Elicitation, metabolomic analysis, and identification of antidiabetic compounds from selected indigenous plants

By

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Submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

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Elicitation, metabolomic analysis, and identification of antidiabetic compounds from selected indigenous plants

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our abilities and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

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ABA	Abscisic acid
ACC	Aminocyclopropane-1-carboxylate
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AsA	Ascorbate
ATM	African traditional medicine
BHT	Butylated hydroxyltoluene
CAM	Complementary and alternative medicine
CAS	Chrome Azurol S
CAT	Catalase
CCaMKs	Calcium/calmodulin-dependent protein kinases
CCE	Cyanidin chloride equivalent
CDPKs	Calcium-dependent protein kinases
CE	Catechin equivalents
CFU	Colony-forming unit
CO_2	Carbon dioxide
COVID-19	Coronavirus disease 2019
CPK	Ca^{2+}-dependent protein kinase
DAB	3′3-diaminobenzidine
DCM	Dichloromethane
DM	Diabetes Mellitus

DMAPP	Dimethylallyl diphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNSA	3,5- dinitrosalicylic acid
DPP-4	Dipeptidyl peptidase-4
DPPH	1,1-diphenyl-2-picrylhydrazyl
EL	Electrolyte leakage
EMP	Embden Meyerhof-Parnas pathway
ERFs	ET-responsive factors
ET	Ethylene
ETI	Effector-triggered immunity
EtOAc	Ethyl acetate
FC	Field capacity
$FeCl_3$	Iron (III) chloride
FIMS	*Fusarium oxysporum* inoculated and moderately stressed
FISS	*Fusarium oxysporum* inoculated and severely stressed
FIWW	*Fusarium oxysporum* inoculated and well-watered
Folin-C	Folin-Ciocalteu
FPP	Farnesyl diphosphate
FRAP	Ferric-reducing antioxidant power
FT-ICR	Fourier Transform Ion Cyclotron Mass spectrometry
FT-IR	Fourier Transform Infrared spectroscopy
Fv/Fm	Photochemical efficiency of photosystem II

GAE	Gallic acid equivalents
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1 analogues
GPP	Geranyl diphosphate
GPX	Guaiacol peroxidase
GR	Glutathione reductase
GSH	Glutathione
H^+	Hydrogen ion
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
HCN	Hydrogen cyanide
HDTMA	Hexadecyltrimethylammonium bromide
HIV	Human immunodeficiency virus
HSPs	Heat shock proteins
IAA	Indole-3-acetic acid
IC_{50}	50 % inhibitory concentration
IPP	Isopentyl pyrophosphate
ISL	Induced Systemic Resistance
ISR	Induced systemic resistance
ITS	Internal transcript spacer

JA	Jasmonic acid
LAR	Local Acquired Resistance
LB	Luria Bertani
LC-MS	Liquid chromatography-mass spectrometry
LEA	Late embryogenesis abundant proteins
LRR	Leucine-rich repeats
MAMPs	Microbial-associated molecular patterns
MAPK	Mitogen-activated protein kinases
MDA	Malondialdehyde
MEP	Methylerythritol phosphate
MHB	Mueller-Hinton broth
mRNA	Messenger Ribonucleic acid
MVA	Mevalonate
MVDA	Multivariate data analysis
NA	Nutrient agar
$NADPH^{+}$	Nicotinamide adenine dinucleotide phosphate
NaOCl	Sodium hypochlorite
NB	Nucleotide-binding domains
NBT	Nitroblue tetrazolium
NCBI	National Centre for Biotechnology Information
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
OD	Optic density

P+FIMS	*Paeonibacillus polymyxa* and *Fusarium oxysporum* inoculated and moderately stressed
P+FISS	*Paeonibacillus polymyxa* and *Fusarium oxysporum* inoculated and severely stressed
P+FIWW	*Paeonibacillus polymyxa* and *Fusarium oxysporum* inoculated and well-watered
PAMPs	Pathogen associated molecular patterns
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PE	Petroleum ether
PEG-6000	Polyethylene glycol 6000
PIMS	*Paeonibacillus polymyxa* inoculated and moderately stressed
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid
PISS	*Paeonibacillus polymyxa* inoculated and severely stressed
PIWW	*Paeonibacillus polymyxa* inoculated and well-watered
pKa	Acid dissociation constant
PLS	Partial least square
pNPG	p-nitrophenyl alpha-D-glucopyranoside
PPFD	Photosynthetic photon flux density
PR-genes	Pathogenesis-related genes
PR-proteins	Pathogenesis-related proteins
PRRs	Pattern recognition receptors

PSI	Phosphate solubilising index
PSII	Photosystem II
PTI	Pathogen associated molecular pattern-triggered Immunity
PVK agar	Pikovskaya's agar
R- proteins	Resistance proteins
R	Rands
RI	Retention index
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rt	Retention time
RWC	Relative water content
SA	Salicylic acid
SAR	Systemic Acquired Resistance
SOD	Superoxide dismutase
T1DM	Type 1 DM
T2DM	Type 2 diabetes
TM	Traditional medicine
UHPLC	Ultra high-performance liquid chromatography
UIMS	Uninoculated and moderately stressed
UISS	Uninoculated and severely stressed
UIWW	Uninoculated and well-watered
USD	United State Dollars
UV	Ultra-violet

vNN	Virtual Network Name
WHO	World Health Organisation
YMB	Yeast malt broth
Zat12	Zinc-finger transcription factor

ABSTRACT

Diabetes mellitus (DM) is an endocrine disorder associated with high blood glucose levels accompanied by disruptions in the metabolism of fat, proteins and carbohydrates. DM is a chronic, non-communicable and medically incurable disease affecting millions of people globally, resulting in high morbidity and mortality rates, especially with the lingering coronavirus disease of 2019 (Covid-19). The use of western antidiabetic medicine has posed many challenges due to their perceived overall safety, treatment failure and cost. Many African communities rely on medicinal plants and their bioactive compounds as sources of medicine as a consequence of the poor state of health facilities, shortage of medical doctors and unaffordability of treatments. For this reason, this study partly evaluated the phytochemical contents, *in vitro* antioxidant and hypoglycaemic potentials of eleven indigenous plants using five different solvents. Putative hypoglycaemic agents from one of the most promising and readily available species were also identified using *in silico* molecular modelling.

Secondary metabolites and their pharmacological activity have been reported as the basis for the wide use of plants in traditional medicine. However, due to the indiscriminate harvesting and environmental pressure, many valuable indigenous plant species have gone into extinction or are at least threatened. Moreover, plants' bioactive compounds are often produced in minute quantities, and prevailing environmental conditions further influence their concentrations in plants. Thus, due to indigenous plants' industrial and medicinal value, deliberate cultivation and elicitation strategies have been adopted for the *en masse* production of uniform indigenous plants and to influence the quality and quantity of their active principles. Thus, this study also assessed the effects of individual and co-inoculation of two isolated drought-resistant and growth-

promoting endophytes on the growth, drought tolerance, medicinal efficacy and metabolome changes in the leaves of *Endostemon obtusifolius*.

In this research, the eleven plants were selected based on the traditional uses of the plants (or their related available species) for treating various ailments, including DM. The preliminary phytochemical quantification results revealed that the highest concentrations of phenolics, flavonoids and tannins were found in the crude extracts of *Combretum krausssii*, *Lippia javanica*, *Psidium guajava*, *Pentanassia prenulloides*, *E. obtusifolius*, *Syzgium cordatum*, *Pachira aquatica* and *Catha edulis*. The inhibitory effects of the crude extracts against the digestive enzymes α-amylase and α-glucosidase also showed that the crude extracts of *C. edulis*, *C. krausssii*, *L. javanica*, *P. aquatica*, *P. guajava*, *P. prenulloides*, *E. obtusifolius* and *S. cordatum* displayed excellent *in vitro* antioxidant and antidiabetic properties. These results validate the extensive use of these plants in the treatment of DM in many African communities.

Furthermore, the 80% ethanol (v/v) leaf extract of *S. cordatum* (one of the most active and readily accessible specie from the previous study) was fractionated into four sub-extracts [petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EtOAc) and water], and their phytochemical content, *in vitro* antioxidant and antidiabetic capacities were evaluated. Although the EtOAc extract was the richest of the sub-extracts in total phenolics, all four sub-extracts of *S. cordatum* showed good *in vitro* free radical scavenging and hypoglycaemic activities. *In silico* modelling evaluation of some (34) bioactive principles found in the Gas Chromatography-Mass Spectroscopy (GC-MS) analysis of the PE, DCM and EtOAc sub-extracts revealed that 21 compounds including andrographolide, benzylidene-iditol, cubenol and deoxyspergualin and bis[3,3,4,7-tetramethyl-1,3-2H-benzofuran-1-yl]-ether returned binding energy scores ≤ -7.5 kcal/mol against α-amylase and α-glucosidase enzymes indicative of their hypoglycaemic potentials. The physicochemical

and toxicological properties of andrographolide, benzylidene-iditol, bis[3,3,4,7-tetramethyl-1,3-2H-benzofuran-1-yl]-ether and cubenol were predicted to be soluble with high gastrointestinal solubility and non-toxic following Lipinski's rule of five and Veber's rule. Thus, these results indicate that these compounds are potential candidates for oral drugs.

The drought tolerance and *in vitro* plant growth-promoting properties of some endophytes isolated from *E. obtusifolius* (another active antidiabetic plant identified from the previous experiment) was evaluated. A total of 26 culturable endophytes (twelve fungi and fourteen bacteria) were isolated from the organs (leaf and root) of *E. obtusifolius*. These endophytic species displayed varying *in vitro* drought stress tolerance and plant-growth-promoting capacities. Two promising drought stress-tolerant and plant-growth-enhancing endophytic species (*Fusarium oxysporum* and *Paenibacillus polymyxa*) were subsequently identified using molecular tools. The identified bacterium (*P. polymyxa*) and fungus (*F. oxysporum*) exhibited a symbiotic relationship in an *in vitro* dual culture experiment.

Paenibacillus polymyxa and *F. oxysporum* individual and co-inoculation differential effects on their host under varying water regimes was further evaluated. The plants were raised with or without endophyte infection under three watering regimes for two months, and their therapeutic efficacy, physiological, biochemical and metabolic responses were assessed. In this study, drought stress markedly affected the growth and hypoglycaemic potentials of *E. obtusifolius*. On the other hand, endophyte inoculation generally enhanced the dry shoot and root biomass, chlorophyll contents and fluorescence, total soluble sugar, relative water content, proline contents and superoxide dismutase activities in the leaves of *E. obtusifolius,* whereas their electrolyte leakage and malondialdehyde contents were lowered. As for phytochemical accumulation, while the total phenolic contents were slightly enhanced by the inoculation of endophytes in the leaves of *E.*

obtusifolius, the flavonoid contents of the plant increased as the water deficit worsened. The EtOAc crude extracts' free radical scavenging capacity across the treatments remained unchanged; their *in vitro* α-glucosidase activity was negatively affected under moderate and severe drought stress but improved with endophyte inoculation.

The metabolome difference between the twelve treatments was evaluated using GC-MS based metabolomics. The bi-plot PCA result revealed that the metabolome of fungal inoculated moderately stressed *E. obtusifolius* correlated less with the other *E. obtusifolius* plants under different treatments. Additionally, a heatmap of eight differential metabolites showed that the most responsive treatment (the co-inoculated severely drought-stressed plants) produced the highest quantities of non-protein amino acids and organic acids known to protect plant cells during abiotic stress.

The leaf extracts of *S. cordatum* and *E. obtusifolius* showed remarkable antioxidant and antidiabetic potentials in this study. Although the putative active principles of these plants were identified using GC-MS analysis, proper isolation and quantification of these compounds can be explored by future studies. Moreover, some culturable endophytic species were isolated from the *E. obtusifolius* organs. *Paenibacillus polymyxa* and *F. oxysporum* showed their drought stress mitigating capacity in *E. obtusifolius* under varying water regimes. Although the concentration of some identified antidiabetic compounds in *E. obtusifolius* were up regulated, the mechanism involved in this observation requires further investigations.

CHAPTER 1: General Introduction

1.1. Medicinal plants and their importance / Introduction

The use of natural products, including plants, animals and microorganisms by man to meet his basic needs such as the production of shelter, food-stuffs, clothing, manure, transportation, spices, fragrances, and medicines, is as old as the existence of man **(GURIB-FAKIM, 2006; NEWMAN et al., 2000; PAULSEN, 2010)**. Plant species have contributed immensely to the development of many nations **(VAN WYK, 2008)**. Medicinal plants, in particular, have been widely used in the traditional medicine systems of different human cultures to provide remedies to ailments affecting man and livestock **(GURIB-FAKIM, 2006; HOAREAU and DASILVA, 1999; NEWMAN et al., 2000)**. Medicinal plants contain bioactive compounds in their organs that have curative properties or are used to develop valuable drugs **(ROY and ROY, 2016)**. Medicinal plants can be defined as "any plant that is used to prevent, ameliorate, or cure diseases or alter the physiological and pathological process in humans and animals" **(MIRANDA, 2021)**. Medicinal plants are the oldest and most widely accepted source of medication **(PAULSEN, 2010)**.

The identification of plant species, peculiar usage and dosage of herbal preparations to ameliorate various diseases were transferred from one generation to the next through verbal accounts **(BALUNAS and KINGHORN, 2005)**. In recent times, active principles from medicinal plants have been identified and isolated following specific extraction and purification procedures, and these compounds can be directly used as drugs (e.g. digoxin) or as precursors (e.g. diosgenin) of other medications **(RATES, 2001)**. Medicinal plants also play an essential role in the economy of many nations. They make an indispensable input to human healthcare, provide income to rural people and are sources of raw materials in industries **(ROY and ROY, 2016)**. Although synthetic

drug usage has increased tremendously since the 19th century, natural products remain the choice of several cultures for a host of health problems, including diabetes in populations throughout the world **(HOAREAU and DASILVA, 1999)**. According to The World Health Organisation (WHO), more than three-quarters of the world's inhabitants in less developed nations still rely mainly on herbal preparations of plants origin for their primary healthcare needs **(GURIB-FAKIM, 2006; WONG, 2001)**. In the last few decades, herbal medicines have gained more recognition from the remaining 20% of the world inhabitants residing in developed countries, partly due to failed attempts to produce relatively inexpensive and safe orthodox medicines **(SAHOO et al., 2010)**. Medicinal plants and their derivatives account for over 25% of all clinical drugs used today to treat various ailments, including malaria, diabetes, cancer, hypertension, and HIV-AIDS **(GURIB-FAKIM, 2006; HOAREAU and DASILVA, 1999)**.

There is a worldwide upsurge of interest in the natural products of indigenous plants. This interest is a direct consequence of the well-documented limitations of orthodox medicine in terms of efficacy, undesirable side effects, affordability, and accessibility **(HEINRICH and ANAGNOSTOU, 2017)**. However, plants' active principles are usually produced in minute quantities by the medicinal plants and are often species or biome specific, thus their persistent harvest mounts pressure on wild populations. Improved indigenous plant cultivation procedures and stimulation of active principle production are necessary to meet the growing demands **(VERPOORTE et al., 2002)**.

1.2. The rationale of the study/problem statement

The richness and diverse applications of traditional medicine in managing human metabolic disorders such as diabetes cannot be overemphasised; however, the continuous availability of

medicinal plants, its integral components, remain under constant environmental threat. The accumulation of active principles in medicinal plants and their overall efficacy strongly correlates with their immediate environmental factors, such as the accessibility of water **(ASKARY et al., 2018; KLEINWÄCHTER et al., 2015)**. Water shortage influences the yield, composition and concentration of active principles in indigenous plants **(GNANASEKARAN and KALAVATHY, 2017)**. The growth of medicinal plants under a limited water supply is usually negatively affected, whereas the concentration of secondary metabolites in most cases under the same conditions are concomitantly up-regulated **(KLEINWÄCHTER and SELMAR, 2015; SELMAR and KLEINWÄCHTER, 2013)**. Consequently, the reported enhancement in secondary metabolite production in drought-stressed medicinal plants may be linked to the overall decrease in the biomass of the plants **(AL-GABBIESH et al., 2015; KLEINWÄCHTER and SELMAR, 2015)**. From the conservation point of view, the global human population explosion, over-reliance of many poor communities on wild medicinal plants, and particularly, the unabating interest of giant pharmaceutical companies in natural products from the wild, accelerates the extinction of many valuable plant species due to overharvesting and habitat degradation **(NETSHILUVHI and ELOFF, 2016; VAN WYK and PRINSLOO, 2018)**. Thus, the cultivation of medicinal plants, as first suggested by Gerstner in 1938 **(CUNNINGHAM, 1988)**, and their deliberate elicitation are alternatives to optimise the synthesis of biologically active secondary metabolites. Plant species are in a vital symbiotic relationship with several endophytes, which enhances their overall wellbeing **(ZHANG et al., 2019)**. The inoculation of medicinal plants with endophytes could be a sustainable approach to improving their resistance to abiotic stress and enhancing their secondary metabolite contents.

1.3. Aims and objectives

Anti-hyperglycemic plants play an integral role in managing type 2 diabetes (T2DM) in many African communities. However, the efficacy and availability of these plants are hampered by biotic and abiotic stress, over-exploitation, seasonal variation, habitat loss, and indiscriminate harvesting. Elicitation can be used as a veritable tool to enhance production, continuous availability, and quality control of these pharmacologically active antidiabetic compounds. Thus, this study aimed to investigate some medicinal plants with anti-hyperglycemic activity and the accumulation of bioactive compounds in plants using an elicitation strategy.

The specific objectives were to:

- Examine the antidiabetic and antioxidative activities and phytochemical constituents of some medicinal plants;
- Identify bioactive constituent(s) from an active medicinal plant using in silico molecular model;
- Evaluate endophytic species of an active medicinal plant;
- Subject endophyte inoculated medicinal plants to varying water stress regimes; and
- Use metabolomic tools to profile the secondary metabolites produced in the plant after elicitation treatments.

1.4. Thesis outline

This thesis consists of eight Chapters, a concise research problem (Chapter 1), and an extensive literature review (Chapter 2). Chapters 3, 4, 5, 6, and 7 represent each of the five experimental

objectives, and the summary of the findings of this study and general conclusions are contained in Chapter 8. The citation and reference style of this study is in line with the South African Journal of Botany guidelines.

CHAPTER 2: Literature Review

2.1. African traditional medicine

Traditional medicine (TM) is an ancient health care system that is culture-driven, and its origin corresponds to the stone age. TM, also known as folklore medicine, native healing, complementary and alternative medicine (CAM), herbal medicine, and ethnomedicine, has been used by humans to combat all forms of diseases that have endangered their wellbeing **(ABE, 2011)**. Due to cultural diversity, there is no single, globally accepted definition for traditional medicine. However, the WHO defined TM as "the total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses" **(WHO, 2000)**. Although TM is known worldwide, it is significantly practised in Japan, China, Thailand, Sri Lanka, Pakistan, India, and many African countries **(HOAREAU and DASILVA, 1999; MUKHTAR et al., 2008)**.

African traditional medicine (ATM) is viewed as the foremost and most abundant of all traditional medicine systems globally **(GURIB-FAKIM, 2006)**. Although most of its claims lack verifiable documentation to date, ATM is very popular and uses over 4000 plant species to manage various forms of illness **(PAYYAPPALLIMANA, 2009)**. ATM includes herbal medicine, spiritual therapies, and manual therapies. ATM practitioners treat people holistically (involving both the body and the mind) in most African community settings by interlacing local religious principles and cultural traditions. Usually, after establishing an ailment, the healers administer medicines, mostly medicinal plants, to ameliorate the symptoms only after treating the spiritual basis of the health condition **(GURIB-FAKIM, 2006)**.

TM has provided background insights into discovering beneficial novel compounds used to treat many human ailments **(GURNANI et al., 2014)**. Most of these bioactive lead compounds were discovered through ethnomedicine and folk knowledge of indigenous people. However, some ATM claims are still awaiting validation despite the advancement of synthetic medicine **(GILANI et al., 2005)**. A Roll Back Malaria investigation revealed that herbal medicine remains the first line of medication for over 60% of children with high fever **(WHO, 2002)** in African countries. Western medicine alone has not yielded a desirable result for Africa; hence herbal medicine may complement western medicine in managing many diseases **(ELUJOBA et al., 2004)**.

Nonetheless, efforts are being made to systematically integrate ATM into western medicine to form a more inclusive health care system. Herbal medicine plays a significant role in lowering mortality, morbidity, and disability rates in conditions like mental disorders, HIV/AIDS, sickle-cell anaemia, malaria, tuberculosis, and diabetes **(ELUJOBA et al., 2004)**. Traditional Medicine reduces poverty by increasing the economic wellbeing of communities and develops health systems by improving the health coverage to the people. The economic influence of ATM is widespread, with TM contributing over R2.9 billion to the South African economy on an annual basis **(MANDER et al., 2007)**.

Globally, over 400,000 species are listed as medicinal plants. Although they are potentially rich sources of bioactive compounds, only a tiny fraction have been investigated for their pharmacological activities **(SHOEMAKER et al., 2005)**. The biodiversity and endemism of Africa's flora are enormous due to its location within the tropical and subtropical climates **(GURIB-FAKIM, 2006)**. African plant species are rich in pharmacologically active compounds, but many of these plant species are rapidly going into extinction **(MAHOMOODALLY, 2013)**. Hence documentation of African medicinal species requires urgent intervention.

2.2. Drug discovery from medicinal plants

Several approaches, including isolation of bioactive compounds from medicinal plants and other natural resources, combinatorial chemistry, molecular modelling, and synthetic chemistry, have been used to obtain pure compounds for drug discovery **(GEYSEN et al., 2003; LEY and BAXENDALE, 2002; LOMBARDINO and LOWE, 2004)**. The recent over-reliance on modern technology such as high-throughput synthesis and combinatorial chemistry by the pharmaceutical industry for the development of new drugs is gradually declining due to unrealised expectations and failure to produce new drugs **(DAVID et al., 2015; KINGHORN et al., 2011; YUAN et al., 2016)**. Consequently, attention has shifted back to natural products in the search for novel drugs to manage debilitating diseases or life-threatening conditions such as cancer, diabetes, neurological disorders, HIV/AIDS and malaria despite the inherent difficulties associated with it **(ATANASOV et al., 2015; BUTLER, 2004; LAHLOU, 2013; NEWMAN et al., 2000; NGO et al., 2013; SHU, 1998)**. The relevance of natural products, especially medicinal plants, in discovering new drugs is unprecedented to date. Medicinal plants are still a significant component of drug discovery **(ATANASOV et al., 2021)** and represent the basis of any drug discovery process **(BUTLER, 2004)** as they occupy a niche of chemical space distinct from synthetic compounds **(SHU, 1998)**. Structural features of natural products (including a high number of chiral centres, high molecular weight, aromatic rings, the complexity of ring systems, degree of molecule saturation, and the ratio of heteroatoms), their greater chemical diversity and biosynthesis associated complexity have been implicated in the high binding affinities of natural products for specific proteins relevant for drug discovery efforts and their overall advantage over synthetic drugs **(ATANASOV et al., 2015;**

CLARDY and WALSH, 2004; FEHER and SCHMIDT, 2003; KOEHN and CARTER, 2005).

Natural products, including medicinal plants, occupy a crucial niche in drug discovery. About 40% of all medicines available in markets today are either natural products or their semi-synthetic derivatives **(JOHN, 2009)**. According to the WHO, about 11% of all primary and essential drugs are exclusively derived from medicinal plants and a substantial number of synthetic drugs are derived from natural products **(RATES, 2001)**. Medicines obtained from natural products contribute substantially to the profits of many companies **(LAHLOU, 2013)**. Some notable drugs that have been isolated from medicinal plants include salicin isolated from *Salix alba* **(SHILPI and UDDIN, 2020)**, codeine isolated from *Papaver somniferum* **(RATES, 2001)**, digitoxin isolated from *Digitalis purpurea* L. (foxglove) **(RATES, 2001)**, quinine isolated from *Cinchona succirubra* **(RATES, 2001)**, pilocarpine found in *Pilocarpus jaborandi* **(VON LINNÉ, 2007)**, triptolide isolated from *Tripterygium wilfordii* **(FIDLER et al., 2003)**, combretastatin A-4 phosphate isolated from *Combretum caffrum* **(HOLWELL et al., 2002)**, galantamine hydrobromide obtained from *Galanthus nivalis* **(HEINRICH and TEOH, 2004)**, artemisinin isolated from *Artemisia annua* **(NEWMAN and CRAGG, 2007)**, vincristine and vinblastine isolated from *Catharanthus roseus* **(RATES, 2001)**.

Identifying and developing therapeutic compounds from indigenous plant species using the conventional bioassay-guided fractionation is a complex, tedious, expensive, and time-consuming exercise **(CHING et al., 2012; RATES, 2001)**. The practice embodies a trans-disciplinary approach based on anthropology, agronomy, botany, biochemistry, biotechnology, chemistry, history, linguistics, pharmacology, and pharmaceutical technology **(FABRICANT and FARNSWORTH, 2001; LEONTI, 2011; RATES, 2001)**. The development of new technologies

such as mass spectrometry (MS) has transformed the screening of natural products for new drug discoveries **(CHING et al., 2012)**. Mass spectrometry is a sensitive, rapid, and high-throughput technology for improving drug discovery from medicinal plants in the post-genomic era **(ZHANG et al., 2018)**. MS is a convenient analytical method commonly used in biomedicine, biochemistry, and biology **(ZHOU et al., 2019)**, and it has been successfully used in determining active compound(s) in crude extracts of medicinal plants **(LIU et al., 2016b)**. Usually, high-quality, high-throughput screening methods such as (ultra) high-performance liquid chromatography ((U)HPLC) or gas chromatography (GC) are integrated or coupled to MS for early screening or de-replication of candidate drug molecules from natural products **(ZHANG et al., 2018)**. Before applying statistical tools, raw analytical data is pre-processed using various commercial software and open-source packages **(JANKEVICS et al., 2012; KAWAGUCHI et al., 2010; PLUSKAL et al., 2010)**. The processed analytical data showing different pharmacological activities are then subjected to multivariate data analysis **(ATANASOV et al., 2015)**.

2.3. Diabetes Mellitus (DM)

DM is an endocrine disorder associated mainly with high blood glucose levels (hyperglycemia) with accompanied disruptions in the metabolism of fat, proteins and carbohydrates resulting from a deficiency in the secretion or action of insulin produced by the pancreatic beta-cells **(BAYNEST, 2015; JEAN-MARIE, 2018)**. DM is further described as a condition where the beta-cells of the pancreas produces little or no insulin or where the liver cells become increasingly unresponsive to its action **(LIU et al., 2010; WILCOX, 2005)**. DM is a chronic, medically incurable disease ill-managed in many patients despite the laudable innovations in pharmacotherapy **(SANYAL, 2013)**. DM patients are in danger of various vascular complications (including nephropathy, coronary

heart disease, retinopathy, and neuropathy) and sometimes can suffer from the diabetic foot **(ASMAT et al., 2016)**. Recently, DM has also been implicated as an underlying factor that affects the development and mortality rate of the coronavirus disease of 2019 (Covid-19) **(MADDALONI and BUZZETTI, 2020)**.

2.3.1. DM prevalence in Africa

In the past, Africans have considered DM as a disease of the rich **(JAKOVLJEVIC and MILOVANOVIC, 2015)**, as the number of diabetic patients on the continent was reasonably low **(KENGNE et al., 2005)**. However, rapid globalisation, urbanisation, and demographic and social changes have led to an increase in Africa's obesity and diabetes epidemic **(JAKOVLJEVIC and GETZEN, 2016; KENGNE et al., 2013)**. A prolonged sedentary lifestyle, population growth, improper nutrition, oxidative stress, and ageing are additional factors predisposing some patients to a familial history of the disease **(ASMAT et al., 2016; WILCOX, 2005)**. In 2019, 351.7 million adults globally (20-64 years of age) had diabetes, and by 2045 about 486.1 million cases are expected to have contracted the disease. About 19 million diabetic patients between 20 and 79 years have been estimated in Africa **(Figure 2.1)**. South Africa (4.6 million), Nigeria (2.7 million), The Democratic Republic of Congo (1.8 million), and Ethiopia (1.7 million) are leading countries in the number of sufferers **(INTERNATIONAL DIABETES FEDERATION, 2019)**.

In 2019, over 4 million deaths were linked to DM globally, and in Africa, DM remains one of the leading causes of non-communicable deaths, with 366,200 deaths recorded in the same year **(INTERNATIONAL DIABETES FEDERATION, 2019)**. The rising incidence of DM in Africa is perturbing as it places untenable expenses on individuals, their careers, the health system, and the continents' economy **(IDEMYOR, 2010)**. The continent spent over USD 9 billion on diabetes-

related health issues. South Africa, for instance, spent 23% of its health budget on DM **(INTERNATIONAL DIABETES FEDERATION, 2019)**.

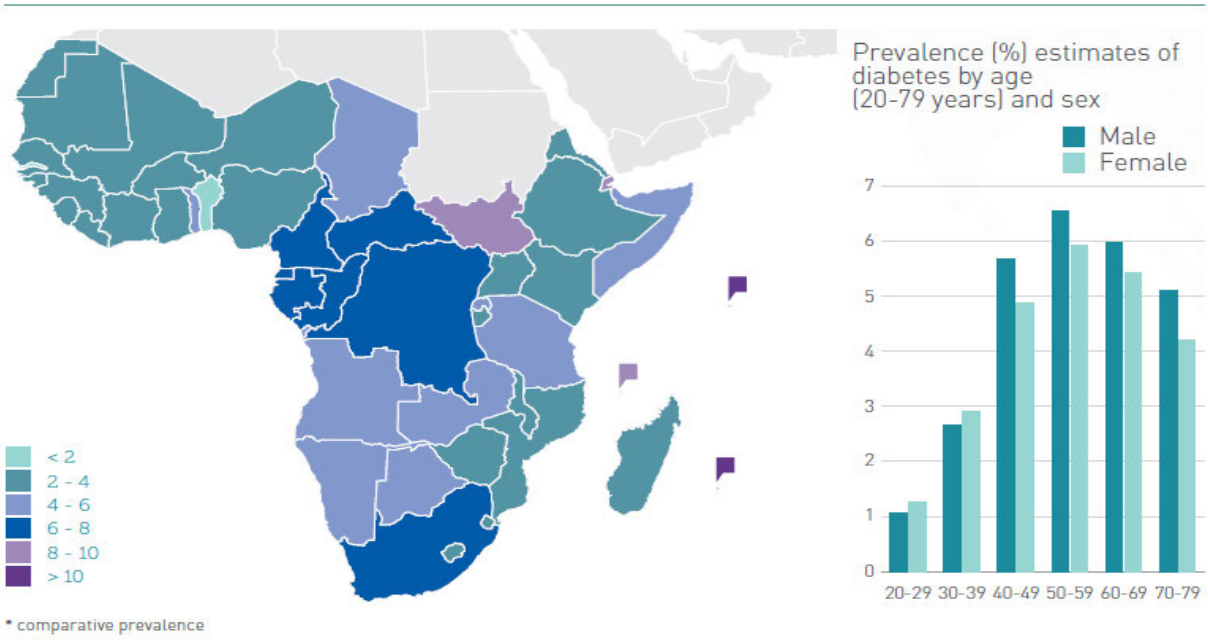

Figure 2.1: A map showing the prevalence of DM within the continent of Africa **(INTERNATIONAL DIABETES FEDERATION, 2019)**.

2.3.2. Types of DM

The classification of DM has been a matter of intense debate. However, four categories (type 1 DM, type 2 DM, gestational DM, and secondary DM) have been identified. Nonetheless, type 1 and 2 DM remain the most prevalent **(FOROUHI and WAREHAM, 2019)**.

I. Type 1 DM

Type 1 DM (T1DM) is also referred to as autoimmune DM. It is characterised by an insufficient or absolute lack of insulin secretion by the pancreas. It is caused by pancreatic beta-cells autoimmune destruction, resulting in hyperglycemia **(DIMEGLIO et al., 2018)**. Type 1 DM accounts for up to 10% of all reported DM cases and is influenced by autoimmunity, family history and environmental factors **(DANEMAN, 2006)**. It is more prevalent during early life, although symptoms can also develop later during adulthood **(KATSAROU et al., 2017)**. Type 1 DM patients become entirely reliant on exogenous insulin throughout their lifetime **(ATKINSON, 2012)**.

II. Type 2 DM

Type 2 DM (T2DM) is characterised by relative insufficient secretion of insulin caused by pancreatic beta-cell malfunction and insulin resistance in peripheral tissue such as muscle, adipose cells and liver **(CHEN et al., 2017)**. Type 2 DM accounts for over 80% of all DM cases globally and is further worsened by an ever-rising excess calorie intake, physical inactivity, obesity **(CHATTERJEE et al., 2017)** and the abuse of drugs such as antibiotics **(FISCHEDICK et al., 2017)** and corticosteroids **(LIGON and JUDSON, 2011)**. Most T2DM patients are overweight; meanwhile, obesity further increases body tissues' resistance to insulin and increases T2DM prevalence **(ASIIMWE et al., 2020)**. Insulin treatment is not needed for the survival of T2DM patients. However, it may be necessary to check hyperglycaemia and prevent serious complications **(JARRETT and KEEN, 1981)**.

2.3.3. Orthodox (Western) treatments of T2DM

Western medicine treatment options for managing T2DM have grown as the knowledge of the fundamental pathophysiological defects have progressed **(THRASHER, 2017)**. While a diabetic curative drug is still elusive **(EDELMAN and POLONSKY, 2017)**, the eight-core defects of diabetes (also known as "the ominous octet") include increased lipolysis, neurotransmitter dysfunction, decreased insulin secretion, increased glucose reabsorption, increased glucagon secretion, decreased glucose uptake, increased hepatic glucose production, and decreased incretin effect are targeted by different therapies to achieve glycaemic control **(DEFRONZO et al., 2013; SCHWARTZ et al., 2016)**.

Currently, a combination of lifestyle changes and the administration of oral and injectable hypoglycaemic drugs are recommended to manage T2DM **(MARÍN-PEÑALVER et al., 2016)**. Glucose lowering agents of different classes including biguanides (e.g. metformin), glucagon-like peptide-1 (GLP-1) analogues (exenatide, lixisenatide, and liraglutide), sodium-glucose co-transporter-2 inhibitors (dapagliflozin and empagliflozin), dipeptidyl peptidase-4 (DPP-4) inhibitors (e.g. sitagliptin, vildagliptin, saxagliptin, and linagliptin), sulfonylurea (e.g. glibenclamide and glimepiride), α-glucosidase inhibitors (e.g. acarbose), thiazolidinediones (e.g. rosiglitazone and pioglitazone), and insulin therapy are widely used individually or in combinations **(KRENTZ and BAILEY, 2005; SCHWARTZ et al., 2016; THRASHER, 2017)**.

Drug resistance, toxicity, and numerous undesirable side effects occur in patients under these western antidiabetic agents **(SALEHI et al., 2019)**. For example, sulfonylurea has been linked with beta-cell death **(TAKAHASHI et al., 2007)**, hypoglycaemia, weight gain, and cardiovascular problems **(MARÍN-PEÑALVER et al., 2016)**. Thiazolidinediones usage may lead to weight gain, heart failure, and kidney toxicity **(CHANG et al., 2013)**, and nausea, abdominal discomfort,

anorexia, flatulence, and diarrhoea may occur in patients on metformin **(MARÍN-PEÑALVER et al., 2016)** and acarbose **(ABE et al., 2011)**. Another major drawback with all current synthetic antidiabetic agents is that they are only designed to alleviate diabetes and not cure it **(CHANG et al., 2013)**. They are also not cost-effective or accessible to the indigent populace of developing countries **(ALEBIOSU and AYODELE, 2005; MARÍN-PEÑALVER et al., 2016)**.

The pattern of antidiabetic management nowadays has shifted from monotherapy to combination therapy. To date, no antidiabetic agent(s), used alone or in combination, has cured this disease in humans. To ensure the wellbeing of diabetes patients, there is an obvious need to develop affordable antidiabetic medicines with satisfactory efficacy and fewer or no adverse side effects. The use of herbal preparations is gathering momentum because of their relative cost-effectiveness, therapeutic efficacy, and fewer significant reported side effects **(GAIKWAD et al., 2014)**. A single medicinal plant contains thousands of phytochemicals; thus, it could serve as a game-changer because many metabolic pathways relating to hyperglycaemia are targeted simultaneously **(CHANG et al., 2013)**. A synergistic approach (a combination of western medicine and herbal medicine) has shown promising results **(KAUR et al., 2013)**; hence, antidiabetic plants or their derived compounds can complement western medicine in searching for a cure to T2DM.

2.3.4. Medicinal plants with established antidiabetic properties used in South Africa

Medicinal plants are good sources of alternative hypoglycaemic drugs and are widely used in several traditional systems of medicine to prevent diabetes **(KOOTI et al., 2015)**. Over a thousand medicinal plants have been documented as therapies for diabetes **(KOOTI et al., 2016)**, and about 500 plants and 800 recipes and compounds have been assessed scientifically **(SINGH et al., 2011)**. The hypoglycaemic properties of medicinal plants have been linked to the ability of their bioactive

principles to enhance the production of insulin or reduce the absorption of glucose by the intestinal walls **(KOOTI et al., 2016)**.

