MHz.

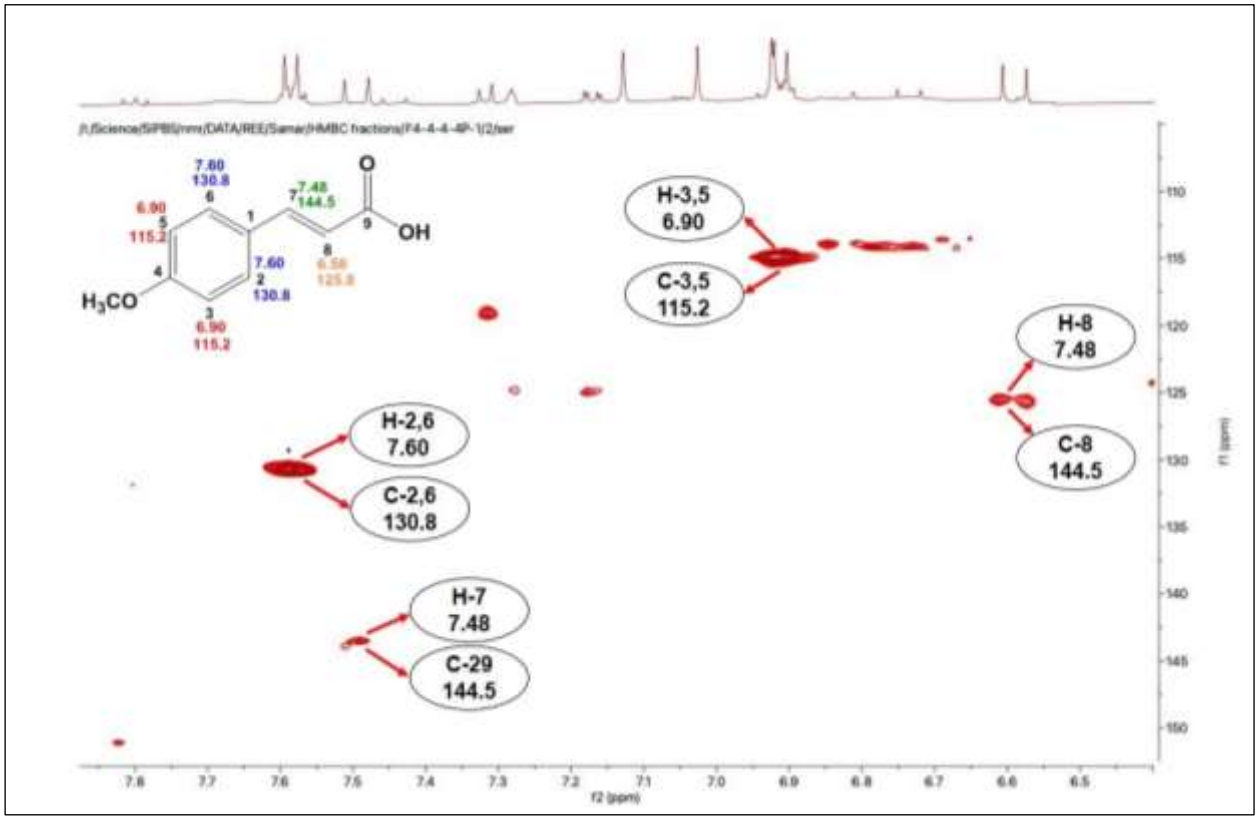


Figure 5.9: HSQC spectrum of compound **2** in DMSO measured at 400 MHz

5.1.3. Structure Elucidation of Compound **3**

Compound **3** was isolated as white amorphous powders (4.2 mg, 3 mg and 3 mg). It is soluble in MeOH and insoluble in $CHCl_3$ and *n*-Hexane. It showed a violet color single spot after heating with *p*-anisaldehyde/H_2SO_4 spray reagent at 110 ºC for 2 min using pre-coated silica gel plates. The molecular ion peak at *m/z* 195.0653 $[M+H]^+$, indicating the molecular weight as 194.0580 g/mol, corresponding to the molecular formula $C_{10}H_{10}O_4$. ^{1}H NMR spectra in **Table 5.3, Figure 5.11** and **5.12** showed resonances for an AMX pattern at δ_H 6.79 (dd, J = 2.0, 8.0 Hz. H-6), δ_H 6.99 (d, J = 8.0 Hz. H-5), and δ_H 7.11 (d, J = 2.0 Hz, H-2), which indicated the presence of a 1',3',4'-trisubstituted phenyl ring. The spectra also showed two *trans* olefinic proton at δ_H 6.42 (d, J = 16.0 Hz. H-8) and at δ_H 7.31 (d, J = 16.0 Hz. H-7) along with a methoxy group at δ_H 3.84 (s). ^{13}C-NMR (**Table 5.3**) as well as HMBC confirmed the positioning of different groups on the molecule. It showed ten carbon atoms present in the compound, and special signals were detected: carbons of olefinic protons at δ_C 145.0 and 119.0 for carbons 7 and 8, respectively, in addition to carbon at δ_C 168.5 ppm was assigned for C-9. Finally, the compound was identified as **ferulic acid** by comparing with the literature (Jain and Surana, 2016).

Table 5.3: NMR spectral data of compound **3** (DMSO, 400, 100 MHz)

Position	δ_H (mult., *J* (Hz)	δ_C
1	-	127.2
2	7.11 d	111.8
3	-	149.0
4	-	147.5
5	6.79 d (8.0)	117.0
6	6.99 dd (2.0, 8.0)	123.8
7	7.31 d (16.0)	145.0
8	6.42 d (16.0)	119.0
9	-	168.5
3-OCH_3	3.72 (s)	56.5