Medicinal plants in South Africa with anti-hyperglycemic properties are well documented **(AFOLAYAN and SUNMONU, 2010; BALOGUN et al., 2016; SABIU et al., 2019)**. Species including *Cannabis sativa, Catha edulis, Gunnera perpensa, Bulbine natalensis, Psidium guajava, Vernonia colorata, Ruta graveolens, Leonotis leonurus, Salvia africana-lutea* and *Sclerocarya birrea* have been widely reported by researchers **(FABRICANT et al., 2005; THRING and WEITZ, 2006; VAN DE VENTER et al., 2008)**. Many putative antidiabetic compounds have also been identified or isolated from some South African plants, including β-sitosterol, β-sitosterol-3-acetate, lupeol, and stigma-4-ene-3-one from *Terminalia sericea* **(NKOBOLE et al., 2011)**, oleanolic acid from *Xylopia aethiopica* fruit **(MOHAMMED et al., 2019)**, and barledinoside from *Barleria dinteri* **(GOLOLO et al., 2017)**.

2.4. Plant secondary metabolites

The sum of all biochemical activities in a plant is known as plant metabolism **(JIMENEZ-GARCIA et al., 2013)**. Plant metabolites are small intermediate molecules or end-products of metabolism **(TIWARI and RANA, 2015)**. Plant metabolites play a variety of roles to ensure the survival of plants. Plant cells' primary metabolic pathways yield a meagre number of products, while secondary metabolic paths diverge into many products. Thus, two categories of metabolites are produced: primary and secondary **(JIMENEZ-GARCIA et al., 2013)**.

In primary metabolism, plant cells undergo key processes (such as photosynthesis, Embden Meyerhof-Parnas pathway (EMP), the citric acid cycle, electron transport, phosphorylation, and energy regulation) and produce primary metabolites, including amino acids, nucleotides,

phytosterols, amino acids, and organic acids **(CANTER et al., 2005)**. Primary metabolites are found in all plants, and they execute vital metabolic responsibilities by participating directly in nutrition and reproduction **(HUSSAIN et al., 2012)**. Secondary metabolites are heterogeneous low molecular weight compounds biosynthetically obtained from limited primary metabolites and are not directly involved in the normal processes of growth and development **(CROZIER et al., 2007; HARBORNE, 1984)**. Plant secondary metabolites are produced in the rough and smooth endoplasmic reticulum, cytoplasm or specific organelles such as the chloroplast **(KUTCHAN, 2005; WINK and HARTMANN, 1982)**. Hydrophilic compounds are commonly deposited in the vacuole **(KUTCHAN, 2005; TERASAKA et al., 2003; YAZAKI, 2005)**, whereas lipophilic compounds are confined in laticifers, resin ducts, glandular hairs, trichomes, thylakoid membranes or on the cuticle, and they do not in any way interact with the plant's metabolism **(KUTCHAN, 2005; WINK, 2013)**. Sometimes, secondary metabolites or phytoalexins of plants are synthesised anew, notably during herbivory or pathogenic attack **(WINK, 2013)**.

Plant secondary metabolites are products of several enzymatic-controlled metabolic pathways, and their metabolism is combined into morphological and biochemical regulatory patterns of plants. Secondary metabolite biosynthesis and accumulation in plants is controlled by environmental factors **(HOLOPAINEN and GERSHENZON, 2010)**. They are unequally distributed in narrow phylogenetic groups within the plant kingdom **(JIMENEZ-GARCIA et al., 2013)**. Secondary metabolites of plants are not the basic framework of plants, and their dearth in plants does not immediately significantly affect the plant's life. However, plants' survival and competitiveness are compromised **(TIWARI and RANA, 2015)**. The evolution, biosynthesis, and accumulation of over one hundred thousand known plant secondary metabolites form the basis for chemotaxonomy/chemosystematics **(IFANTIS et al., 2013; WINK, 2003; WINK et al., 2018)**,

chemical defence systems against herbivores and diseases **(KUŚNIERCZYK et al., 2007; MAZID et al., 2011; TIAGO et al., 2017; WAR et al., 2011; WINK, 2010)**, signal compounds to attract seed-dispersing animals and pollinators, antioxidants against reactive oxygen species (ROS) and UV protectants **(WINK, 2013; WINK, 2015)**. Pharmacologically, secondary metabolites of plants represent interesting, abundant biogenic resources that humans have used as medicines to treat various ailments and disorders, poisons, toxins, pesticides, dyes, polymers, fibres, glues, oils, waxes, flavouring agents and perfumes **(NUNNERY et al., 2010; WINK, 2010)**.

Plant secondary metabolites are structurally diverse, and they can be broadly divided into three main groups viz: terpenes, phenolic compounds, and nitrogen-containing compounds based on their biosynthetic pathways **(IRCHHAIYA et al., 2015)**.

2.4.1. Terpenes

Terpenes make up the largest and most structurally diverse group of secondary metabolites with more than 40,000 compounds **(SACCHETTINI and POULTER, 1997)**. Terpenes are linked by their common biosynthetic source from acetyl-coA or glycolytic intermediates **(BOHLMANN and KEELING, 2008; TONG, 2013)**. Terpenes are lipid-soluble compounds, and their basic structure includes one or more 5-carbon isoprene units, which are universally produced by all organisms through two potential pathways, methylerythritol phosphate (MEP) and mevalonate (MVA) **(Figure 2.2)**. Both MEP and MVA pathways ultimately produce the intermediate general precursors for terpene biosynthesis, namely isopentyl pyrophosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) **(MATSUMI et al., 2011; OKADA, 2011)**. Progressive condensation of IPP and DMAPP produce geranyl diphosphate (GPP) or farnesyl diphosphate

(FPP), which can be converted to monoterpenes and sesquiterpenes in the presence of terpene synthases **(THOLL, 2006)**. Terpenoids undergo decomposition at high temperatures to give off the alkene gas isoprene. The isoprene units polymerise under suitable conditions to form several terpenoid skeletons; thus, terpenoids are also regarded as isoprenoids **(SANCHEZ and DEMAIN, 2011)**. Terpenoids are classified according to the number of isoprene units they contain; isoprene, which itself is synthesised and released by plants, comprises one unit and is classified as a hemiterpenes C5, monoterpenes include iridoids (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (including steroidal saponin and cardiac glycosides) (C30), tetraterpenes (C40) and polyterpenes (more than C40) **(ASHOUR et al., 2018; KO et al., 2014)**.

Terpenoids are versatile and take part in important plant primary metabolism, including photosynthesis as pigments (phytol and carotenoids), electron carriers (sidechains of the plastoquinones and ubiquinones), as membrane structures (phytosterols) and growth and development regulators (strigolactones, gibberellins, cytokinins, abscisic acid) **(THOLL, 2015; THOMAS et al., 2005)**. Terpenoids also serve as protection against abiotic stress **(BERTAMINI et al., 2019)**, as a defence against herbivorous insects, mammals and pathogens **(ASHOUR et al., 2018; DAS et al., 2013)**, as scents to mediate plant communication with their mutualistic pollinators and animals that disperse pollens or seeds **(THOLL, 2015)**, or as agents of allelopathy **(THOLL, 2015; WINK, 2010)**.

Terpenoids have been used commercially to produce many products, including dyes, flavours, fragrance agents, beverages, soaps, adhesives, coatings, emulsifiers, insecticides **(ASHOUR et al., 2018)**, and lately in the development of biofuels **(THOLL, 2015)**. Terpenes have great biological activities such as: anticancer (lycopene, squalene, paclitaxel), antimicrobial (α-terpineol, cineole, terpinen-4-ol) **(ASHOUR et al., 2018)**, antidiabetic (2,3-seco-20(29)-lupene-2,3-dioic acid)

(LAOTHAWORNKITKUL et al., 2012), anti-inflammatory (1,8-cineole, camphor) **(LIU et al., 2002)**, antimalaria and antiviral (artemisinin) **(YOUNS et al., 2009)** agents.

2.4.2. Phenolic compounds

Over 8000 phenolic compound structures have been identified, and they are the most widely distributed secondary plant metabolites in the plant kingdom **(LATTANZIO, 2013)**. Phenolics contain at least one aromatic ring bearing one (phenol) or more (polyphenol) hydroxyl groups, including their functional derivatives (e.g. esters and glycosides) **(CROZIER et al., 2007)**. In the biosphere, 40% of all organic carbon recycled are phenolics. They are produced mainly via either the shikimic acid/phenylpropanoid pathway or the malonate/acetate pathway (polyketide pathway), and related biochemical pathways **(LATTANZIO, 2013)**. Phenolic compounds influences growth and development of plants **(QUIDEAU et al., 2011)**.

In the phenylpropanoid pathway, phenylalanine, an amino acid, is produced in the shikimate pathway **(Figure 2.2)**. Phenylalanine is then enzymatically modified gradually to hydroxycinnamic acids and esters, which are then altered by reductases, oxygenases, and transferases into cinnamic acid, salicylic acid (SA), caffeic acid, coumaric acid, lignin, and many more compounds **(VOGT, 2010)**. Tyrosine or tryptophan also sometimes serve as the starting molecule. The polyketide pathway combines p-coumaroyl CoA, an intermediate of the phenylpropanoid pathway, to malonyl-CoA in the presence of enzyme chalcone synthase to produce naringenin chalcone. This undergoes various modifications to produce flavonoids **(YU and JEZ, 2008)**.

Phenolic compounds range from simple, aromatic-ringed, small molecular-weight compounds (phenolic acids) to large and complex tannins and derived polyphenols **(CROZIER et al., 2007;**

DAI and MUMPER, 2010). Generally, phenolics are divided into soluble phenolics such as phenolic acids, flavonoids and quinones, and non-soluble compounds such as condensed tannins, lignins, and cell-wall bound hydroxycinnamic acids **(KRZYZANOWSKA et al., 2010)**. Phenolic compounds have a wide range of functions in plants, including pigments and scents that attract pollinators, cell wall components, and defence compounds against stress factors **(LATTANZIO et al., 2008)**. Several phenolic compounds have dietary value in the human diet and have been associated with the therapeutic values shown by some plants. They function as antioxidants (rosmarinic acid) **(ZHENG and WANG, 2001)**, anticancer (kaempferol) **(LEUNG et al., 2007)**, anti-inflammatory (*p*-coumaric acid) **(PRAGASAM et al., 2013)**, antibacterial and antiviral (gallic acid and methyl gallate) **(CHOI et al., 2008)** and antidiabetic (ferulic acid) **(NARASIMHAN et al., 2015)** compounds.

2.4.3. Alkaloids

Alkaloids are naturally occurring chemical compounds containing a basic nitrogen atom. They are derived principally from plants and less frequently from fungi and animals **(SCHLÄGER and DRÄGER, 2016)**. Alkaloids (more than 12,000) make up a diverse group of nitrogen-containing secondary metabolites found in one-fifth of all angiosperms and are derived mainly from amino acids **(Figure 2.2) (ZIEGLER and FACCHINI, 2008)**. The alkaloids are a class of secondary metabolites that have medically, socially, politically, and economically influenced human history. They have been used extensively as narcotics, hallucinogens, poisons, analgesics, and antibiotics **(FALCÃO et al., 2008)**.

Our understanding of how alkaloids are biosynthesised in plants remains weak, partly due to the trace quantity of alkaloids isolated thus far from plants **(KISHIMOTO et al., 2016)**. Based on

their chemical structure, alkaloids are broadly classified into heterocyclic (typical alkaloids) and non-heterocyclic alkaloids (atypical alkaloids). Heterocyclic alkaloids contain nitrogen atoms in the heterocycle, e.g. pyrrole, pyridine, quinoline and indole alkaloids. Non-heterocyclic alkaloids lack nitrogen atoms in their heterocycle, e.g. ephedrine, cathinone, and colchicine **(AMIRKIA and HEINRICH, 2014)**.

Alkaloids serve as signal compounds between plants and other organisms. Alkaloids such as nicotine and quinolizidine are important plant defence compounds against grazing mammals and pathogenic attacks. When some insects ingest pyrrolizidine, it is enzymatically modified within the insect and is used either as pheromones to attract mates or as toxins to ward off predators **(SANCHEZ and DEMAIN, 2011)**. Alkaloids also have pharmacological activities. Some alkaloids have been used as analgesics (codeine) **(O'CONNOR, 2010)**, antioxidants (berberine, quinoline) **(ZUO et al., 2006)**, muscle relaxants (papaverine) **(O'CONNOR, 2010)**, anticancer (vinblastine and vincristine) **(BHANOT et al., 2011)**, antimicrobial (sanguinarine and berberine) **(O'CONNOR, 2010)**, antidiabetic and antiemetic (isovaleric acid) **(GRYNKIEWICZ and GADZIKOWSKA, 2008)** agents.

2.4.4. Glycosides

Glucosinolates and cyanogenic glycosides are nitrogen-containing compounds found in plants aside from alkaloids. Glucosinolates and cyanogenic glycosides usually turn into volatile toxic substances when the plant organs are crushed **(VERMA and SHUKLA, 2015)**. Glucosinolates are mainly found in the Brassicaceae and other related families, and they are responsible for the unique odour and taste in vegetables such as radish, cabbage, and broccoli **(TAÍZ and ZEIGER,**

2006). Cyanogenic glycosides are a widely distributed group of compounds found in over 100 families, including the Fabaceae, Rosaceae, Linaceae, and Compositae **(HARBORNE, 1993)**.

Cyanogenic glycosides release cyanohydric acid or hydrogen cyanide (HCN), a poisonous gas when enzymatically hydrolysed. In an undamaged plant, the enzyme and the cyanogenic glycoside remain compartmentalised, but when plant tissue is damaged, both come in contact and HCN is released **(GRUHNERT et al., 1994)**. HCN gas is highly poisonous to many organisms due to its capacity to link up with metals which serve as functional groups of many enzymes, thereby inhibiting vital processes such as oxygen reduction in the cytochrome respiratory chain, electron transport in photosynthesis, and the activity of enzymes such as oxidase **(TAÍZ and ZEIGER, 2006)**.

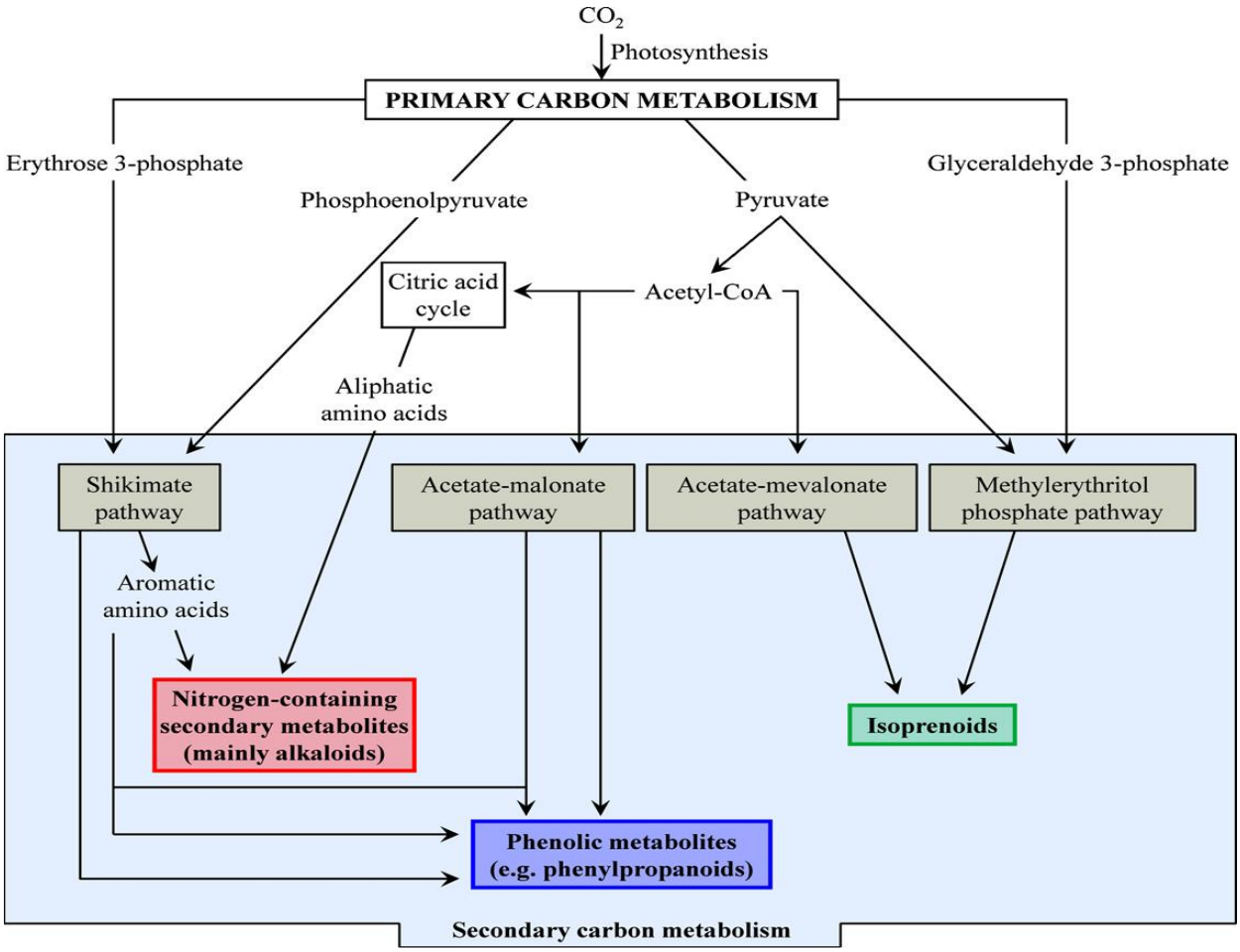

Figure 2.2: Schematic network showing different biosynthetic pathways leading to the formation of secondary metabolite classes adapted from **GROßKINSKY et al. (2012)**.

2.5. Effects of environmental stress on plant secondary metabolite biosynthesis

Plants are often exposed to environmental pressures intensified by progressive global climate change. Environmental stress is defined as an alteration in plant growth condition(s) that adversely interrupts their normal metabolic activities **(SHULAEV et al., 2008)**. Plants, including indigenous medicinal species, are greatly affected by stress caused by both natural (biotic and abiotic components) and anthropogenic factors (e.g. mining, irrigation, nuclear power), thereby barring plants from reaching their full inherent capacities and outputs **(GROVER et al., 2011)**. Although

plants lack locomotory organs, they have evolved numerous mechanisms for perceiving, processing, and translating environmental stimuli into adaptive responses (secondary metabolites) to enable functional flexibility and survival under stressful conditions **(BEN REJEB et al., 2014; BERINI et al., 2018; NCUBE et al., 2012)**.

Abiotic stresses, including high light intensity, temperature (high and low), drought, heavy metal toxicity, salinity, nutrient deficiency, and ultraviolet radiation, have been implicated in losses in yield and quality of crops **(KHAN et al., 2016)**. These stresses also affect secondary metabolites (quality and quantity) and other compounds that medicinal plants synthesise. Plants also exhibit defence responses against biotic stress factors representing a barrage of attacks and damages orchestrated by pathogens and pests **(BILGIN et al., 2010; HARTMANN, 2004)**. Each kind of stress activates a complex defence response system within plants to prevent the destruction of vital molecules. Thus, plants are modified to tolerate stressful conditions **(JONES and DANGL, 2006)**.

Under typical field conditions, plants are rarely exposed to single stressors; instead, they commonly encounter a combination of stressors concurrently; thus, different adaptive responses are anticipated for each stress combination **(HEWEZI et al., 2008; MITTLER, 2006)**. Several predictions indicate that abiotic stress (in the form of heatwaves) in plants is likely to become prevalent in coming decades as a result of increased fluctuations in climatic factors **(MITTLER and BLUMWALD, 2010)** and as a consequence, plants will be more susceptible to pathogens and pest attacks **(ATKINSON and URWIN, 2012; MITTLER and BLUMWALD, 2010)**. Medicinal plants can adapt to the changing environment by adjusting their metabolic pathways. This metabolic pathway elasticity may affect metabolite production, which is the fulcrum of their therapeutic activity **(MISHRA et al., 2016)**. Thus, understanding the effects of combined stress

on secondary metabolite biosynthesis in medicinal plants is essential in meeting the demand for therapeutic plant secondary metabolites.

2.5.1. Abiotic stress

Plants require specific environmental resources and variables such as wind, temperature, relative humidity, light, water, mineral nutrients, and CO_2 in an optimal range for their survival **(MITOVA et al., 2017)**. Any deviation from the optimal external conditions is considered abiotic stress **(DITTMER and KANOST, 2010)**. Abiotic stress is caused by a disparity in the environment's physical and chemical components, which affect the normal functioning of plants **(LATA and PRASAD, 2011; PRADHAN et al., 2017)**. Abiotic stresses such as high light intensity, extreme temperature, floods, drought, salinity, and nutrient deficiency are interrelated, thereby expressing similar deleterious effects on plant productivity and prompting similar molecular responses **(KERCHEV et al., 2020)**. Abiotic stress exerts oxidative stress, i.e. accumulation of reactive oxygen species (ROS) such as superoxide (O_2^{-1}), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and the hydroxyl radical (OH). These molecules pose a threat to plant cells as they cause the peroxidation of lipids, impairment of nucleic acids, enzyme inhibition, and initiation of programmed cell death **(PETROV et al., 2015; SHARMA and DUBEY, 2005)**.

Plant species have evolved several adaptive mechanisms to cope and curtail the debilitating effects of abiotic stress **(MINOCHA et al., 2014)**. These adaptive mechanisms can be categorised into avoidance, tolerance, and acclimation. Stress avoidance relies on strategic adjustments to reduce the negative consequences of stress to tissues (such as waxy cuticle and stomatal closure to prevent excessive water loss) **(DITTMER and KANOST, 2010; VERSLUES et al., 2006)**. The stress tolerance mechanism helps the plant withstand stress such as stress perception, signalling,

production of compatible osmolytes and effector proteins, accumulation of metabolites, and induction of ROS detoxification **(VERSLUES et al., 2006)**. Plants activate their acclimation mechanism, also known as hardening when tolerance mechanisms cannot reduce the negative impacts of stressful conditions. The acclimation mechanism involves physiological and biochemical changes controlled by genes and hormones, which enable the plant to become less sensitive to stress **(VERSLUES et al., 2006)**. Thus, abiotic factors, including drought, are crucial determinants of the composition and concentration of therapeutic secondary metabolites in medicinal plants **(RAMAKRISHNA and RAVISHANKAR, 2011; VERMA and SHUKLA, 2015)**.

2.5.2. Drought stress and responses of plants

Drought stress or water-deficit stress is a condition where poor water accessibility reduces water potential and turgor to a point where usual physiological activities of plants are compromised **(LISAR et al., 2012)**. Drought stress is one of the most widely investigated stress types because of its devastating effects on crop and medicinal plant cultivation, particularly in dry regions of the world **(MCKIERNAN et al., 2014)**.

Plants require sufficient water all through their life cycle. A limited water supply affects a plants' growth and development, including germination, cell division, respiration, synthesis of organic compounds, and several metabolic activities **(TAÍZ and ZEIGER, 2006)**. Thus, water deficit brings about several changes, which vary with taxonomic groupings **(CASER et al., 2018; PRADHAN et al., 2017; ZHOU et al., 2017)**. The quality and quantity of all significant secondary metabolites produced by indigenous plant species are also affected by limited water supply **(KLEINWÄCHTER and SELMAR, 2014)**.

Drought stress is a physiological burden to plants. During water shortage, stomata are closed, and the photosynthetic metabolism of plants, such as photochemical efficiency of photosystem II (PSII) and enzyme Rubisco activities, are downregulated **(BARTA et al., 2010)**. Stomatal closure disrupts the uptake of CO_2, transpiration, and water absorption, thereby eventually impeding photosynthesis. The closure of stomata is principally controlled by the change in turgor pressure of the guard cells in response to signals from dehydrated roots. Plant hormones such as abscisic acid (ABA) is the signal compound **(CHENG et al., 2018; TAÍZ and ZEIGER, 2006)**. Consequently, the amount of H^+ and $NADPH^+$ accessible for the critical processes of the Calvin cycle is decreased, and the concentration of $NADP^+$ and the electron receptor capability for the electron transport chain declines. This eventually initiates ROS accumulation **(ALBERGARIA et al., 2020; SELMAR and KLEINWÄCHTER, 2013)**. The persistence of water-deficit stress negatively affects cell expansion, cell elongation, and the vegetative phase of plants **(JALEEL et al., 2009)**. For example, there was a reduction in shoot length, root length, leaf growth, fresh and dry biomass production and photosynthetic pigments of *Catharanthus roseus* subjected to drought stress **(JALEEL et al., 2008)**.

At the molecular level, plants also respond to water stress by activating signalling transduction pathways and inducing genes with various functions to ensure tolerance to water deficit **(KUMAR et al., 2018)**. Although the precise mechanism employed by plants to recognise osmotic stress changes is largely unknown **(HARFOUCHE et al., 2014)**. Certain transmembrane osmo-sensors such as membrane-bound histidine kinase and aquaporins have been implicated to potentially detect changes in plant cell osmolarity **(NONGPIUR et al., 2020)**. Sensors serve as pioneering molecules in perceiving stress stimuli and relaying the signals to downstream molecules to initiate the signal transduction pathway(s) **(ZHU, 2016)**. The sensed stress signals activate transduction

processes, including mitogen-activated protein kinases (MAPK) and Ca^{2+}-dependent protein kinase (CPK) cascades **(BREDOW and MONAGHAN, 2019; DE ZELICOURT et al., 2016; LUAN et al., 2002; WEINL and KUDLA, 2009**). These induce drought-responsive gene expression which encodes two proteins, namely functional and regulatory molecules **(SHINOZAKI and YAMAGUCHI-SHINOZAKI, 2007)**. Functional molecules such as chaperones, water channel proteins, sugar and proline transporters, late embryogenesis abundant (LEA) proteins, and osmolytes biosynthesis enzymes are involved in drought stress alleviation via osmotic adjustment, the protection of cell membranes and other cellular proteins. Regulatory molecules including transcription factors, protein kinases, protein phosphatases, enzymes involved in ABA synthesis and other signalling molecules (ethylene (ET) and SA) regulate stress responses and organise downstream processes **(DOS REIS et al., 2016; KAUR and ASTHIR, 2017; SHINOZAKI and YAMAGUCHI-SHINOZAKI, 2007; YOSHIDA et al., 2014)**.

Under limited water supply, ROS concentrations increase in plant cells, causing oxidative degradation of RNA and DNA, lipid peroxidation (membrane injuries), and enzyme inactivation. Thus, oxidative stress is induced **(MITTLER, 2002; ZLATEV and LIDON, 2012)**. Medicinal plants, like other plants, combat oxidative stress by synthesising various secondary compounds and step up the production of endogenous enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and glutathione reductase (GR) and non-enzymatic antioxidants like ascorbate (AsA), glutathione (GSH), to reduce the effect of ROS **(KUMAR and SHARMA, 2018)**. Plants also synthesise alcohols, sugars, proline, and glycine betaine under drought stress to maintain cell turgor and protect proteins from osmotic damage **(SEKI et al., 2007)**.

2.5.3. Biotic stress: Pathogen recognition and signalling

Fossil records have established that biotic components have interacted with plants for over 450 million years **(HASSANI et al., 2018)**. Biological agents that influence plant productivity and survival include viruses, bacteria, fungi, weeds, parasitic plants, nematodes, and insects **(ASHRAF et al., 2018; SERGEANT and RENAUT, 2010)**. Interactions of plants with other biological entities can result in biotic stress. Biotic stress, especially those caused by pathogens, lead to changes in the plant's metabolic activities and manifest in signs and symptoms such as chlorosis, necroses and eventually death **(SADDIQUE et al., 2018; SERGEANT and RENAUT, 2010)**. Plants are devoid of a well-defined immune system; however, they respond to biotic stress by activating the innate immune system of each cell on perceiving stress signals **(SCHULZE-LEFERT and PANSTRUGA, 2011)**. Generally, in plants, two distinct responses can occur - the early Local Acquired Resistance (LAR), which is restricted to the infection site and the later systemic resistance, i.e. Systemic Acquired Resistance (SAR) or Induced Systemic Resistance (ISL) which appear in distant uninfected tissues **(DAVID et al., 2019)**.

As a form of protection and resistance, plants have evolved several sophisticated machineries such as the secretion of a diverse group of phytoalexin and phytoanticipins (secondary metabolites) to counter the infestations of pathogens **(JONES and DANGL, 2006; MASSALHA et al., 2017; VAN DAM and BOUWMEESTER, 2016)**. In conjunction with the production and exudation of antimicrobial secondary metabolites, the first layer of a plants active defence against pathogens is the LAR which involves complex defence strategies to perceive attack signals of pathogens and decipher these signals into a suitable and effective defence response **(MATILLA, 2018; ZHAO et al., 2005)**. Thus, pathogen infection in plants activates both the basal or primary innate immune responses, which helps mainly to curb the spread of pathogens as well as activating the second

plant innate immune response tailored to individual types of a pathogen **(GAO et al., 2014; JONES and DANGL, 2006; TENA et al., 2011)**. Conserved pathogen recognition provokes the basal response- or microbial-associated molecular patterns (PAMPs or MAMPs) such as lipopolysaccharides, flagellins, and peptidoglycans by pattern recognition receptors (PRRs) found on the plasma membrane of plant cells. This response is MAMP- or PAMP-triggered Immunity (PTI) **(JONES and DANGL, 2006; RANF, 2017)**. PTI responses are controlled by a web of interrelated signal transduction pathways, and plant growth regulators, including jasmonic acid (JA), SA, and ET, participate actively **(VIDHYASEKARAN, 2015)**.

The second specific response is activated to recognise specific pathogen effector proteins by the host encoded resistance (R) proteins, which grants an additional resistance layer. This is termed R gene- or effector-triggered immunity (ETI) **(GAO et al., 2014; JONES and DANGL, 2006)**. R-genes of plants are polymorphic, containing nucleotide-binding (NB) domains and leucine-rich repeats (LRR). R-genes products are useful in recognising pathogen effectors and the induction of various hormonal controlled signalling pathways. This confers LAR (hypersensitive response or programmed cell death) and SAR or ISL in distal plant tissues **(MATILLA, 2018; SCHULZE et al., 2019)**. SAR is an induced mechanism of defence that confers long-time protection against a wide range of pathogens and prepares the plant to react promptly to subsequent pathogen attacks **(SCHULZE et al., 2019)**. SAR is usually activated by the production of mobile signal molecules, particularly SA. Salicylic acid is associated with the activation of pathogenesis-related (PR) genes and accumulation of PR-proteins (such as chitinases and β-1,3-glucanases) which mediate protection to plant cells **(DERKSEN et al., 2013; HAMMERSCHMIDT, 2009)**. Another form of systemic resistance is the induced systemic resistance (ISR), which, unlike SAR, is triggered by non-pathogenic microbes such as rhizobacteria and some endophytes

(CHOUDHARY et al., 2007; MISHRA et al., 2018; PIETERSE et al., 2014). ISR relies on jasmonate or ET mediated pathways, and PR-proteins or genes are not involved in its activities **(ANJUM et al., 2019)**.

2.5.4. The plant-endophyte relationship

The plant-endophytes interaction has a significant impact on plant growth and secondary metabolism. Endophytes are associated with many plant species and have been extensively studied in the field and laboratories, making them an excellent model to investigate biotic stress in plants. The pioneering report on endophytes was published in the early 20th century **(FREEMAN, 1904)**, describing a non-spore producing fungus on *Lolium temulentum* seeds suggestive of a mutualistic relationship between the host and the fungus **(THOMAS et al., 2016; WILSON, 1995)**. Endophytes are regarded as microbes (often fungi and bacteria) that can colonise inner tissues of healthy plants without the manifestation of any disease symptoms **(WANI et al., 2015)**. However, they may become pathogenic when the host plant undergoes senescence **(RODRIGUEZ and REDMAN, 2008)**. The two partners are not perceptibly harmed during plant-endophyte interactions, and their gains depend on their taxonomy **(KUSARI et al., 2012)**. The scope of this multifaceted interaction extends from extremely total mutualism to parasitism, saprophytism or exploitation, with the capacity to advance to a more complicated interaction **(ZUCCARO et al., 2011)**.

Endophytic microbes are microorganisms present in nonvascular and vascular plants **(SUN and GUO, 2012)**. They adopt a similar approach to plant pathogens to enter the host plant tissues **(SIEBER, 2007)**. Although endophytes infect plant organs (aerial and underground) just like pathogens, they differ from pathogens in that their infection is harmless and asymptomatic

672 **(KUMAR et al., 2017; ZIMMERMAN and VITOUSEK, 2012)**. The colonisation of plants by
673 endophytic microbes depends on several factors such as endophyte taxon and strain type, plant
674 tissue type, plant genetic composition and prevailing environmental conditions **(HARDOIM et**
675 **al., 2015)**. Endophytes ingress their host generally through the phyllosphere, rhizosphere, seeds,
676 root hairs, flowers, fruits, stem and leaf surfaces, lenticels, stomata, hydathodes, scratches with
677 soil particles, abiotic stress damage and wounds caused by insects **(COMPANT et al., 2010;**
678 **LATA et al., 2018; LIDOR et al., 2018; PHILIPPOT et al., 2013)**.

679 Like other plant-microbe interactions, endophytism is also preceded by a physical engagement
680 between the host plant and the potential endophytes, followed by several physical and chemical
681 hurdles that must be crossed to conclusively establish the association **(KUMAR et al., 2017)**.
682 There are contrasting theories to explain the plant-endophyte relationship, including the balanced
683 antagonism theory, mosaic effect theory and acquired immune system theory **(ARNOLD et al.,**
684 **2003; KUMAR et al., 2017; SCHULZ and BOYLE, 2005)**. The understanding of the molecular
685 mechanism involved in establishing a plant-endophyte interaction and response is limited
686 **(STRAUB et al., 2013; WANI et al., 2015)**. These mechanisms include the induction of plant
687 systemic resistance (SAR and ISR) **(LASTOCHKINA et al., 2019; PEREZ et al., 2017)**, the
688 accumulation of ROS **(BACON and WHITE, 2016; WHITE and TORRES, 2010)**, and the
689 production of various secondary metabolites including plant growth regulators **(PASTERNAK et**
690 **al., 2005; TORRES et al., 2012)** by endophytes within plants. These mechanisms have been
691 suggested as possible means towards establishing endophytism **(WANI et al., 2015)**. Due to the
692 absence of virulent genes in endophytic microbes, they are recognised as “minor pathogens” in the
693 earlier phase of the interaction **(SCHULZ and BOYLE, 2005; WANI et al., 2015)**, and this limits

the activation of full defence responses by the host plants. However, the host becomes primed for a resistance response against subsequent attack **(CONN et al., 2008)**.

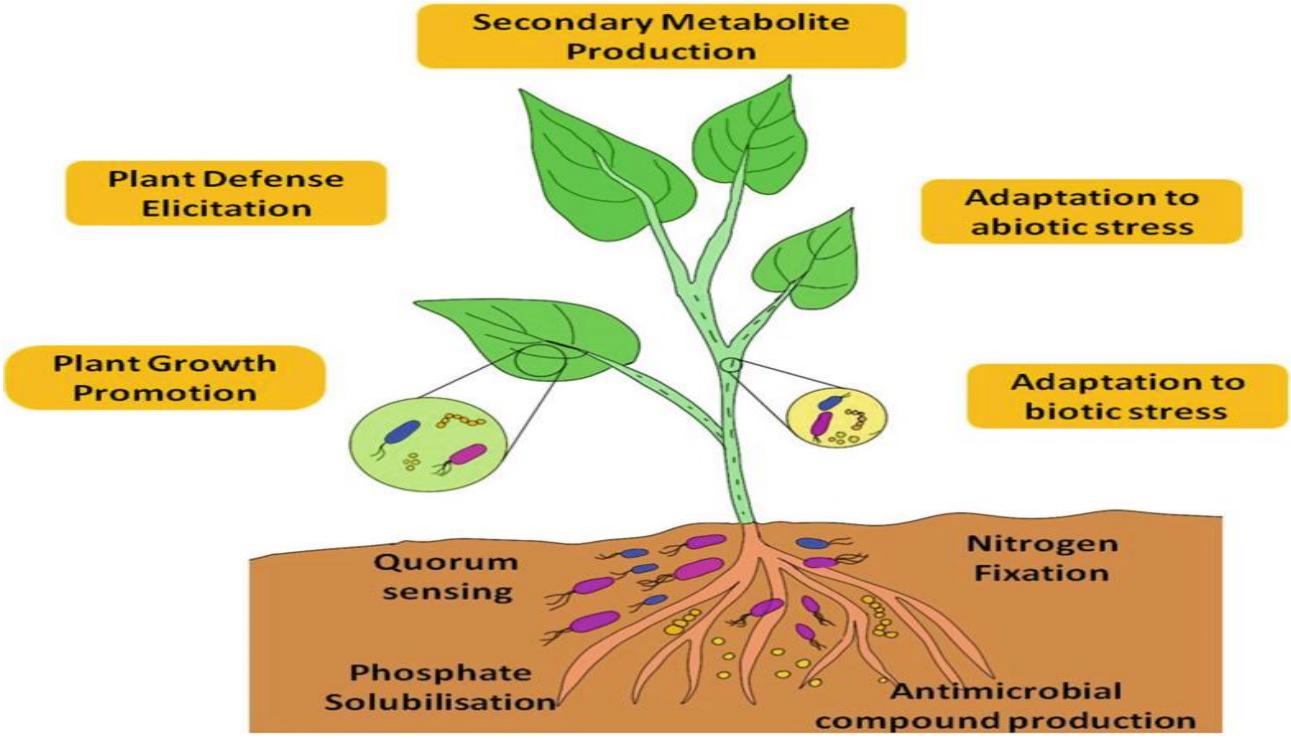

Figure 2.3: Plant stress management and growth-promoting properties of endophytes **(SINGH et al., 2020)**.

Due to the endophytes' niche in the host microenvironment, they obtain nourishment, shelter from the host plants and are protected from the excessive competition of other microbes **(PATLE et al., 2018; SAIKKONEN et al., 1998)**. As shown in **Figure 2.3**, endophytes are biological factors that assist plants in maintaining their healthy metabolism and survival under stress **(COSME et al., 2016; KHARE et al., 2018; LATA et al., 2018)**. They can also synthesise siderophores and plant growth regulators such as ET, gibberellins, ABA, cytokinins, and auxins, which are essential for

regulating seed germination, plant growth, and development **(FIRÁKOVÁ et al., 2007; VIJAYABHARATHI et al., 2016)**. Under normal conditions, endophytes also help promote host plant growth, increase the absorption of nutrients, reduce the debilitating effects of diseases, and improve host resistance against environmental stresses via secondary metabolite accumulation **(KHARE et al., 2018; SAIKKONEN et al., 2010)**.

2.5.5. Stress in combination/Multiple stressors

The consequences of individual stress factors on plants and the molecular process controlling the responses of plants have been extensively investigated **(ABUQAMAR et al., 2009; TORRES-RUIZ et al., 2015)**. Plants have developed an accurate method of perceiving and responding to specific environmental stresses enabling them to adapt appropriately. Although less investigated, plants are commonly subjected to multiple concomitant stresses within or across the biotic and abiotic stress spectrum under natural conditions **(HUBER and BAUERLE, 2016; NCUBE et al., 2012; RAMEGOWDA and SENTHIL-KUMAR, 2015)**. Due to fluctuations in seasonal climatic factors, diverse types of stress combinations are expected to elicit erratic and complex plant responses **(HOLOPAINEN and GERSHENZON, 2010)**. These responses may be unique or represent cohesive signalling cascades. These responses require more experimental considerations to understand the plant's responses to multiple stressors **(HUBER and BAUERLE, 2016)**. Transcriptome analysis suggests that plants show specific responses to various concurrent stresses, which cannot be directly deduced from the results obtained when either stress factor was applied individually **(ATKINSON et al., 2013; RAMEGOWDA and SENTHIL-KUMAR, 2015; SUZUKI et al., 2014)**.

The sensitivity of plants to multiple stressors varies. It depends on the prevailing environmental conditions, the severity of the stress factors, the genetic makeup of the plants, and its developmental stage **(BOSTOCK et al., 2014; SUZUKI et al., 2014)**. The defence responses of plants to multiple stressors also relies on the stress combinations and the degree of concomitancy **(RAMEGOWDA and SENTHIL-KUMAR, 2015; SUZUKI et al., 2014; TANI et al., 2018)**. The effects of multiple stressors on the plant under field conditions are usually interactive, showing the versatility of combined simultaneous stress **(NCUBE et al., 2012)**. The simultaneous multiple stress effects on plants could be additive, idiosyncratic (completely different from the single stress responses), dominant (response very close to one of the stressors), synergistic, or antagonistic **(BEN REJEB et al., 2014; PRASCH and SONNEWALD, 2015)**. In addition to this, multiple stressors influence the vulnerability or tolerance of plants to other kinds of stressors **(PANDEY et al., 2015; RAMEGOWDA and SENTHIL-KUMAR, 2015)**. Thus, multiple and concurrent stressors may impact plants negatively or positively **(HUSSAIN et al., 2018a; MITTLER, 2006; PANDEY et al., 2017; SUZUKI et al., 2014)**.

The effects of every single specific combination of stressors should be appreciated and explored **(MITTLER and BLUMWALD, 2010)**, especially on the accumulation of highly coveted secondary metabolites produced by medicinal plants. The impacts of different abiotic and biotic stress combinations on plant secondary metabolite accumulation in medicinal plants can enhance our understanding of how these metabolites confer resistance to the plants under stressful conditions and, at the same time, provide us insights on the optimum yields and quality which are required by industries.

2.5.6. Abiotic and biotic stress interaction signalling pathways

Plants often sustain an energy cost balance between growth and defence responses to environmental stress to ensure survival and continuity. The plant's growth and defence responses to environmental stress are costly processes whose demands can rarely be met simultaneously. Growth-defence trade-offs occur depending on extrinsic and intrinsic factors, with plants prioritising their limited energy towards defence or growth **(HUOT et al., 2014)**. The activation of defence mechanisms, including the production of defence compounds such as secondary metabolites, becomes imperative in many stress factors. This usually compromises plant growth **(HUOT et al., 2014; KEMPEL et al., 2011; MELDAU et al., 2012)**.

Plants respond to multiple stresses concurrently. This entails crosstalk between different stress-response pathways and gives room for stress response prioritisation (trade-offs) and increased plant fitness **(BERENS et al., 2019; VERMA et al., 2013)**. Stress response trade-offs are a means of balancing the high cost of activating and sustaining various kinds of stress responses given the limited resources of plants **(BERENS et al., 2017; SPOEL and DONG, 2008; WOLINSKA and BERENS, 2019)**.

The response pathways of plants to stress factors under normal conditions where resources are often limited are specific. Plants prioritise the most efficient defence responses against more life-threatening stress, and less significant defence responses are not readily activated to conserve resources **(ANDERSON et al., 2004)**. Usually, when plants are exposed to simultaneous abiotic and biotic stress, abiotic stress responses often exhibit overriding effects and further predispose the plant to biotic pressure **(KIRÁLY et al., 2008; LUO et al., 2005)**, notably less virulent facultative microorganisms **(DESPREZ-LOUSTAU et al., 2006)**. Sporadic abiotic stress occurring just before infection predisposes plants to diseases **(BOSTOCK et al., 2014;**

KISSOUDIS et al., 2014; THALER and BOSTOCK, 2004), suggesting a hormonal imbalance, reduced defence genes expression, disruption in primary metabolism **(PRASCH and SONNEWALD, 2015)** and trade-off in responses **(BOYER, 1995)**. The cross-tolerance in plants between abiotic and biotic stresses has been described. For example, water-deficit stressed plants accumulated ABA, which simultaneously leads to an improved resistance of the plants to microbes **(ACHUO et al., 2006; ASSELBERGH et al., 2008)**. However, there are conflicting reports on the prioritised defence responses in plants under simultaneous abiotic and biotic stress. The prioritised responses of plants to multiple stress varies in a natural setting. It ultimately relies on the intensity and exposure length of each stress and the species of the biotic component **(GUPTA et al., 2016; TAKATSUJI, 2017)**. This may influence the subsequent symbiotic interactions of plants in their microbiome **(BARRETT et al., 2009; VOS et al., 2013)**.

The distinct responses (stress signalling pathways) of plants, as shown in **Figure 2.4** to different stress conditions (abiotic and biotic), share common elements **(PANDEY et al., 2015)**. This crosstalk is mediated by plant growth regulators, MAPK cascades, calcium ions, transcription factors, and ROS **(GASSMANN et al., 2016; GROßKINSKY et al., 2016; KU et al., 2018)**. Ca^{2+} and ROS serve as secondary messengers in early stress responses to abiotic and biotic stress **(Fu et al., 2011)**. During biotic and abiotic stress, the concentration of cytoplasmic Ca^{2+} increases rapidly due to the influx of calcium from the external pool **(CAO et al., 2017; VERMA et al., 2013)**. The increase in cytoplasmic Ca^{2+} concentration is perceived by calcium-binding proteins (calcium sensors), and this activates other calcium-interacting proteins such as calcium-dependent protein kinases (CDPKs) and calcium/calmodulin-dependent protein kinases (CCaMKs) **(ARIMURA and MAFFEI, 2010; VERMA et al., 2016)**. This triggers the activation of several concurrent pathways, which eventually mediate certain transcription factors that control

797 expression levels of stress-responsive genes **(VERMA et al., 2013; VIRDI et al., 2015)**. Calcium
798 ion is a key factor in signalling cascades (MAPKs, CDPKs and ROS production) elicited by both
799 biotic and abiotic stress **(ATIF et al., 2019; EVANS et al., 2016)**, and this omnipresent feature of
800 Ca^{2+} in stress signalling validates its role in the crosstalk between pathways **(CHINNUSAMY et**
801 **al., 2004; FRAIRE-VELAZQUEZ et al., 2011)**.

802 Similarly, ROS also accumulates rapidly in plants (oxidative burst) in response to stress, and it is
803 a toxic by-product in aerobic respiration **(NOSTAR et al., 2013)**. ROS are generated by the plasma
804 membrane-bound NADPH-oxidase, and they serve as a response signal to abiotic and biotic stress
805 **(SUZUKI et al., 2012)**. Zinc-finger transcription factor (*Zat12*) was implicated as a regulator in
806 ROS scavenging mechanism mediating both abiotic and biotic stress responses **(DAVLETOVA**
807 **et al., 2005)**. ROS can also induce antioxidant enzymes, transcription factor dehydrins, HSPs, and
808 PR-proteins **(GECHEV and HILLE, 2005)**.

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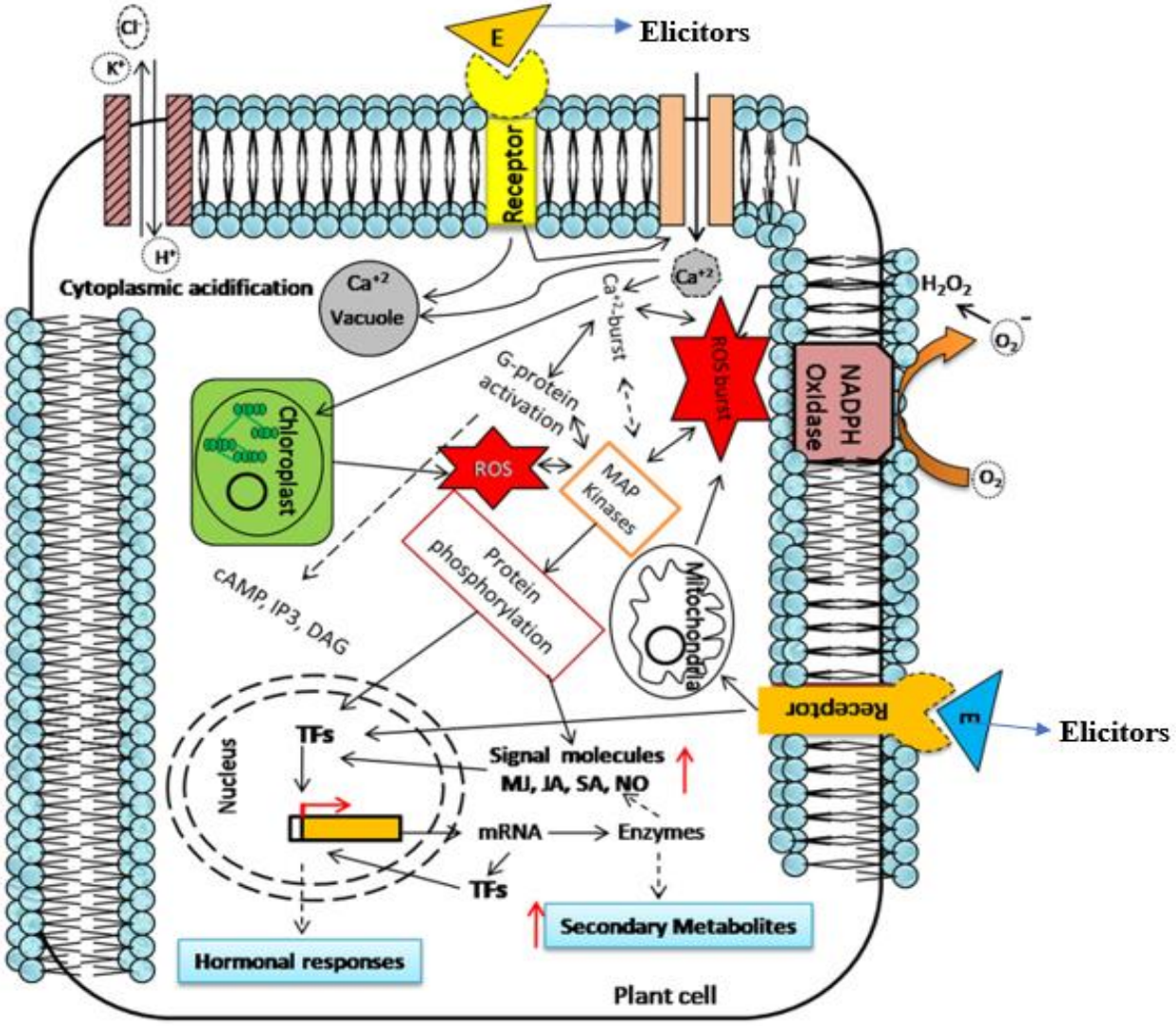

Figure 2.4: Schematic representation of plant cell responses to elicitors **(HALDER et al., 2019)**.

MAPK cascades are well conserved among eukaryotic organisms **(XU and ZHANG, 2015)**. They are significant stress response signalling pathways and translate diverse environmental signals and developmental cues into adaptive intracellular responses **(JAGODZIK et al., 2018)**. MAPK cascades are triggered by ROS such as H_2O_2. It enables plant cells to respond to a wide range of stresses, including water-deficit, salinity, heavy metal, diseases, wounding, and oxidative stress **(DE ZELICOURT et al., 2016)**. MAPK cascades are also involved in mediating antioxidant

defence mechanisms and hormonal responses of plants to different types of stress **(SINHA et al., 2011)**. MAPK cascades regularly mediate a series of downstream processes and contribute immensely to cross-tolerance between abiotic and biotic stress responses **(FRAIRE-VELAZQUEZ et al., 2011; JALMI and SINHA, 2015)**.

Plant growth regulators act as mediators in plant stress crosstalk and trade-offs, enhancing a plant's responses to simultaneous multiple stresses **(BERENS et al., 2017; NGUYEN et al., 2016)**. However, understanding the synergistic or antagonistic interactions and their synchronised overall signalling networks are rudimentary and require further investigation **(BERENS et al., 2017; SAIJO and LOO, 2020)**. ABA, SA, JA and ET play significant roles in mediating plant defence responses against pathogens and abiotic stresses **(BERENS et al., 2017)**. Other hormones, including brassinosteroids, auxins, cytokinins, gibberellins, and strigolactones, also participate in the complex crosstalk processes **(VERMA et al., 2016)**. ABA principally controls responses to abiotic stress **(CUMING and STEVENSON, 2015; MCADAM et al., 2016)**, whereas an antagonistic interplay mediates defence against biotic stress between the JA/ET and SA signalling pathways **(BARI and JONES, 2009; LIU et al., 2016a; VERMA et al., 2016)**.

ABA negatively regulates disease resistance by antagonising SA, JA and ET **(ANDERSON et al., 2004; BERENS et al., 2019; VERMA et al., 2016)** and disrupts the accumulation of defence compounds such as lignins and phenylpropanoids **(YASUDA et al., 2008)**. ABA's suppression of biotic defence responses is widely reported in land plants, and the mechanisms involved are species-specific **(XU et al., 2013; YASUDA et al., 2008)**. The ABA biosynthesis capability and/or host ABA production machinery is hijacked by some pathogens **(BERENS et al., 2017)**. However, ABA also positively influences disease resistance via the stimulation of callose deposition **(LUNA et al., 2011)** and defence gene expression **(ALAZEM et al., 2017)**.

Plant responses to abiotic and biotic stress involve several changes controlled by key genes encoding special regulatory proteins called transcription factors which may activate or repress stress-induced genes and pathways **(BAILLO et al., 2019; BEN REJEB et al., 2014)**. Transcription factors assist in the modulation of indispensable processes in plant metabolism such as cell differentiation, tissue/organ development, seed dormancy and germination, hormone coordination, and defence responses to environmental stress **(GONZALEZ, 2015)**. Transcription factors include myeloblastosis (MYB) **(LIU et al., 2015)**, WRKY genes **(JIANG et al., 2017)**, ABA activator (basic helix-loop-helix (bHLH)) **(CASTILHOS et al., 2014)**, ET-responsive factors (ERFs) **(MÜLLER and MUNNÉ-BOSCH, 2015)**, and dehydration-responsive transcription factor NAC **(FUJITA et al., 2004; NURUZZAMAN et al., 2013)**. They all mediate different hormone signalling pathways and the crucial crosstalk signalling in simultaneous abiotic and biotic stress responses **(VERMA et al., 2016)**.

2.5.7. Endophytes interactions with drought-stressed medicinal plants

Abiotic stress, including drought, influences plant growth, development, and the biosynthesis of bioactive constituents in medicinal plants. For example, water-stressed medicinal plants including *Salvia. officinalis* **(NOWAK et al., 2010)**, *S. fruticosa* **(CHRYSARGYRIS et al., 2016)**, *Lippia alba* **(DE CASTRO et al., 2020)**, *Melissa officinalis* **(OZTURK et al., 2004)** and *Petroselinum crispum* **(PETROPOULOS et al., 2008)** showed a significant rise in the concentration of secondary metabolites (monoterpenes) while the vegetative biomarkers such as biomass and plant height were reduced. Medicinal plants interact uniquely with their microbiome, including endophytes, due to their ability to synthesise structurally diverse compounds responsible for the complex and highly specific interaction **(QI et al., 2012)**.

The well-coordinated responses or adjustments of medicinal plants to environmental stresses such as drought might be a consequence of their modulated symbiotic relationship with endophytes **(DUPONT et al., 2015; WANI et al., 2015)**. Although the mechanisms by which endophytes modify the impacts of drought stress on their host is poorly understood, they protect and enhance the survival of their hosts **(JALEEL et al., 2009)**. Endophytes of medicinal plants influence the synthesis and accumulation of secondary metabolites in the tissues of their hosts, and they influence the functioning of antioxidant enzymes, which in turn activate the defence signal cascade. The activated defence signals further stimulate the up-regulation of gene expression of essential enzymes during the synthesis of secondary metabolites **(DE ZÉLICOURT et al., 2018; LATA et al., 2018)**. Endophytes also produce additional phytohormones, antioxidants, osmoprotectants, promoter elements and transcription factors for their hosts under stress **(NAIK, 2019; TORRES et al., 2012; VAISHNAV et al., 2019; WANI et al., 2015)**.

Generally, as annotated in **Figure 2.5**, plants infected with endophytes are often healthier, less threatened by the harmful effects of abiotic stress, such as drought **(SAIKKONEN et al., 2010)**. Plants infected by the endophytic fungi *Epichloë* spp. showed diverse responses to drought compared to endophyte-free plants **(FAETH et al., 2010; HAMILTON et al., 2009)**. Two endophytic bacteria *Pseudomonas aeruginosa* and *Pseudomonas pseudoalcaligenes* improved the yield, growth, and essential oil content of *Hyptis suaveolens* under stress **(JHA, 2019)**. Similarly, the endophytic bacteria *Bacillus pumilus* alleviated drought-stressed *Glycyrrhiza uralensis* **(ZHANG et al., 2019)**.

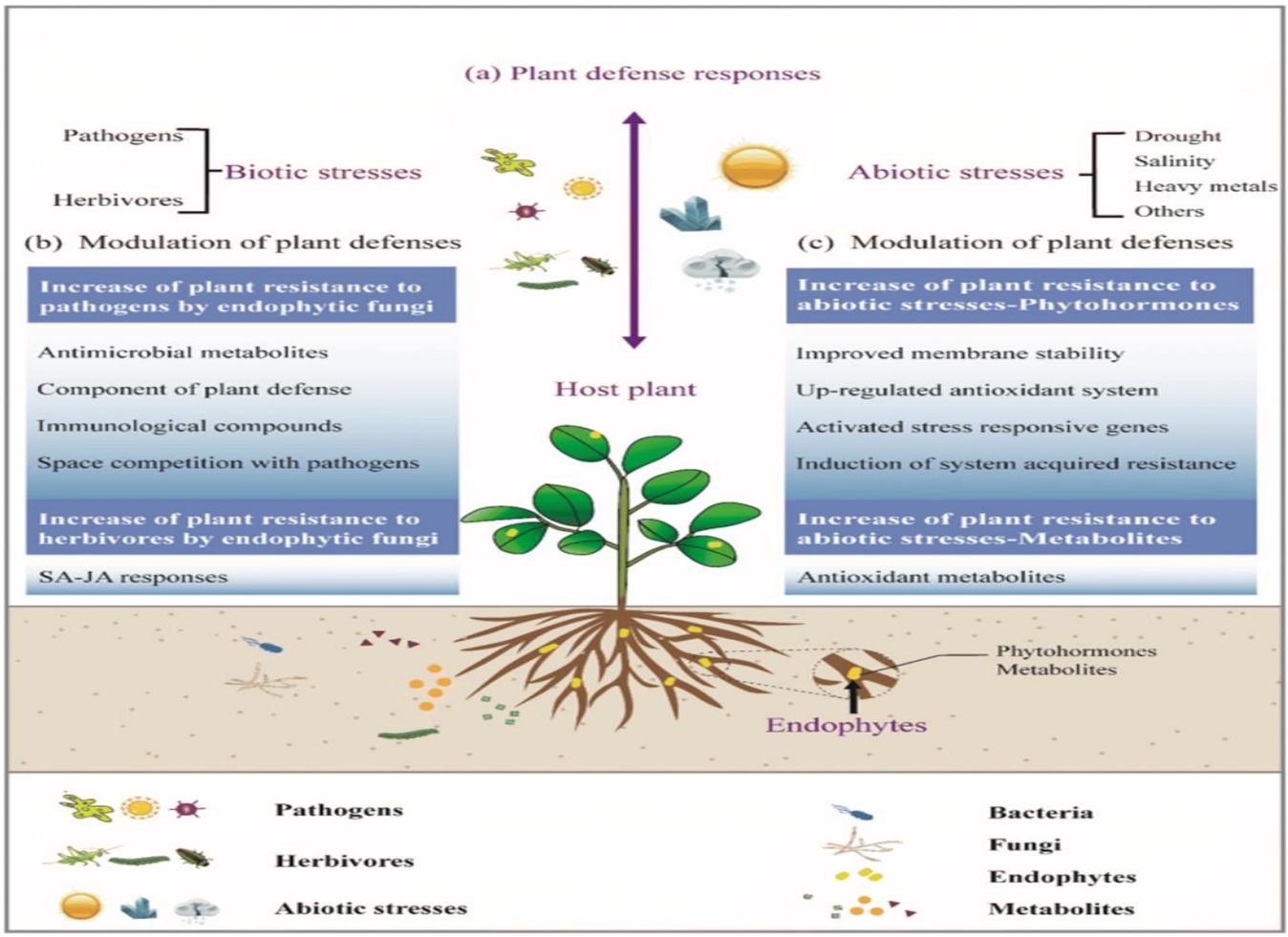

Figure 2.5: Endophytes' colonisation of plants triggers defence responses in both abiotic and biotic stresses **(YAN et al., 2019)**.

2.6. Secondary metabolites production techniques

Societal interests in plant secondary metabolites have risen significantly because of their massive impact as direct therapeutic compounds or precursors to produce new drugs and products **(HUSSAIN et al., 2012)**. Chemical synthesis of plant secondary metabolites for industrial use is not realistic due to the complex metabolic pathways, complicated structures, and chirality exhibited by these compounds **(PYNE et al., 2019)**. Poor efficacy from chemically synthesised

compounds has also been reported **(SHILPA et al., 2010)**. The commercial demand for these bioactive compounds can only be met by obtaining them directly from the wild or cultivation fields. However, the quantity of the active principles produced by medicinal plants in their natural habitat is usually low, and it depends on the plant's physiological state, age, and environmental factors. Moreover, efficient extraction of the desired compounds may require complete harvesting of the plant part(s) or the whole plant, subsequently leading to indiscriminate harvesting of medicinal plants and rapid extermination of several valuable species of medicinal plants **(TILMAN et al., 2017)**. Additionally, some valuable plant secondary metabolites are confined within certain species or genera **(PICHERSKY and LEWINSOHN, 2011; WINK, 2016)**; hence, such compounds are inevitably scarce and highly sought after.

Owing to the high global demand for valuable plant secondary metabolites and the challenges of over-exploitation of medicinal plants from the wild, different approaches including, *in vitro* micropropagation **(TIWARI and RANA, 2015)** and *in vivo* cultivation of plants **(PEREIRA et al., 2019)**, have been investigated as dependable substitutes for the production of economically valuable plant secondary metabolites. Poor yields of the cultures have hampered the large-scale production required by most pharmaceutical industries **(HALDER et al., 2018)**. Biotechnological approaches such as the fortification of culture media composition and physical constants, extensive cultivation in bioreactors, elicitation, precursor feeding, metabolic engineering, selection of high yielding lines, hairy root culture, plant cell immobilisation, and biotransformation are being investigated for their effectiveness in overcoming the poor yield in cultures **(HALDER et al., 2018; ZHAO et al., 2005)**.

2.5.8. Elicitation

Elicitation is the stimulation of stress response or enhancement of the biosynthesis of plant secondary metabolites due to the addition of minute quantities of elicitors **(BAENAS et al., 2014; NARAYANI and SRIVASTAVA, 2017; RADMAN et al., 2003)**. Elicitors are biotic or abiotic substances of diverse origin that stimulate the biosynthesis and accumulation of specific plant secondary metabolite(s) in both *in vitro* cultures **(RAMAKRISHNA and RAVISHANKAR, 2011)** and *in vivo* cultures **(KUZEL et al., 2009**). Elicitation is regarded as the most efficacious biotechnological strategy for the extensive production of plant secondary metabolites both *in vitro* and *in vivo* due to the responses of plant tissues to elicitors **(POULEV et al., 2003; RAMIREZ-ESTRADA et al., 2016)**. Most of the biotic elicitors employed to scale up the production of secondary metabolites of plants are either of exogenous or endogenous microbial origins, while abiotic elicitors are of non-living sources, either chemical (inorganic salts, heavy metal salts, osmotic stress) or physical (heat stress, ultra-violet radiation) in nature **(VASCONSUELO and BOLAND, 2007)**.

Some *in vitro* and *in vivo* medicinal plant elicitation studies using drought and endophytic elicitors have been reported. For example, polyethylene glycol (PEG) induced *in vitro* drought stress that led to a significant increase in the secondary metabolites content of the callus culture *Agave salmiana* **(PUENTE-GARZA et al., 2017)** and *Taxus baccata* **(SARMADI et al., 2019)**. The secondary metabolite content of *S. dolomitica* (especially sesquiterpenes) was enhanced during water shortage stress in a pot experiment **(CASER et al., 2019)**. Polysaccharide elicitors isolated from four endophytic fungi of *Dendrobium catenatum* stimulated the accumulation of flavonoids and phenolics in *D. catenatum* plantlets **(ZHU et al., 2018)**. The meristem culture of *Atractylodes lancea* accumulated a higher quantity of volatile oil under the influence of an exopolysaccharide

elicitor isolated from the endophytic fungus *Gilmaniella* sp. **(CHEN et al., 2016)**. Scientific reports on the roles played by medicinal plant endophytic species when the host is under abiotic stress conditions such as drought are scarce. Likewise, information on the combined effects of endophytic and abiotic elicitors on the accumulation of plant secondary metabolites in medicinal plants is also limited.

2.7. Metabolomics

A metabolome is a total collection of all low molecular mass compounds (primary and secondary metabolites) produced under a given condition and time by a particular cell(s) or organism **(TUGIZIMANA et al., 2013)**. The word metabolomics was coined in 2002 by Oliver Fiehn. It is a complete qualitative and quantitative profiling of all chemical compounds present in a given organism at a particular time under a given physiological state **(FARAG et al., 2017)**. Metabolomics is a member of "omics" technologies (others include transcriptomics and proteomics), and it serves as a complementary tool in system biology, biotechnology, and functional genomics **(SAITO and MATSUDA, 2010)**. Transcriptomics and proteomics profiling capacity in the prediction of gene function is limited. The biochemical phenotypes of organisms do not always relate to changes in the proteome or transcriptome, and at the same time, the enzymatical functionality of translated proteins is not definite **(SUMNER et al., 2003)**. The nonexistence of a universal database has also narrowed down or revealed insufficient information since mRNA and proteins during transcriptome or proteome profiling are identified through sequence similarity or a database matching **(JEAN-FRANGOIS et al., 2013)**.

On the other hand, metabolomics provides the most efficient data set because a given metabolite possesses a unique structure identified and is well defined **(JEAN-FRANGOIS et al., 2013)**.

Integrating all these approaches is ideal for understanding better gene functions **(JEAN-FRANGOIS et al., 2013)**. Generally, there are two methods to metabolomics; targeted metabolic profiling, designed to identify and quantify certain metabolites for a given purpose, and non-targeted metabolomics which aims at holistic information on all metabolites **(BERKOV et al., 2011)**. Metabolomics is driven mainly by data generated from highly selective and sensitive analytical and bioinformatics tools **(SACCENTI et al., 2014; SUMNER et al., 2015)**. There is no certified method to detect the metabolome, and suitable extraction and detection techniques affect the outcome of the final analysis **(KIM and VERPOORTE, 2010)**. The physiochemical properties of plant metabolites vary, and their variation is mainly evident in solubility, volatility, size, polarity, quantity and stability **(DUNN and ELLIS, 2005)**. Thus, a wide range of metabolomics strategies, including metabolite profiling, metabolite target analysis, and metabolite fingerprinting are employed to analyse plant metabolites **(TUGIZIMANA et al., 2013)**. As shown in **Figure 2.6**, a typical metabolomic investigation involves three major experimental stages: preparation of sample, data collection using analytical tools, and data processing and interpretation using chemometric methods **(TUGIZIMANA et al., 2013)**.

Sample preparation includes harvesting of plants at the right time, followed by quenching (such as freeze-drying) to avoid enzymatic degradation of metabolites **(KIM and VERPOORTE, 2010)**, an appropriate extraction method to ensure a complete metabolites extraction **(VILLAS-BÔAS et al., 2005)**, and sample preparation for analysis **(VILLAS-BÔAS et al., 2005)**. Analytical techniques such as gas chromatography-mass spectrometry (GC-MS) **(HILL and ROESSNER, 2013)**, liquid chromatography-mass spectrometry (LC-MS) **(ZHOU and YIN, 2016)**, nuclear magnetic resonance (NMR) **(KIM et al., 2011)**, and Fourier Transform Infrared spectroscopy (FT-IR) **(KHAIRUDIN et al., 2014)** are employed to analyse the impact of various factors such as

season and environmental stress on hundreds of metabolites simultaneously **(KRÁL' OVÁ et al., 2012)**. MS platforms (including combined chromatography-MS such as GC-MS and LC-MS) is the most extensively used technology in metabolomics. It provides a mix of quick, sensitive, and selective qualitative and quantitative analyses with the ability to identify metabolites **(ARBONA et al., 2013)**. GC-MS is suitable for targeted analysis of derivatised primary metabolites **(GHATAK et al., 2018)** and non-targeted metabolite profiling of volatile and thermally stable non-polar or derivatised polar metabolites **(MORGENTHAL et al., 2007)**. LC-MS is flexible and may be adapted to many compounds **(ARBONA et al., 2009)**. It is commonly employed in profiling plant secondary metabolites with no prior derivatisation **(WANG et al., 2017)**. Other analytical techniques, such as NMR and FT-IR, do not require any separation technique and are used only for fingerprinting purposes **(ARBONA et al., 2013)**.

Metabolomic analysis generates extensive data, which is challenging to analyse. Automated software is used to identify peaks from raw data, align the peaks of different samples, and replicate to identify and quantify each metabolite **(DOERFLER et al., 2013)**. Bioinformatics and statistical tools are used to process, mine and analyse data **(SUN and WECKWERTH, 2012)**. The datasets generated by the metabolomic investigation are usually high-dimensional and complex, and as such, they can neither be analysed nor interpreted using univariate statistical tools **(GHATAK et al., 2018)**. Multivariate data analysis (MVDA) and statistical tools such as principal component analysis (PCA), ANOVA, and partial least square (PLS) are used to obtain useful information **(GHATAK et al., 2018)**. Web-based applications such as MetaboAnalyst **(XIA et al., 2015)**, MetaGeneAlyse **(DAUB et al., 2003)**, MetaMapp **(BARUPAL et al., 2012)**, MetiTree **(ROJAS-CHERTÓ et al., 2012)**, and metaP-Server **(KASTENMÜLLER et al., 2011)** are used in metabolomics analysis from data pre-processing to biological interpretation **(GHATAK et al.,**

2018). Compound identification is the final and a critical step in metabolite analysis. It involves the biochemical interpretation of metabolomic data, and it depends on the availability of a well-structured database to identify the metabolites **(OKAZAKI and SAITO, 2012)**. The title of putative compounds relies on molecular properties such as the mass spectral pattern and accurate mass to define molecular and empirical formulae from which the metabolite can be identified **(DOERFLER et al., 2014)**. Its identification is based on a definitive compound's retention index (RI), retention time (Rt), mass spectral fragmentation, and NMR spectral shift. The identified compound is confirmed through a similar library search, *in vivo* labelling methods, or authentic chemical standards **(GHATAK et al., 2018)**.

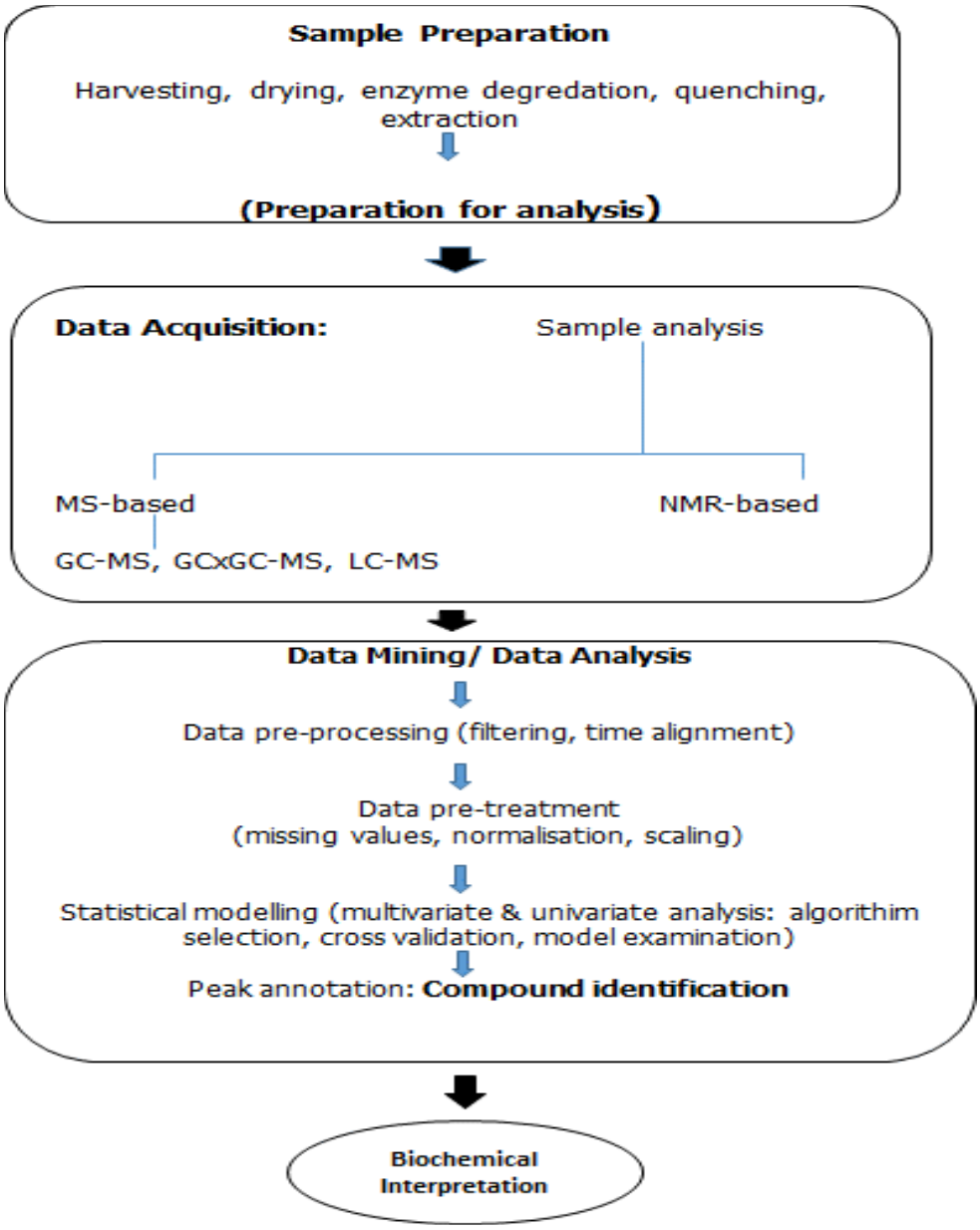

Figure 2.6: Schematic flowchart of the metabolomics workflow **(TUGIZIMANA et al., 2013)**.