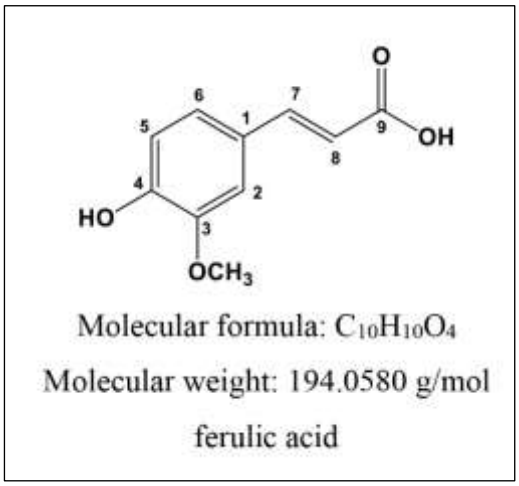


Figure 5.10: Chemical structure of compound **3**

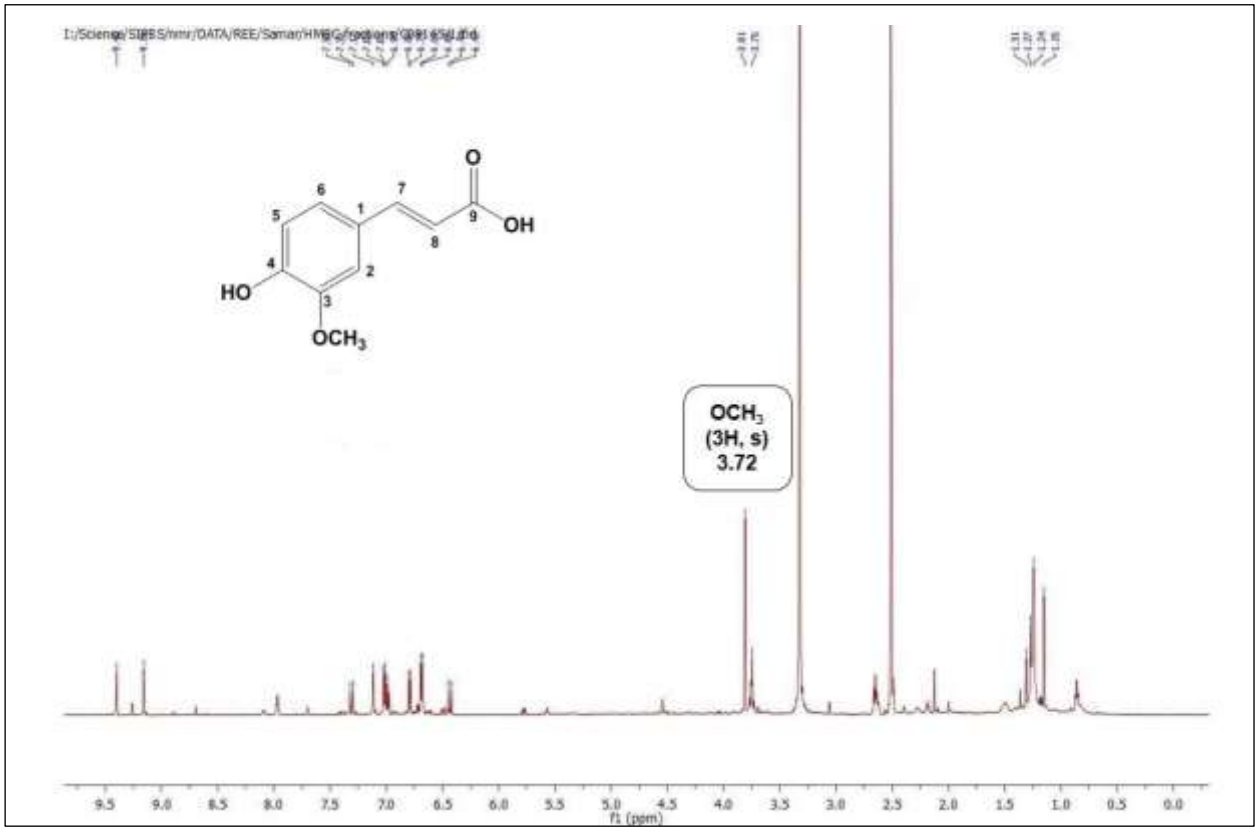


Figure 5.11:[1]H-NMR spectrum of compound **3** in DMSO measured at 400 MHz

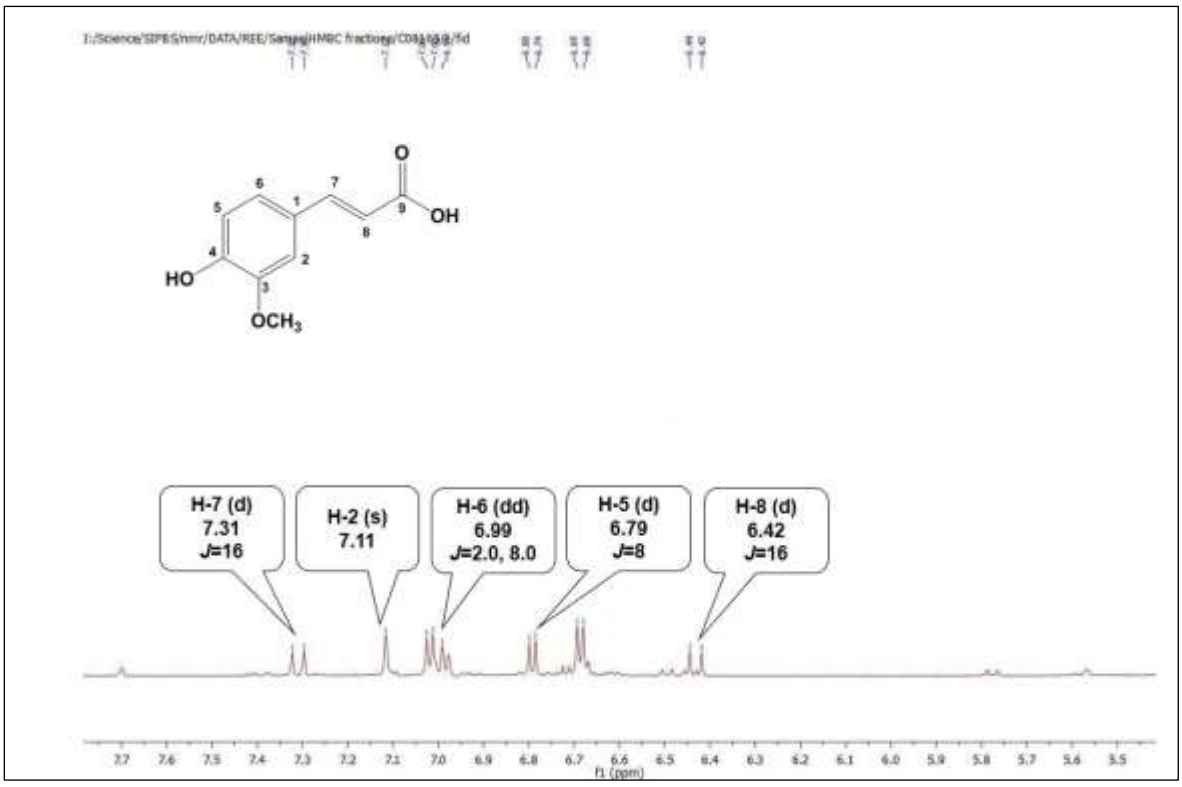


Figure 5.12:[1]H-NMR spectrum expansion of compound **3** in DMSO measured at 400 MHz.

5.1.4. Structure Elucidation of Compound **4**

Compound **4** was obtained as white powder (6.3 mg). It is soluble in MeOH and insoluble in $CHCl_3$ and *n*-Hexane. It showed a violet color single spot after heating with *p*-anisaldehyde/H_2SO_4 spray reagent at 110 ºC for 2 min using pre-coated silica gel plates. Molecular ion peak at *m/z* 143.150 $[M+H]^+$, indicating the molecular weight as 142.10 g/mol, corresponding to the molecular formula $C_8H_8O_2$. The ^{1}H-NMR spectrum of compound **4** (**Table 5.4, Figure 5.14**) showed signals resonating in the region from 7-8 ppm that give indication of the presence of benzene ring in compound **4**. Protons H-3 and H-5 appear as doublet at δ_H 7.12 (d, J = 8.0 Hz). Moreover, H-2 and H-6 appear as doublet at δ_H 7.87 (d, J = 8.0 Hz), each two chemically and magnetically equivalent.

The ^{13}C-NMR spectrum of compound **4** (**Table 5.4**) detected the presence of eight carbons (6 benzyl carbons and aldehyde group and one methoxyl group), 6 of them were benzylic C-2, 6 at δ_C 133.6, C-3, 5 (116.5), C-4 (165.4), C-1 (130.3) in addition to aldehyde carbon at δ_C 192.7. The signal of the carbon spectrum at δ_C 130.3 (C-1) and 116.5 (C-5) attributed to carbons carrying aldehyde group and methoxyl group as elucidated from HMBC spectra (**Figure 5.15**). Therefor, compound **4** was detected as **4-anisaldehyde** by comparing with the literature (Altun et al., 2017).

Table 5.4: NMR spectral data of compound **4** (DMSO, 400, 100 MHz)

Position	δ_H (mult., J (Hz)	δ_C
1	-	130.3
2	7.87 d (8.0)	133.6
3	7.12 d (8.0)	116.5
4	-	165.4
5	7.12 d (8.0)	116.5
6	7.87 d (8.0)	133.6
7	9.87 (s)	192.7
OCH_3	3.89 (s)	55.0