2.7.1. Metabolomics as an investigative tool in elicitation treatments

The application of metabolomics is prevalent in plant stress physiology, as it is a useful tool to analyse the metabolomic profile of biological samples **(AYOUNI et al., 2016)**. It has been successfully used to investigate variations of metabolites between different organs of a plant or

between plant species **(ZAHMANOV et al., 2015)**, drug discovery and quality control of natural products **(SKALICKA-WOŹNIAK et al., 2017)**, examine the metabolic changes in plants under various environmental stress **(FERNANDEZ et al., 2016)**, chemotaxonomic findings **(GEORGIEV et al., 2011; KIM and VERPOORTE, 2010)**, and in studying the activities of medicinal plants **(MODARAI et al., 2010)**.

The metabolomic analysis serves as an investigative tool in responses of medicinal plants to environmental perturbations **(ALLWOOD et al., 2008)**, *in vitro* cell cultures **(WESTON et al., 2015)**, and elicitors' treatments **(FISCHEDICK et al., 2015; KRÁL' OVÁ et al., 2012)**. It has been used to elucidate the role of endophytes in plant responses to drought stress at the metabolic level **(KHARE et al., 2018; KUMAR et al., 2018; ZAIDI et al., 2014)**. **MAGGINI et al. (2019)** used GC-MS analysis to reveal the differences in the volatile oils build-up between *in vitro* endophyte-infected and non-infected *Echinacea purpurea* cells. GC-MS and Fourier Transform Ion Cyclotron Mass spectrometry (FT-ICR) analysis was used to show the accumulation of various metabolites by thyme under drought stress **(MORADI et al., 2017)**. However, such studies using metabolomics tools on medicinal plants are still limited.

CHAPTER 3: Phytochemical quantification, in vitro antioxidation and retardation of key carbohydrate hydrolysing enzymes by some indigenous plants

This chapter was written following the format of the Journal of Herbal Medicine

3.1. Introduction

Diabetes mellitus (DM) is a lingering and complex disruption in the normal metabolism of proteins, lipids, and carbohydrates in the body's cells **(BHATIA et al., 2019)**. DM is caused by either inadequacy in insulin secretion by the β-cells of the pancreas (type 1 DM) or the insensitivity of body tissues (muscles or liver) to secreted insulin (type 2 DM), consequently resulting in hyperglycaemia **(ELEKOFEHINTI et al., 2018; SOLAYMAN et al., 2016)**. Typically, diabetes symptoms include excessive urination, thirst and appetite, inexplicable weight loss, skin itches, tachycardia, and hypotension **(RAMACHANDRAN, 2014; SURYA et al., 2014)**. Type 2 DM is the most prevalent kind of DM, accounting for over 80% of all DM cases, and it has been associated with obesity, age, excess intake of calories and a sedentary lifestyle **(CHATTERJEE et al., 2017)**. Persistent or uncontrolled high blood glucose levels over time lead to urinary problems, stroke, kidney failure, neuropathy, oxidative stress, vision loss, myocardial infarction, and sexual dysfunction **(CHAUDHURY et al., 2017; GIOVANNINI et al., 2016; NAZARIAN-SAMANI et al., 2018)**.

Oxidative stress is a crucial determinant in the development of complications in T2DM **(PHAM-HUY et al., 2008)**. Chronic hyperglycemic conditions trigger the production of mitochondrial and non-mitochondrial reactive oxygen species (ROS) **(OYEDEMI et al., 2017)**. In T2DM, an increase in ROS concentration could be a result of alterations in the production of enzymatic/non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione

peroxidase (GSH-Px) **(ASMAT et al., 2016)**. The increase in the synthesis of ROS and fluctuation in the amount of these antioxidants impacts negatively on the insulin signalling cascade resulting in mitochondrial and β-cells dysfunction, the resistance of tissues to insulin, and the development of various problems associated with T2DM **(OYEDEMI et al., 2017)**.

Globally, diabetes remains one of the most common and fastest rising non-communicable illness of the endocrine system affecting about 465 million people living in developed and developing regions of the world, with about 19.5 million residing in Africa **(IDM'HAND et al., 2020; INTERNATIONAL DIABETES FEDERATION, 2019)**. Many years ago, unlike other regions of the world, Africa had few reported cases of DM **(KENGNE et al., 2013)**; thus, DM was misconstrued by Africans as the illness of the privileged **(JAKOVLJEVIC and MILOVANOVIC, 2015)**. However, in the last few decades, the prevalence of DM in Africa has risen tremendously, and it is projected to increase by 145% by the year 2045, with about 50 million cases expected **(INTERNATIONAL DIABETES FEDERATION, 2019)**. In Africa, the health care system is mostly weak and inefficient, and with rising DM cases, it may become overstretched **(MUTYAMBIZI et al., 2018)**. The rising incidence of DM in Africa is disturbing as it places untenable expenses on individuals, their careers, the health system, and the continents' economy **(IDEMYOR, 2010)**. The continent spent over USD 9 billion on diabetes-related health issues, and South Africa, for instance, spent 23% of its health budget on DM **(INTERNATIONAL DIABETES FEDERATION, 2019)**.

A pragmatic and therapeutic approach to managing T2DM is by controlling the post-prandial blood glucose levels. Low blood sugar level can be attained by hindering the activities of carbohydrates-hydrolysing enzymes, especially pancreatic α-amylase, and intestinal α-glucosidase enzymes **(KATO-SCHWARTZ et al., 2020)**. The retardation in the activities of these digestive enzymes

has been proven to slow down the rates of oligosaccharide and disaccharide digestion, hold back the absorption of monosaccharides (glucose) into the intestine, and ultimately reduces blood glucose levels especially after a meal **(YIN et al., 2014)**. Currently, synthetic carbohydrates-hydrolysing enzymes inhibiting drugs such as miglitol, acarbose and voglibose are used widely to manage T2DM and its complications; however, certain drawbacks such as indigestion, abdominal cramps, diarrhoea, and hypoglycaemia have been experienced by patients **(RENGASAMY et al., 2013)**. These adverse effects have been linked to their excessive retardation of pancreatic α-amylase, which results in an aberrant microbial breakdown of undigested food in the large intestine **(KWON et al., 2008).** Thus, the most beneficial and effective strategy to manage T2DM focuses on drug candidates or natural products with high intestinal α-glucosidase inhibitory potentials and moderate action against pancreatic α-amylase to maintain optimal glucose levels in the blood **(KWON et al., 2008)**.

Many indigenous plants and their active principles are commonly used to prevent and manage DM in many cultures globally **(KOOTI et al., 2016)**. The active principles of medicinal plants, including alkaloids, terpenoids, flavonoids, phenolics, glycosides, and carotenoids, have been reported as hypoglycaemic agents **(AFRISHAM et al., 2015)**. The blood glucose-lowering properties of some indigenous plants are due to the ability of their active principles to enhance insulin secretion or lessen glucose absorption by the intestinal walls **(KOOTI et al., 2016)**. Nonetheless, only a few of these plants have been scientifically proven to be effective in reducing blood sugar levels. Hence, it has become a necessity to search further for effective antioxidants and safer carbohydrates-hydrolysing enzyme inhibitors from natural products (medicinal plants) to overcome T2DM and related complications. Eleven relatively common and easy-to-come-by medicinal plants were selected for this study. These plants have been used in folk medicine to treat

a broad spectrum of human diseases, whereas little is known about the antioxidant and antidiabetic properties of some of the plants. Thus, this study was designed to examine the antioxidant and antidiabetic potentials of eleven medicinal plants using five different solvents.

3.2. Materials and Methods

3.2.1. Chemicals and reagents

Acarbose, p-nitrophenyl alpha-D-glucopyranoside (pNPG), α-glucosidase, porcine pancreatic α-amylase, 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, soluble starch, trichloroacetic acid, 3,5-dinitrosalicylic acid (DNSA), dimethyl sulfoxide (DMSO), Folin-Ciocalteau phenol are products of Sigma-Aldrich, USA. Ferric chloride aluminium chloride, sodium hydroxide and sodium carbonate were products from Merck Chemical Company, Germany. Butylated hydroxyltoluene (BHT), potassium ferricyanide, sodium potassium tartrate tetrahydrate, and sodium nitrite were acquired from BDH Biochemicals, England. Other reagents and chemicals used in this study were of analytical grade.

3.2.2. Plant materials

Healthy leaves of eleven plant species were harvested from the surrounding regions of the Umgungundlovu district (29.5101° S, 30.3436° E) of Pietermaritzburg (PMB), South Africa, between August and September 2019. A voucher specimen of each plant species was identified and deposited **(Table 3.1)** in the University of KwaZulu-Natal (UKZN), PMB, Bews Herbarium. The leaf materials were carefully rinsed under tap water to remove dirt and dust, after which they were dried to a constant weight in an oven at 40 °C. The dried leaf materials were pulverised into powders using an electric blender and kept at room temperature in airtight containers.

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1154 **Table 3.1:** List of the eleven tested plant species studied with their traditional uses and herbarium specimen numbers.

Plant specie	Family	Voucher number	Common name	Global distribution	Traditional uses	References
Catha edulis (Vahl) Forssk.	Celastraceae	NU0087099	Khat	Angola, Eritrea, Ethiopia, Kenya, Sudan, South Africa, and Yemen	Asthma, tuberculosis, lessening of hunger, obesity, stimulant, and antidepressant.	**KASSIM and CROUCHER (2006); ODENWALD et al. (2009)**
Ruta graveolens L.	Rutaceae	NU0087098	Common Rue	Widely distributed in both temperate and tropical regions	Rheumatism, aches, eye problems, contraceptives, flu, cough, and dermatitis.	**COELHO-FERREIRA (2009); COLUCCI-D'AMATO and CIMAGLIA (2020); HALE et al. (2004)**
Endostemon obtusifolius (E. Mey. ex Benth.) N.E. Br.	Lamiaceae	NU0087097	-	South Africa, Zimbabwe, Tanzania, Botswana, and Angola	The leaves are commonly used for culinary purposes.	**SADASHIVA et al., (2013)**

Combretum kraussii Hochst.	Combretaceae	NU0087096	Forest bushwillow	Swaziland, South Africa, and Zimbabwe	Fever, stomach problems, wounds, snakebite antidote, easing of labour, and inflammation.	**BROOKES et al. (1999); ELDEEN et al. (2005)**
Celtis africana Burm.f.	Cannabaceae	NU0087095	White stinkwood	Widely distributed in tropical Africa	Cancer, fever, headache, indigestion, and oedema.	**KRIEF et al. (2005**)
Pachira aquatica Aubl.	Malvaceae	NU0087094	Guiana chestnut	It is found in Central and South America as well as Africa	Wound healing, allergic itching, postpartum and low stamina.	**SUNDAY et al. (2019); YAKUB et al. (2019)**
Vernonia amygdalina Del.	Asteraceae	NU0087093	African bitter leaf	Widely distributed in tropical Africa, Yemen, and Brazil	Antihelmintic, antimalarial, purgative, enema, cough expectorant, worm expeller and fertility inducer.	**BURKILL (1985); ERASTO et al. (2006)**
Psidium guajava L.	Myrtaceae	NU0087092	Guava	It is found in tropical and sub-tropical regions including Central America, the Caribbean, Africa, and Asia	Diarrhoea, fever, dysentery, gastroenteritis, hypertension, diabetes, caries, pain relief, and wounds.	**HEINRICH et al. (1998); HOLETZ et al. (2002); LEONTI et al. (2001)**

Pentanisia prunelloides (Klotzsch ex Eckl. & Zeyh.) Walp.	Rubiaceae	NU0090107	Wild verbena	It is found in Southern Africa and Tanzania.	Burns, heartburn, fever, tuberculosis, muscle relaxant and haemorrhoid.	**KAIDO et al. (1997); LINDSEY et aL. (1998)**
Lippia javanica (Burm.f.) Spreng	Verbenaceae	NU0090106	Fever tea	It is found in South and Central America and Tropical and Southern Africa.	Cough, colds, malaria, branchial problems.	**GOVERE et al. (2000)**
Syzygium cordatum Hochst. ex Krauss.	Myrtaceae	NU0090105	Water berry tree	Commonly found in Eastern and Southern Africa.	Gastrointestinal disorders, malaria, tuberculosis, colds and cough.	**CHIGORA et al., (2007); NAIDOO et al., (2013; NANYINGI et al., (2008)**

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3.2.3. Sample preparation

Plant extracts were prepared following the reported protocols of **GHUMAN et al. (2016)**. Pulverised plant samples were extracted using different solvents, namely, ethanol (ETH), 50% aqueous-ethanol (AE), ethyl-acetate (EA), distilled water (DW), and boiled distilled water (BDW). All extracts except the BDW extracts were prepared by vigorously shaking 20 g of pulverised powdered leaf samples with 200 ml of different solvents for 24 h on a mechanical shaker and then sonicated for 1 h in an ice containing sonicator (Branson Model 5210, Branson Ultrasonics BV, Soest, Netherlands). Afterwards, the crude extracts were filtered through Whatman No. 1 filter paper under vacuum, and the resulting extracts were concentrated below 50 °C using a rotary evaporator. Subsequently, concentrated extracts were dried under a lab fan for 3 min, weighed, and stored at 4 °C till they were needed for the assays. Boiled distilled water extracts were prepared by mixing 20 g of the fine plant powders with 200 ml of boiled distilled water, and the mixture was immediately placed in a water bath at 95 °C for 15 min. After cooling, the extracts were filtered as described above, and all filtered water-based extracts were carefully transferred into well labelled and pre-measured glass containers and frozen overnight. The frozen extracts were then lyophilised using a top freeze dryer (Virtis Bench), and their weights were recorded.

One gram of each pulverised plant material was extracted with 50% aqueous ethanol (20 ml) for 20 min in an ice containing sonicator was used to estimate the condensed tannins, flavonoids, and total phenolics in the test plants. The resultant hydro-ethanolic extracts were filtered through Whatman No. 1 filter paper under vacuum, and the filtrates were used instantly.

3.2.4. *In vitro* antioxidant assays

3.2.4.1. 1-1- Diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity

The ability of all test plants' crude extracts to scavenge the stable radical ($DPPH^-$) was assayed using a modified protocol published by **SRIDHAR and CHARLES (2019)**. Briefly, in absolute methanol 0.1 mM, DPPH solution was prepared. Then equal volume (1 ml) of the DPPH solution was mixed with varying (5-100 µg/ml) concentrations of sample extracts dissolved in 50% aqueous methanol. The mixture was gently vortexed and incubated in a dark room for half an hour at 25 ± 2 °C. The changes in optic density (OD) of the final mixtures were measured using a Cary 50 UV-visible spectrophotometer (Varian, Australia) spectrophotometer at 517 nm. This assay was done in triplicate, and butylated hydroxytoluene (BHT) was used as the standard.

The inhibition percentage was derived using the equation:

$$\% \text{ Inhibition} = [(A0 - AS)]/(A0)] \times 100$$

where A0 is the absorbance of DPPH radical without sample, AS is the absorbance of DPPH radical with sample extracts /standard.

The 50% inhibitory concentration (IC_{50}) values of extracts were derived using a non-linear regression curve of the percentage of scavenging activity against the logarithm of concentrations.

3.2.4.2. Ferric-reducing antioxidant power (FRAP) assay

The iron reducing power of all the plant sample extracts was evaluated as elucidated by **MOYO et al. (2013**). Briefly, in 96-well micro-plates, 30 µl of the standard (BHT) or each extract in triplicate were mixed with 40 µl 0.2 M phosphate buffer (pH 7.2) and serially diluted to obtain different concentrations (0.039 - 0.625 µg/ml). Thereafter, 1% (w/v) potassium ferricyanide (40

µl) was added and incubated at 50 °C for half an hour. Each reaction mixture post-incubation was acidified with 40 µl of trichloroacetic acid (10% in phosphate buffer, w/v), after which distilled water (150 µl) and 30 µl ferric chloride (0.1% in phosphate buffer w/v) were added successively. The absorbance of the reaction mixtures was measured at 630 nm using a microplate reader (Opsys MRTM micro-plate reader, Dynex Technologies Inc.). The samples' OD values were plotted against their concentration gradient, and the slope values of each sample were calculated.

3.2.5. *In vitro* antidiabetic assays

3.2.5.1. α-Amylase inhibitory activity assay

The α-amylase inhibitory effects of the sample plant extracts were done following a modified procedure published by **WICKRAMARATNE et al., (2016).** In brief, the extracts were dissolved in a solvent {5% dimethyl sulfoxide (DMSO) and 95% phosphate buffer (0.02 M, pH 6.9) containing 0.006 M NaCl to yield varying concentrations (10 - 100 µg/ml)}. Then an equal volume (250 µl) of α-amylase (from *Aspergillus oryzae*) enzyme solution (2 U/ml) and the extract were mixed in a test tube, and the resultant mixture was incubated for 10 min at room temperature. Afterwards, 1% starch solution (250 µl) in the same phosphate buffer was added to the mixture to initiate the reaction, and the resultant solution was further incubated for 10 min at 25 ºC. Thereafter, the reaction was halted by adding 1 ml 3,5-dinitrosalicylic acid (DNSA) reagent, and the mixture was subsequently boiled in a water bath for 10 min at 95 ºC, cooled to room temperature, and 3 ml of distilled water was finally added just before measuring the OD at 540 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia). The standard and control were prepared following the steps taken above, but sample plant extracts were substituted for acarbose

and phosphate buffer, respectively. The enzyme inhibition percentage was calculated with the equation shown below.

$$\% \text{ α-amylase inhibition} = 100 \times ((\text{Abs Control} - \text{Abs Sample}) / \text{Abs Control})$$

The IC_{50} values, which denote the concentrations of sample plant extracts that lead to 50% enzyme inhibition, were derived by graphical extrapolation.

3.2.5.2. α-Glucosidase inhibitory activity assay

The inhibitory effects of sample plant extracts against α-glucosidase were estimated, as explained by **RENGASAMY et al. (2013)**. Briefly, in 96-well microplates, equal volumes (20 µl) of each plant extract was dissolved in DMSO at different concentrations (0.016 - 0.125 mg/ml) and yeast α-glucosidase (0.1 U/ml) in phosphate buffer (0.1 M, pH 6.8) were mixed. Then, 40 µl of the substrate (0.375 mM p-nitrophenyl-a-D-glucopyranoside (pNPG) in 0.1 M phosphate buffer at pH 6.8) was added to the mixture to initiate the reaction. Thereafter, the reaction mixture was incubated for 40 min at 37 ºC. After the incubation period, 80 µl of 0.2 M sodium carbonate in potassium phosphate buffer (0.1 M, pH 6.8) was added to each well to halt the reaction. The intensity of the colour change in the reaction mixture which signifies the amount of p-nitrophenyl produced was measured at 405 nm using an Opsys MR 96-well microplate reader. Wells containing phosphate buffer in place of sample extracts were regarded as the control, whereas acarbose was used as a standard. The enzyme inhibition percentage was calculated as follows:

$$\text{Percentage α-glucosidase inhibition} = ((\text{Abs Control} - \text{Abs Sample}) / \text{Abs Control}) \times 100$$

The IC_{50} of each sample was determined by graphical extrapolation.

3.2.6. Phytochemical quantification

3.2.6.1. Estimation of total phenolics

The total phenolic content of each test plant was evaluated as per a modified method of **DRAGOVIĆ-UZELAC et al. (2007)**. In brief, 500 µl of 1N Folin-Ciocalteu (Folin-C) reagent was added to 2.5 ml of 2% w/v sodium carbonate, 50 µl of each plant sample filtrate, and 950 µl of distilled water. Afterwards, the mixtures were vortexed and incubated for 30 min at room temperature for colour development. The optical density of each reaction mixture was measured at 725 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia), and all measurements were performed in triplicate. The total phenolic contents were quantified as mg/g gallic acid equivalents (GAE) using the equation: y = 0.0238x + 0.0535, R2 = 0.9731 derived on the calibration curve.

3.2.6.2. Estimation of total flavonoids

The total flavonoid content of each test plant was estimated using the colourimetric aluminium chloride method as described by **DRAGOVIĆ-UZELAC et al. (2007)** with some modifications. Briefly, 250 µl of each plant sample filtrate was mixed with 750 µl of distilled water. Subsequently, 75 µl of aluminium chloride (10%), 75 µl of sodium nitrate (5%), 500 µl of sodium hydroxide and 600 µl of sterile distilled water were added to each reaction mixture. Instantly, the optical density of the reaction mixture was measured using a Cary 50 UV-visible spectrophotometer (Varian, Australia) at 510 nm. The quantity of flavonoids in the plant samples was expressed as mg/g catechin equivalents (CE) using the equation: y = 0.0016x - 0.0001, R^2 = 0.989 derived from a calibration curve.

3.2.6.3. Estimation of total condensed tannins

The total condensed tannins were quantified using the modified procedure of Makkar (2000). In brief, 3 ml of butanol-HCl reagent (95:5 v/v) was added to 500 µl of each plant filtrate and 100 µl of ferric reagent (0.2% w/v ferric ammonium sulphate in 2N HCl). The reaction mixture was vortexed and incubated in a boiling water bath for 1 h. Thereafter, the absorbance of each incubated reaction mixture against the blank was measured at 550 nm using a Cary 50 UV-visible spectrophotometer (Varian, Australia). Cyanidin chloride was used to prepare the standard curve. The concentration of condensed tannins was quantified as cyanidin chloride equivalents mg/g (CCE) using the equation: $y = 0.0022x + 0.0111$, $R^2 = 0.9322$ derived from the calibration curve.

3.2.7. Statistical analysis

Data derived from these experiments were analysed using the one-way analysis of variance (ANOVA), and results are expressed as mean ± standard error of means of triplicates. The significance of means was calculated using Dunnett's multiple comparison Test, and P values < 0.05 were deemed to be statistically significant.

3.3. Result

The crude extract yield from the test plants is shown in **Table 3.2**. The 50% hydro-ethanol had the highest average extraction yield, followed by ethanol, boiled distilled water, distilled water, and ethyl acetate. The 50% aqueous ethanol extract of *P. guajava* gave the highest overall percentage yield (32.55%), followed by the ethanol extract of *R. graveolens* (27.7%), while the lowest percentage yield was obtained from the ethyl acetate extract of *P. prunelloides* (2.48%).

The total phenolic, flavonoid, and condensed tannin contents of the eleven test plants are presented in **Figures. 3.1, 3.2** and **3.3**, respectively. The highest quantity of total phenolics (451.60 ± 6.16 mg GAE /g of plant material), flavonoids (1759.82 ±79.00 mg CE/g of plant material), and condensed tannins (523.00 mg CCE/g of plant material) were found in the leaf extracts of *P. guajava*. Other test plants with significant phenolic, and flavonoid contents include *C. kraussii*, *S. cordatum, C. edulis*, *L. javanica*, *P. prunelloides,* and *E. obtusifolius*. In contrast, only *C. edulis*, *P. prunelloides* and *S. cordatum* exhibited significant contents of condensed tannins aside from *P. guajava.*

The results of the antioxidant activity of each plant extract against DPPH and their FRAP are presented in **Tables 3.2** and **3.3**, respectively. As presented in **Table 3.2**, the highest free radical (DPPH) scavenging activity in the ethanolic and 50% aqueous-ethanolic crude extracts was observed in *L. javanica* extracts with IC_{50} values of 2.50±1.13 µg/ml and 3.40±2.14 µg/ml, respectively, and they were approximately 3.3 and 2.4 folds less than the standard BHT with an IC_{50} value of 8.26 ± 0.42 µg/ml. The ethyl acetate crude extract of *C. edulis* (IC_{50} = 16.14±0.37 µg/ml), distilled water extract of *P. aquatica* (IC_{50} = 2.79±0.49 µg/ml) and the boiled distilled water extract of *E. obtusifolius* (IC_{50} = 3.26±0.08 µg/ml) all showed good DPPH radical scavenging potentials comparable to BHT. Overall, the boiled distilled water extracts showed the

best DPPH radical scavenging capabilities compared to other solvents employed in this study. As presented in **Table 3.3**, the FRAP slope values of the crude extracts range between 0.54 ± 0.07 - 7.00 ± 0.40. The result also revealed that the highest slope values in the crude ethanolic, 50% aqueous-ethanolic and ethyl acetate extracts were found in *S. cordatum* (5.91 ± 0.50), *C. kraussii* (6.44 ± 0.37) and *E. obtusifolius* (2.98 ± 0.16), respectively. Furthermore, in the distilled water and boiled distilled water extracts *P. guajava* extracts significantly showed the highest ferric reduction in antioxidant power with slope values of 7.00±0.40 and 6.07±0.31, respectively.

The correlation coefficients between the phytochemical contents of the eleven tested plants and their antioxidant activities are shown in **Table 3.5**. Overall, the total phenolic contents exhibited the strongest relationship with the two antioxidant assays expressed by the highest coefficients in absolute values. The total flavonoid and total phenolics were negatively correlated with the IC_{50} values of all the extracts in the DPPH assay, consequently enhancing the antioxidant activities of the extracts. In contrast, the strongest correlation coefficients (r^2 = 0.917, 0.880, 0.851 and 0.758) in this study were recorded between solvent extracts of the FRAP assay and the total phenolic contents **(Table 3.5)**.

Table 3.2: Percentage yield (%), DPPH percentage radical scavenging activity (RSA) and IC_{50} of solvent crude extracts of the eleven tested plant species against DPPH.

	Ethanol			50 % Aqueous ethanol			Ethyl acetate			Distilled Water			Boiled distilled water		
Plant species	% Yield	DPPH % RSA at 100 µg/ml	IC_{50} (µg/ml)	% Yield	DPPH % RSA at 100 µg/ml	IC_{50} (µg/ml)	% Yield	DPPH % RSA at 100 µg/ml	IC_{50} (µg/ml)	% Yield	DPPH % RSA at 100 µg/ml	IC_{50} (µg/ml)	% Yield	DPPH % RSA at 100 µg/ml	IC_{50} (µg/ml)
C. edulis	19.05	98.80±0.48^{a-d}	12.86±0.38^{m-r}	26.90	98.55±0.30^{a-e}	9.71±0.17^{n-r}	5.00	98.75±0.24^{a-d}	16.14±0.37^{l-p}	15.65	94.39±0.12^{a-i}	8.02±0.01^{o-r}	19.55	95.22±0.11$^{a-i\ i}$	6.18±1.76^{p-r}
C. africana	6.30	60.50±1.20^{mn}	94.85±1.94^{ab}	26.90	47.03±1.56^{o}	> 100	8.00	46.64 ±2.60^{op}	> 100	12.40	55.72±0.60^{n}	68.36±0 30^{c}	18.00	65.70±0.36^{l}	51.95±0.40^{de}
C. kraussii	17.30	99.40±0.12^{a}	8.50±0.03^{o-}	15.30	90.10±0.24^{h-j}	8.83±0.02^{o-r}	3.35	76.30±0.72^{k}	47.55±0.04^{d-f}	22.90	97.51±0.24^{a-g}	4.64±1.74^{qr}	21.95	97.20±0.0^{a-g}	5.42±0.70^{p-r}
L. javanica	16.30	97.00±0.50^{a-g}	2.50±1.13^{r}	17.75	99.17±0.72^{ab}	3.40±2.14^{qr}	3.00	90.64±0.12^{h-j}	35.54±1.70^{g-i}	11.70	77.13±0.42^{k}	32.26±1.40^{g-i}	12.60	92.52±0.50^{g-j}	8.21±1.43^{o-r}
P. aquatica	11.00	98.75±0.24^{a-d}	19.66±1.30^{j-n}	11.85	93.00±0.48^{g-j}	13.68±4.30^{m-q}	4.20	41.79±0.48^{op}	> 100	6.00	93.35±0.25^{f-j}	2.79±0.49^{r}	9.05	94.39±0.36^{a-i}	6.20±0.66^{p-r}
P. prennuloides	17.65	98.03±0.06^{a-f}	3.94±1.93^{qr}	17.15	95.22 ±0.36^{a-i}	10.31±0.42^{m-r}	2.48	43.67±2.02^{op}	> 100	5.00	90.23±0.05^{ij}	26.57±2 94^{j-l}	5.00	89.19±1.68^{j}	42.28±0.54^{e-g}
P. guajava	26.10	98.44±0.30^{a-e}	8.01 ± 1.75^{o-r}	32.55	97.00±0.35^{a-g}	4.94±1.20^{qr}	3.15	79.21±0.24^{k}	65.61±3.16^{c}	11.50	94.18±0.21^{b-j}	8.40±1.37^{o-r}	10.35	98.96±0.36^{a-c}	10.13± .36^{m-r}
R. graveolens	27.70	98.96±0.12^{a-d}	18.76±0.62^{k-n}	24.40	79.63±1.10^{k}	54.04±2.50^{d}	6.16	43.04±0.42^{op}	> 100	14.30	76.33±0.36^{k}	37.93±0.48^{f-h}	15.60	92.72±0.12^{g-j}	20.51±0.06^{j-m}
E. obtusifolius	11.72	97.71±0.36^{a-i}	3.35±1.45^{qr}	16.80	95.29±0.14^{a-i}	15.86±1.70^{l-p}	4.67	34.03±1.45^{pq}	> 100	5.20	75.76±0.18^{k}	5.85±2.95^{p-r}	6.40	94.39±0.36^{a-i}	3.26±0.08^{qr}
S. cordatum	14.70	98.75±0.63^{a-d}	15.16±2.98^{l-p}	16.95	93.62±0.14^{e-j}	5.98±3.11^{p-r}	2.68	99.00±0.58^{a-c}	32.55±0.68^{g-i}	10.00	96.54±0.37^{a-g}	16.98±1.73^{l-p}	9.00	95.56±0.45^{a-h}	15.24±3.46^{l-p}
V. amgydalina	17.00	94.00±0.12^{c-j}	30.02±0.48^{h-k}	20.09	97.50±0.24^{a-g}	27.60±0.19^{h-l}	3.80	46.60±2.65^{op}	> 100	20.70	64.66±2.16^{lm}	70.56±0 32^{c}	25.00	93.76±0.48^{d-j}	13.84±2.05^{m-q}
BHT		99.37±0.12^{a}	8.26±0.42^{o-r}												

Data are mean ± SE (n=3). Means with different lower-case letters in the same column are significantly different at $P < 0.05$ (ANOVA, followed by Dunnett's multiple comparison test).

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1331 **Table 3.3:** Ferric-reducing antioxidant power (FRAP) of the eleven tested plant species.

	Ethanol		50% Aqueous ethanol		Ethyl acetate		Distilled Water		Boiled Distilled Water	
Plant specie	**Slope**	**R^2**	**Slope**	**R^2**	**Slope**	**R^2**	**Slope**	**R^2**	**Slope**	**R^2**
C. edulis	$4.25\pm0.09^{c\text{-}k}$	0.92	$4.41\pm0.20^{c\text{-}j}$	0.94	$2.75\pm0.12^{g\text{-}o}$	0.97	$4.35\pm0.40^{c\text{-}j}$	0.92	$3.13\pm0.14^{g\text{-}o}$	0.85
C. Africana	0.91 ± 0.03^{o}	0.93	0.54 ± 0.07^{o}	0.93	$1.29\pm0.22^{l\text{-}o}$	0.99	0.91 ± 0.04^{no}	0.99	0.62 ± 0.07^{no}	0.97
C. kraussii	$5.04\pm1.01^{a\text{-}h}$	0.90	$6.44\pm0.37^{a\text{-}c}$	0.87	$1.53\pm0.05^{l\text{-}o}$	0.96	6.96 ± 0.29^{ab}	0.84	$5.96\pm0.43^{a\text{-}d}$	0.85
L. javanica	$3.66\pm0.23^{d\text{-}m}$	0.88	$1.95\pm0.15^{j\text{-}o}$	0.98	$2.01\pm0.13^{j\text{-}o}$	0.95	0.74 ± 0.03^{o}	0.95	$1.80\pm0.09^{k\text{-}o}$	0.96
P. aquatica	$2.09\pm0.02^{j\text{-}o}$	0.94	$2.42\pm0.05^{i\text{-}o}$	0.97	$1.21\pm0.05^{m\text{-}o}$	0.99	$2.39\pm0.05^{i\text{-}o}$	0.93	$2.42\pm0.06^{j\text{-}o}$	0.95
P. prennuloides	$2.43\pm0.74^{i\text{-}o}$	0.82	$3.18\pm0.20^{e\text{-}o}$	0.70	$2.49\pm0.61^{i\text{-}o}$	0.94	$4.03\pm0.16^{c\text{-}l}$	0.91	$2.00\pm0.16^{j\text{-}o}$	0.87
P. guajava	$4.46\pm2.06^{b\text{-}j}$	0.87	$5.65\pm0.34^{a\text{-}e}$	0.83	$2.18\pm0.13^{j\text{-}o}$	0.96	7.00 ± 0.40^{a}	0.85	$6.07\pm0.31^{a\text{-}d}$	0.89
R. graveolens	1.07 ± 0.18^{no}	0.91	$1.55\pm0.04^{l\text{-}o}$	0.88	$2.45\pm0.06^{i\text{-}o}$	0.88	1.24 ± 0.01^{no}	0.81	0.89 ± 0.31^{no}	0.69
E. obtusifolius	$2.65\pm0.25^{h\text{-}o}$	0.82	$3.31\pm0.41^{e\text{-}n}$	0.91	$2.98\pm0.16^{g\text{-}o}$	0.86	$2.35\pm0.26^{j\text{-}o}$	0.96	$1.96\pm0.15^{j\text{-}o}$	0.89
S. cordatum	$5.91\pm0.50^{a\text{-}d}$	0.81	$5.54\pm0.38^{a\text{-}f}$	0.84	$1.51\pm0.13^{l\text{-}o}$	0.98	$5.26\pm0.15^{a\text{-}g}$	0.93	$4.91\pm0.14^{a\text{-}i}$	0.92
V. amgydalina	$2.00\pm0.08^{j\text{-}o}$	0.92	$2.59\pm0.03^{h\text{-}o}$	0.90	1.02 ± 0.11^{no}	0.87	$1.52\pm0.03^{l\text{-}o}$	0.91	$1.63\pm0.06^{l\text{-}o}$	0.98
BHT	7.34 ± 0.69^{a}	0.96								

Data are mean ± standard error (n = 3). Ferric-reducing power of samples increases with higher slope values. Slope values with the same lower-case letters (a-o) in the same column are not significantly different at $P \leq 0.05$ (ANOVA, followed by Dunnett's multiple comparison test). R^2 signifies the fitness of the curve and the nearer the R^2 of a sample is to 1, the higher its reliability.

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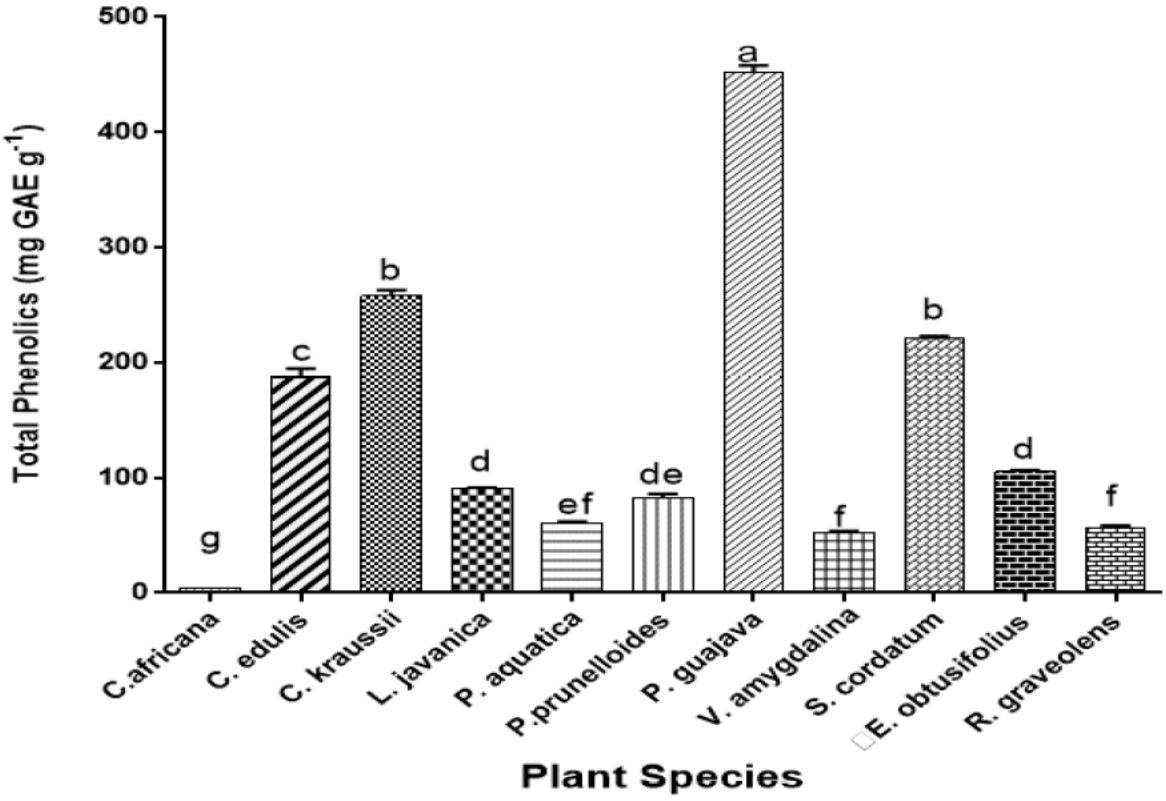

Figure 3.1: Total phenolic content (mg GAE g^{-1}) of the leaves of the eleven tested plant species. Bars with the same lower-case letters (a-g) are not statistically different at $P \leq 0.05$ (ANOVA, followed by Dunnett's multiple comparison).

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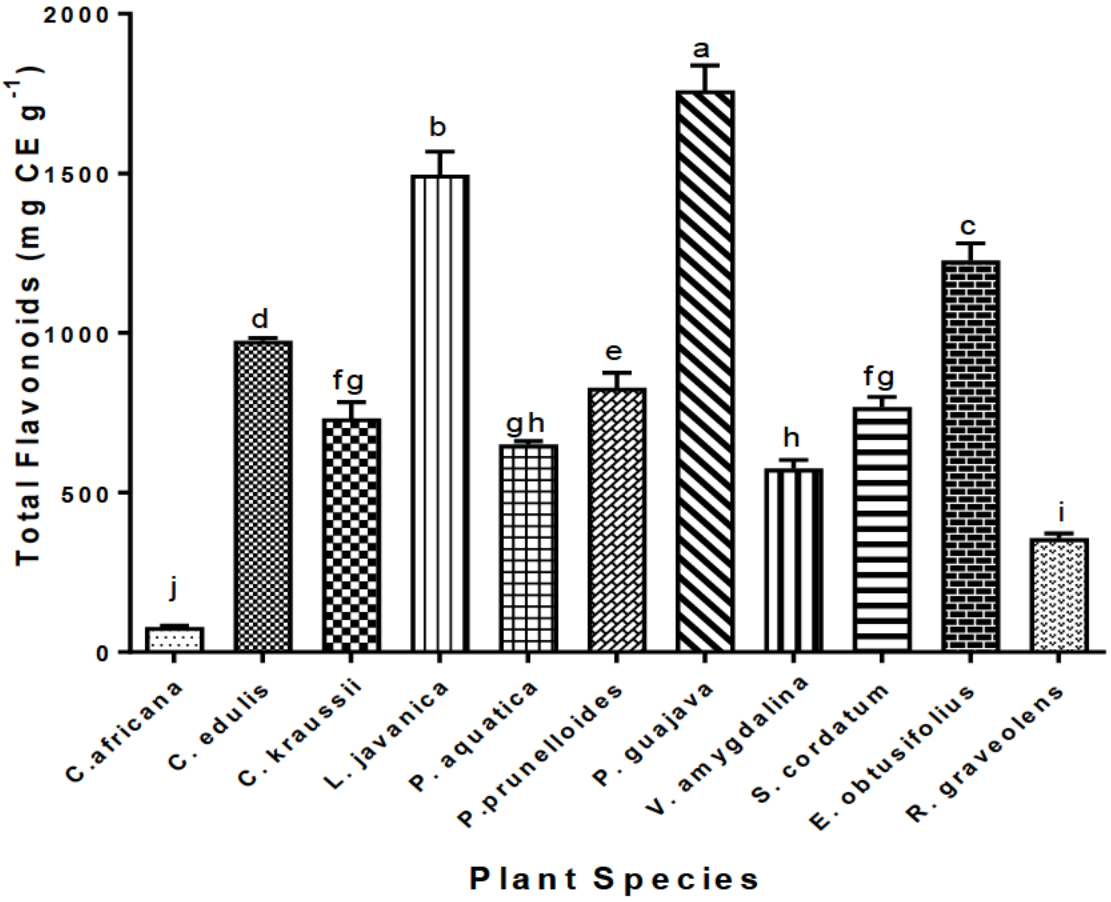
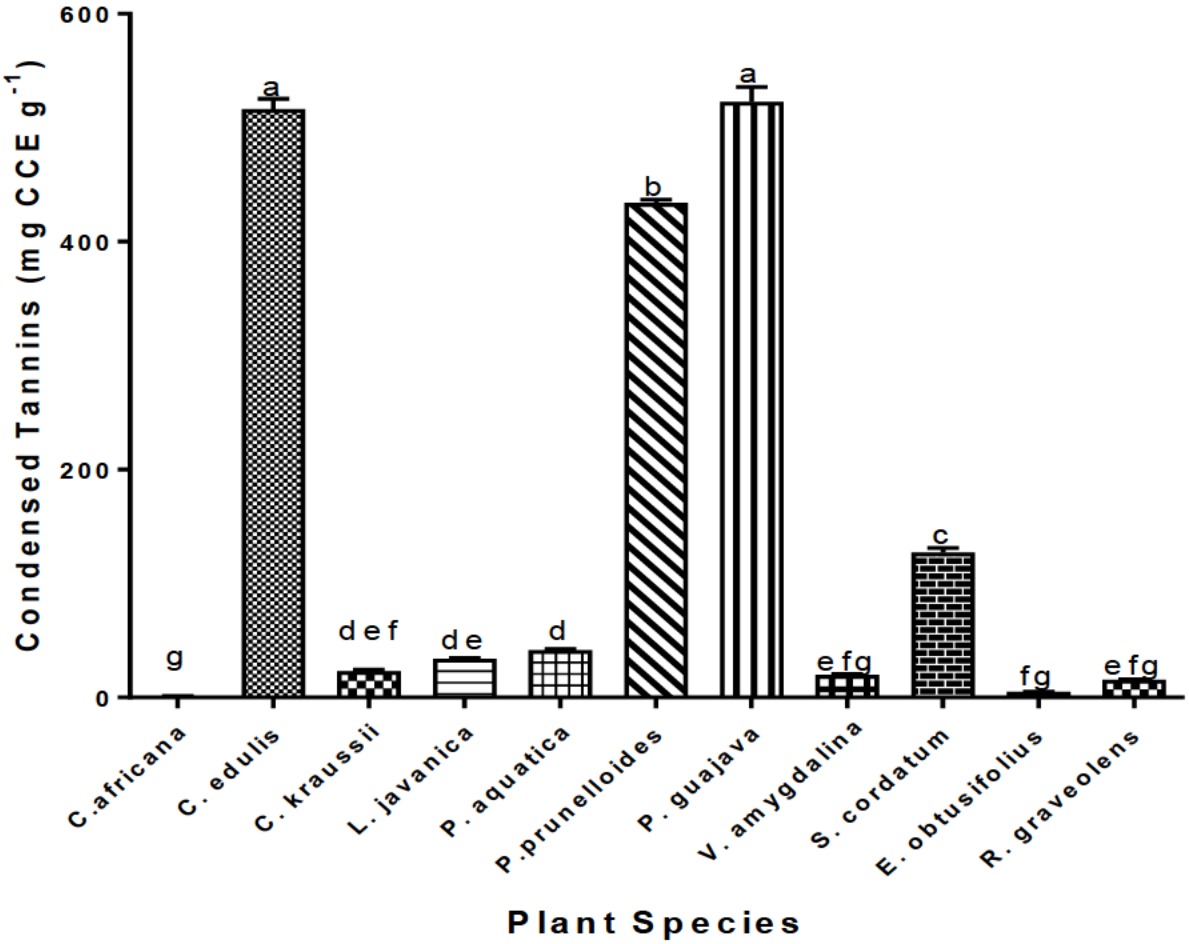


Figure 3.2: Flavonoid content (mg CE g^{-1}) of the leaves of the eleven tested plant species. Bars with the same lower-case letters (a-g) are not statistically different at $P \leq 0.05$ (ANOVA, followed by Dunnett's multiple comparison).



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1343 **Figure 3.3:** Condensed tannin content (mg CCE g^{-1}) of the leaves of the eleven tested plant species.
1344 Bars with the same lower-case letters (a-g) are not statistically different at $P \leq 0.05$ (ANOVA,
1345 followed by Dunnett's multiple comparison).

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The inhibitory effects of the test plant crude extracts against the two digestive enzymes (α-amylase and α-glucosidase) are presented in **Table 3.4**. Our results showed that the ethanolic, aqueous-ethanolic, and ethyl acetate crude extracts of extracts *C. edulis* exhibited the highest inhibition of α-amylase in their respective solvent category with IC_{50} values of 9.53±1.94 µg/ml, 16.95±4.41 µg/ml, and 5.31±2.23 µg/ml, respectively. Similarly, distilled water and boiled distilled water crude extracts of *L. javanica* displayed higher α-amylase inhibition than the positive control, acarbose (IC_{50} = 24.46±4.11 µg/ml) with lower IC_{50} values of 11.93±1.27 µg/ml and 17.72±0.65 µg/ml, respectively. In the α-glucosidase inhibitory result, there was no significant difference between IC_{50} values of the ethanolic crude extracts of *P. guajava* (7.00±2.15 µg/ml), *C. kraussii* (9.05± 3.50 µg/ml), *C. edulis* (10.18±4.68 µg/ml), *L. javanica* (11.53±2.00 µg/ml) and *S. cordatum* (12.87±2.43 µg/ml), which were all lower than the positive control (28.26±2.52 µg/ml). Additionally, in the 50% aqueous-ethanol crude extracts, *P. guajava* showed the best α-glucosidase inhibition with the IC_{50} value of 4.74±1.27 µg/ml, whereas the ethyl acetate crude extracts of *L. javanica* (4.33± 1.62 µg/ml) and *E. obtusifolius* (4.40 ± 0.45 µg/ml) showed impressive α-glucosidase inhibition potential. The distilled water and boiled distilled water extracts of *C. edulis, S. cordatum,* and *C. kraussii* also showed promising inhibitory effects against α-glucosidase.

Table 3.4: Inhibitory concentration (IC_{50}) of solvent extracts of the eleven tested plant species on α-amylase and α-glucosidase activities.

	Ethanol		50% Aqueous ethanol		Ethyl acetate		Distilled Water		Boiled Distilled Water	
	IC_{50} (µg/ml)		IC_{50} (µg/ml)		IC_{50} (µg/ml)		IC_{50} (µg/ml)		IC_{50} (µg/ml)	
Plant specie	**α-Amylase**	**α-Glucosidase**	**α-Amylase**	**α-Glucosidase**	**α-Amylase**	**α-Glucosidase**	**α-Amylase**	**α-Glucosidase**	**α-Amylase**	**α-Glucosidase**
C. edulis	9.53 ± 1.94^{qr}	$10.18\pm4.68^{m-q}$	$16.95\pm4.41^{o-r}$	$27.50\pm3.50^{i-p\ -r}$	5.31 ± 2.23^{r}	$9.13\pm0.10^{n-q}$	$109.29\pm3.22^{a-c}$	$9.92\pm3.94^{m-q}$	$102.50\pm2.82^{b-d}$	$17.75\pm2.62^{k-q}$
C. africana	129.25 ± 11.98^{ab}	$69.14\pm3.10^{a-d}$	$106.96\pm19.07^{b-d}$	$69.36\pm1.30^{a-d}$	$79.30\pm2.57^{d-f}$	87.73 ± 4.80^{ab}	$45.48\pm0.55^{g-l}$	$68.36\pm0.303^{c-f}$	$36.51\pm1.34^{k-p}$	$79.68\pm1.08^{a-c}$
C. kraussii	$31.53\pm3.84^{i-q}$	$9.05\pm3.50^{n-q}$	$76.60\pm1.05^{e-g}$	$18.46\pm2.40^{k-q}$	$14.87\pm1.93^{p-r}$	$47.55\pm0.04^{d-f}$	$65.58\pm2.01^{f-j}$	$20.69\pm0.45^{j-q}$	$62.77\pm1.80^{f-k}$	$10.01\pm3.86^{m-q}$
L. javanica	$65.25\pm3.95^{f-j}$	$11.53\pm2.00^{m-q}$	$54.00\pm2.39^{g-l}$	$50.87\pm2.68^{d-h}$	$21.38\pm2.64^{n-r}$	4.33 ± 1.62^{q}	11.93 ± 1.27^{qr}	$30.75\pm0.77^{h-n}$	17.72 ± 0.65^{or}	$27.88\pm2.19^{h-o}$
P. aquatica	$110.79\pm1.95^{a-c}$	$42.52\pm3.61^{f-j}$	$106.54\pm1.86^{b-d}$	88.61 ± 5.65^{ab}	$56.92\pm1.25^{g-k}$	$78.05\pm4.56^{a-c}$	$73.02\pm0.80^{e-i}$	$70.34\pm8.88^{a-d}$	$75.40\pm1.10^{e-h}$	90.34 ± 4.76^{a}
P. prennuloides	$92.82\pm2.86^{c-e}$	$29.00\pm7.29^{h-o}$	127.65 ± 3.96^{ab}	$32.40\pm3.16^{h-m}$	$62.83\pm5.24^{f-k}$	$59.10\pm0.60^{c-g}$	$17.86\pm6.80^{o-r}$	$37.40\pm1.29^{g-l}$	$22.35\pm1.77^{o-r}$	$32.82\pm2.70^{h-m}$
P. guajava	$43.68\pm0.18^{j-n}$	$7.00\pm2.15^{o-q}$	$41.54\pm2.42^{j-o}$	4.74 ± 1.27^{pq}	$29.04\pm4.37^{l-r}$	$39.87\pm10.07^{g-k}$	$82.78\pm2.00^{d-f}$	$18.97\pm0.91^{k-q}$	$82.48\pm4.10^{d-f}$	$28.26\pm2.52^{k-q}$
R. graveolens	$60.06\pm2.42^{f-k}$	97.87 ± 5.62^{a}	$54.34\pm1.71^{g-k}$	85.95 ± 3.87^{ab}	$55.80\pm2.34^{g-k}$	$62.71\pm4.83^{c-f}$	$50.00\pm0.64^{g-l}$	$50.66\pm2.89^{d-h}$	$86.94\pm1.08^{d-f}$	$63.71\pm3.40^{b-f}$
E. obtusifolius	$23.63\pm1.58^{n-r}$	$56.53\pm3.37^{c-g}$	$73.08\pm2.59^{e-i}$	$47.22\pm4.57^{e-i}$	$38.86\pm1.60^{k-p}$	4.40 ± 0.45^{q}	$50.00\pm0.64^{i-m}$	$25.93\pm2.13^{i-q}$	$50.26\pm1.29^{h-m}$	$14.17\pm3.42^{m-q}$
S. cordatum	$45.45\pm2.74^{j-n}$	$12.87\pm2.43^{m-q}$	$74.32\pm4.12^{e-i}$	$10.33\pm4.50^{m-q}$	$40.00\pm2.36^{k-p}$	$63.73\pm1.01^{c-f}$	$22.83\pm4.55^{n-r}$	$12.11\pm1.62^{m-q}$	$30.64\pm5.59^{\ i-q}$	$15.04\pm5.74^{i-q}$
V. amgydalina	133.80 ± 3.38^{a}	$72.75\pm7.30^{a-d}$	$114.31\pm3.38^{a-c}$	87.42 ± 8.512^{ab}	$25.36\pm1.00^{m-r}$	$78.21\pm3.75^{a-c}$	$39.50\pm5.04^{k-p}$	$66.04\pm0.82^{b-e}$	$57.71\pm2.43^{f-k}$	$63.32\pm3.31^{c-f}$
Acarbose	$24.46\pm4.11^{n-r}$	$28.26\pm2.52^{h-o}$								

Data represent mean values ± SE (n=3). Means with the same lower-case letters in the same column are not significantly different at $P < 0.05$ (ANOVA, followed by Dunnett's multiple comparison).

Table 3.5: Pearson correlation among total polyphenol content, total tannin content, total flavonoid content and antioxidant activity (ferric reducing power (FRAP) and 2, 2-diphenyl-1-picryl hydrazyl (DPPH)).

	Total phenolic contents	**Total flavonoid contents**	**Total condensed tannin contents**
DPPH ethanolic extracts	- 0.424	- 0.680	- 0.313
DPPH 50% aqueous-ethanolic extracts	- 0.531	- 0.729	- 0.391
DPPH ethyl acetate extracts	- 0.514	- 0.421	- 0.347
DPPH distilled water extracts	- 0.567	- 0.538	- 0.355
DPPH boiled distilled water extracts	- 0.416	- 0.552	0.032
FRAP ethanolic extracts	0.758	0.540	0.357
FRAP 50% aqueous-ethanolic extracts	0.851	0.459	0.435
FRAP ethyl acetate extracts	0.164	0.450	0.459
FRAP distilled water extracts	0.880	0.380	0.565
FRAP boiled distilled water extracts	0.917	0.481	0.397

3.4. Discussion

The exploration of potent active principles of plant origin with good antioxidant properties, hormonal signalling, and digestive enzyme inhibitory potentials has gained momentum in recent times over synthetic drugs in managing DM and its intricacies **(JUSTINO et al., 2017)**. The present study investigated the preliminary quantification of phytochemical constituents, *in vitro* antioxidant, and *in vitro* inhibition of α-amylase and α-glucosidase digestive enzymes by the crude extracts of 11 medicinal plants.

Plants synthesise varieties of low molecular weight phytochemicals which are not primarily involved in their wellbeing but confer resistance and protection against environmental pressures, including herbivory and pathogens **(WINK, 2018)**. Besides their beneficiary effects on plants, secondary plant metabolites represent the active core principles eliciting various biological and pharmacological effects on humans and animals **(EDEOGA et al., 2005)**, and they have been widely used in traditional medicine over the years **(HUSSEIN and EL-ANSSARY, 2019; WINK, 2015)**. Flavonoids, phenolics, and condensed tannins are some of the main therapeutic secondary metabolites of medicinal plants with an incredible array of antioxidant, antitumor, antimicrobial, antimalaria, and antidiabetic properties **(DURAZZO et al., 2019; LIN et al., 2016).** This current study showed that the leaves of *C. edulis*, *C. kraussii*, *L. javanica*, *P. guajava*, *E. obtusifolius*, *S. cordatum* and *V. amygdalina* contained considerable amounts of phenolics, flavonoids and condensed tannins. Thus, these species are potentially rich sources of pharmacologically important bioactive compounds, and it justifies their age-long use in traditional medicine in treating several ailments. As observed in this study, similar total flavonoid, phenolic and condensed tannin contents were reported by different researchers in *P. guajava* **(ANBUSELVI and REBECCA,**

2017), *C. edulis* **(SAGAR et al., 2018)**, *L. javanica* **(OSUNSANMI et al., 2019)**, *S. cordatum* **(MULAUDZI et al., 2012)** and *Combretum* spp. **(HAMAD et al., 2019)**.

The other essential part of this study is the antioxidant properties of the tested plant species. Antioxidants are molecules or compounds (even at low concentrations) that shield biological systems from injurious effects of oxidation chain reactions generated by free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) **(KARADAG et al., 2009)**. Free radicals often generated in biological systems either endogenously or exogenously are highly reactive low molecular weight diffusible molecules containing unpaired electron(s) **(WAJID et al., 2017)**, and they accumulate in cells causing oxidative stress. Free radicals play crucial roles in the development of diseases, such as DM, cancer, cardiovascular diseases, and neurodegenerative problems **(HALLIWELL, 2012)**. Specifically, the pancreas is highly vulnerable to oxidative stress, and it may lead to pancreatic β-cells dysfunction and insulin resistance within peripheral tissues **(WANG and WANG, 2017)**. Natural antioxidants of medicinal plants, including condensed tannins, phenolics and flavonoids, help ameliorate oxidative stress-induced diseases such as DM **(BAHAR et al., 2017)**. These phytochemicals owe their antioxidant efficiency to their ability to donate hydrogen ions, scavenge free radicals, and assist in the quenching of decomposing peroxides and ROS **(BORGES et al., 2017)**. Furthermore, the antioxidant potential of each tested plant can be ascribed to its active principles' composition and a likely synergistic effect of these compounds **(AL-OWAISI et al., 2014)**.

The 1,1-diphenyl-β-picrylhydrazyl (DPPH) is an organic, stable and nitrogen-free radical compound that can be reduced by free radical scavengers **(CHAKRABORTY et al., 2020)**. DPPH is a reliable and widely employed substrate used to determine the antioxidant capacity of chemical compounds **(SANTOS and GONÇALVES, 2016)**, and it can transform into a stable diamagnetic

molecule on receiving an electron **(KEDARE and SINGH, 2011)**. A lower IC_{50} value in DPPH-assay corresponds to an intense scavenging activity, and a DPPH radical scavenging activity above 50% is considered significant **(HUSSAIN et al., 2018b)**. *C. edulis*, *C. kraussii*, *L. javanica*, *P. guajava*, *P. aquatica*, *E. obtusifolius*, and *S. cordatum* extracts exhibited high free radical scavenging activities (lowest IC_{50} values) and this may be due to their phytochemical constituents. Several reports have suggested that the antioxidant properties of plants or their derived compounds are mainly due to the radical scavenging ability of their flavonoids, tannins, and phenolic constituents **(AIRAODION et al., 2019; REBAYA et al., 2015)**. This DPPH assay is accurate and reliable because neither metals nor enzyme inhibition influences its outcome; however, it does not completely elucidate the actual reactivity of the antioxidant when considered alone **(YADAV et al., 2014)**

The iron reducing power assay estimates the reduction capacity of an antioxidant (reducing agent) to reduce the colourless Fe^{3+} complex of 2,4,6-tripyridyl-s-triazine (TPTZ) into a deep blue ferrous form Fe^{2+} TPTZ **(BENZIE and STRAIN, 1996)**. Higher antioxidant capacity of a compound or an extract is characterised by higher FRAP absorbance values at λ 630 nm **(STEENKAMP et al., 2006)**, and this is due to the reduction of ferric ions by the antioxidants **(KHEZRILU BANDLI et al., 2017)**. From our results, it was noted that phenolic-rich plants (*C. edulis*, *C. kraussii*, *P. guajava* and *S. cordatum*) exhibited the highest antioxidant capacity. Furthermore, the relationship between total phenolic contents and the antioxidant capacity of medicinal plants has been widely investigated **(BENABDALLAH et al., 2016; HATAMI et al., 2014; ULEWICZ-MAGULSKA and WESOLOWSKI, 2019)**. From our findings, there is a strong and positive direct link between the FRAP values and the total phenolic contents, and this concurs with the earlier findings of many researchers that reported positively related linearity between total phenolic contents of many

indigenous plants and FRAP values **(BENABDALLAH et al., 2016; KHEZRILU BANDLI et al., 2017; KOCZKA et al., 2016)**. The iron reducing power assay is comparatively very fast and easy to conduct, however its reaction is non-specific, and any compound with a suitable redox potential may drive Fe^{3+}-TPTZ reduction **(BENZIE and STRAIN, 1996)**. Many researchers have linked the pathogenesis of DM, and its resulting complications, including retinopathy, to oxidative stress **(LI et al., 2017a)** and antioxidants have been prescribed as valuable remedies **(ALCUBIERRE et al., 2015)**. Thus, it can be argued that the free radical scavenging capacity of phytochemicals may be the basis for the reported hypoglycaemic activities of many indigenous plant species. This study has shown the antioxidant capabilities of *C. edulis*, *C. krausssii*, *L. javanica*, *P. aquatica*, *P. guajava*, *P. prenulloides*, *E. obtusifolius*, and *S. cordatum*, which is comparable to BHT, a reference antioxidant.

Alpha-amylase and α-glucosidase are two of the key enzymes in the human digestive system. They are responsible for breaking dietary polysaccharides and disaccharides into simpler and absorbable monosaccharides such as glucose **(CASIROLA and FERRARIS, 2006)**. Due to the rapid surge in the cases of T2DM and its complications around the globe, the development of novel hypoglyceamic agents from plants for the effective regulation of post-meal elevation in blood sugar has been noted in recent times **(BAHMANI et al., 2014)**. Consequently, the inhibition of carbohydrates-hydrolysing enzymes is an efficient means of retarding carbohydrate digestion, thereby regulating the rate of sugar absorption into the bloodstream **(KALITA et al., 2018)**. *In vitro* α-amylase and α-glucosidase assays are easy enzymatic assays suitable for evaluating antidiabetic phytocompounds, however, the limits of detection are too low **(VHORA et al., 2020)**. From our findings, crude extracts of *C. krausssii*, *L. javanica*, *P. guajava*, *P. prenulloides*, *E. obtusifolius*, *S. cordatum*, and *C. edulis* displayed higher α-glucosidase inhibition and lower

retardation of α-amylase than acarbose. Our results agree with prior studies on several medicinal plants including *P. guajava* **(SIMÃO et al., 2017)**, *C. krausssi* **(MADIKIZELA et al., 2017)**, *P. prunelloides* **(MAKHUBU et al., 2019)**, *C. edulis* **(PIERO and JOAN, 2011)** and *S. cordatum* **(MUSABAYANE et al., 2005)**. Condensed tannins, flavonoids and phenolic compounds are known to bind with digestive enzymes and directly modulate the decomposition of polysaccharides and disaccharides **(AMOAKO and AWIKA, 2016)**. Interestingly from our results, plant species with higher contents of flavonoids, condensed tannins and total phenolics showed the highest inhibitory activities against the digestive enzymes.

The noticeable variations between the investigated plant extracts in this study as per their enzyme inhibition properties and antioxidant potentials may be due to the inherent variations in their chemical compositions and their respective extraction solvent polarity. Disparities in the polarity of extraction solvents impact the overall pharmacological activities of the resultant plant extracts **(MAZOUZ et al., 2020; RACHA et al., 2018)**. Polar solvents including acetone, ethanol, ethyl acetate, and methanol used singly and in combination facilitate the extraction of several polyphenols **(SAHA et al., 2017)** whose anti-hyperglycaemic and antioxidant capabilities have been well reported **(ALAM et al., 2020; SOLAYMAN et al., 2016)**.

3.5. Conclusion

The phytochemical constituents, *in vitro* antioxidant, and antidiabetic capacity of eleven indigenous plants were investigated to further validate the therapeutic values of these species in this research. The *in vitro* α-amylase and α-glucosidase inhibition potential of *E. obtusifolius* crude extracts was reported for the first time as far as we know. *Endostemon obtusifolius* leaves possessed moderate total phenolics, high flavonoids, high antioxidant activities, moderate α-

amylase inhibitory activities, and high α-glucosidase inhibitory activities; thus, it can be considered as a valuable source in the indigenous medicinal system. Furthermore, findings from this study indicate that highly efficacious hypoglycaemic agents could possibly be derived from indigenous plants, particularly *E. obtusifolius*.

CHAPTER 4: GC-MS analysis, *in vitro* and *in silico* antidiabetic potentials of *Syzygium cordatum* leaf extracts

Preface

This chapter was written following the short communication format of Natural Product Research Journal.

Going by the seasonal unavailability (winter) of *Endostemon obtusifolius* leafy materials and the abundance of *Syzygium cordatum* leaves, *S. cordatum* (with an impressive antioxidant and antidiabetic results in **Chapter 3**), was selected for further investigation in this Chapter.

4.1. Introduction

Syzygium cordatum Hochst. ex Krauss (Water berry) is a widely distributed eastern and southern African water-loving tree species of the Myrtaceae family **(MAROYI, 2018)**. It is a highly valued tree commonly used in agroforestry, livelihoods (food, fruits, timber, dyes), energy sources, and medicines **(DLAMINI and GELDENHUYS, 2009; KATUMBA et al., 2004)**. *Syzygium cordatum* is widely used in folklore medicine to manage various ailments, including coughs **(KIGEN et al., 2016)**, malaria **(RAMADHANI et al., 2015)**, wounds **(TUGUME et al., 2016)**, tuberculosis **(CHIGORA et al., 2007)**, sexually transmitted diseases **(DE WET et al., 2012; NAIDOO et al., 2013)** and digestive issues **(NANYINGI et al., 2008**).

Type 2 Diabetes mellitus (T2DM) is one of the fastest-growing public health concerns globally, affecting people of diverse cultural backgrounds **(BLIND et al., 2018)**. Owing to the risk factor status of T2DM sufferers to viral infections **(ABU-ASHOUR et al., 2018)**, the morbidity and

mortality rates of the lingering COVID-19 crisis have been exacerbated by the disease **(WU et al., 2020)**. Oxidative stress is a crucial mechanism through which the pathogenesis of T2DM can be explained **(GALICIA-GARCIA et al., 2020)**. Oxidative stress impedes the normal functioning of pancreatic beta-cells and induces insulin resistance in the liver, adipose cells and muscles **(YARIBEYGI et al., 2020)**. Several phyto-active products from plants have been documented as remarkable sources of antioxidants and anti-hyperglycaemia agents with high efficacy and minimal side effects **(GOTHAI et al., 2016)**.

Moreover, *S. cordatum* leaf extracts elicited high efficacy against mild DM but were found to be less effective against severe cases of high blood sugar levels **(DELIWE and AMABEOKU, 2013; MUSABAYANE et al., 2005)**. Certain pharmacologically active principles include 6,10,14-trimethyl-pentadecane-2-one, 2,3-butanediol diacetate, hexadecanoic acid methyl ester, n-hexadeconoic acid, isopentyloxyethyl acetate, triacetin, 2-furanone, and naphthalene,1,6-dimethyl-4-(1-methyl-ethyl) have been identified from the essential oils of *S. cordatum* leaves by **CHALANNAVAR et al. (2011)**. However, the antioxidant properties and the putative hypoglyceamic properties of *S. cordatum* leaf extracts, as far as we know, remain elusive.

Furthermore, molecular docking studies have not been reported in developing lead hypoglyceamic molecules isolated or identified from *S. cordatum*. Thus, this study investigated the antioxidant and antidiabetic potential of *S. cordatum* leaf extract fractions. In addition, the active principles in the fractions were identified and quantified using GC-MS, and an *in silico* assessment of some of the identified potential antidiabetic compounds of *S. cordatum* were evaluated.

4.2. Materials and Methods

4.2.1. Collection of plant materials

Matured leaves of *S. cordatum* were collected as described in **Section 3.2.2** of **Chapter 3**.

4.2.2. Extraction and fractionation procedure of plant material

The ground leaves (200 g) were extracted with 1 L of ethanol 80% (v/v) for 24 h through maceration on a mechanical shaker at room temperature. The resultant crude extract was then sonicated for 1 h in an ice-filled sonication bath (Branson Model 5210, Branson Ultrasonics B.V., Soest, Netherlands), filtered *in vacuo*, though a Büchner funnel and Whatman No. 1 filter paper and concentrated at 30 °C using a rotary evaporator (Heidolph vv 2000, Germany). The residue was further extracted twice at room temperature for 12 h. Consequently, 34 g of the crude extract representing 17% of the total extract yield was obtained and used for fractionation using different solvents. Subsequently, the crude extract was re-suspended in 500 ml ethanol/water (1:1) and partitioned using a separating funnel using petroleum ether (3×250 ml), followed by dichloromethane (3×250 ml) and ethyl acetate (3×250 ml). Finally, the left-over aqueous extract was freeze-dried to obtain the aqueous fraction.

4.2.3. Estimation of total phenolic content

The total phenolic contents of the *S. cordatum* fractions were quantified as described in **Section 3.2.6.1** of **Chapter 3**.

4.2.4. Estimation of total flavonoids

The *S. cordatum* fractions' flavonoid contents were quantified as described in **Section 3.2.6.2** of **Chapter 3**.

4.2.5. 1-1- Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The radical scavenging activities of *S. cordatum* fractions were carried out as described in **Section 3.2.4.1** of **Chapter 3**.

4.2.6. Ferric-reducing antioxidant power (FRAP) assay

The iron reducing power of the *S. cordatum* fractions were carried out as described in **Section 3.2.4.1** of **Chapter 3**, but ascorbic acid was used as the standard.

4.2.7. α-Amylase inhibitory activity

The α-amylase inhibitory activities of the *S. cordatum* fractions were carried out as described in **Section 3.2.5.1** of **Chapter 3**.

4.2.8. α-Glucosidase inhibitory activity assay

The α-glucosidase inhibitory activities of the *S. cordatum* fractions were done as described in **Section 3.2.5.2** of **Chapter 3**.

4.2.9. Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis

The GC-MS analysis of the PE, DCM, and EtOAc fractions were done using a Shimadzu QP-2010 SE Gas Chromatography coupled with (an Agilent) 5973 Mass Selective detectors driven by Agilent Chemstation software. A Zebron ZB-5MSplus capillary column (30 m × 0.25 mm internal

diameter, 0.25 µm film thickness) was used. Ultra-pure helium with a linear velocity of 37 cm/s and a 1.0 ml/min flow rate was used as a carrier gas. Three microliters of each fraction sample were injected into the column with the injector temperature set at 250 ℃. The initial oven temperature was set at 60 ℃, which was automated to increase at 10 ℃ per min to 280 ℃, with a holding time of 3 min at each increment. The mass spectrometer (MS) was operated in the electron ionisation mode at 70 eV, and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230 ℃, quadrupole temperature 150 ℃, solvent delay 4 min and scan range 50–700 amu. The compounds were identified by direct comparison of the mass spectrum of the analyte at a particular retention time to that of reference standards found in the 2011 National Institute of Standards and Technology (NIST) library. The area percentage of each component was calculated by comparing its average peak area to the total area obtained.

4.2.10. *In silico* molecular modelling

Since the fractionation extracts of *S. cordatum* leaf extracts displayed significant inhibitory activity towards α-glucosidase and α-amylase, the compounds detected by GS-MS were modelled *in silico* to determine their molecular interactions with the enzymes. High-resolution X-ray diffraction crystal structures of α-glucosidase (PDB ID: 5NN8; 2.45 Å) and α-amylase (PDB ID: 5E0F; 1.40 Å) were downloaded from the Protein Data Bank and were prepared for molecular docking. All nonstandard molecules, including H_2O and acarbose, were removed, and polar hydrogen atoms and Gasteiger charges were added using AutoDock Tools. The ligands were prepared by drawing three-dimensional structures from the SMILES structures using the Open Babel Server **(O'BOYLE et al., 2011)**. Energy optimisations were carried out using the PRODRG server, and

the Gromos 96 Forcefield was used to minimise the energy of the ligand structures. Coordinates of 30, 30 and 30 in x, y and z dimensions were used to construct grid boxes containing active site residues. Interacting residues from each complex were determined using PyMol Software (Version 1.7.4) and Discovery Studio.

4.2.11. Physicochemistry, pharmacokinetics and *in silico* toxicology of compounds detected by GC-MS

A comprehensive investigation of the physicochemical properties of the compounds detected in *S. cordatum* were conducted using the SwissADME server **(DAINA et al., 2017; PIRES et al., 2015)**. Physicochemical properties were assessed to determine their accordance with Lipinski's rule of five and Veber's rule. The pharmacokinetic properties of the compounds, including human intestinal absorption, solubility and oral bioavailability, were investigated employing the SwissADME and FAF-Drugs4 servers **(LAGORCE et al., 2017)**. The toxicity of the compounds in terms of probable mutagenicity (AMES), hepatotoxicity, carcinogenicity and cytotoxicity was assessed using the ProTox-II web-server **(BANERJEE et al., 2018)**.

4.2.12. Statistical analysis

The phytochemical contents, antioxidant activities and enzyme inhibitory activities data of this study were analysed using the one-way analysis of variance (ANOVA) on GraphPad prism 7. The significance of means was determined using Tukey's Multiple Range Test at P values < 0.05, and results are expressed here as the mean ± standard error of means (S.E.M).

4.3. Results and Discussion

4.3.1. Phytochemical composition of the fractions

As can be deduced from **Table 4.1**, the total flavonoid contents of *S. cordatum* leaf fractions can be ordered as follows: PE < Water < EtOAc < DCM. In comparison, a slightly different ranking (PE < DCM < Water < EtOAc) was noticed in the total phenolic contents of the same fractions. The ethyl acetate fraction contained the highest phenolic content (726.40 mg GAE/g), significantly differing from the other fractions. Phenolic contents of plant parts are considered high if it exceeds 50 mg GAE/g (**CHEW et al., 2011**), indicating high total phenolic contents in the quantified fractions. **HIDAYATI et al. (2020)** reported similar phenolic content values for their ethyl acetate (645.16 mg GAE/g) and methanol (532.93 mg GAE/g) fractions of *S. cumuni* leaves. On the other hand, though the DCM fraction (668.19 mg CE/g) had the highest flavonoid content, it was not statistically different from that of the EtOAc fraction (576. 57 mg CE/g). Consequently, the appreciable amount of flavonoids detected in all the fractions of *S. cordatum* in this study can be linked to the wide distribution of flavonoids in plants which further lay credence to their therapeutic and ecological significance **(HOENSCH and OERTEL, 2015; PANDEY and RIZVI, 2009; SAMANTA et al., 2011)**.