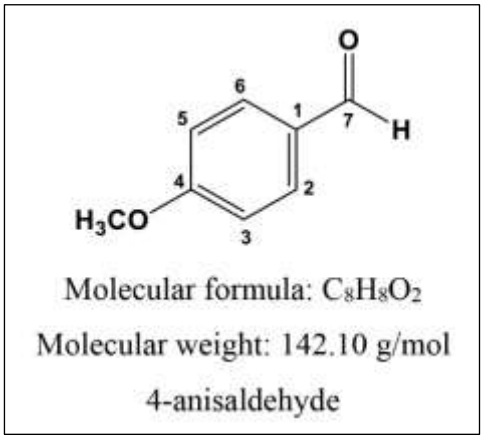


Figure 5.13: Chemical structure of compound **4**

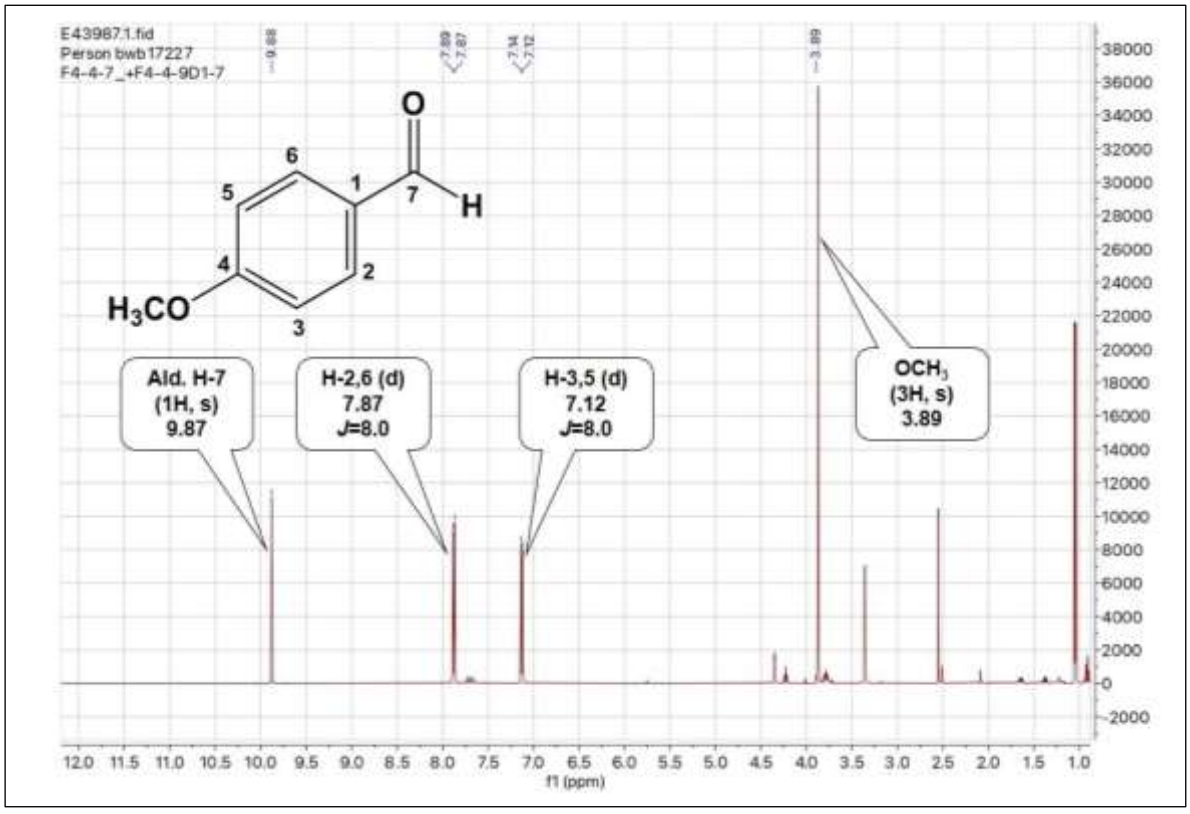


Figure 5.14:[1]H-NMR spectrum of compound **4** in DMSO measured at 400 MHz

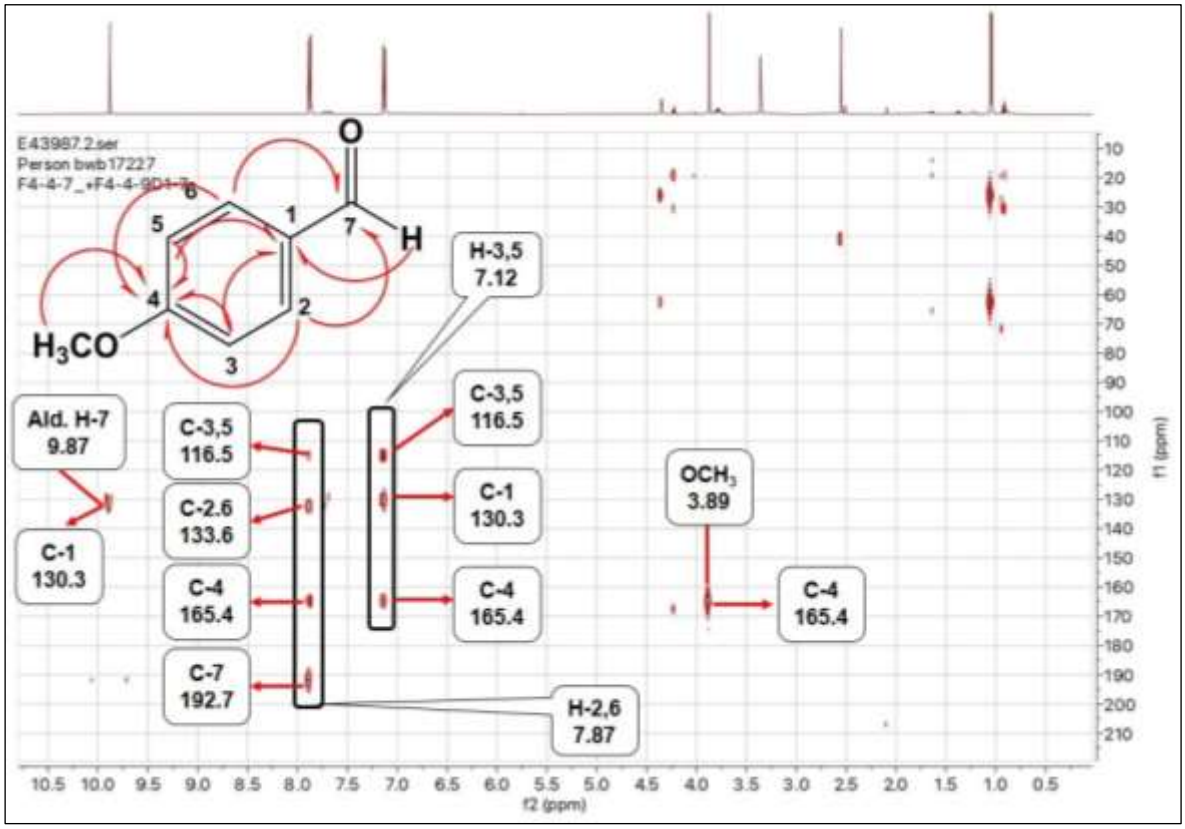


Figure 5.15: HMBC spectrum of compound **4** in DMSO measured at 400 MHz

5.1.5. Structure Elucidation of Compound **5**

Compound **5** was isolated as yellow amorphous powder (8.8 mg) that showed molecular ion peak at *m/z* 479.1183 $[M+H]^+$, indicating the molecular weight as 478.11g/mol , corresponding to the molecular formula $C_{22}H_{22}O_{12}$. ^{1}HNMR spectrum (**Figure 5.17, 5.18 and Table 5.5**) showed signals of B- ring typical to that of isorhamnetin. It displayed H-5` proton $\delta_H$ 6.89 ppm, as doublet with *J* (*ortho*) = 8.0 Hz. The H-2` proton was displayed at δ_H 7.5 ppm while H-6` proton appeared as ortho coupled doublet at δ_H 7.71 ppm = 2.0, 8.0 Hz. The methoxy protons were displayed at δ_H 3.86 ppm as singlet equivalent to 3 protons. The ring A- protons gave rise to two meta coupled protons at δ_H 6.20 and δ_H 6.4 ppm assigned to H-6 and H-8, respectively. The spectra displayed also anomeric protons of sugar unit appeared at δ_H 5.5 ppm (that confirmed presence of sugar at C-3) with *β*- configuration which evidenced by 8.0 Hz coupling constant. Their upfield shift confirmed that sugar is not in C-7 (Abdallah and Esmat, 2017).

^{13} C NMR spectrum (**Figure 5.19 and 5.20**) of compound **5** displayed the characteristic signals of isorhamnetin. Sugar part was confirmed to be galactose (Khalfallah et al., 2014).

Attachment of sugar unit to C-3 of flavonoid was confirmed by cross peak in HMBC (**Figure 5.22**) between anomeric sugar proton at δ_H 5.5 ppm and carbon C-3 at δ_c 133.0 ppm.

Comparing the obtained spectral and published data (Khalfallah et al., 2014; Abdallah and Esmat, 2017) compound **5** was identified as: **isorhamnetin -3-*O*-*β*-D-galactopyranoside**.

Table 5.5: NMR spectral data of compound **5** (DMSO, 400, 100 MHz).

Position	δ_H (mult., *J* (Hz)	δ_C
1	-	-
2	-	156.7
3	-	133.0
4	-	178.0
5	-	161.7
6	6.2 (d, 2.0 Hz)	99.3
7	-	164.8
8	6.4 (d, 2.0 Hz)	94.2
9	-	157.0
10	-	104.4
1`	-	121.6
2`	7.5 (d, 2.0 Hz)	122.4
3`	-	150.3
4`	-	147.7
5`	6.89 (d, 8.0 Hz)	115.7
6`	7.71 (d, 2.0, 8.0 Hz)	129.0
OCH_3	3.86 (s)	56.5
1``	5.50 (d, 8.0 Hz)	102.1
2``	3-4.5 ppm	71.8
3``		٧٦,٧
4``		67.9
5``		73.8
6``	3.38 (m), 3.48 (m)	60.8

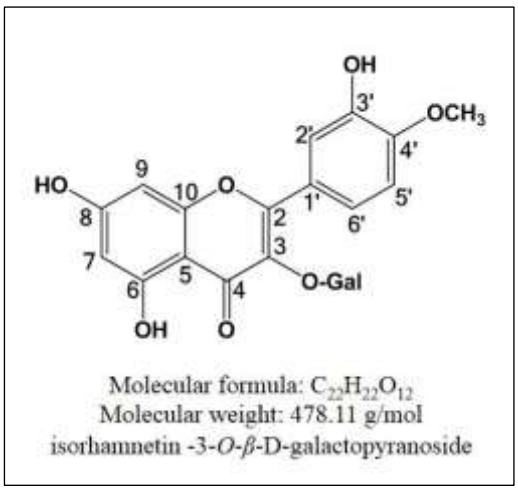


Figure 5.16: Chemical structure of compound **5**

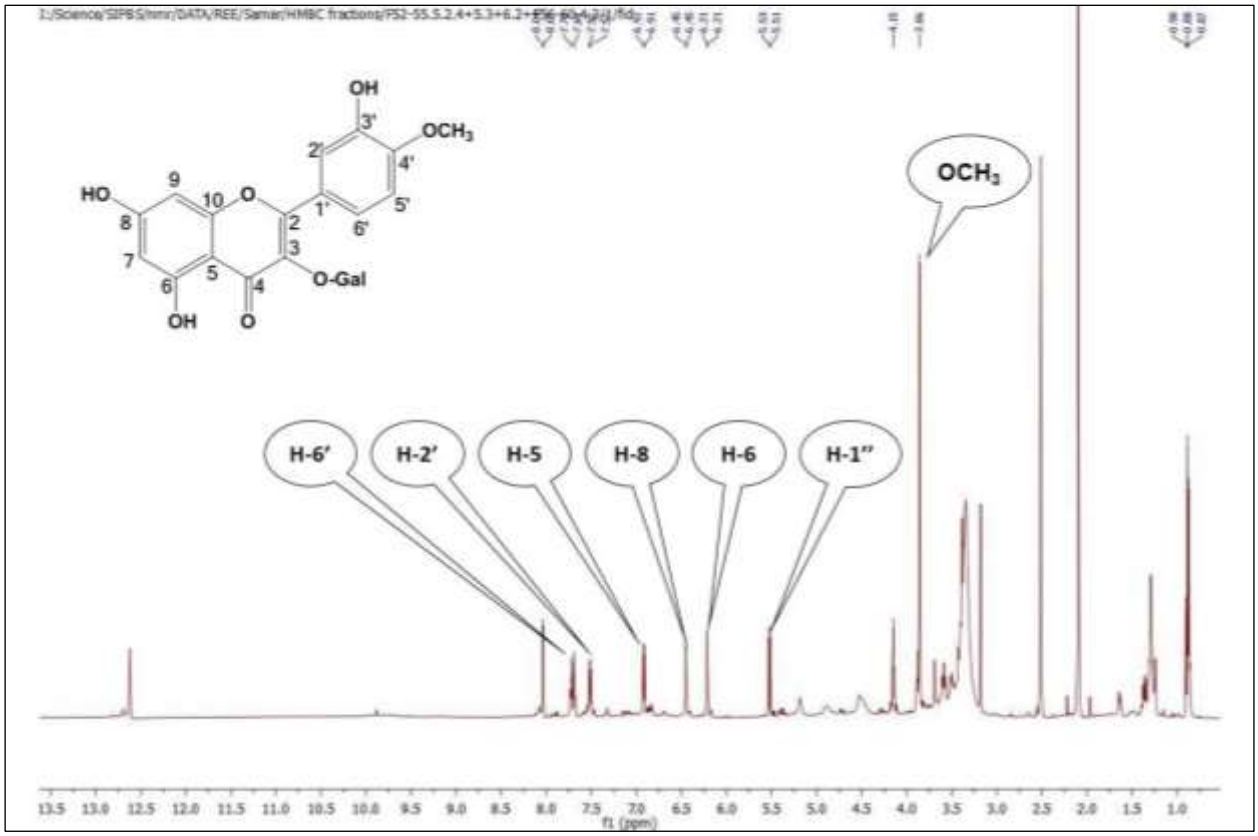


Figure 5.17:[1]H-NMR spectrum of compound **5** in DMSO measured at 400 MHz

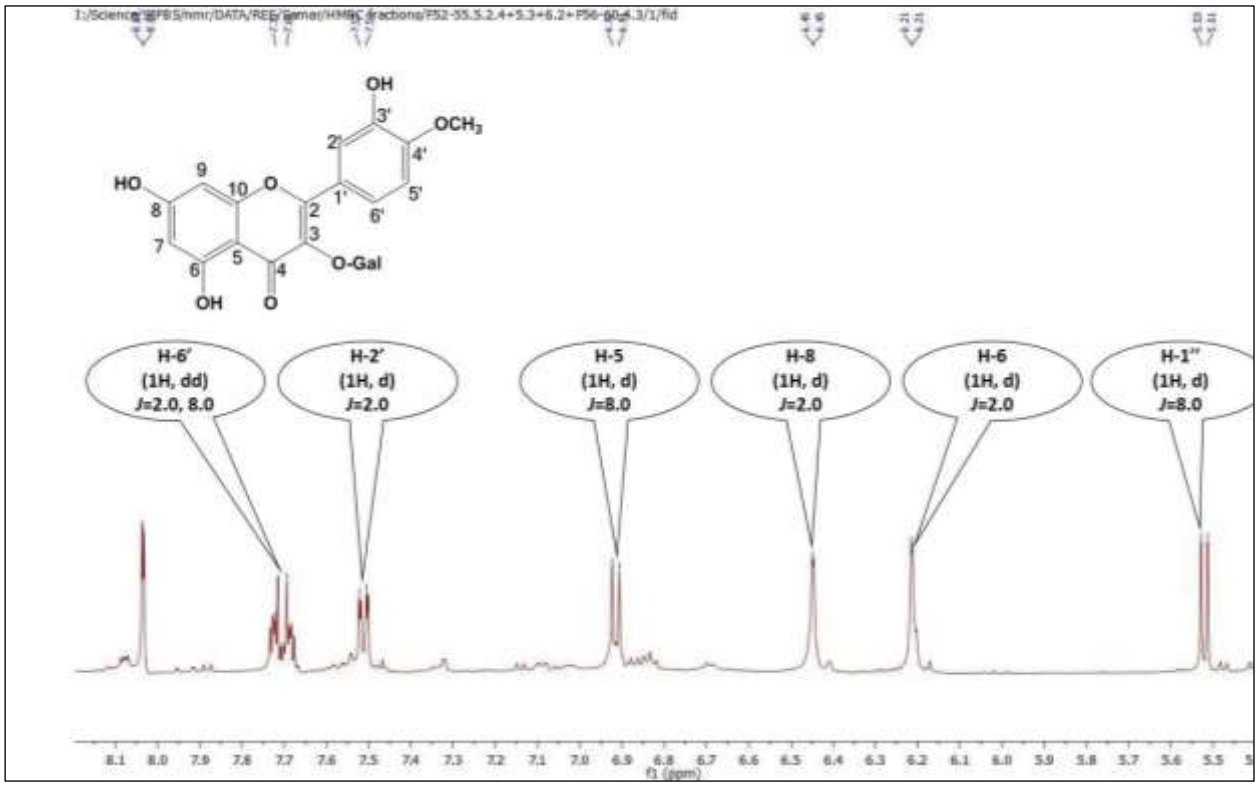


Figure 5.18:[1]H-NMR spectrum expansion of compound **5** in DMSO measured at 400 MHz

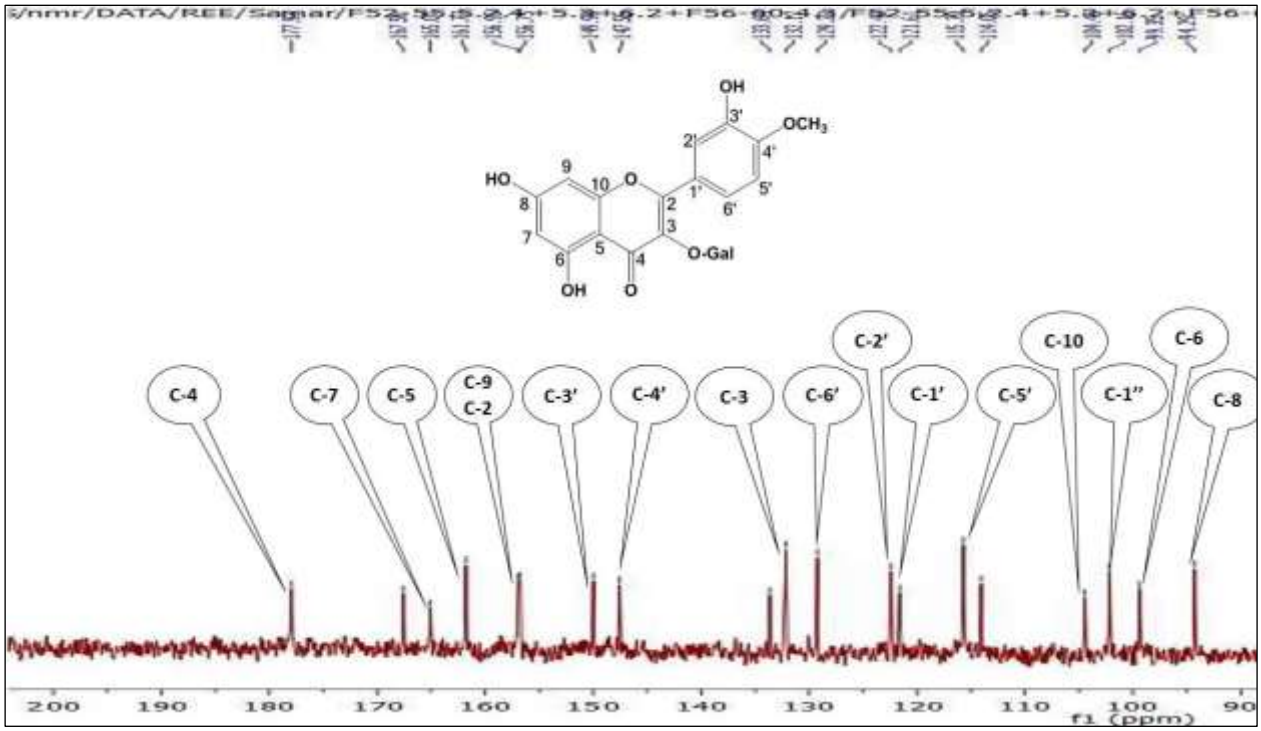


Figure 5.19: [13] C NMR spectrum expansion-1 of compound **5** in DMSO measured at 400 MHz

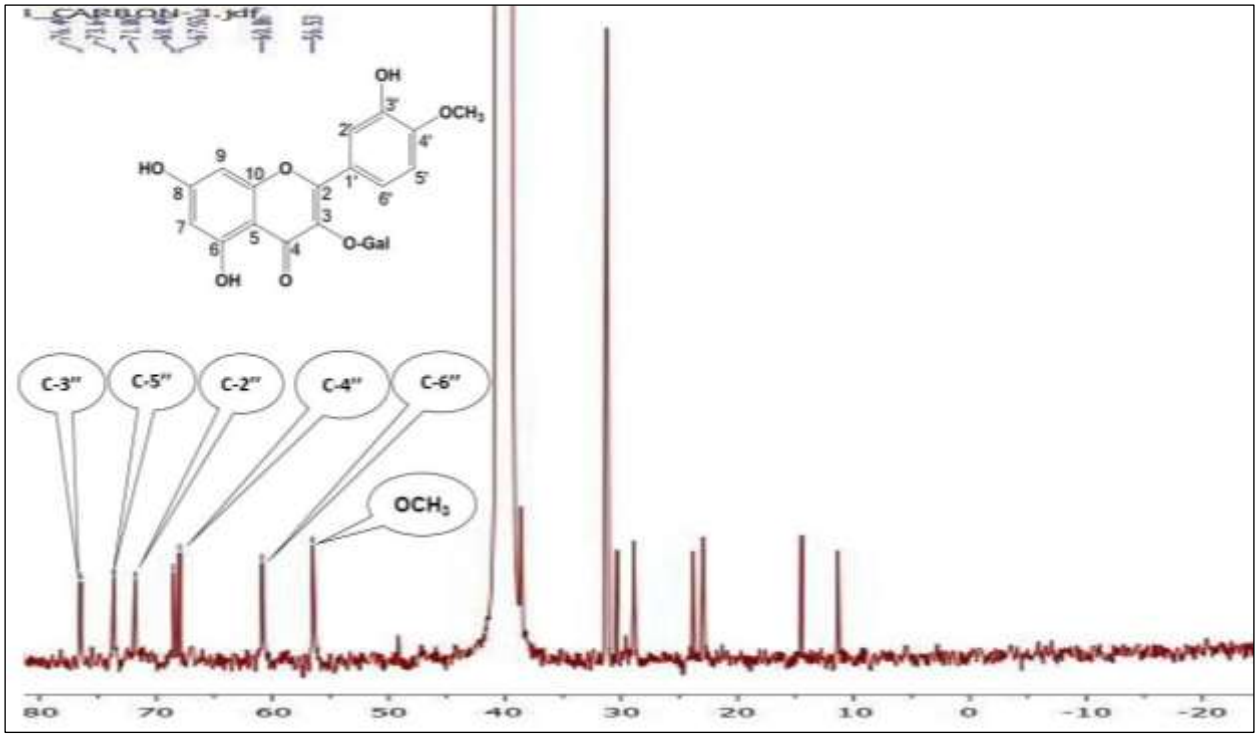


Figure 5.20: [13]C NMR spectrum expansion-2 of compound **5** in DMSO measured at 400 MHz.

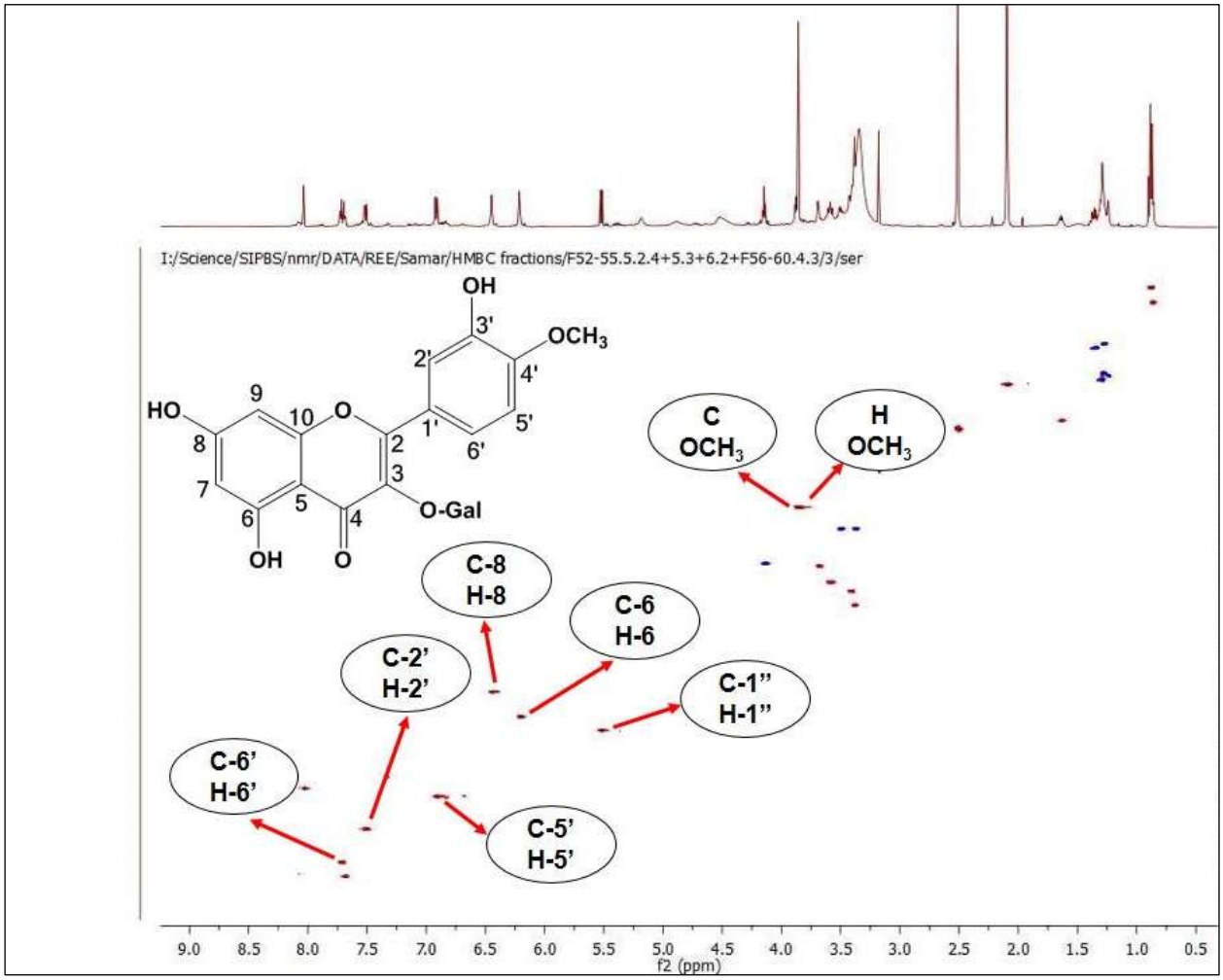


Figure 5.21: HSQC spectrum of compound **5** in DMSO measured at 400 MHz.