4.3.2. The antioxidant activities of the fractions

The DPPH radical scavenging capacities of *S. cordatum* leaf fractions significantly differed at $P < 0.05$, as revealed by the results presented in **Table 4.1**. The EtOAc fraction exhibited the highest inhibition percentage (99.61%) and the lowest IC_{50} value of 0.66 µg/ml, which is approximately six-fold lower than the standard (BHT). In the same vein, the EtOAc fraction showed the highest reducing power through its absorbance values (2.40 at 630 nm) in the FRAP assay; however, it

was not significantly different from the standard (ascorbic acid) and the water fraction **(Figure 4.1)**.

Interestingly, the richest fraction (EtOAc) in total phenolics showed the highest ferric ion reducing power and DPPH radical scavenging capacity from our data. The high antioxidant effectiveness displayed by the EtOAc fraction of *S. cordatum* is perhaps due to the medium-polarity nature of its bioactive molecules, and this concurs with the earlier findings of **WATHSARA et al. (2020)** and **ZHI et al. (2008)**, who reported high antioxidant potency of ethyl acetate fractions of *S. caryophyllatum* and *S. cumini* leaves respectively.

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Table 4.1: Enzyme inhibitory activities, phytochemical contents, and DPPH scavenging capacity of *Syzygium cordatum* leaf fractions.

Fraction	Total flavonoid content (mg CE/g)	Total Phenolic content (mg GAE/g)	DPPH radical scavenging activity		α-amylase inhibitory activity IC_{50} (µg/ml)	α-glucosidase inhibitory activity IC_{50} (µg/ml)
			IC_{50} (µg/ml)	% Inhibition		
PE	453.29 ± 2.64^{c}	59.33 ± 0.40^{d}	44.17 ± 0.52^{a}	86.56 ± 0.67^{b}	67.88 ± 0.93^{d}	54.82 ± 0.05^{a}
DCM	668. 19 ± 54.71^{a}	160.89 ± 11.71^{c}	15.52 ± 0.17^{b}	98.98 ± 0.34^{a}	116.87 ± 0.54^{a}	54.23 ± 0.05^{a}
EtOAc	576. 57 ± 2.80^{ab}	726.40 ± 12.23^{a}	0.66 ± 0.04^{e}	99.61 ± 0.00^{a}	79.53 ± 0.70^{c}	47.87 ± 0.05^{b}
Water	473.69 ± 12.70^{bc}	520.60 ± 10.45^{b}	9.321 ± 0.32^{c}	97.18 ± 0.56^{a}	84.81 ± 0.42^{b}	54.24 ± 0.05^{a}
BHT/Acarbose	---	---	6.30 ± 0.39 d	98.18 ± 0.93^{a}	63.38 ± 0.52^{e}	44.00 ± 0.04^{b}

All values are presented as mean ± standard mean of errors (SEM). Different letter(s) in each column indicate significant differences between means at $P < 0.05$ (one way ANOVA followed by Tukey's Multiple Range Test).

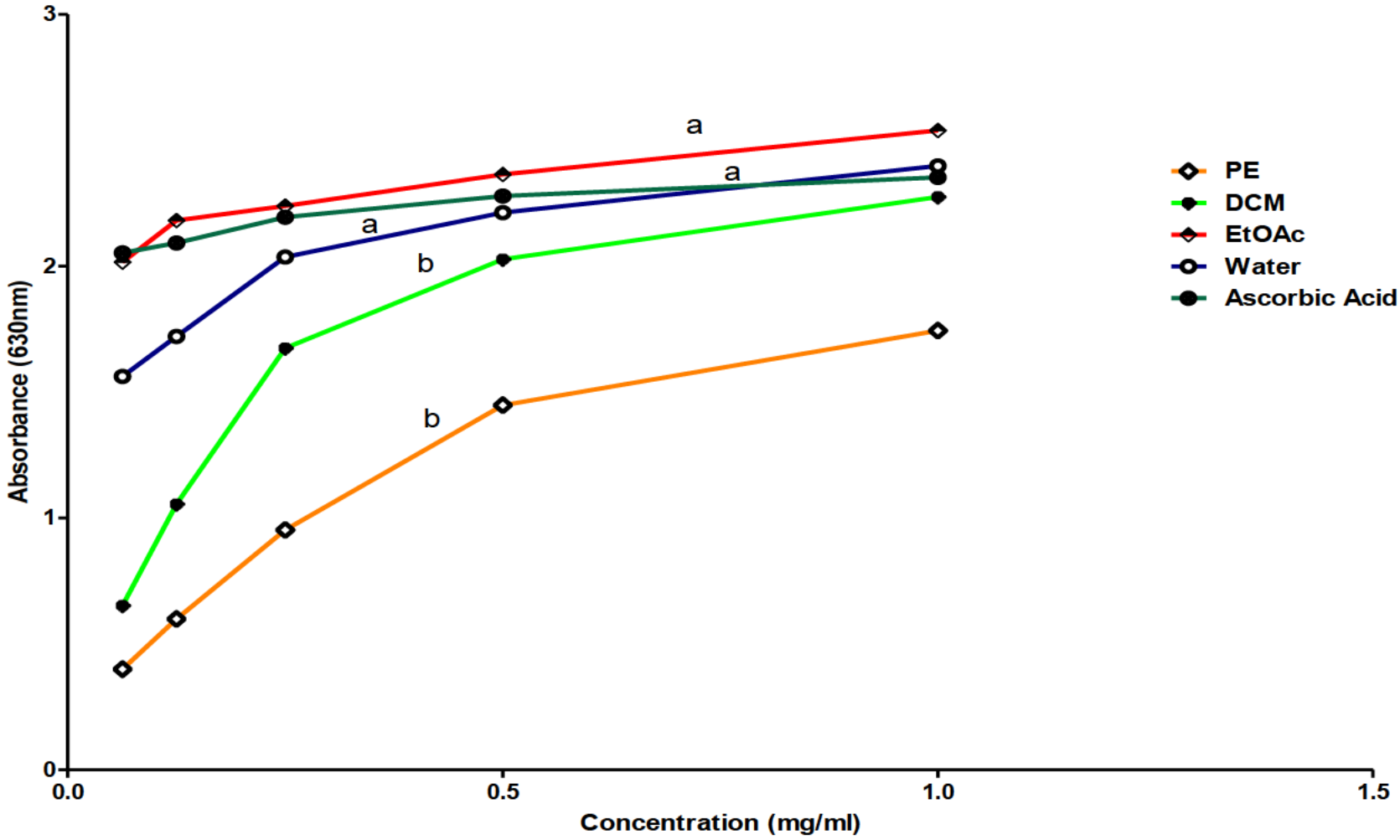

Figure 4.1: The dose-dependent ferric ion-reducing power (FRAP) of *Syzygium cordatum* fractions and ascorbic acid. Different letter(s) indicate significant differences between samples.

4.3.3. *In vitro* antidiabetic activities of the fractions

As represented in **Table 4.1**, all the fractions of *S. cordatum* leaves considerably repressed the activity of α-amylase *in vitro*. The PE fraction showed the best inhibitory activity with an IC_{50} value of 67.88 µg/ml. However, acarbose showed the highest α-amylase inhibitory activity (IC_{50} = 63.38 µg/ml), which differed significantly from the *S. cordatum* fractions (PE < EtoAc < Water < DCM). The *in vitro* inhibitory α-glucosidase IC_{50} values of the EtOAc fraction of *S. cordatum* (IC_{50} = 47.87 µg/ml) obtained in this study is slightly higher but comparable statistically to acarbose (IC_{50} = 44.00 µg/ml), and lower than the IC_{50} values of the other fractions **(Table 4.1)**. The delay in the digestion of polysaccharides (starch and other disaccharides) and the disruption of glucose absorption into the bloodstream via the retardation of α-amylase and α-glucosidase is a well-known strategy in combating the menace of postprandial hyperglycaemia **(OJO et al., 2018)**. This study shows that fractions of *S. cordatum* retarded the activities of the digestive enzymes with lower α-amylase and stronger α-glucosidase inhibitory activities, which could be attributed to the polyphenol contents of the fractions. Polyphenolic compounds are excellent antioxidants, and their ability to bind to α-amylase and α-glucosidase slows down the metabolic activities of these enzymes **(DAS et al., 2017)**, hence they are regarded as carbohydrate inhibitors or blockers **(MOEIN et al., 2017)**.

4.3.4. *In silico* assessment of the molecular interactions

Inhibitors of α-glucosidase mediate the absorption of carbohydrates from the small intestine, which reduces postprandial blood glucose and insulin levels. α-Amylase, on the other hand, breaks down polysaccharide molecules into glucose and maltose, thereby increasing postprandial and blood

glucose levels **(KAUR et al., 2021)**. Alpha-glucosidase and α-amylase inhibitors have thus become essential treatment regimens for patients inflicted with type 2 diabetes. In this Chapter, *in vitro* assessments of extracts of *S. cordatum* revealed encouraging glucosidase inhibitory activity. Compounds with the most active extracts (PE, DCM, and EtOAc) were detected by GC-MS and subsequently used to predict binding to the α-glucosidase and α-amylase enzymes *in silico*. Detailed information pertaining to the binding energy scores of the compounds detected by GC-MS with the α-glucosidase and α-amylase enzymes are presented in **Table 4.2** and **4.3**. Compounds exhibiting binding energy scores ≤ -7.5 kcal/mol were considered active **(NAIDOO et al., 2020)**. Of the 35 compounds that were screened, 21 compounds returned binding energy scores ≤ -7.5 kcal/mol. It was evident that Cubenol **(**compound **18, Figure 4.2, Table 4.2)** displayed the most encouraging binding energy score against both α-glucosidase (-8.6 kcal/mol) and α-amylase (-9.2 kcal/mol). Cubenol is a tertiary alcohol, sesquiterpenoid and a major essential oil component of many plants. Sesquiterpenoids include thousands of compounds with over 200 skeletal structures, thus forming the largest group of terpenoids **(CHEN et al., 2019)**. Several sesquiterpenoid have displayed interesting pharmacological activities as potent enzyme inhibitors. For instance, **BELHADJ et al. (2020)** described the inhibition of α-amylase by two sesquiterpenoid isolated from *Zygogynum pancheri*. Rizvi and colleagues recently isolated gorgonane sesquiterpenoid that was found to be more potent than the standard drug (acarbose) in *in vitro* assessments **(RIZVI et al., 2019)**. In the current study, compound **18** interacted with α-glucosidase through hydrogen bonding (Ala229) and hydrophobic interactions, including Van der Waals forces (Met 302, Glu231, Asn301, Leu227, Leu300, Gly228 and Asp333), and pi-alkyl interactions (Pro230, Val334, Phe297, Val335, Phe397). Compound **18** also interacted with α-amylase through hydrophobic interactions (Trp58, His305, Trp59, Gln63, Leu165, Leu162, Asp197, Arg195,

His299, Tyr62). Apart from these covalent interactions, a noncovalent interaction was also noted between compound **18** and Gln63 of α-amylase that may provide more stability to the complex **(DANDEKAR et al., 2021)**. Several essential catalytic residues, including Asp197, Glu233 and Asp300, characterise the active site of α-amylase. Compound **18** was capable of interacting with Asp197, which plays a key role in the hydrolysis of polymeric substrates. The intermolecular interactions between complexes of compound **18**-α-glucosidase and compound **18**-α-amylase are presented in **Figure 4.3**.

Additionally, compound **18** also formed interactions with Trp59 and Leu162, two residues that are also targeted by myricetin, a commercially available α-amylase inhibitor. Interestingly, andrographolide (compound **14**), a diterpenoid that was first isolated from *Andrographolis paniculata,* was also detected in *S. cordatum* **(ISLAM, 2017)**. Andrographolide is a versatile compound that has been evaluated for its ability to influence inflammatory, thrombotic, hypertensive and atherosclerotic pathways **(AMROYAN et al., 1999)**. The compound has also been evaluated in *in vivo* assays as a potential drug against type 2 diabetes. Subramanian and colleagues revealed that andrographolide significantly reduced peak blood glucose and urea in diabetic rats **(SUBRAMANIAN et al., 2008)**. In the current study, andrographolide returned binding energy scores of -7.9 kcal/mol and -8.2 kcal/mol against α-glucosidase and α-amylase, respectively. Hence it is feasible to suggest that the *in vitro* inhibition of these enzymes may be due to the presence of andrographolide. In addition to Asp197, several of the compounds detected in *S. cordatum,* including andrographolide, could interact with Glu233 and Asp300 **(Table 4.3)**. Amino acid residue Asp300 optimises the orientation of the substrate, while Glu233 plays a role in acid-based catalysis during its hydrolysis **(WILLIAMS et al., 2012)**. The ability of the compounds detected in *S. cordatum* to interact with these residues and form hydrogen or

hydrophobic (and covalent interactions with Gln63) interactions validates the extracts *in vitro* inhibitory activity towards α-glucosidase and α-amylase and suggests the potential for use as treatment regimens for Type 2 diabetes.

Table 4.2: Bioactive compounds identified from the leaf extracts (fractions) of *Syzygium cordatum* quantified using GC-MS.

Compound	Name	Fraction	Peak	Similarity index %	Retention time (RT)
1.	2,3-Bis(1-methylallyl)pyrrolidine	EtOAc	94	72	16.05
2.	2,6-Dichloro-4-nitrophenyl-.beta.-phenylpropionate	PE	58	68	15.63
3.	2-(2-Hydroxy-2-methyl-4-phenyl-but-3-ynylamino) hexanoic acid	EtOAc	32	45	9.97
4	2-Methyl-3,5-dinitrophenyl-3-phenylpropanoate	PE	58	69	15.63
5	2-Methyl-4-pentyltetrahydro-2H-thiopyran-1,1-dioxide	EtOAc	42	70	11.04
6	3,3,5,6-Tetramethyl-1-indanone	EtOAc	43	62	11.17
7	3-Ethyl-3-hydroxyandrostan-17-one	PE	47	83	14.74
8	4-Chloro-2-nitrophenyl-beta-phenylpropionate	PE	58	68	15.63
9	4-Methyl-2,6-dihydroxyquinoline	PE	52	73	15.05
10	4-tert-Butylbenzaldehyde-((E)-amino [oxido (oxo) hydrazono] methyl) hydrazone	PE	61	61	15.97
11	5-(1-Ethylvinyl)-4-methyl-5-(2-methyl-2-propenyl)-2(5H)-furanone	EtOAc	93	72	15.90
12	6-epi-shyobunol	EtOAc	97	71	16.44
13	7-Azabicyclo[4.3.0]nonan-8-one,_5-benzyloxy-	EtOAc	101	53	17.32
14	Andrographolide	PE	31	57	13.56
15	Aspidofractinine-3-methanol	EtOAc	106	76	18.23
16	Benzylidene-iditol	DCM	15	64	7.35

17	Bis[3,3,4,7-tetramethyl-1,3-2H-benzofuran-1-yl]-ether	PE	52	74	15.05
18	Cubenol	PE	40	84	14.14
19	Cyclo-(S-2-mercaptopropionyl-S-phenylalanyl-S-prolyl)	EtOAc	70	63	13.51
20	Deoxyspergualin	EtOAc	38	61	10.57
21	Ethyl-iso-allocholate	DCM	39	62	11.39
22	Imidazole-5-carboxylic-amide,-N-methyl	EtOAc	46	63	11.33
23	Longiverbenone	PE	46	75	14.64
24	Methyl-3-butyl-4-nitro-4-pentenoate	PE	9	72	11.21
25	Methyl-N-(N-benzyloxycarbonyl-beta-l-aspartyl)-beta-d-glucosaminide	DCM	15	65	7.35
26	N-(3-Imidazol-1-yl-propyl)-N'-(4-isopropyl-phenyl)-oxalamide	EtOAc	66	60	13.07
27	Pregna-1,4,7,16-tetraene-3,20-dione	PE	31	59	13.56
28	Pregnane-3,17,20-triol,_(3.alpha.,5.beta.,20S)-	DCM	81	63	17.43
29	Spiro[7H-benz[e]indene-7,1'-[2]cyclopentene]- 4',9(8H)-dione	PE	31	57	13.56
30	[1,2,4]Oxadiazole,-5-benzyl-3-(thiophen-2-yl)-	EtOAc	70	62	13.51
31	cis-4-methoxy-thujane	EtOAc	81	67	14.49
32	p-Toluidine,_N-methyl-N-nitroso-	EtOAc	31	80	9.85
33	tau-Cadinol	PE	42	93	14.28
34	trans-Sesquisabinene-hydrate	PE	40	84	14.14

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Table 4.3: Molecular interaction results of α-glucosidase and α-amylase enzyme proteins with the bioactive compounds of *Syzygium cordatum* quantified using GC-MS.

Compound	α-Glucosidase		α-Amylase	
	Binding Energy Score (kcal/mol)	**Interacting Residues**	**Binding Energy Score (kcal/mol)**	**Interacting Residues**
1	-5,8	Phe206, Phe166, Asp333, Thr203, Gly273, Phe297, Gly228, Ile146, Val334, Arg400, Phe147, Tyr489, Asp202, Arg200, Glu271	-5,4	His101, His299, Asp197, Try58, Leu162, Tyr62, Leu165, Gln63, Thr163, Try59, Ala198
2	-7,5	Asp62, Arg400, Tyr65, Asp333, Phe155, Thr203, Glu271, Phe206, Gly273, Gly273, Thr226, Asp274, Leu244, **Tyr235**, Gly228, Ile146, Ala229, Tyr389, Phe297, His105, Asp202, Phe147, Gln170	-6,8	Leu165, His305, Try59, Arg195, Tyr62, Trp58, His299, Asn298, Asp197, Ile235, Glu233, Ala198, Leu162
3	-8,2	**Arg400**, Gly273, Thr203, Asp202, Phe166, Phe147, Leu227, Leu300, Asn301, Arg340, Val335, Phe397, Ala229, Pro230, Tyr389, Phe206, Asp333, Glu271	-6,3	**Asn301**, **His305**, **Asn298**, His299, Trp58, Arg195, Asp197, His201, Ala198, Ile235, Lys200, Leu162, Tyr62, Glu233, Phe256, Ala307, Gly306, His305
4	-8,4	**Arg400**, Phe397, Phe297, Gly228, Tyr389, Arg400, Phe147, Ile146, Phe206, Gly273, Phe166, Thr203, Asp202, His105, Val334, Glu271, Asp333, Ala229, Leu227, Pro230, Asn301	-7,6	Try59, Arg195, Phe256, Glu233, Asp197, Asn298, Ile235, His299, Gly306, Asn301, His305, His201, Tyr62, Ala198, Trp58
5	-6,6	**Gly228**, Arg200, Arg400, Phe147, Phe297, Gly228, Tyr389, Ile146, Ala229, Glu271, Phe206, Gly273, Asp333, His105, Thr203, Gln, 170, Asp62, Phe166, Tyr65, Asp202	-5,8	**Asn301**, **Asn298**, **His305** gly306, His299, Glu233, Tyr62, Arg195, Try58, Asp197, Trp59, Ile235, Phe256, Ala307

6	-6,5	**Leu300**, Tyr389, Leu227, Phe397, Asp333, Phe297, Ala229, Gly228, Pro230, Val335, Val334, Arg340, Asn301	-7	Try59, Leu165, His299, Asp197, Arg197, Arg195, Asn298, Glu233, Ile235, Ala198, Leu162, Trp58, Tyr62
7	-7,6	Ala229, Leu227, Asn301, Phe297, Val334, Arg340, Lys398, Glu396, Pro395, Val335, Phe397, Pro230	-8,3	Trp59, Trp58, His305, Asn298, Glu233, Arg195, Tyr62, His299, Leu162, Leu165, Thr163, Gln63, *Gln63*,
8	-7,6	**Arg400**, Phe397, Tyr389, Asp333, Phe147, Ile146, Phe166, Glu271, Phe206, Thr203, Gly273, Gly228, Val334, Phe297, Pro230, Ala229	-7,1	**Arg389**, **Lys322**, Gln390, Glu484, Val383, Arg343, Trp388, Ala318, Arg392, Thr376, Arg387, Thr377
9	-7,4	**Glu271**, Asp202, Phe166, Thr203, Ile272, Phe297, Ile146, Phe206, Gly273, Gly228, Phe147, Tyr389, Asp333	-6,1	**Arg398**, **Thr11**, **Ser289**, Thr336, Asp402, Pro332, Gly334, Asp290, Arg252, Phe335
10	-7,5	Phe206, Ile146, Gly228, Arg400, Val334, Tyr389, Phe397, Pro230, Val335, Asp333, Glu271, Phe166, Phe297, Thr203, Gly273, Ile272	-7	**Asn301**, **Asn298**, Gly306, Ala307, His299, His305, Phe256, Ile235, Glu233, Tyr62, Leu162, Leu165, Thr163, Gln63, Trp59, Trp58, Arg195, Asp197
11	-5,8	Met302, Asn301, Arg340, Pro230, Phe397, Ala229, Tyr389, Gly228, Asp333, Phe297, Val334, Leu300, Val335	-6,1	**Arg195**, **His299**, Asp197, His101, Ala198, Leu165, Leu162, Gln63, Tyr62, Trp59, rp58, His305
12	-6,6	Tyr389, Phe147, Phe166, Asp333, Arg400, His105, Arg200, Asp202, Thr203, Glu271, Ile146, Phe206, Gly228, Gly273, Phe297	-6,3	Trp59, His299, Trp58, Ala198, Tyr62, Arg195, Asp197, Leu162, His101, Leu165, Gln63, *Gln63*
13	-7,7	**Arg400**, Tyr389, Ile146, Gly228, Phe206, Gly273, Thr203, Gly273, Glu271, Arg200, His332, Tyr65, Asp62, Asp202, Gln170, Asp333, Phe147	-7,3	Arg195, His299, Asp197, Tyr62, His101, Leu165, Leu162, Tyr151, His201, Lys200, Ile235, Lys200, Ala198, His305, Asn298Trp58

14	-7,9	**Lys398**, **Leu300**, Glu396, Glu231, Met302, Phe397, Ala229, Pro230, Gly228, Asn301, Leu227, Phe297, Val334, Asp333, Arg340, Val335, Gly399	-8,2	**Asn301**, **His299**, **Trp59**, Asn298, Glu233, Trp58, Arg195, Leu165, Thr163, Trp59, Leu162, His101, Tyr62, Asp197, His305, Gly306
15	-6,6	Arg456, Phe463, Val12, Asp48, Asn4, Trp7, Phe463	-5,8	His305, Gly306, Asn301, Ala307, Asn298, Phe256, Ile235, Glu233, Ala198, Asp300, Arg195, Asp197, Leu162, Tyr62, Try58, Trp59, His299
16	-8,2	**Asn301**, Asp333, Phe397, Arg340, Val335, Val334, Leu300, Phe297, Leu227, Met302, Ala229, Thr226, Glu231, Lys225, Pro230	-8,5	**His305**, **Arg195**, **Glu233**, **Ala198**, Gly306, Ala307, Asn298, His299, Trp58, Gln63, Tyr62, Trp59, Leu165, Leu162, Ile235
17	-7,9	**Asn301**, Leu227, Thr226, Lys225, Ala229, Asn301, Leu300, Arg340, Val335, Val334, Phe397, Pro230, Glu231, Met302	-8	Thr377, Gln390, Trp233, Lys322, Arg343, Ala318, Val383, Phe315, Trp316
18	-8,6	**Ala229**, Met302, Glu231, Pro230, Asn301, Leu227, Leu300, Val334, Phe297, Gly228, Val335, Asp333, Phe397	-9,2	Trp58, His305, Trp59, Gln63, Leu165, Leu162, Asp197, Arg195, His299, Tyr62
19	-6,8	**Val335**, Met302, Asn301, Phe297, Gly228, Ala229, Tyr389, Arg340, Val334, Asp333, Phe397, Pro230, Glu396, Glu231	-6,7	**Pro332**, **Ser298**, **Gly334**, Gly403, Arg398, Arg421, Asp402, Pro4, Phe335, Tyr333, Thr11, Arg252, Asp290
20	-7,5	**Gly399**, **Asp333**, **Gly228**, **Tyr235**, **Gly273**, Val334, Arg340, Pro230, Asn301, Val335, Leu300, Lue227, Tyr389, Phe297, Arg400, Ala229, His240, Leu244, Asp275, Asp274, Asn205, Glu271, Thr226, Phe206, Thr203, Ile146, Phe166, Phe397, Glu377, Cys403	-8	**Thr6**, **Asp402**, **Arg421**, **Gly403**, **Pro332**, Arg398, Gln8, Asn5, Thr11, Val401, Phe335, Ser289, Tyr333, Gly9, Pro4

21	-6,8	Phe147, Gly228, Leu300, Asp333, Pro230, Asn301, Leu300, Leu227, Asn301, Arg340, Phe397, Met302, Glu396, Lys398, Phe297, Val335, Val334, Ala229, Tyr389, Arg400	-5,2	**Gly403**, **Arg421**, **Asn5**, **Thr6**, Ser289, Gly334, Phe335, Arg10, Gly9, Gln8, Pro4, Thr11, Asp402, Ser3, Pro332, Arg398, Val401, Arg252
22	-7,5	**Glu271**, **Asp333**, **Asp202**, His332, Arg200, Tyr65, Thr203, Phe166, Arg400, Phe147, Phe297	-8	**Pro332**, **Gly334**, **Asp402**, Arg421, Tyr333, Arg252, Ser289, Phe335, Thr336, Thr11, Gly403
23	-5,1	**Asp333**, Ala229, Arg400, Lys398, Met302, Val335, Leu300, Arg340, Phe397, Asn301, Val334, Pro230	-4,7	**Arg195**, Asn298, Leu162, Phe256, Glu233, Ile235, Ala198, His101, His305, Tyr62, His299, Asp197, Trp58
24	-7,2	**Arg400**, Asp333, Gly273, Tyr389, Ile146, Gly228, Phe297, Glu271, Thr203, Phe166, Asp202, Tyr65, Asp62, Gln170, His105, Phe147	-7,4	**Ser289**, **Arg421**, Arg252, Val401, Asp402, His331, Pro332, Arg398, Gly403, Phe335, Thr11, Gly334
25	-5,8	**Asp333**, Phe297, Val334, Pro230, Ala229, Gly228, Arg400, Tyr389	-5,2	**Arg195**, **Asn298**, **Asn301**, His201, Ile235, Ala198, Lys200, Glu233, Phe256, His299, Gly306, Ala307, Asp197, Trp58, His305, His101, Tyr62, Gln63, Leu165, Trp59, Tyr151, Leu162
26	-7,6	**Gly228**, Asp202, Thr203, Arg200, Glu271, Asp333, Val334, Phe397, Val335, Leu227, Asn301, Phro230, Ala229, Tyr389, Ile146, Arg400, Phe166, Phe147, Asp62, Thr203	-7	**Thr163**, Ala198, Asp197, Arg195, His299, Tyr62, Trp58, Trp59, Gln63, Gly104, Ala106, Asn105, Gly164, Leu165, His101
27	-8,2	Glu396, Met302, Arg340, Leu300, Asn301, Val334, Phe334, Phe297, Ala229, Leu227, Val335, Gly228, Phe397, Pro230, Asp333	-6,8	**His305**, **Asn301**, Trp59, Phe256, Asp197, Glu233, Asn298, Ile235, His299, Gly306, Leu162, Tyr62, His201, Trp58, Ala198
28	-8,1	**Leu227**, **Glu396**, Phe397, Met302, Val335, Pro230, Pro395, Lys398, Arg340, Leu300, Val334,	-5,9	**Trp59**, **Asn301**, Leu165, Tyr62, Thr163, Gln63, Glu233, Trp58, Ile235, Ala307, His305, Gly306, His299, Asn298

29	-8,1	Asp333, Leu300, Met302, Phe297, Val334, Arg340, Asn301, Glu231, Ala232, Glu396, Phe397, Pro230, Val335, Ala229	-8,3	**Asp197**, **Glu233**, **Arg195**, His101, Leu165, Gln63, Thr163, Trp59, Leu162, Ile235, His299, Ala198, Tyr62
30	-7,9	Asp333, Arg340, Met302, Glu231, Ala232, Glu396, Phe397, Pro230, Val335, Ala229, Leu227, Phe297, Val334, Leu300, Asn301	-8,2	Ala198, Thr163, Leu162, His101, Asp197, Glu233, Arg195, His299, Tyr62, Trp58, Trp59, Gln63, Leu165
31	-7,8	Gly273, Glu271, Ile146, Phe297, Phe147, Tyr389, Arg400, Asp333, Gly228, Phe166, Phe206, Thr203	-5,3	**His299**, Trp58, Trp59, Leu165, Leu162, His101, Ala198, Tyr62, Asp197, Arg195, Asn298
32	-5,8	Gly228, Tyr389, Ile146, Phe147, Arg400, Thr203, Asp202, Tyr65, His332, Asp333, Glu271, Tyr295, Phe166	-5,6	**Gly334**, Pro4, Thr336, Thr11, Asp402, Phe335, Arg252
33	-6,6	Gly399, Arg400, Val335, Val334, Phe397, Phe297, Leu227, Leu300, Gly228, Arg340, Ala229	-6,9	Tyr62, Leu165, Trp58, Trp59, Leu162, Thr163, Gln63
34	-6,8	**Gly228**, Thr203, Phe166, Gly273, Ile146, Phe206, Glu271, Tyr389, Asp333, Arg400, Phe147, Tyr65, Asp62, Gln170, Asp202, Phe297, His105	-7,2	**Gly334**, Pro332, 421, Arg398, Thr11, Ser3, Pro4, Arg252, Phe335, Asp402

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Table 4.4: Physicochemical, pharmacokinetic and toxicological properties of the bioactive compounds of *Syzygium cordatum* quantified using GC-MS.

Compound	Mol. Wt. (dalton or g/mol)	No. Rotatable Bonds	No. Hydrogen Bond Acceptors	No. Hydrogen Bond Donors	cLog P	Solubility	GI Absorp-tion	Toxicity			
								Mutagenicity	Hepatotoxicity	Cytotoxicity	Carcinogenicity
1	179.30	4	1	1	2.89	Soluble	High	Inactive	Inactive	Inactive	Inactive
2	340.16	6	4	0	3.50	Moderate	High	Active	Inactive	Inactive	Active
3	289.37	7	4	3	1.55	Soluble	High	Inactive	Inactive	Inactive	Inactive
4	330.29	7	6	0	2.09	Moderate	High	Active	Inactive	Inactive	Inactive
5	218.36	4	2	0	2.94	Soluble	High	Inactive	Inactive	Inactive	Inactive
6	188.27	0	1	0	3.21	Moderate	High	Inactive	Inactive	Inactive	Inactive
7	318.49	1	2	1	4.22	Moderate	High	Inactive	Inactive	Inactive	Inactive
8	305.71	6	4	0	3.09	Moderate	High	Active	Inactive	Inactive	Active
9	175.18	0	2	0	1.54	Soluble	High	Active	Active	Inactive	Inactive
10	263.30	5	4	2	1.40	Soluble	High	Active	Active	Inactive	Active
11	206.28	4	2	0	3.15	Soluble	High	Inactive	Inactive	Inactive	Inactive
12	222.37	3	1	1	3.79	Soluble	High	Inactive	Inactive	Inactive	Inactive
13	245.32	3	2	1	2.10	Moderate	High	Inactive	Inactive	Inactive	Inactive
14	350.45	3	5	3	2.33	Soluble	High	Inactive	Inactive	Inactive	Inactive
15	310.43	1	2	2	2.65	Moderate	High	Inactive	Inactive	Inactive	Inactive
16	358.39	4	6	2	1.69	Soluble	High	Inactive	Inactive	Inactive	Inactive

17	366.49	2	3	0	5.09	Moderate	High	Inactive	Inactive	Inactive	Inactive
18	222.37	1	1	1	3.52	Soluble	High	Inactive	Inactive	Inactive	Inactive
19	332.42	2	3	1	1.61	Moderate	High	Inactive	Inactive	Inactive	Inactive
20	387.52	19	6	7	-0.35	Soluble	Low	Inactive	Inactive	Inactive	Inactive
21	452.67	6	5	3	3.01	Soluble	High	Inactive	Inactive	Inactive	Inactive
22	125.13	2	2	2	-0.20	Soluble	High	Inactive	Inactive	Inactive	Inactive
23	218,33	0	1	0	3.42	Soluble	High	Inactive	Inactive	Inactive	Inactive
24	215.25	8	4	0	1.71	Soluble	High	Active	Inactive	Inactive	Inactive
25	442.42	12	10	6	-1.00	Soluble	Low	Inactive	Inactive	Inactive	Inactive
26	314.38	9	3	2	1.82	Moderate	High	Inactive	Inactive	Inactive	Inactive
27	263.04	2	6	3	-1.25	Soluble	High	Inactive	Inactive	Inactive	Inactive
28	308.41	1	2	0	3.67	Soluble	High	Inactive	Inactive	Inactive	Active
29	336.51	1	3	3	3.37	Soluble	High	Inactive	Inactive	Inactive	Inactive
30	370.48	2	4	0	3.62	Moderate	High	Inactive	Inactive	Inactive	Active
31	168.28	2	1	0	2.80	Soluble	High	Inactive	Inactive	Inactive	Inactive
32	150.18	2	2	0	2.03	Soluble	High	Active	Inactive	Inactive	Active
33	222.37	1	1	1	3.44	Soluble	High	Inactive	Inactive	Inactive	Inactive
34	222.37	4	1	1	3.79	Soluble	High	Inactive	Inactive	Inactive	Inactive

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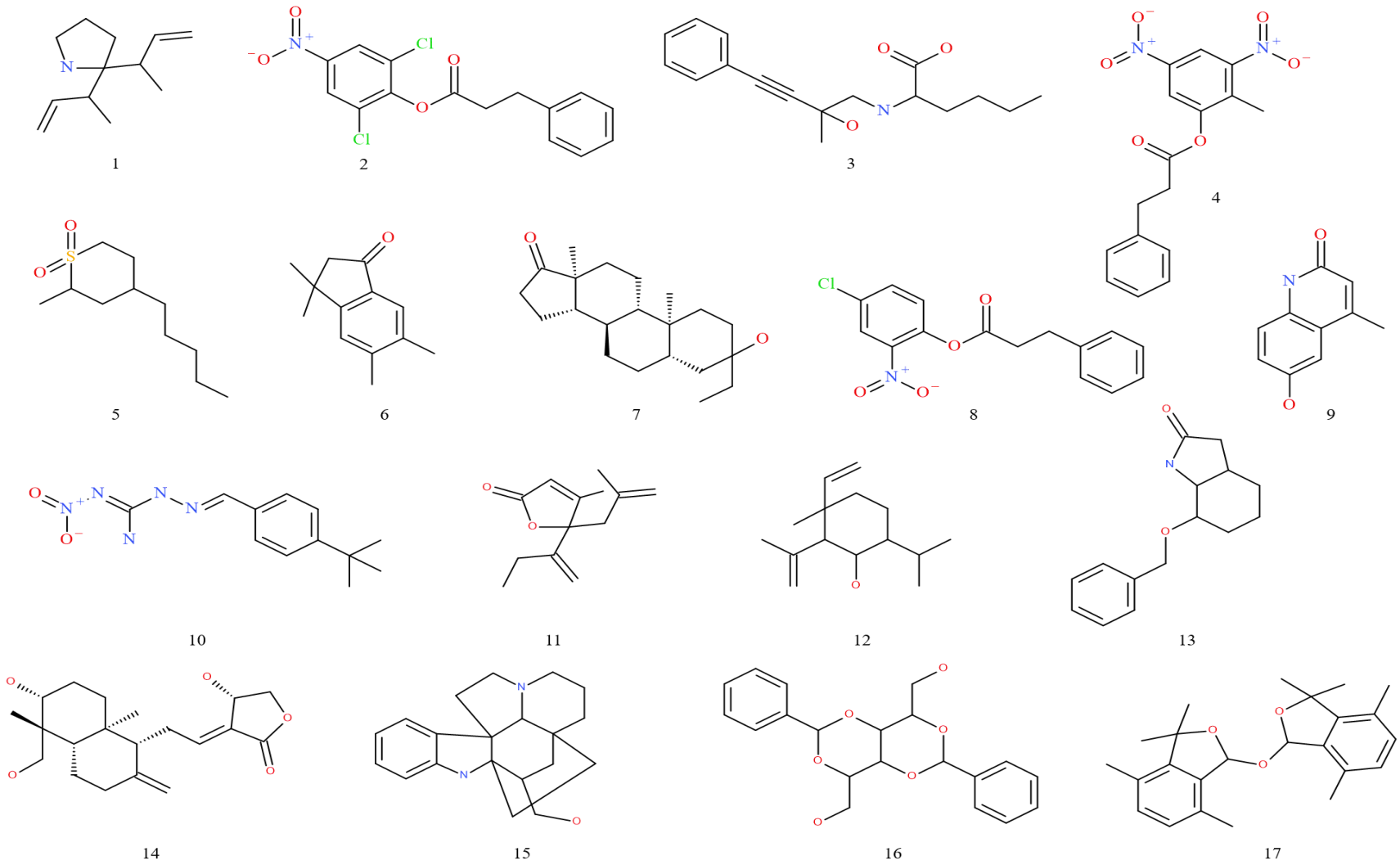
4.3.5. Physicochemistry, pharmacokinetics and *in silico* toxicology of compounds detected by GC-MS

The pharmacokinetics of drug leads are most frequently estimated by assessing the physicochemical properties of a compound **(NAIDOO et al., 2020)**. We evaluated the physicochemical properties of the compounds detected in extracts of *S. cordatum* in accordance with Lipinski's rule of five and Veber's rule. Lipinski's rule of five considers the logarithm of partial coefficient (cLog$P \leq 5$), molecular weight (not more than 500 dalton or g/mol), number of hydrogen bond acceptors (not more than 10) and donors (not more than 5) and the number of rotatable bonds (not more than 10) to make accurate predictions of the oral bioavailability and membrane permeability of a drug compound **(LIPINSKI et al., 2012)**. The compounds detected in *S. cordatum*, with the exception of compounds **20** and **25**, seemed to conform to Lipinski's rule of five **(Table 4.4)**. Despite its encouraging affinity for both α-glucosidase (-7.5 kcal/mol) and α-amylase (-8.0 kcal/mol), compound **20** violated the limit for the number of hydrogen bond donors (7) and as a result, the compound was predicted to display poor gastrointestinal absorption. Additionally, although several of the compounds were found not to violate the rule of five, a few of these compounds were predicted to be toxic. For instance, 4-Methyl-2,6-dihydroxyquinoline (**9**) was predicted to be mutagenic and hepatotoxic. The Virtual Network Name (vNN) server predicts mutagenicity based on an Ames mutagenicity model consisting of 6512 compounds. The model predicts mutagenicity with an overall accuracy of between 79 and 85%. Hydroxyquinolines have several uses in the pharmaceutical and agribusiness sectors, however, mutagenicity within this group has previously been reported for 8-hydroxyquinoline that tested positive against *Salmonella* tester strains TA92 and TA100 **(SKEGGS and COOK, 1978)**.