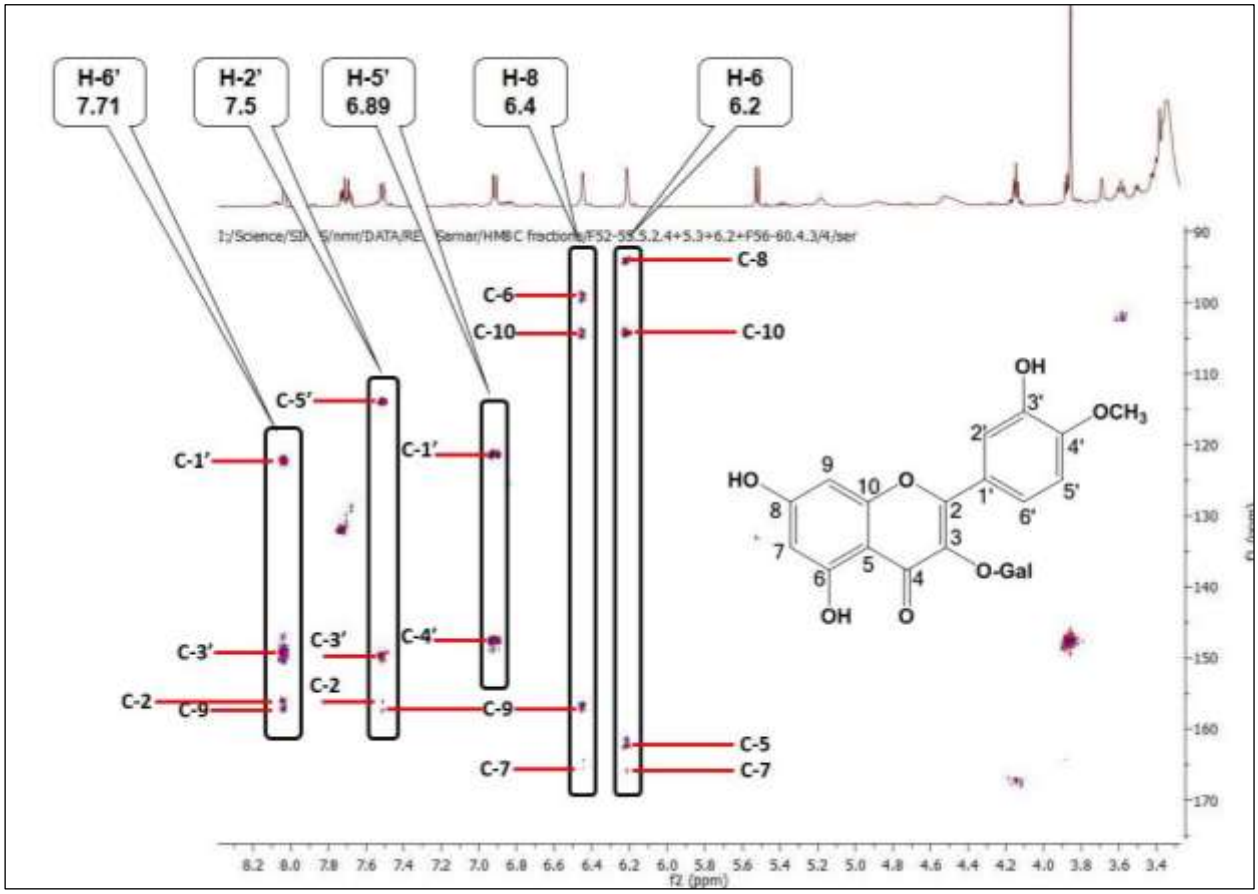


Figure 5.22: HMBC spectrum of compound **5** in DMSO measured at 400 MHz

CHAPTER VI: GENERAL DISCUSSION

6. General Discussion

6.1. LC-HRMS Analysis of *Salsola kali* Fractions

Genus *Salsola* is an important genus in the plant kingdom and is one of the most notorious plant genera from the taxonomical point of view (ElNaggar et al., 2022). The genus has been reported as a rich source of diverse phytochemical classes, such as alkaloids, cardenolides, triterpenoids, coumarins, flavonoids, isoflavonoids, and phenolic acids (ElNaggar et al., 2022) being involved within diverse traditional, industrial, and environmental applications. Dereplication is defined as the process of implementing spectroscopy in the identification of known metabolites in the early stages of isolation. This is achieved by using LC-HRMS, where hits with certain *m/z* values are compared to available databases like Dictionary of Natural Products (DNP). Along with multivariate analysis that pinpoints the active compounds, this allows prioritising fractions for further purification and helps save time and money in the process of isolating novel bioactive compounds. Within our study, the metabolites of *S. kali* were thoroughly analyzed *via* high resolution LC-ESI-HRMS analytical method to put an emphasis on its phytochemical profile. Tentative chemical identification of *S. kali* metabolites was achieved by comparing the *m/z* values of the molecular ions, MS/MS transitions and retention times with those in the literature and high-resolution mass spectral records and databases. Our results demonstrated that more than ten types of substances are present within *S. kali* with a total of 43 hits putative metabolites being detected within *S. kali* extract. Detected metabolites would fall under the chemical classes of phenolics, more especially flavonoids and their derivatives, organic acids, nitrogenous compounds, and sugar derivatives. Main metabolites of *S. kali* were also highlighted as flavonoids, phenolic acids, lipids, amino acids, and their derivatives. Our results would greatly enrich the understanding of the chemical composition in this *Salsola* spp (**Table 4.20, Figure 4.19**).

The HR-ESI-MS analysis of the crude extract showed the $[M+H]^+$ signal at *m/z* 329.1262, corresponding to the molecular formula ($C_{18}H_{19}NO_5$) which was identified as *N*-(4'-Methoxy-cinnamoyl)-norepinephrine or *N*-[2-(3,4-dihydroxyphenyl)-2-hydroxyethyl]-3-(4-methoxyphenyl)prop-2-enamide (Hit **ID**, **P2735**). The ion at *m/z* 311.1157 ($C_{18}H_{17}NO_4$) was due to the loss of H_2O $[M-H_2O]^+$ from the molecular ion and identified as *N*-[2-(3,4-dihydroxyphenyl)-2-hydroxyethyl]-3-(4-methoxyphenyl)prop-2-enamide (Hit **ID, P2637**). Both two hits were previously reported (Khan et al., 2003).

Hit **ID, P7873** showed the $[M+H]^+$ signal at molecular ion *m/z* 343.142 corresponding to the molecular formula ($C_{19}H_{21}NO_5$) which was identified as *N*-(3',4'-Dimethoxy-cinnamoyl)-norepinephrine or *N*-[2-(3-hydroxy-4-methoxyphenyl)-2-hydroxyethyl]3-(4-methoxyphenyl)-prop-2-enamide which was previously reported (Khan et al., 2003). It is worth noting that Hit **ID, P7873** is only different from Hit **ID, P2735** *via* the presence of a methoxy group instead of a OH group.

Moving towards other nitrogenous-based metabolites, (Hits **ID, P5816**, **P2656** and **P2657**) have been detected and identified as moupinamides (*N-trans*-feruloyltyramine) and showed the $[M+H]^+$ signal at *m/z* 313.1313, corresponding to the molecular formula ($C_{18}H_{19}NO_4$). Moupinamides were reported from different *Salsola* spp. in both free and combined glucosidic forms. The nitrogenous-based metabolites possess the skeleton of *N-trans*-feruloyltyramine or *N-trans*-feruloyldopamine structures. The structures of *N-trans*-feruloyl-3-*O*-methyldopamine and *N-trans*-feruloyl-3'''-methoxydopamine 4'-*O*-*β*-D-glucopyranoside, have been previously reported within *S. collina* (Xiang et al., 2007a).

On the other hand, both *N-trans*-feruloyltyramine and 7'-hydroxy *N-trans*-feruloyltyramine were identified within *S. collina* and *S. tetrandra* (Oueslati et al., 2006; Xiang et al., 2007a; Jin et al., 2011). Furthermore, *trans-N*-feruloyl tyramine-4'''-*O*-*β*-D-glucopyranoside was reported by Elsharabasy *et al*. as being isolated from *S. inermis* Forssk (Elsharabasy and Hosney, 2013). The only reported moupinamide derivative with a "*cis*" double bond configuration for its cinnamoyl moiety was *cis-N*-feruloyltyramine being previously isolated from the aerial parts of *S. baryosoma* (Hussein and El-Bassuony, 2004; ElNaggar et al., 2022).

Steroids are a group of natural products biosynthesized from the isoprenoid pathway *via* the 2,3-oxidosqualene (C_{30}) route. The reported steroids in the genus *Salsola* comprised several phytosterols with diversity in the alkyl side chains at C-17, including campesterol, cholesterol, and desmosterol from *S. collina* (Mayakova et al., 1984), *β*-sitosterol, stigmastanol, and stigmasterol, in addition to a combined phytosterol, stigmasterol-3-*O*-*β*-D-glucopyranoside from the aerial parts of *S. inermis* (Elsharabasy and Hosney, 2013). Here within our study several sterols were reported including the (Hit **ID, P5929**) depicting the $[M+H]^+$ signal at molecular ion *m/z* 414.3868 corresponding to the molecular formula ($C_{29}H_{50}O$) and identified as *β*-sitosterol which was previously reported (Zaikov et al., 1992; Tundis et al., 2008; Elsharabasy and Hosney, 2013; Alturkistani et al., 2017). Another of steroids, (Hits **ID, P1863**), have been detected and identified as stigmasterol showing the $[M+H]^+$ signal at *m/z* 412.3719, corresponding to the molecular formula ($C_{29}H_{48}O$) which was previously reported (Karawya et al., 1972; Salt and Adler, 1985; Syrchina et al., 1989; Zaikov et al., 1992; Elsharabasy and Hosney, 2013). Finally,

the (Hit **ID, P1873**) showed the $[M+H]^+$ signal at molecular ion *m/z* 427.3979 corresponding to the molecular formula ($C_{30}H_{50}O$) and identified as lupeol which being previously detected within the petroleum ether extract of *S. kali* (Alturkistani et al., 2017)

The existence of fatty acid esters or acylated sterols was reported by Mayakova *et al*. from the genus *Salsola*. They investigated the contents of the saponified acylsterols fraction of the pentane extract of *S. collina*. The neutral fraction indicated the presence of four sterols, including *β*-sitosterol, stigmasterol, cholesterol, and campesterol, whereas the acyl fraction of the hydrolysed esters composed of stearic, palmitic, and oleic acids (Mayakova et al., 1984). In our *S. kali* extract, the oleic acid (Hit **ID, N665**) was detected and showed the $[M-H]^-$ signal at molecular ion *m/z* 282.2558 corresponding to the molecular formula ($C_{18}H_{34}O_2$) which was previously reported (Karawya et al., 1972; Zaikov et al., 1992; Rasheed et al., 2013; Elsharabasy et al., 2015; Ghorab et al., 2017).

Coumarins are another bioactive secondary metabolites biosynthesized in plants from the phenylpropanoid (C_6C_3) pathway by cyclisation of cinnamic acid contributing to a diverse of biological activities, such as anticoagulant, antimicrobial, antiviral, and anticancer activities (Stringlis et al., 2019). Several studies reported the presence of simple coumarins in members of the genus *Salsola*. These reported coumarins are either free or glycosylated with mostly methoxylated C-6 and oxygenated C-7 positions. In our *S. kali* extract, the Umbelliferone (Hit **ID, P2014**) was detected and showed the $[M+H]^+$ signal at molecular ion *m/z* 163.0389 corresponding to the molecular formula ($C_9H_6O_3$) which was previously reported (Elsharabasy and Hosney, 2013).