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1784 **Figure 4.2:** Structures of some of the identified compounds from the GC-MS analysis of *Syzygium cordatum* leaf extracts.

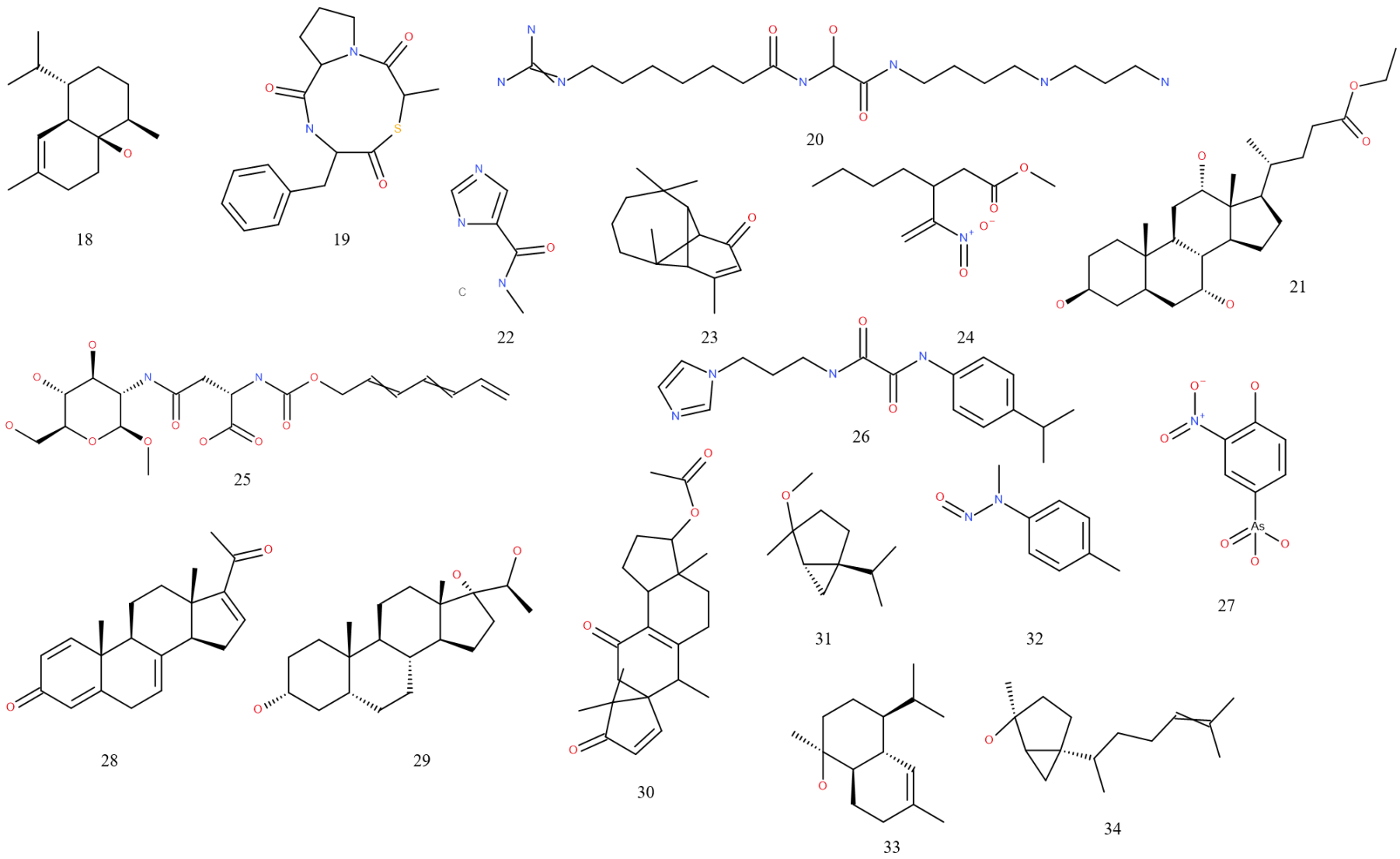

Figure 4.2: continued.

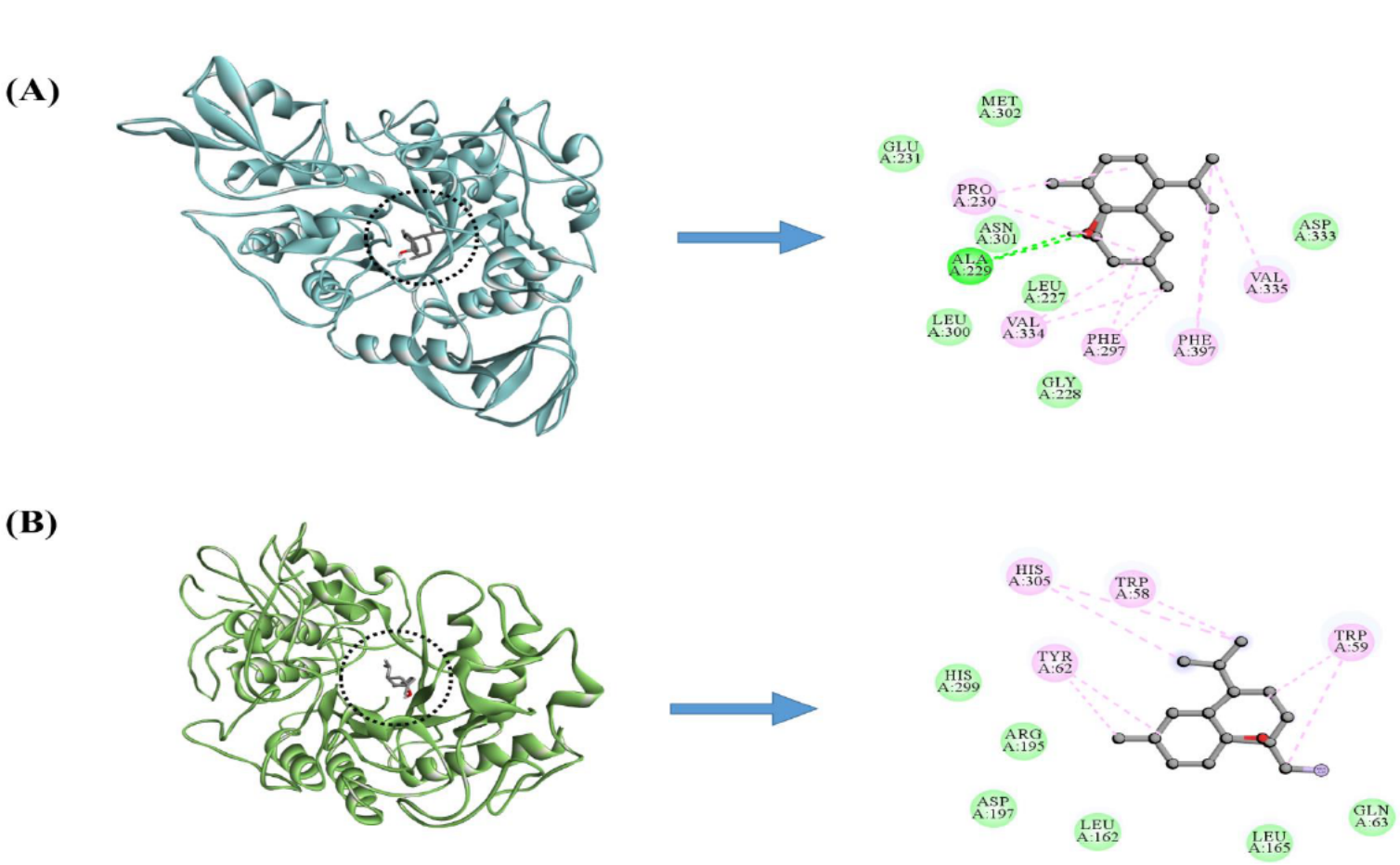

Figure 4.3: Interactions of compound **18** with important enzymes involved in the pathophysiology of type 2 diabetes. (A) α-glucosidase is represented by a cyan ribbon. (B) α-amylase is represented by a green ribbon. Compound **18** is represented by a grey ball and stick figure. Hydrogen bonds are represented by dotted green lines, while hydrophobic interactions are represented by dotted purple lines. Van der Waals forces are represented by a light green sphere.

On the other hand, based on physicochemical properties, compound **18** was predicted to be soluble and to display high gastrointestinal solubility. The compound was also predicted to be non-toxic in terms of mutagenicity, hepatotoxicity, carcinogenicity and cytotoxicity. These results and the affinity of compound **18** for α-glucosidase and α-amylase, as depicted earlier, suggest an important role for the compound in treating type 2 diabetes and prompt further experimental work.

4.4. Conclusion

Syzygium cordatum leaf extract fractions exhibited encouraging *in vitro* antioxidant and hypoglycemic activities in this study. The GC-MS analysis of the non-polar and mid-polar fractions showed that phytocompounds such as Andrographolide, Bis[3,3,4,7-tetramethyl-1,3-2H-benzofuran-1-yl]-ether, Cubenol, Deoxyspergualin and 3-Ethyl-3-hydroxyandrostan-17-one are some of its likely antidiabetic active principles. The *in silico* physicochemistry, pharmacokinetics and toxicity predictions of some bioactive phytocompounds revealed that Andrographolide, Bis[3,3,4,7-tetramethyl-1,3-2H-benzofuran-1-yl]-ether and Cubenol are soluble and displayed high gastrointestinal solubility. These compounds did not equally show any hepatoxic, mutagenic, cytotoxic or carcinogenic properties. For further studies, proper isolation (from *S. cordatum*), identification (using techniques such as NMR) and *in vivo* hypoglycemic activity evaluation of these compounds should be considered.

CHAPTER 5: Drought tolerance and plant-growth-promoting potentials of endophytes isolated from *Endostemon obtusifolius* (E. Mey. ex Benth.) N. E. Br.

This chapter was written following the format of the South African Journal of Botany.

5.1. Introduction

Fluxes in environmental factors predispose plant species, including valuable indigenous plants, to different types of abiotic stress such as water deficit, salinity, heavy metal toxicity, high and low-temperature stress, UV radiation stress, and nutrient deficiency **(IMADI et al., 2015)**. Generally, abiotic stress causes havoc and influences the growth, development, and related metabolic pathways responsible for accumulating valuable phytochemicals in medicinal plants **(RAMAKRISHNA and RAVISHANKAR, 2011)**. Water scarcity is the most familiar and significant environmental issue affecting many world regions **(DIKILITAS et al., 2016)**. Drought stress is unique because it is directly linked to other stress types, including salinity and heat stress **(DIKILITAS et al., 2016)**. Researchers have widely reported the negative impacts of water deficit on plants **(ABUQAMAR et al., 2009; XU et al., 2010)**, and its consequences are predicted to be more devastating in the coming decades **(JOETZJER et al., 2014)**. In plants, severe drought stress causes water loss, stomatal closure, limits gaseous exchange, disrupts nutrient uptake, impairs metabolic activities and cell division, stimulates ROS accumulation in cells and sometimes leads to cell damage or death **(JALEEL et al., 2008)**. Consequently, many valuable indigenous species in some parts of the world have been lost to extreme drought conditions **(LIU et al., 2019)**. However, depending on the drought intensity, exposure period, genetic makeup and growth stage of the plants, certain plants tolerate or survive drought with a series of biochemical and physiological processes mediated by their mutualistic association with microbial endophytes **(SINGH et al., 2017; OGBE et al., 2020)**. Moreover, plant-endophyte interactions mainly promote plant growth and health **(SILVA et al., 2020)**, consequently improving a plants' resistance abilities to combat environmental stress **(EGAMBERDIEVA et al., 2017; GLICK, 2012)**.

Endophytes are primarily endosymbiotic bacteria and fungi that colonise plants' interior tissues and may be cultured *in vitro* after a series of sterilisation procedures **(FIGUEIREDO et al., 2009)**. Specifically, several medicinal plants' associated endophytes have demonstrated capability of synthesising a range of natural products that are useful in promoting plant growth under moderate or stressful environmental conditions **(NAVEED et al., 2014)**. Medicinal plants' associated endophytes are highly remarkable because of their diversity and probiotic biosynthesis **(WICAKSONO et al., 2017)**. Endophytes stimulate plant growth, development, and productivity by solubilising inorganic phosphates **(NIMAICHAND et al., 2016)**, decreasing ethylene production in the plants **(VURUKONDA et al., 2016)**, and fixing inert atmospheric nitrogen **(NIMAICHAND et al., 2016)**. The beneficial impacts of endophytes may also be through the synthesis of hydrolytic enzymes, plant growth regulators (such as auxins) **(VURUKONDA et al., 2018)**, ammonia **(YAISH et al., 2015)**, and siderophores **(LIN and XU, 2013)**.

Globally, plant species in the Lamiaceae (mint) family are the most explored indigenous plants owing to their use as herbs and spices **(MARWAT et al., 2011**), memory boosters and antioxidants **(ORHAN et al., 2007)**. *Endostemon obtusifolius* (E. Mey. *ex Benth*.) N.E.Br., a relatively unknown and under-explored member of the mint family and its pharmacological activities have not been verified. **SADASHIVA et al. (2014)** reported the free radical scavenging and acetylcholinesterase inhibiting properties of *E. obtusifolius*. Nonetheless, the endophytic composition of its organs, the drought tolerance capability, and plant growth-promoting properties of the endophytes remain elusive. Therefore, this study aimed at exploring the bacterial and fungal endophytes in the leaves and roots of *E. obtusifolius* and to investigate the *in vitro* plant growth-promoting properties and drought tolerance of the endophytic species.

5.2. Materials and Methods

5.2.1. Plant sample collection

Following COVID-19 restrictions and the bottlenecks encountered while attempting to raise *S. cordatum* seedlings in the greenhouse, *E. obtusifolius* was selected as a test plant in **Chapters 5, 6** and **7**. *Endostemon obtusifolius* is a fast-growing shrub and it showed a good antioxidant and antidiabetic capabilities in **Chapter 3**.

Healthy and disease-free leaves and roots of *E. obtusifolius* plants were harvested from the University of KwaZulu-Natal (UKZN), Pietermaritzburg (PMB) Campus Botanical Garden. The plant parts were transferred to the lab in sterile biosafety plastic bags filled with ice.

5.2.2. Surface sterilisation and isolation of endophytes

The plant materials were surface sterilised following a modified procedure of **HASSAN (2017)**. The detached infection-free leaves and roots were initially cleaned under running tap water repeatedly for half an hour to remove epiphytic microorganisms, dust and other adhering soil particles, followed by washing with Tween 20 detergent (3 drops) for 1 min. Thereafter, the plant materials were dipped in 0.1% carbendazim solution with constant agitation for 20 min and then washed with sterile distilled water four times. Then, the root and leaf explants were separately immersed into 70% ethanol for 60 s, followed by a treatment in 2% NaOCl for another 60 s, and they were again treated with 70% ethanol for 30 s and finally washed five times with sterile distilled water to remove all traces of the sterilising agent left on the explants. Afterwards, the explants were dried on sterile paper towels and dissected into 2 cm pieces and then imprinted (pressed) onto freshly prepared Oxoid™ Nutrient agar (NA) and Potato dextrose agar (PDA) as a disinfection control. To further ascertain the effectiveness of this sterilisation protocol, aliquots

(30 µl) of sterile distilled water were used in the final rinse of the explants were also plated onto the culture media. The success of the surface sterilisation method was confirmed by the absence of microbial growth on the culture media.

Culturable fungal endophytes were isolated from the internal tissues of the sterilised leaves. Root explants were cultured on plates containing freshly prepared PDA amended with streptomycin (50 mg/ml) and incubated at 30 ± 2 ºC for 7 days following a modified method described by **KHAN et al. (2015)**. The bacterial endophytes were isolated on freshly prepared NA as reported by **JIMTHA et al. (2014)** and incubated at 30 ± 2 ºC for 3 days. After incubation, endophytic bacterial colonies found adjacent to the explants, and endophytic fungi filaments that emerged from the internal tissues, were carefully transferred to fresh NA and PDA plates respectively, and pure cultures and sub-cultures were obtained and stored. All the sterilisation and inoculation activities were carried out aseptically on a laminar flow cabinet. Pure culture plates were stored at 4 ºC till further use.

5.2.3. Extracellular metabolite extraction from endophytes

Microbial secondary metabolites were extracted from the isolated endophytic fungi and bacteria following the modified methods described by **HIGGINBOTHAM et al. (2013)** and **DELJOU and GOUDARZI (2016)**. Briefly, for endophytic fungi, a single disc of each isolated fungus was cautiously excised from actively growing pure culture tips and transferred aseptically into a freshly prepared 100 ml of Yeast malt broth (YMB) in 500 ml Schott bottles and incubated at 25± 2 ºC for 10 days on an orbital shaker at 150 rpm. Similarly, isolated bacterial endophytes were also cultured in 200 ml Mueller-Hinton broth (MHB) and incubated at room temperature for 48 h on an orbital shaker at 150 rpm. After the incubation period, each broth culture with apparent microbial growth was mixed with an equal volume of EtOAc (100 ml). The resulting mixture was vigorously mixed for 5 min, poured into a separating funnel, and allowed to separate and thereafter

filtered with Whatman No. 1 filter paper. The aqueous phase of the mixtures were discarded while the organic solvent was removed using a rotary evaporator, and the extracts in amber bottles were stored at 4 ºC until used.

5.2.4. Quantification of secondary metabolites

5.2.4.1. Estimation of total phenolics

The total phenolic contents of the crude extracts obtained from the endophytes were quantified as described in **Section 3.2.6.1.** of **Chapter 3**.

5.2.4.2. Estimation of total flavonoids

The total flavonoid contents of the crude extracts obtained from the endophytes were quantified as described in **Section 3.2.6.2.** of **Chapter 3**.

5.2.5. Antioxidant activity of crude extracts produced by the endophytes

5.2.5.1. 1-1- Diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity of crude extracts obtained from the endophytes

The radical scavenging activities of the endophytes' crude extracts was carried out as described in **Section 3.1.4.1.** of **Chapter 3**.

5.2.6. Evaluation of plant-growth-promoting properties and drought resistance of selected endophytic isolates

Endophytic isolates (fungi and bacteria) of *E. obtusifolius* with free radical scavenging capabilities (low IC_{50} values) and an appreciable quantity of secondary metabolites were further assessed for their drought stress tolerance and *in vitro* plant growth-promoting potential.

5.2.6.1. Screening of endophytic isolates for drought stress tolerance

Selected endophytic isolates were evaluated for their drought stress tolerance *in vitro* following a modified method described by **EKE et al. (2019)**. Briefly, polyethylene glycol (PEG) 6000 at different concentrations [0, 10, 20, 30 and 40% (w/v)] was employed as a drought stress stimulator and added to culture broth media. Fifty millilitres of MHB (bacteria) and YMB were prepared in Schott bottles and amended with the stated PEG concentrations. In triplicate, a loopful of each bacterial isolate was inoculated into the PEG supplemented MHB and incubated on an orbital shaker (180 rpm) for 4 days at 25±2 ºC, whereas a single disc of fungal isolates (about 1 cm) was excised from freshly prepared fungal isolates, inoculated in the PEG amended YMB and incubated at 25±2 ºC on an orbital shaker (180 rpm) for 10 days. After the incubation period, the growth of the microbial isolates at different water-deficit levels was estimated spectrophotometrically at optical density (OD) 600 nm and compared to 0% PEG cultures. Endophytic isolates with high OD values were considered as water stress-tolerant species.

5.2.6.2. Ammonia production

The production of ammonia by selected *E. obtusifolius* endophytic isolates was investigated using a modified, published protocol of **HASSAN (2017)** and **PASSARI et al. (2016)** using Nessler's reagent in peptone broth. Briefly, in triplicate, each of the freshly prepared endophytic bacteria and fungi cultures were aseptically inoculated into their respective labelled test tubes containing 10 ml of peptone liquid media. They were incubated for 7 days at 28±2 ºC on an orbital shaker at 150 rpm. Thereafter, 1 ml of Nessler's reagent was added to each test tube, and the appearance of

brown to yellow colour indicated a positive test for ammonia production and absorbance was measured at 530 nm using a spectrophotometer. The concentration of ammonia was evaluated using the standard curve ($y = 1.488x + 0.022$) generated from the standard (ammonium sulphate), and the amount of $(NH_4)_2SO_4$ was expressed in mM.

5.2.6.3. Hydrogen cyanide production

The ability of the selected endophytic bacteria and fungi to synthesise hydrogen cyanide was evaluated based on the described methods of **BAKKER and SCHIPPERS (1987)**. Each bacterial culture was streaked on petri-dishes containing Luria Bertani (LB) agar supplemented with 4.4 g/l of glycine. For the fungal species, discs were aseptically placed on plates containing PDA augmented with 4.4 g/l of glycine. A sterilised Whatman No. 1 filter paper pre-soaked in 0.5% picric acid in 2% sodium carbonate for 1 min was gently stuck on the lids of each of the petri-dishes. The plates were then sealed with parafilm and incubated for 7 days at 28±2 °C. A change in colour of the filter papers from yellow to deep orange or reddish-brown indicated a positive result. Three replicates were made for each selected endophytic isolate.

5.2.6.4. Phosphate solubilisation activity

The phosphate solubilisation capability of the selected endophytic bacteria and fungi was examined as per the published method of **YADAV et al. (2016)**. Briefly, 10 µl each of the freshly prepared selected bacteria endophytic isolates were spot inoculated on Pikovskaya's (PVK) agar plates amended with 5% tri-calcium phosphate whereas, fungal discs (5 mm) excised from actively growing tips of the selected fungal strains were carefully inoculated in plates containing PVK agar incorporated with 5% tri-calcium phosphate and the phosphate solubilising capacity of each isolate, in triplicate, was determined as phosphate solubilising index (PSI). Inoculated plates were incubated at 27±2 ºC for 7 days, and plates were closely monitored for the development of clear

zones around the bacterial and fungal colonies. PSI was calculated on PVK plates as per the formula of **EDI PREMONO et al. (1996)**. Un-inoculated agar plates were used as control, and the experiment was done in triplicate for each endophytic isolate.

PSI = Colony diameter + Clear zone diameter/Colony diameter

5.2.6.5. Indole-3-acetic acid (IAA) production

The modified methods of **XINXIAN et al. (2011)** and **CHAND et al. (2020)** were employed to assay IAA production by the selected bacterial and fungal endophytes. In brief, 40 µl of each freshly prepared selected bacterial culture was inoculated in Schott bottles containing 40 ml of LB liquid medium fortified with 5 mg/l L-tryptophan and incubated at 25±2 ºC on an orbital shaker (180 rpm) for 7 days. As for the fungal isolates, 1 cm of each freshly cultivated fungal isolate was excised and inoculated in 40 ml of YMB supplemented with 5 mg/l L-tryptophan and incubated for 10 days on an orbital shaker (180 rpm) at 25 ºC. After the incubation, 5 ml each of the fungal and bacterial cultures was transferred into sterile centrifuge bottles and centrifuged at 10,062 g for 10 min, and the supernatants were collected. One millilitre of each collected isolate supernatant was mixed with 2 ml of Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$), and the reaction mixture was incubated in the dark for 30 min. The experiments were conducted in triplicate and the production of IAA by the microbial isolates was confirmed by the appearance of pink colour and the absorbance of the reaction mixture was measured at 530 nm using UV spectrophotometer. The concentration of IAA in µg/ml was estimated by using a standard curve: $y = 0.1167x + 0.1686$, $R^2 = 0.9132$ derived from 0,1,5,7, and 10 µg/ml of standard IAA (Sigma) diluted in sterile distilled water.

5.2.6.6. Aminocyclopropane-1-carboxylate (ACC) deaminase activity of endophytes

The selected bacterial and fungal endophytic isolates were evaluated for their ability to use ACC as their exclusive source of nitrogen using a modified protocol of **JASIM et al. (2013)**. Briefly, the bacterial and fungal isolates were inoculated on plates containing Dworkin and Foster (DF) minimal salts agar augmented with 3 mM ACC as the only nitrogen source. In triplicate, the plates were incubated at 28±2 ºC for 5 days, and the manifestation of bacterial or fungal growth afterwards was considered a positive result.

5.2.6.7. Siderophore production

Siderophore production was qualitatively investigated by Chrome Azurol S (CAS) agar for all the selected bacterial and fungal endophytic isolates using a modified method of **MILAGRES et al. (1999)**. CAS-blue agar was prepared by dissolving 60.5 mg of CAS into 50 ml of distilled water which was then mixed with 10 ml of iron (III) solution (1 mM $FeCl_3$. $6H_20$ in 10 mM HCl). The mixture was gently mixed using a magnetic stirrer to 72.9 mg of hexadecyltrimethylammonium (HDTMA) bromide dissolved in 40 ml distilled water. The resulting dark-blue mixture was then autoclaved at 121 ºC for 15 min. Simultaneously, a mixture of 30.24 g of piperazine-N, N'-bis (2-ethanesulfonic acid (PIPES), 15 g of agar, 900 ml of distilled water, and 50% (w/w) of NaOH adjusted the pKa of PIPES to 6.8 was also autoclaved. Finally, the two mixtures were added gently and agitated under the laminar flow bench to avoid foaming and thereafter poured aseptically into plates. Upon solidification, freshly prepared selected bacterial cultures and 1 cm discs of fungal isolates excised from the growing hyphal tips were spot inoculated in their respective plates and incubated for 7 days at 25±2 ºC. The appearance of yellow/orange or purple halo around the microbial colonies was regarded as a positive result for siderophore production. The experiment was conducted in triplicate.

5.2.7. Endophytes' antagonistic check using dual culture method

Two endophytes (leaf fungi-5 and root bacteria-2) that exhibited remarkable *in vitro* plant growth-promoting potentials and drought stress tolerance were evaluated for any possible incompatible growth pattern against each other on PDA plates using the dual antagonistic culture method as illustrated by **NAIK et al. (2009)**. A five-millimetre disc of the fungal isolate was aseptically laid at the middle of the PDA plates while the bacterial isolate was inoculated at an equal distance from the plate periphery. The organisms were incubated for 7 days at 27±2 ºC and observed for any inhibition zone. Compatibility between the two organisms is confirmed by the absence of inhibition zone while its establishment implies antagonism between the species. The experiment was conducted in triplicate, and the control plates were inoculated with either of the organisms.

5.2.8. Molecular identification of endophytes

To minimise cost, two dominant endophytic species from all our isolates with promising *in vitro* plant growth-promoting properties and drought stress tolerance were identified using molecular tools. The isolated fungi (LF5), which displayed good plant growth-promoting attributes and drought stress tolerance, were identified using molecular tools. The fungus was grown of PDA media at 27±2 ºC for 7 days, after which the fungal mycelia was carefully scraped out and suspended in autoclaved distilled water under sterile conditions. DNA extraction and purification were done using Quick-DNA Fungal/Bacterial Kit (Zymo Research, India) following the manufacturer's instructions. The universal internal transcript spacer (ITS) regions of the 18S rRNA (recombinant deoxyribose nucleic acid) genes of the fungus were amplified using polymerase chain reaction (PCR) and ITS primers ITS1-5′-TCC GTA GGT GAA CCT GCG G-3 (forward primer) and ITS4 - 5′-TCC TCC GCTTAT TGA TAT GC-3′ (reverse primer). Each reaction mixture continued 1µl of the extracted DNA in 20 µl PCR reaction mixture. PCR was done using a thermocycler under the following conditions: min initial denaturation at 94 ºC, 35 cycles of 30 s

denaturation at 94 ºC, 30 s min annealing at 50 ºC and 1 min extension at 68 ºC, and 5 min final extension at 68 ºC.

Endophytic bacteria (RB2) showing excellent drought stress tolerance and plant growth promotion was characterised using 16S rRNA gene sequences. Similarly, the DNA of the bacteria was extracted and purified as done for the fungal species above. The PCR composition was the same as mentioned above except for the primers that were 907-R and 1492-R. PCR was done using thermocycler with the following conditions: 5 min initial denaturation at 95 ºC, 30 cycles of 1 min denaturation at 94 ºC, 1 min annealing at 55 ºC and 1.5 min extension at 72 ºC, and 10 min final extension at 72 ºC.

The amplified products were electrophoresed in 1% w/v agarose gel electrophoresis with (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Blue light DNA Dye. The cleaned products were injected on an Applied Biosystems ABI 3500XL Genetic Analyser or Applied Biosystems ABI 3730XL Genetic Analyser with a 50 cm array, using POP7, whereas the sequence chromatogram analysis was performed using Finch TV analysis software and Bio Edit Sequence Alignment Editor v.7.0. The amplified sequence was deposited in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.gov/BLAST). The sequences thus obtained were submitted to the GenBank for their accession numbers.

5.2.9. Statistical analysis

Numerical data obtained from the different assays in this study were analysed using the one-way analysis of variance (ANOVA) using GraphPad Prism 7 (GraphPad Software, Inc. California USA), and results are expressed as mean ± standard error of means of triplicates. The significance of means was calculated using Duncan's Multiple Range Test at P values < 0.05.

5.3. Results

5.3.1. Sterilisation, isolation, and purification of endophytes

The endophytic microbial community of healthy leaves and roots of *E. obtusifolius* were evaluated in this study using surface sterilised explants cultivated on PDA and NA **(Figure 5.1)**. The two sterilisation check methods employed in this study, namely culturing of aliquots of water from the last explants rinse onto culture media and imprinting of the sterilised explant surfaces onto nutrient media were comparable, effective and no microbial growth was observed on these control culture plates; thus, mixed microbial cultures were devoid of epiphytic microbes and were considered as endophytes. Colonies with different morphology were selected and sub-cultured on newly prepared media, and 26 pure cultures were obtained. Five endophytic fungi and eight endophytic bacteria designated LF-1-LF-5 and LB-1-LB-9 were isolated from the leaves, whereas seven endophytic fungi and six endophytic bacteria designated RF-1-RF-9 and RB-1-RB-6 were obtained from the roots as shown in **Figure 5.2** and **5.3**.

5.3.2. Total phenolics (TP) and flavonoid contents estimation

The total phenolic and flavonoid contents of ethyl acetate extracts of all the endophytic pure cultures were evaluated, and the results are shown in **Table 5.1**. The flavonoid and TP concentrations in the crude extracts of endophytic isolates differed considerably. The TP contents of the extracts range from 0.27 to 9.80 mg GAE/ mg^{-1} DW of extracts. The highest content of TP was in the extracts of LF-5 with 9.80 ± 0.03 mg GAE/ mg^{-1} DW of extracts, followed by RF-1 (6.74 ± 0.30), and RB-2 (4.22 ± 0.05). Notably, the fungal isolate RF-1 had the highest amounts of flavonoids (69.04 ± 2.83 mg CE/mg^{-1} DW of extracts), whereas LB-5 with 7.60 ± 0.72 mg CE/mg^{-1} DW of extracts.

Table 5.1: Quantities of flavonoids, total phenolics and IC_{50} values of the crude extracts obtained from the isolated endophytic species.

Endophytes	% DPPH Radical Scavenging abilities at 100 µg/ml	DPPH IC_{50} (µg/ml)	Flavonoids (mg CE/mg^{-1} DW of extracts)	Total Phenolics (mg GAE/ mg^{-1} DW of extracts)
RB-1	75.52 ± 1.13^{de}	48.95 ± 1.09^{hij}	27.66 ± 0.72^{de}	4.00 ± 0.02^{c}
RB-2	81.90 ± 0.79^{bc}	35.68 ± 0.87^{ij}	32.26 ± 1.10^{cd}	4.22 ± 0.05^{c}
RB-3	10.00 ± 1.08^{o}	707.48 ± 10.89^{b}	16.582 ± 0.60^{ghijk}	-
RB-4	20.86 ± 2.94^{m}	256.26 ± 59.23^{cd}	17.21 ± 1.27^{ghijk}	-
RB-5	25.86 ± 2.88^{kl}	195.03 ± 18.34^{def}	18.04 ± 0.75^{ghij}	-
RB-6	20.86 ± 1.58^{m}	245.47 ± 24.07^{cde}	16.08 ± 0.75^{ghijk}	-
LB-1	$68.27 \pm 1.77f^{g}$	70.76 ± 2.00^{ghij}	19.30 ± 0.21^{ghi}	$1.55\ 0.08^{g}$
LB-2	15.49 ± 0.66^{n}	245.30 ± 2.79^{cde}	14.28 ± 1.46^{hijklm}	0.28 ± 0.02^{k}
LB-3	67.37 ± 0.66^{gh}	66.22 ± 1.23^{ghij}	$10.73\ 1.00^{klmno}$	0.27 ± 0.23^{k}
LB-4	80.30 ± 0.84^{cd}	32.37 ± 0.84^{ij}	$56.\ 918 \pm 6.90^{b}$	2.00 ± 0.05^{f}
LB-5	12.18 ± 1.31^{no}	352.41 ± 20.74^{c}	7.60 ± 0.72^{no}	-
LB-6	$24.81 \pm 1.08l^{m}$	215.36 ± 14.17^{def}	19.72 ± 0.21^{ghi}	-
LB-7	35.94 ± 0.94^{j}	136.79 ± 7.17^{j}	12.611 ± 1.90^{jklmn}	-
LB-9	41.20 ± 0.40^{i}	118.19 ± 2.65^{i}	11.57 ± 1.30^{jklmn}	-
LF-1	63.10 ± 1.24^{h}	57.31 ± 1.25^{ij}	21.60 ± 4.00^{efg}	1.35 ± 0.07^{h}
LF-2	29.83 ± 1.21^{k}	172.12 ± 6.08^{defg}	8.43 ± 0.60^{mno}	-
LF-3	72.24 ± 1.64^{ef}	47.77 ± 0.68^{hij}	20.76 ± 0.00^{fgh}	1.00 ± 0.04^{h}
LF-4	22.93 ± 1.08^{lm}	224.78 ± 12.34^{def}	9.69 ± 0.21^{lmno}	-
LF-5	86.44 ± 1.88^{ab}	$26.\ 67 \pm0.889^{j}$	15.33 ± 0.80^{ghijkl}	9.80 ± 0.03^{a}
RF-1	90.38 ± 0.40^{a}	23.43 ± 0.18^{ij}	69.04 ± 2.83^{a}	6.74 ± 0.30^{b}
RF-3	23.76 ± 4.06^{lm}	222.08 ± 42.44^{def}	38.00 ± 6.88^{c}	3.70 ± 0.05^{d}
RF-4	23.16 ± 1.59^{lm}	221.44 ± 22.07^{def}	13.45 ± 1.50^{ijklmn}	-
RF-5	66.62 ± 2.77^{gh}	62.57 ± 1.65^{hij}	16.60 ± 0.42^{ghijk}	2.80 ± 0.02^{e}
RF-6	$21.05 \pm 1.30l^{m}$	248.67 ± 19.55^{cd}	$16.60\ 1.80^{ghijk}$	-
RF-8	64.36 ± 1.45^{gh}	64.63 ± 2.067^{ghij}	26.82 ± 2.54^{def}	2.12 ± 0.02^{f}
RF-9	41.05 ± 3.26^{i}	147.44 ± 10.74^{defgh}	17.63 ± 0.63^{ghij}	0.62 ± 0.02^{j}
BHT	86.21 ± 1.53^{ab}	42.98 ± 3.86^{hij}	-	-

Data represent mean values ± standard error of means. Columns with similar letter(s) show non-significant results, and columns with different letter(s) show significant results. - = not detected.