Flavonoids and isoflavonoids are predominant plant polyphenols having a C6-C3-C6 skeleton and are considered as one of the frequently studied plant phytochemicals (Wang et al., 2020a). Flavonoids are yellow-colored compounds possessing a highly distinctive biosynthetic pathway as they are synthesized from the mixed phenylpropanoid and polyketide pathway (Falcone Ferreyra et al., 2012). In the genus *Salsola*, the reported flavonoids can be clustered into several classes including; (1) flavones (e.g. apigenin, chrysin, luteolin-7-*O*-*β*-D-glucoside, and tricin) from *S. imbricate* Forssk, *S. kali* L., and *S. collina* Pall., respectively (Xiang et al., 2007b; Jin et al., 2011; Shehab et al., 2015; Boulaaba et al., 2019). (2) Flavonols (e.g. isorhamnetin, quercetin, and kaempferol derivatives), (3) flavanols (e.g. catechin), and (4) flavanones (e.g. hesperidin, hesperitin, and naringenin). The free flavonol aglycone, kaempferol was incompletely identified by UPLC/qTOF-MS analysis of the aerial parts and roots of *S. vermiculata* and *S. tetrandra* plants (Rasheed et al., 2013). In our *S. kali* extract, the flavone apigenin (Hit **ID, N834**) was detected and showed the $[M-H]^-$ signal at molecular ion *m/z* 269.0454 corresponding to the

molecular formula ($C_{15}H_{10}O_5$) which was previously reported (Shehab and Abu-Gharbieh, 2014). Other flavonoids such as quercetin, rutin, hesperitin, epicatechin, kaempferol, isorhamnetin and its derivatives were also identified in our *S. kali* extract (**Table 4.20, Figure 4.19**).

Simple phenols represent a minor class of natural products defined as aromatic compounds with at least one hydroxyl group attached to a benzene ring, such as catechol, resorcinol, and phloroglucinol. On the other hand, phenolic acids and their derivatives comprise a major class of plant-derived natural products being categorized into benzoic acids (e.g. protocatechuic and gallic acids; C6-C1) as well as cinnamic acids (e.g. caffeic and coumaric acids; C6-C3) (Kougan et al., 2013).

Various free cinnamic acids and their esters were reported from the plants of *Salsola* genus. Previous phytochemical studies on *S. kali*, *S. imbricate*, *S. vermiculata*, *S. tetrandra*, *S. cyclophylla*, and *S. collina* showed the presence of free cinnamic acids such as caffeic, cinnamic, *p*-coumaric, and ferulic acids within these species (Sokolowska-Krzaczek et al., 2009; Jin et al., 2011; Rasheed et al., 2013; Shehab et al., 2015; Osman et al., 2016; Mohammed et al., 2021).

Our current investigation highlighted the presence of simple phenols and phenolics phytochemicals such as vanillic, syringic, ursolic, linolenic, caffeic, tricosanoic, rosmarinic, neochlorogenic and ferulic acids within our *S. kali* extract (**Table 4.20, Figure 4.19**).

The results from the biological assays of our extract were incorporated with the LC-MS data for multivariate analysis. The extracts were grouped according to their bioactivity. The crude extract in our study was fractionated and tested for their biological activity. The preliminary detection of our isolated compounds in OPLS-DA plot guided us for further purification of the isolated compounds. All the isolated compounds were pinpointed by multivariate analysis to exhibit the putative bioactivity. The isolated target compounds were all known compounds and based on the reported data, our metabolites were identified as; lupeol (**1**), *trans* 4-methoxy cinnamic acid (**2**), ferulic acid (**3**), 4-anisaldehyde (**4**) and isorhamnetin-3-*O*-*β*-D-galactopyranoside (**5**).

From LC-MS analysis, the total of 43 hits presents within *S. kali* (**Table 4.20, Figure 4.19**), only compounds **1**, **2**, **3** and **5** were detected while compound **4** did not detected because its *m/z* molecular ion peak was fall out of the measuring range we used. Due to the very low yield of the detected hits in LC-MS analysis, we were not able to isolate all of them.

6.2. Correlating the Biological Assay of Genus *Salsola* Fractions and Isolated Compounds

Plants of the genus *Salsola* are widely used in the folk medicine of different countries for their antihypertensive, anti-inflammatory, and immunostimulant activities. Research studies showed that extracts of different *Salsola* spp. and compounds isolated from them exert a wide range of variable pharmacological activities. One of the genus highly reported species is *Salsola kali* being well-known for its medicinal benefits. Current evidence supported the clinical applications of *Salsola kali* through several reported pharmacological activities, including analgesic, antipyretic, antioxidant, cytotoxic, hepatoprotective, contraceptive, antidiabetic, neuroprotective, and antimicrobial activities (ElNaggar et al., 2022).

6.2.1. Antioxidant Activities

Antioxidant potentiality is one of the most extensively studied activities of *Salsola* species. Flavonoids and their glucosidal derivatives are mostly the responsible compounds for antioxidant activities of plant-origin extracts (ElNaggar et al., 2022). On the other hand, other metabolites including essential oil components, alkaloids, and biphenylpropanoids only show moderate free-radical scavenging activities (ElNaggar et al., 2022). Our *S. kali* extract showed prominent antioxidant activity through free-radical α,α-diphenyl-*β*-picrylhydrazyl (DPPH) scavenging assay. It could be concluded from our reported results that the used plant parts comprising relevant metabolites as well as the extraction solvent could greatly contribute to the furnished antioxidant activity. A plethora of reported data have highlighted the potential biological significance of different *Salsola* spp. as antioxidants. Boulaaba *et al.* investigated the antioxidant activity of the methanolic extract of different plant parts of *S. kali* using the same methods used for *S. cyclophylla* extracts (Boulaaba et al., 2019). Leaf and stem extracts showed the highest antioxidant activity (Boulaaba et al., 2019). Oueslati *et al*. investigated the antioxidant activity of biphenylsalsonoids A and B isolated from the ethyl acetate fraction of the roots of *S. imbricata* using DPPH and 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{+}$) assay methods. The two compounds showed moderate antioxidant activity (Oueslati et al., 2017). *Trans-N*-feruloyltyramine derivatives isolated from *S. foetida* exhibited moderate antioxidant activity with IC_{50} ranging from 378 to 427 mM using DPPH radical scavenging assay (Khan et al., 2003).

Another study investigated the antioxidant activity of the ethyl acetate extract of *S. komarovii* aerial parts following HPLC separation. Extract components being suggested responsible for the antioxidant activity were identified by HPLC-MS as the flavonoids, isorhamnetin, astragalin, isoquercitrin, and rutin (Lee et al., 2012).

Concerning another *Salsola* spp., the ethyl acetate fraction of *S. baryosma* showed 77% DPPH radicals scavenging activity while as other tested fractions showed lower activities below 57% (Ahmed et al., 2006). This result is quite contradictory with that obtained by Khacheba *et al*. who reported weak antioxidant activity of *S. baryosma* ethyl acetate extract using DPPH assay (Khacheba et al., 2014). The antioxidant activity of 80% (v/v) aqueous-methanol extracts of *S. vermiculata* and *S. baryosma* in addition to other Algerian herbs was tested using the hydroxyl nitroxide and ($ABTS^{+}$) radicals scavenging assays. The results showed that *S. baryosma* exhibited the highest antioxidant activity in hydroxyl radical assay with an EC_{50} of 0.26 ppm despite its low phenolic content (Djeridane et al., 2015).

Beyaoui *et al*. investigated the antioxidant activity of two compounds, tetranins A and B, isolated from the ethyl acetate extract of *S. tetrandra* roots using DPPH and ABTS assays. The dihydrostilbene, tetranin A exerted higher antioxidant activity than the identified isoflavonoid, tetranin B, yet both compounds showed lower activity than the standard antioxidant, trolox (Beyaoui et al., 2012). Finally, the ethanol extract of *S. collina* Pall also demonstrated anti-oxidant activity through DPPH radical scavenging capacity (Oh et al., 2014).

6.2.2. Cytotoxic Activity

Our study highlighted the significant cytotoxic activities of *S. kali* methanolic crude extract being superior to those of EtOAc crude extract (low-value IC_{50s}) across the three different tested cancerous cell lines. Through surveying current literature, only few studies have been identified for investigating the cytotoxic activity of limited number of *Salsola* spp., including *S. cyclophylla*, *S. oppositifolia*, *S. collina* Pall, and *S. baryosma* (Taha and Alsayed, 2000; Ahmad et al., 2006; Tundis et al., 2008; Oh et al., 2014; Mohammed et al., 2021).

The cytotoxic activity of 95% aqueous-ethanolic extract of the aerial parts of *S. cyclophylla* was investigated using MTT assay against cancer cells, and the normal human fibroblast cells. The aqueous ethanolic extract of *S. cyclophylla* showed low to moderate cytotoxic activity only at high concentrations (50–400 mg/mL) against the tested cell lines and no significant cytotoxic effect was observed at low concentration (< 50 mg/mL) (Mohammed et al., 2021).

Different fractions obtained from the extract of the aerial parts of *S. oppositifolia* were screened for cytotoxic activity against a panel of cancer cell lines (Tundis et al., 2008). The *n*-hexane fraction showed the highest cytotoxic activity on lung carcinoma (COR-L23) and amelanotic melanoma (C32) cell lines with IC_{50} values of 19.1 µg/mL and 24.4 µg/mL, respectively. The dichloromethane fraction also demonstrated cytotoxic activity against these two cell lines with IC_{50} values of 30.4 µg/mL and 33.2 µg/mL for COR-L23 and C32 cell lines, respectively. The

ethyl acetate fraction exhibited a selective moderate cytotoxic activity against breast cancer, MCF-7 cells (IC_{50} 67.9 µg/mL). The major constituents isolated from the ethyl acetate fraction, isorhamnetin-3-*O*-glucopyranoside and isorhamnetin-3-*O*-rutinoside also demonstrated a potential activity against MCF-7 with IC_{50} values of 18.2 and 25.2 µg/mL, respectively. Additionally, isorhamnetin-3-*O*-rutinoside showed high activity against the hormone-dependent prostate carcinoma cell line (LNCaP) with an IC_{50} value of 20.5 µg/mL (Tundis et al., 2008). The ethanol extract of *S. collina* Pall showed cytotoxic activity against human colon carcinoma cells (HT29). It resulted in a reduction in the number and size of the cells through cell cycle regulation and caused cell arrest in the G2/M phase (Oh et al., 2014). The ethanol extract of *S. baryosma* whole plant showed no significant cytotoxic activity when tested with other plant extracts using the brine shrimp method (Taha and Alsayed, 2000). The same result was reported by Ahmed *et al.* while 80% ethanol extract of *S. baryosma* did not exhibit cytotoxic activity against brine shrimp larvae and only the ethyl acetate fraction showed 50% cytotoxic activity. However, all tested fractions of *S. baryosma* showed phytotoxicity against *Lemnaminor* plant growth (Ahmed et al., 2006). This was quite different from our results since our methanolic extract fractions of *S. kali* were more prominent than the ethanolic extract across different cancer cell lines. This could be related to the different tested cancerous cell lines highlighting the differential anti-proliferative activity profiles for tested extracts.

6.3. Role of Isolated Compounds from *S. kali* Fractions for their Antioxidant and Anticancer Activities

Salsola is an important halophytic genera of the family Amaranthaceae and is considered genera of plants containing antioxidants compounds with low caloric composition (Chauhan et al., 2018). *Salsola* species have been studied for their antioxidant potential in greater detail than any other. The results that have been published suggest that the antioxidant activity may be significantly impacted by the plant parts that were used as well as the extraction solvent. Most of the compounds responsible for antioxidant activities are flavonoids and their glucosidal derivatives. However, other substances with only moderate activities included as biphenyl propanoids, alkaloids, and components of essential oils (ElNaggar et al., 2022).

Polyphenols such as flavonoids and phenolic acids are a class of secondary metabolites found in plants that have been widely investigated. Polyphenols are distinguished by the presence of numerous phenol (benzene) rings in their structure (Sung et al., 2016). Polyphenols have a wide range of biological roles, including anticancer action, due to their structural diversity (Madunić et al., 2018). Flavonoids exert their anticancer effect by inducing apoptosis in cancer cells

(Kopustinskiene et al., 2020). However, studies have demonstrated that high flavonoid consumption is related to a lower risk of several forms of cancer. Aside from anticancer action, flavonoid-mediated health advantages include antioxidant activity by eliminating free radicals, which may damage lipids, proteins, and DNA (Simon et al., 2000; Sak, 2014; Sung et al., 2016).

Based on the reported data, our identified metabolites including lupeol (**1**), *trans* 4-methoxy cinnamic acid (**2**), ferulic acid (**3**), 4-anisaldehyde (**4**) and isorhamnetin-3-*O*-*β*-D-galactopyranoside (**5**) are suggested to greatly contribute within the obtained antioxidant and cytotoxic activities of *S. kali* extracts within the presented study.

Lupeol (1) is a pentacyclic triterpenoid; that was isolated previously from *S. kali* (Alturkistani et al., 2017); occurs in the skin of lupin seeds, as well as in the latex of fig trees and of rubber plants. In the last decade, a plethora of studies on the pharmacological activities of lupeol have been conducted and have demonstrated that lupeol possesses an extensive range of pharmacological activities such as anticancer (liver cancer, lung cancer, colorectal cancer, bladder cancer and osteosarcoma), antioxidant, anti-inflammatory, and antimicrobial activities (Liu et al., 2021). The identified triterpenoid exerted a significant synergistic cytotoxic effect when combined with low-dose cisplatin without side effects. Results by Lee *et al* suggested that lupeol may be an effective agent either alone or in combination for treatment of advanced tumors. The reports also provide solid evidence that lupeol may be a novel therapeutic agent that is clinically applicable for the treatment of cancers in which nuclear factor-κB (NF-κB) plays a significant role (Lee et al., 2007). Studies demonstrate that lupeol is an anti-angiogenic compound that inhibit endothelial cell proliferation, migration, and capillary network formation through the disruption of the tumor necrosis factor-α-vascular endothelial growth factor receptor-2 (TNF-α-VEGFR-2) axis, and is effectively suppress growth of cholangiocarcinoma xenografts by downregulating inflammatory cytokine production, macrophage recruitment and tumor angiogenesis (Kangsamaksin et al., 2017). Specifically, it was shown to possess significant antitumor activity in a two-stage model of mouse skin carcinogenesis, inducing cell cycle arrest and apoptosis in melanoma cell line 451Lu cells (Saleem et al., 2004). A recent study by Jiang *et al.* demonstrated the reduced cellular viability of HCT-116 and SW620 colon cancer cell lines by **lupeol** where the latter can suppress the cancer migration and invasion through inhibiting the Ras homologous gene family member A - Rho-related coiled-coil containing protein kinase1 (RhoA-ROCK1) pathway (Jiang et al., 2020). **Lupeol and lupeol acetate** (**LA**) have been shown to exhibit higher anti-inflammatory activity than the commonly used nonsteroidal anti-inflammatory drug indomethacin in rat and mouse models of inflammation (Saleem, 2009). Despite the promising anti-inflammation and antitumor capability reported by **lupeol**, poor

bioavailability limits applications in living subjects. (LA) was developed showing Lupeol's comparable biological activities yet with better pharmacokinetics (Wang et al., 2016).

***Trans* 4-methoxy cinnamic acid (2)** is one of the most studied phenylpropanoids with high importance not only in the wide spectrum of therapeutic activities but also its potential application for the food industry. This natural compound derived from plants exhibits a wide range of biologically useful properties; therefore, during the last two decades it has been extensively tested for therapeutic and nutraceutical applications (Płowuszyńska and Gliszczyńska, 2021). One of the first papers describing the antitumor potential of **compound (2)** was published in 2000 by Hudson *et al* and presented the results of an investigation of the activity of the phenol fraction of brown rice on the proliferation and growth of human breast and colon cancer cells (Hudson et al., 2000). The authors documented the potential inhibitory properties of phenolic acids on colon and breast cancers as well as **compound (2)** extracted from cooked rice bran and brown rice. They indicated that phenols might be a good candidate nutraceutical with colon or particularly breast cancer chemopreventive activity. **Compound (2)** obtained from brown rice and applied in a concentration of 50 μM showed the activity to inhibit the proliferation of colon tumor cells of the HT 29 line and human colon cells of the HCEC line, and the obtained results were similar to those observed for the positive controls genistein (30 μM) and tricine (50 μM).

It was also reported that **compound (2)** interfered with the colony formation of colon cancer cell line SW480 with a mechanism of anticancer action being evaluated by Gunasekaran *et al*. The authors confirmed that this phenylpropanoid induces apoptosis *via* an increase in caspase 3 and caspase 9 activities, resulting in the release of cytochrome C to cytosol (Gunasekaran et al., 2015). Chemopreventive activity of **compound (2)** has also been evaluated within preclinical models of colon cancer against 1,2-dimethylhydrazine-induced (DMH) rat colon carcinogenesis (Gunasekaran et al., 2019). Supplementation with **compound (2)** decreased the size and incidence of tumors in the colon of carcinogen-treated rats (Sivagami et al., 2012). It was shown that this phenylpropanoid acid in a dose of 40 mg/kg b.wt. ameliorating anticancer effects by altering multiple processes, including proliferation, angiogenesis, invasion, and induction of cell death in DMH-induced rat colon carcinogenesis. However, the molecular mechanism of the action of **compound (2)** still needs more advanced studies as it seems that the putative antioxidant, anti-inflammatory, and anticarcinogenic properties are crucial to this point (Gunasekaran et al., 2019).

Ferulic acid (3) (4-hydroxy-3-methoxycinnamic acid), an effective component of Chinese medicine herbs such as *Angelica sinensis, Cimicifuga heracleifolia* and *Lignsticum chuangxiong*,

is a ubiquitous phenolic acid in the plant kingdom. The phytochemical is a ferulic acid derivative consisting of *trans*-cinnamic acid bearing methoxy and hydroxyl substituents at positions 3 and 4, respectively, on the phenyl ring. Pharmacokinetic profile of **compound (3)** has been highlighted with low toxicity, feasible absorption, and adequate metabolism in the human body. It was identified in *S. kali* previously as well as in *S. collina*, *S. imbricate* and *S. vermiculata* (Sokolowska-Krzaczek et al., 2009). Clinical evidence associated **compound (3)** with strong antioxidant (Tundis et al., 2007; Sokolowska-Krzaczek et al., 2009), antimicrobial (Ou and Kwok, 2004; Wang et al., 2004; Wang et al., 2020b), anti-inflammatory (Ou and Kwok, 2004; Sokolowska-Krzaczek et al., 2009; Osman et al., 2016), anti-thrombosis, and anti-cancer activities (Ou and Kwok, 2004; Sokolowska-Krzaczek et al., 2009). The cinnamic acid derivative was reported with significant activity for protection against coronary disease, lowers cholesterol and increases sperm viability.

Compound (3) was also reported to serve as an antioxidant, an anti-inflammatory agent, an apoptosis inhibitor and a cardioprotective agent. It is a conjugated acid of a ferulate. Ayna *et al* demonstrated that the use of antioxidants as nutritional supplements and, in particular ferulic acid, protected cells and reduced apoptosis-induced cell death in the immune system, liver, heart and kidneys (Ayna et al., 2020). Overall, the pharmaceutical potential of **compound (3)** can be attributed to its ability to scavenge free radicals. However, recent studies have revealed that ferulic acid presents pharmacological properties beyond those related to its antioxidant activity, such as the ability to competitively inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and activate glucokinase, contributing to reduce hypercholesterolemia and hyperglycemia, respectively (Paiva et al., 2013).

4-anisaldehyde (4) or *P*-methoxybenzaldehyde is a member of the class of benzaldehydes consisting of benzaldehyde itself carrying a methoxy substituent at position 4. Methoxybenzaldehydes have shown promising inhibitory activity against cancerous cells. Study by Arulvasu *et al* investigate the antioxidant and anticancer property of **compound (4)** *in vitro*. The free radical scavenging activity was evaluated using DPPH, iron chelating, reducing power assay and the cytotoxicity was assessed by MTT method using human cancer cell lines. The lowest DPPH free radical scavenging activity of **compound (4)** obtained was 20% at 400 µg/mL and the highest DPPH free radical scavenging activity obtained was 21% at 800 µg/mL concentrations. The maximum chelation of metal ions obtained was 27% at 1000 µg/mL and the reductive capability was high in 23% at 1000 µg/mL.

Anti-cytotoxic activity of **compound (4)** has been evaluated against various cancer cell lines such as human breast adrenocarcinoma cell line (MCF-7), liver adrenocarcinoma (HepG2) and

epidermoid carcinoma (ME 180). The IC_{50} values of 400, 600 and 800 µg/mL at MCF-7, HepG2 and ME 180 cell lines, respectively. In this result the **compound (4)** showed a superior effect on MCF-7 compared to other cell lines. Therefore, the **compound (4)** proved to be a potent antioxidant and anticancer property against human cancer cell lines (Arulvasu et al., 2014).

Isorhamnetin-3-O-β-D-galactopyranoside (5), as natural flavonol compounds, are primarily extracted from various plant-based foods or medicinal plants (Ma et al., 2016; Antunes-Ricardo et al., 2017; Wang et al., 2019; Wang et al., 2023). Isorhamnetin glycosides (IGs) are biologically important flavonols with proven beneficial properties that give them medicinal value (Hyun et al., 2006; Abdel Motaal et al., 2020). They possess diverse biological and pharmacological properties, such as antioxidant, anti-inflammatory, anti-cancer, antidiabetic, anti-obesity, and hepatoprotective properties (Kim et al., 2012a; Cho et al., 2013; Ku et al., 2013; Antunes-Ricardo et al., 2019; Hussain et al., 2019). Due to their beneficial biological activities, IGs have been considered a significant potential class of phytonutrients, and an increasing number of products containing IGs are circulating on the market in many countries, including the United States, Canada, Mexico, China, India, and some European countries (Barba et al., 2022; Wang et al., 2022). **Compound (5)** was isolated previously from *Salsola imbricata* Forssk (Osman et al., 2016) and *S. oppositifolia* but reported for the first time in *S. kali*.

Compound (5) showed significant antioxidant activity, as determined *via* DPPH assay, with IC_{50} values of 11.76 and 9.01 µM in DPPH assay, and 3.34 and 2.56 µM in the peroxynitrite assay (Hyun et al., 2006). **Compound (5)** act as free radical scavengers and chain-breaking antioxidants of DPPH, with IC_{50} values of 4.84 and 4.51 µM (Demirkiran et al., 2013). Evaluation of the antioxidant properties of IGs were also carried out using various cell type experiments and animal models. IGs had the ability to inhibit the formation of H_2O_2-induced radicals in the surrounding environment of intestinal epithelial cells (Abdallah and Esmat, 2017). Moreover, **compound (5)** demonstrated strong inhibition of reactive oxygen species (ROS) production in the oxidative burst activity of whole blood, neutrophils, and mononuclear cells (Yeskaliyeva et al., 2006). Interestingly, almost 100 µg/mL concentration of **compound (5)** exhibited no cytotoxicity in RAW 264.7 macrophage cells (Wu et al., 2018). Meanwhile, it demonstrated a potential activity against MCF-7 with IC_{50} values of 18.2 µg/mL (Tundis et al., 2008).

CHAPTER VII: CONCLUSION, RECOMMENDATIONS & FUTURE WORK

7. General Summary and Conclusion

7.1. Conclusion

Cancer is the main cause of deaths globally. Curing this disease has been the potential target for novel natural products by providing a huge diversity of chemical profiles. Nowadays, a great deal of effort is being expended to find effective antioxidants for the treatment or prevention of free radical-mediated deleterious effects. The principal objective of this study was to identify a traditional medicinal plant that would exhibit strong combined antioxidant and anticancer activities. Additionally, the study aimed to explore the potential of such plant to provide natural compounds with potential therapeutic benefits.

The utilization of natural products in the treatment of ailments is widely recognized as an initial step in the process of drug discovery. Natural products have long been recognized as a valuable source of drugs. Generally, the utilization of natural resources for managing diseases represents an appealing opportunity for discovering novel therapeutic agents. The latter is primarily due to the vast chemical space and diversity of these compounds being the result of the involvement of millions of species within their production. Compounds, derived from various natural sources such as plants, animals, and microorganisms, have provided the foundation for numerous pharmaceuticals. Their diverse chemical structures and biological activities make them promising candidates for drug discovery and development. Moreover, the revival of interest in the use of plants as sources of food and medicine also encouraged the researchers to explore plant based natural antioxidants and bioactives as ingredients of functional food and nutraceuticals.

Development of novel drugs from natural source is considered challenging owing to the lots of drawbacks related to the natural source of bioactive compounds. Challenges include the inaccessibility to mass biosynthesize the secondary metabolites, limited access to the natural source of the active compounds, expensive isolation schemes for affording the purified active compounds, and elucidation difficulties encountered for complex chemical structure all of which hamper the successful translation of natural compounds within pharmaceutical industries. Fortunately, the steady emergence of new technologies in analysis and biological screening procedures have speeded up and greatly facilitate the drug discovery pipeline for natural source compounds.

Concerning one of the most promising discovery tools, metabolomics has been considered as a powerful tool in providing concrete guidelines for an isolation scheme based on HR-LCMS and NMR. Metabolomics tools such as MZmine 2.53, In-house EXCEL Macro customized with the Dictionary of Natural Products (DNP) 2017, and SIMCA-P V 17.0 assisted the prediction and interpretation of results. The natural-based dictionary is efficiently used in early prediction of the bioactive secondary metabolites and prioritizing the fractions that will go further for purification work the thing that would highlight the precursor metabolites being used within the active metabolites' biosynthesis.

This study focuses on investigating the medicinal potential and bioactive compounds of one of the traditional medicinal plants from Saudi Arabia belonging to genus *Salsola*. A bio-guided fractionation approach was employed to identify bioactives present in the plant and assess their medical properties.

Genus *Salsola* is an important halophytic genera of the family Amaranthaceae and is considered genera of plants containing antioxidants compounds with low caloric composition. It is known to be widely spread all over the world, has a history of medicinal uses against different diseases in the folk medicine system of several civilizations. Unfortunately, the plants of this genus are less explored and under-utilized and rarely characterized for their biochemical principles. This bolstered the need to investigate the biochemical prospects of these multipurpose plants.

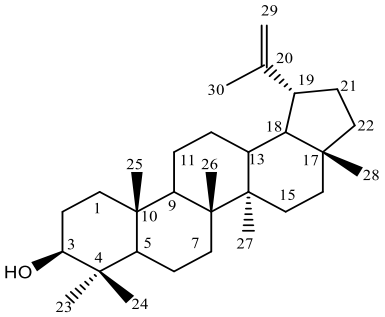
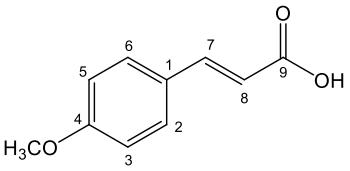
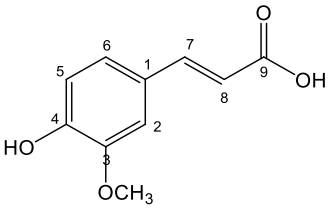
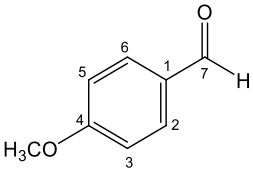
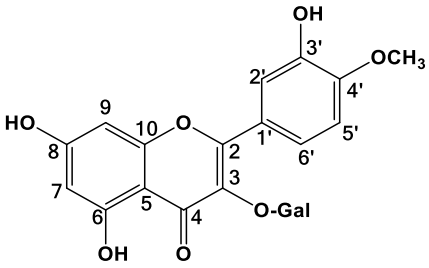
In this work, *Salsola kali* was extracted separately with methanol and ethyl acetate to give **KM** and **KE** extracts respectively. Prepared extracts were tested for their antioxidant as well as anticancer activities using different human cell lines (HepG2, MCF-7, HCT-116). DPPH assay was conducted at concentration of 30 µg/mL of sample using ascorbic acid as positive control. As a result, KE had higher antioxidant activity compared to KM. Moreover, KE was further fractionated on MPLC to give 15 fractions that were tested again for their antioxidant activity. Fractions F4, F5, F6, F7-9, F52-55, F56-60 and F61-76 have significant antioxidant activity within the range from 41% up to almost 65% compared to ascorbic acid 40%. The *in vitro* EC_{50} values for the promising active fractions = 49.72, 19.98, 17.92, 20.37, 18.64, 17.02, 28.63, respectively compared to 5.09 µg/mL for ascorbic acid.

In addition, *in vitro* MTT cytotoxic bioassay was carried out for *S. kali* crude extracts (KM and KE) against three tested human cancerous cell lines (HepG2, MCF-7 and HCT-116). KM and KE showed modest anticancer activity with preferential activity against HepG2 with IC_{50} 104.5 and 34.5 µg/mL, respectively. Thus, the antioxidant activity of *S. kali* activity could be referred to as its major isolated compounds.

Bioactive fractions were further fractionated using high-throughput chromatography Biotage® flash system that enhanced the purification process of the bioactive metabolites and minimized the number of isolation steps. Also, a conventional column employing a normal silica gel column with variable mobile phases was used. A combination of different chromatographic techniques was used for purification of the compounds. The structural determination of the isolated compounds was performed by study of their physical and spectroscopic data including UV, 1D and 2D NMR studies. Five isolated compounds are identified as lupeol (**1**), *trans* 4-methoxy cinnamic acid (**2**), ferulic acid (**3**), 4-anisaldehyde (**4**) and isorhamnetin-3-*O*-*β*-D-galactopyranoside (**5**) which was reported for the first time in *S. kali*. These compounds were subjected to 1D, 2D NMR analysis to be elucidated in accordance to the data reported in the literature. The isolated compounds **1-5** showed considerable reported cytotoxic and antioxidant activities. The structures of the isolated compounds and their biological activities were listed in **Table 7.1.**

Additionally, a metabolomics-guided approach was applied by using NMR and LC-HRMS as profiling tools to afford an intensive chemical profile of the *S. kali* to target the bioactive metabolites. The spectral data was processed using Xcalibur, MZmine 2.53, in-house MS-Excel macro, and Dictionary of Natural Products for dereplication studies. (**KE**) fractions were subjected to extensive metabolic studies. A supervised multivariate analysis was done by orthogonal partial least squares discriminant analysis (OPLS-DA) in SIMCA-P V 17.0 to predict and pinpoint the plausible bioactive components. The analysis of the extract revealed the presence of 43 hits, indicating the occurrence of bioactive phenolics, particularly flavonoids and their derivatives. Additionally, organic acids, nitrogenous compounds and sugar derivatives were also detected in the extract. A combination of metabolomics- and bioassay-guided approaches gave an access to a shorter and faster route to highlight active metabolites highly correlated to the bioactivity during the first stage of fractionation, which might be produced in a very small amount, solving the mystery behind the fact that purified major compounds could be not found active.

Table 7.1: Isolated compounds and their biological activities from *Salsola kali* in this study

Name of Isolated Compound	Structure of Isolated Compound	Chemical Formula	MWt (g/mol)	Fraction number
Compound 1 (lupeol)		$C_{30}H_{50}O$	426.3906	F4
Compound 2 (*trans* 4-methoxy cinnamic acid)		$C_{10}H_{10}O_3$	178.0631	F4
Compound 3 (ferulic acid)		$C_{10}H_{10}O_4$	194.0580	F4 F5 F6-9
Compound 4 (4-anisaldehyde)		$C_8H_8O_2$	142.10	F4
Compound 5 (isorhamnetin -3-O-*β*-D-galactopyranoside)		$C_{22}H_{22}O_{12}$	478.11	F52-55 F56-60

7.2. Recommendations and Future Work

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

compounds will also require rigorous toxicological and pharmacokinetic testing as well as clinical verification of the findings obtained *in vitro*.

Further investigation for other fractions obtained from the ethyl acetate extract of *Salsola kali* (KE) is recommended to isolate other biological active compounds. A study comparing the chemical profiles of other *Salsola* species could help provide a better understanding of cinnamic acids biosynthesis and/or accumulation patterns within that genus. Enrichment of hydroxy fatty acids suggests potential antimicrobial and anti-inflammatory effects in *Salsola* extracts, which has yet to be examined. It is also necessary to investigate the synergistic effects of phytochemicals and optimal extraction techniques. Overall, more research is warranted to validate the traditional medicinal uses of *Salsola kail* and develop evidence-based herbal remedies. Thus, further isolation and testing of these metabolites will help provide more evidence for possible medicinal uses of *Salsola*.

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