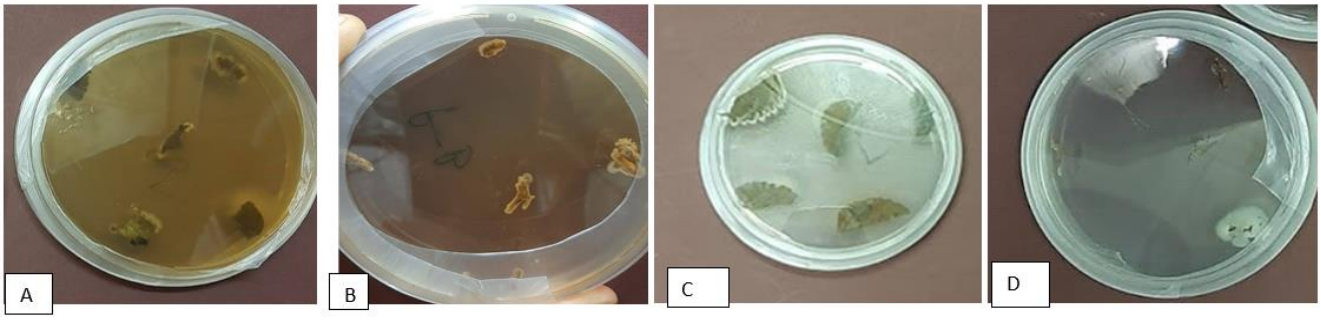

Figure 5.1: Growth of endophytic bacteria (A, B) and fungi (C, D) emerging from the leaves and roots of *Endostemon obtusifolius.*

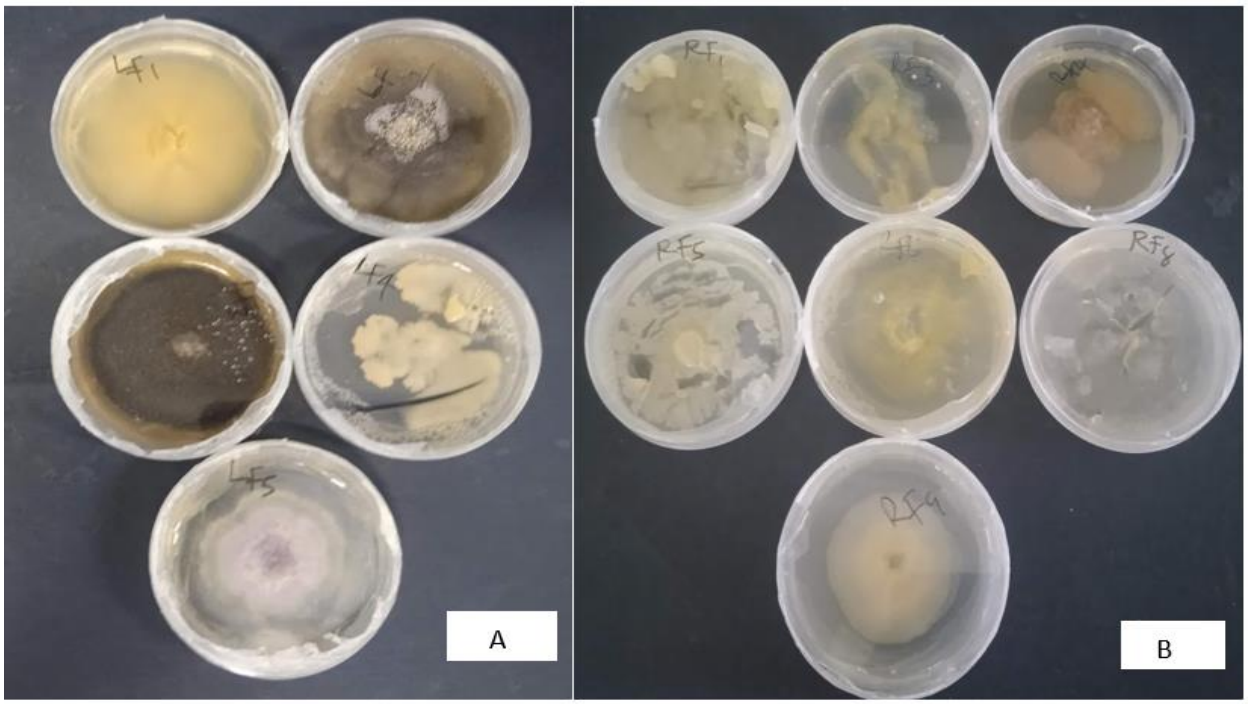

Figure 5.2: Pure culture plates of fungi isolated from the leaves (A) and roots (B) of *Endostemon obtusifolius.*

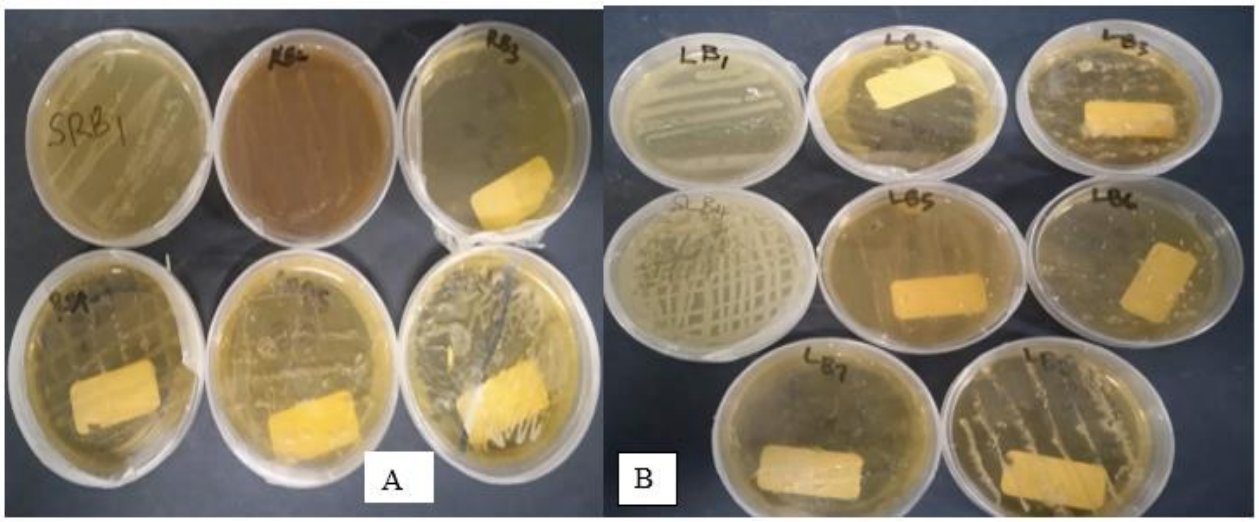

Figure 5.3: Pure culture bacteria from the roots (A) and leaves (B) of *Endostemon obtusifolius*.

5.3.3. DPPH radical scavenging abilities

The result of the DPPH radical scavenging abilities of the ethyl acetate crude extracts obtained from the pure culture isolates are presented in **Table 5.1**. The results showed that RF-1, LF-5, and RB-2 had the highest scavenging power against DPPH radicals at 100 µg/ml ranging from 81.90 to 90.38%, which were not significantly different from the control BHT (86.21%) at $P < 0.05$. The IC_{50} (concentration of sample required to scavenge 50% of free radicals) values of the endophytes' crude extracts varied widely, and RF-1 (23.43 ± 0.18 µg/ml) had the lowest IC_{50} values, which is not significantly different from BHT (42.98 ± 3.86 µg/ml) at $P < 0.05$. Additionally, there was a positive correlation relationship between the free radical scavenging capacity of the endophyte extracts and the TP ($R^2 = 0.705$) and flavonoid content ($R^2 = 0.674$). Eleven endophytic isolates RF-1, RF-5, RF-8, LF-1, LF-3, LF-5, RB-1, RB-2, LB-1, LB-3, and LB-4 with low IC_{50} values and appreciable quantities of flavonoids and total phenolics were further investigated for their drought tolerance and plant growth-promoting capacities.

5.3.4. The water-deficit resistance potential of selected endophytes

The result from this study revealed that all the eleven selected endophytic isolates, at varying degrees, resisted water-deficit stress initiated by the addition of 10-40% PEG 6000 to broth media. A general decline in the optical density of all the endophytic isolates monitored at 600 nm was observed as the concentration of the PEG 6000 increased from 10% to 40%. At the highest concentration (40%) of PEG 6000 treatment, RB-1 (0.178 nm), RB-2 (0.125 nm), RF-5 (0.148 nm), LF-1 (0.283 nm), and LF-5 (0.235 nm) exhibited significant OD values indicating microbial cell multiplication and drought stress tolerance potency, whereas there was a complete disruption in the growth of RF-8 and LB-3 under the same condition **(Figure 5.4)**.

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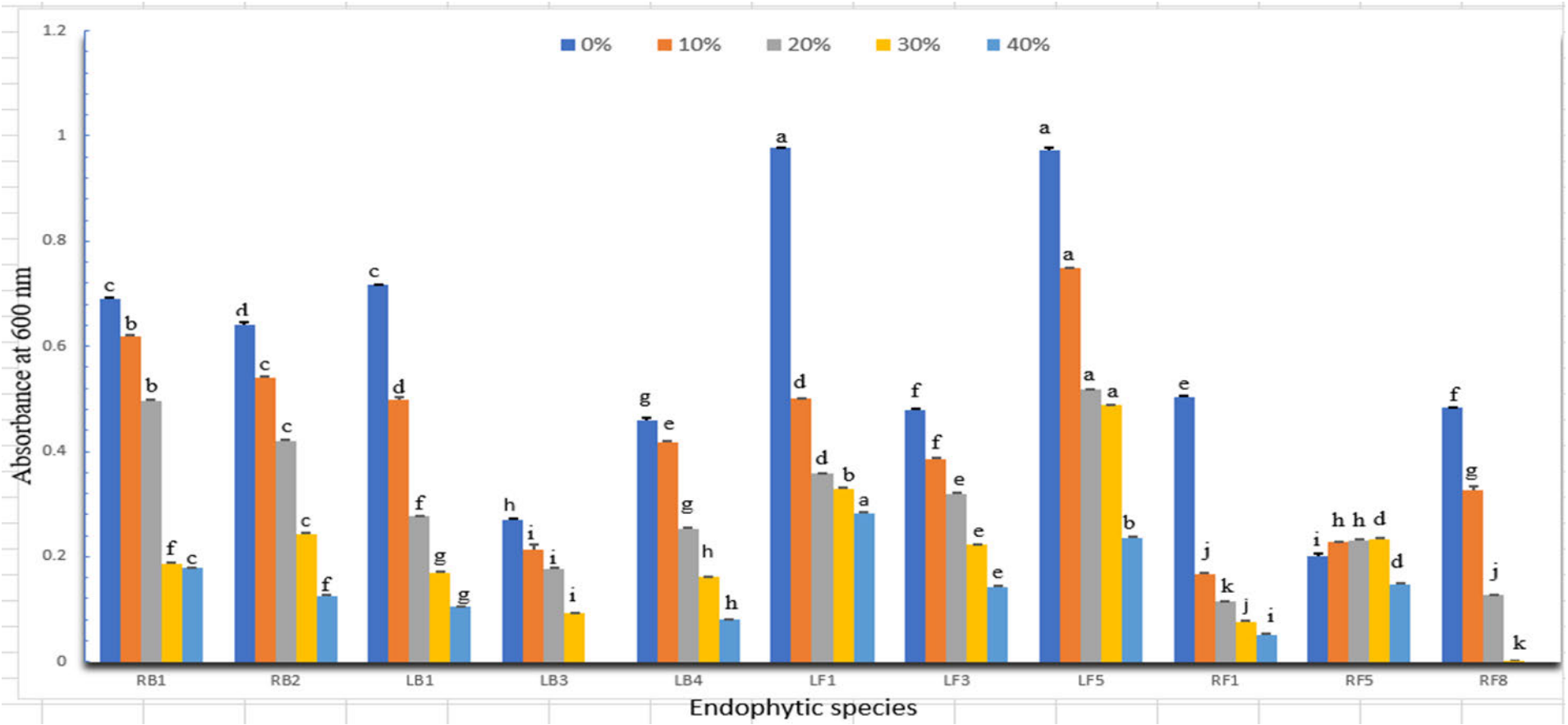

Figure 5.4: The growth of selected endophytic isolates under non-stressed (0%) and water-deficit conditions treated with increasing (10-40%) PEG 6000 concentrations. Data are mean ± standard error of means. Similar letter(s) show non-significant results at $P < 0.05$.

5.3.5. Plant-growth-promoting characteristics of selected endophytic isolates

This study showed that all isolates had the capacity to produce ammonia, as shown in **Table 5.2**. Isolates RB-2, LB-1, LB-4, RF-1 and LF-5 showed the most vigorous colour intensity indicating ammonia production, while the weakest colour intensity was observed in isolates LB-3, RB-1, and RF-8 **(Figure 5.8)**. The results of quantitative ammonia determination further revealed that the quantity of ammonia produced by isolates ranged from 0.51 to 3.88 mM. Isolate RF-1 (3.88 mM) showed the highest ammonia producing capacity **(Figure 5.9)**. All the selected endophytic isolates except LB-3 tested positive for HCN production **(Table 5.1)**. The picric acid pre-soaked filter papers placed underneath the incubated petri-dish lids of isolates changed from yellow to deep red or orange, indicating the ability of the isolates to produce HCN. RB-2, LF-1, LF-3, and LF-5 were the highest producers **(Table 5.2, Figure 5.5)**. This study further revealed that two fungal isolates (LF-3 and LF-5) of the six selected fungal isolates and all the bacterial isolates utilised ACC as the exclusive nitrogen source **(Table 5.2)**. The ability of these isolates to grow on DF minimal salt media supplemented with ACC confirmed their ACC deaminase activity **(Figure 5.6)**. Siderophore producing capacity was also found in all isolates, albeit to variable extents, as indicated by the formation of orange halo zones around colonies on CAS agar plates **(Table 5.2)**. As shown in **Figure 5.7**, RB-2, LB-1, LF-1 and LF-5 exhibited the capacity to produce maximum siderophore; nonetheless, LF-5 had the largest halo zone around its colonies. **Table 5.2** further demonstrated that 10 of the selected endophytic isolates solubilised tricalcium phosphate on PVK agar plates. These isolates produced halo zones around their colonies **(Figure 5.10)**, indicating their phosphate solubilisation potency. The phosphate solubilisation index (PSI) results showed that PSI values ranged from 0.00 to 3.02 cm **(Figure 5.9)**. Isolate LF-3 PSI value (3.02 cm) was significantly higher than other PSI values.

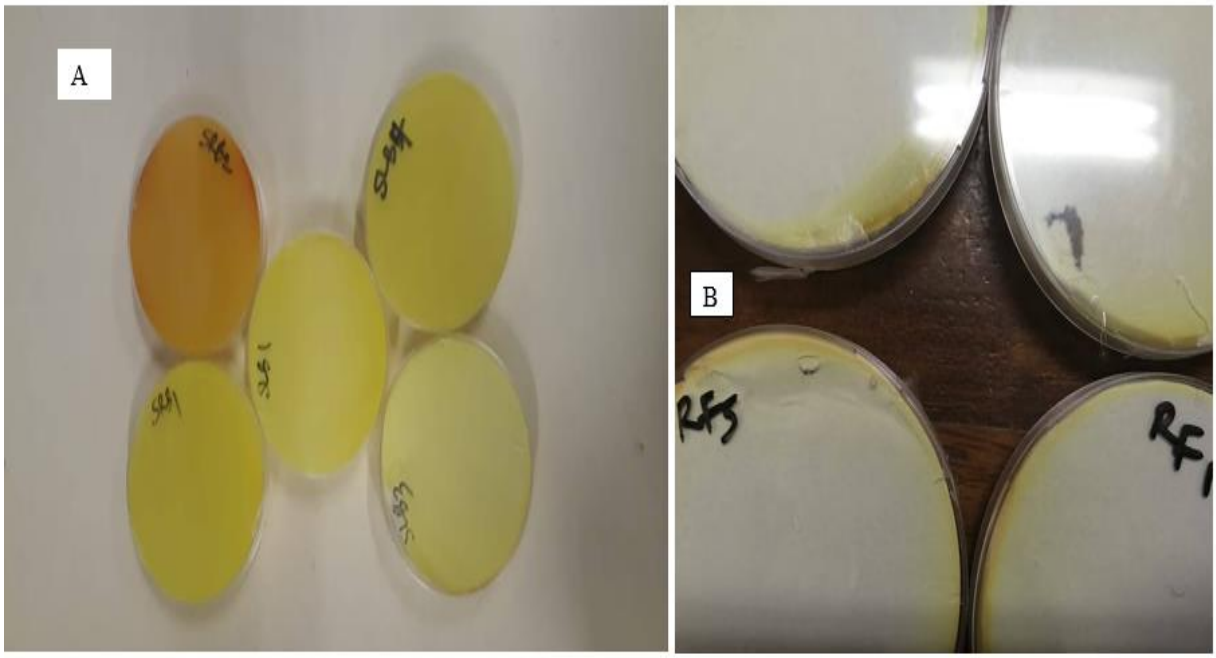

Figure 5.5: Hydrogen cyanide synthesis potential of selected bacterial (A) and fungal (B) endophytic isolates.

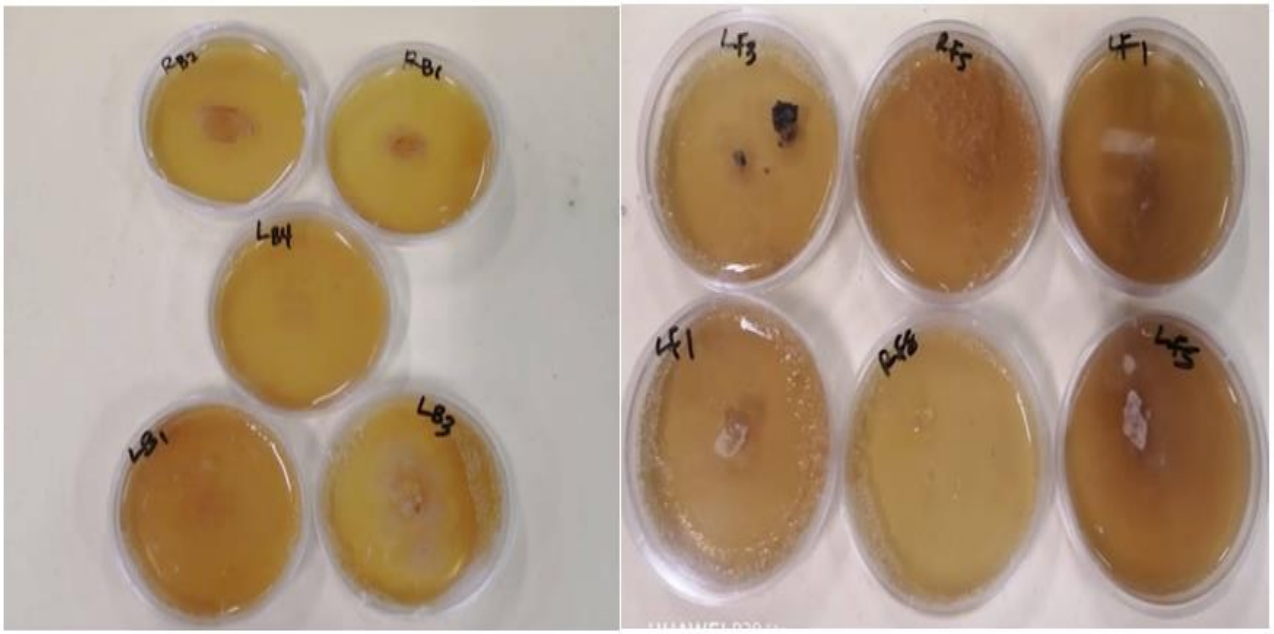

Figure 5.6: ACC deaminase activity in the selected bacterial (A) and fungal (B) endophytic isolates on DF minimal salt media.

2212 **Table 5.2:** Qualitative plant growth stimulating capacities of endophytic isolates.

2213

PGP characters	**RB-1**	**RB-2**	**LB-1**	**LB-3**	**LB-4**	**RF-1**	**RF-5**	**RF-8**	**LF-1**	**LF-3**	**LF-5**
Ammonia production	+	+++	+++	+	+++	+++	++	+	++	++	+++
Siderophore production	++	+++	+++	+	+	++	++	+	+++	++	+++
IAA production	+	+	+	-	+	+	-	-	-	+	+
Phosphate solubilisation	+	+	+	-	+	+	+	+	+	+	+
Hydrogen cyanide production	+	+++	+	-	+	+	+	+	++	++	++
ACC deaminase activity	+	+	+	+	+	-	-	-	-	+	+

- = no activity + = low activity ++ = medium activity +++ = strong activity

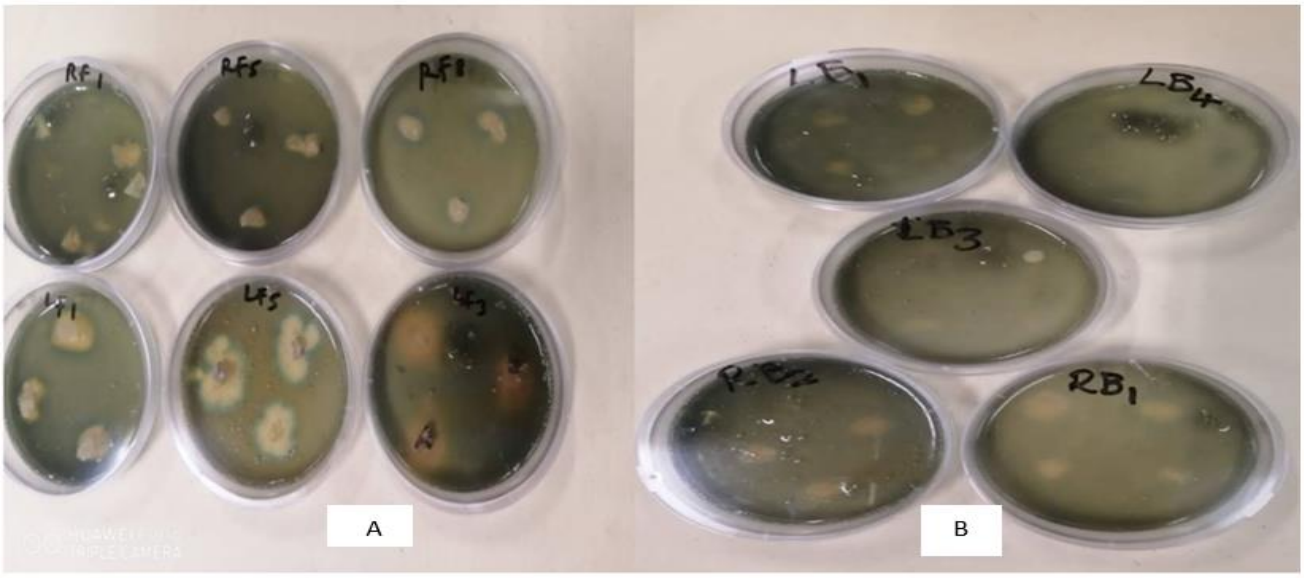

Figure 5.7: Siderophore production activity of the selected fungal (A) and bacterial (B) endophytic isolates.

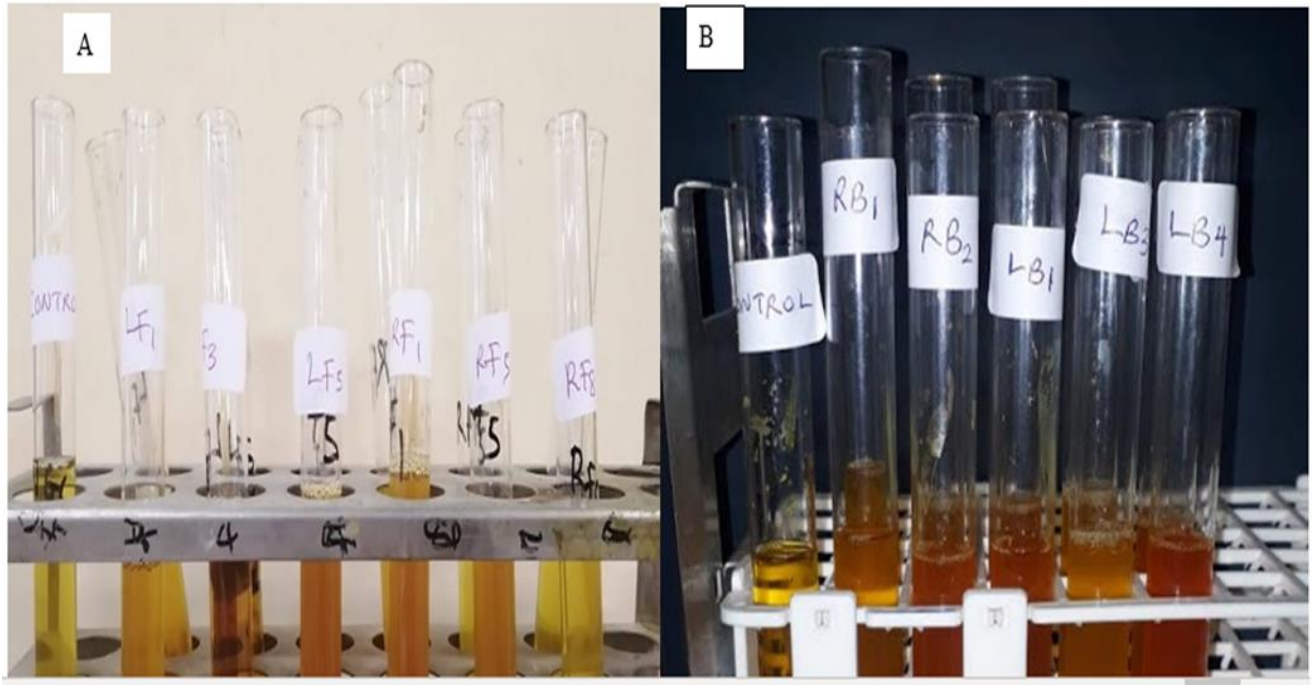

Figure 5.8: Qualitative test of ammonia in selected fungal (A) and bacterial (B) endophytic isolates.

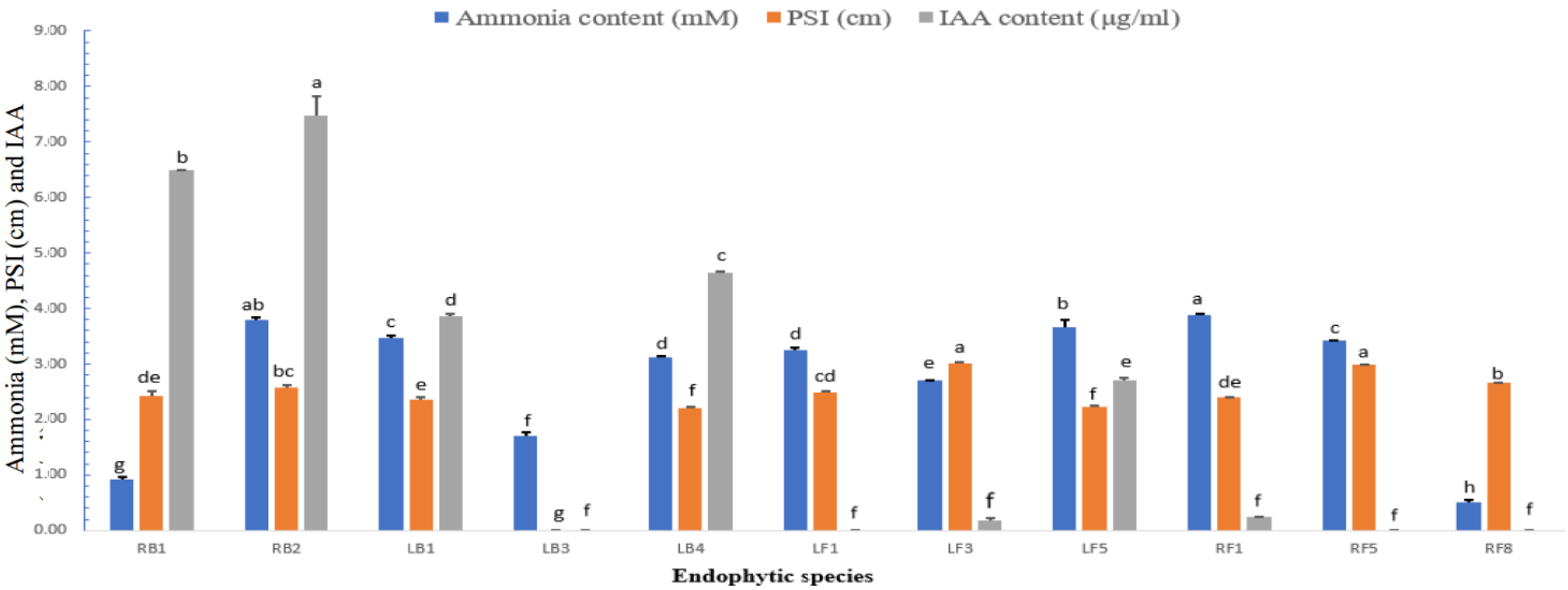

Figure 5.9: Phosphate solubilisation index measurement, IAA production, and ammonia production quantitative measurement in selected endophytic isolates. Data are mean ± standard error of means. The means with similar letter(s) show non-significant results at $P < 0.05$.

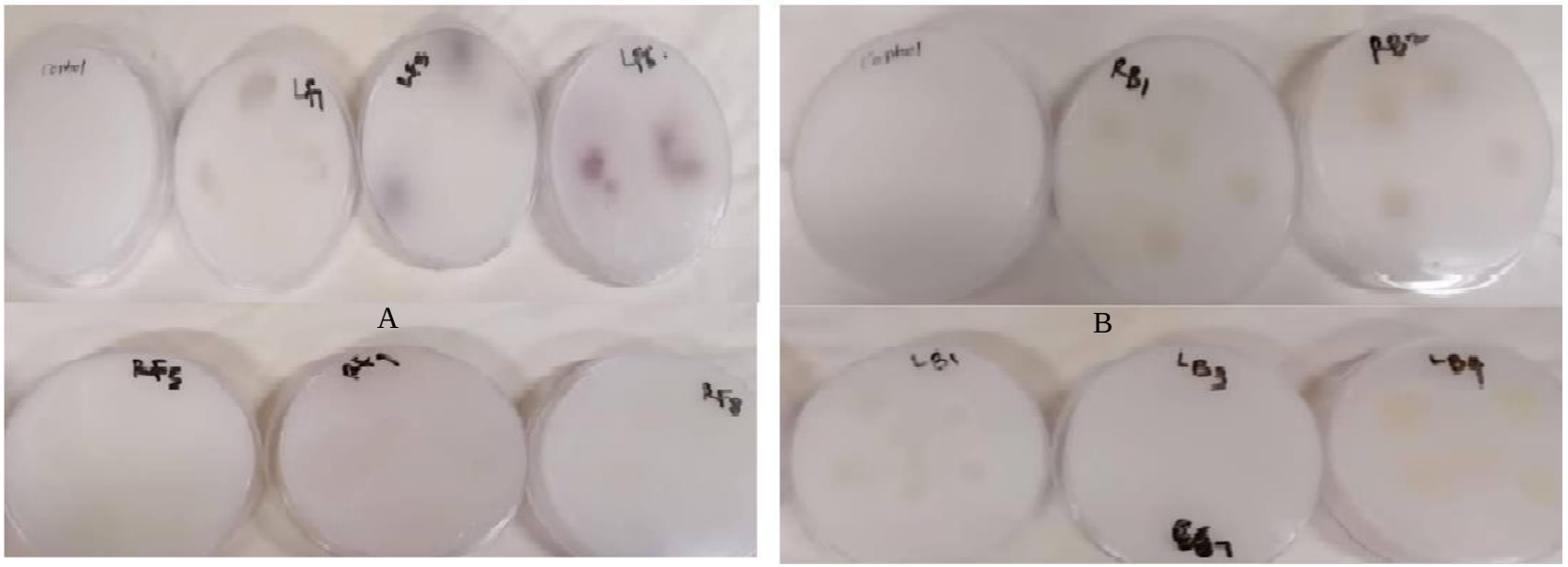

Figure 5.10: Tricalcium phosphate solubilisation capacity of selected bacterial (A) and fungal (B) endophytic isolates on PKV media.

The production of IAA was detected in seven (four bacterial and three fungi isolates) of the eleven selected endophytic isolates. Isolates RB-1, RB-2, LB-1, LB-4, RF-1, LF-3 and LF-5 developed a pinkish colour on the addition of Salkowski reagent, as shown in **Figure 5.11**. The results of the quantitative estimation of IAA as presented in **Figure 5.9** showed that RB-2 synthesised the highest quantity of IAA (7.47 µg/ml) followed by RB-1 (6.45 µg/ml), LB-4 (4.64 µg/ml), LB-1 (3.85 µg/ml), LF-5 (2.70 µg/ml), RF-1 (0.24 µg/ml) and LF-3 (0.12 µg/ml).

In this study, the isolates RB-2 and LF-5 appeared to be compatible symbionts as they grew *in vitro* with minimal antagonism **(Figure 5.12)**. Following the molecular characterisation of RB-2 and LF-5 using 16S rRNA and ITS rRNA sequence analysis, respectively, the bacterial isolate (RB-2) was identified as *Paenibacillus polymyxa* (MT163461.1), whereas the fungal endophyte (LF-5) was found to be close homologs of *Fusarium oxysporum* (MT560381.1). The BLAST results further revealed 100% and 99.6% identity of the bacterial and fungal endophytes with the rRNA sequences of the related species **(Table 5.3)**. Assigned GenBank accession numbers of the submitted sequence data are presented in **Table 5.3**.

Figure 5.11: Indole-3-acetic acid production quantification of selected bacterial (A) and fungal (B) endophytic isolates.

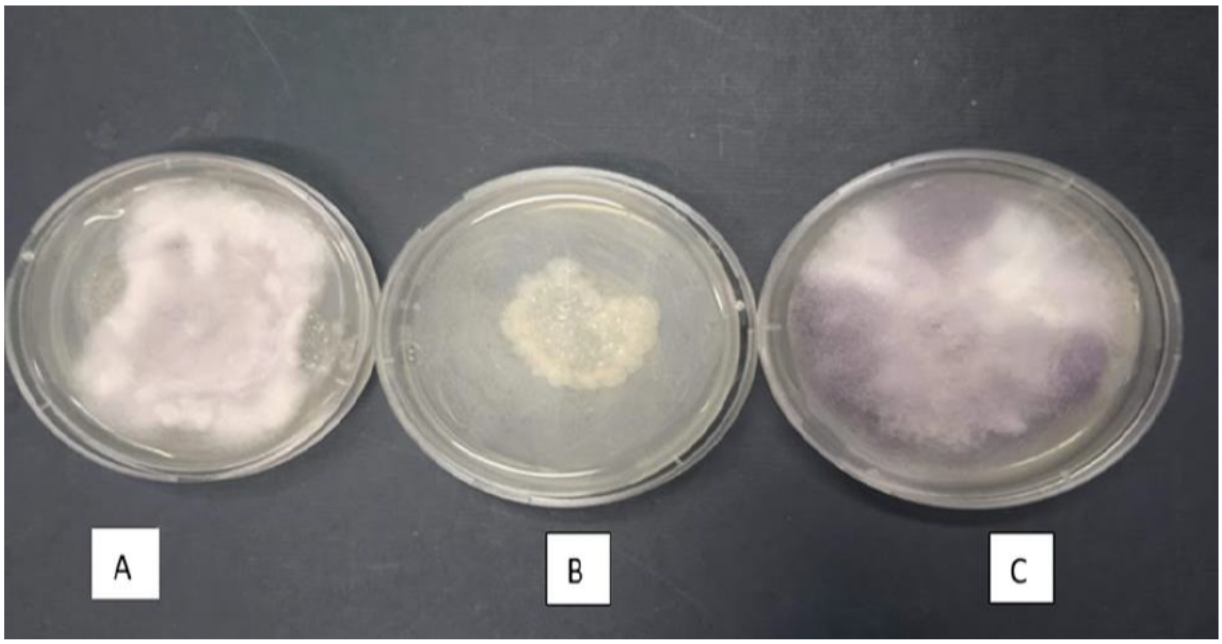

Figure 5.12: *In vitro* compatibility test of RB-2 and LF-5. Dual culture plate of the RB-2 and LF-5 on PDA (A.), RB-2 on PDA (B), and LF-5 on PDA (C).

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Table 5.3: Molecular identification of two drought resistant and plant growth-promoting endophytes isolated from *Endostemon obtusifolius*.

Isolate code	Most closely related homologue sequence (accession number)	Sequence Identity (%)	GeneBank Accession number
EORB-2	*Paenibacillus polymyxa* (MT163461.1)	100.00	OL619995
EOLF-5	*Fusarium oxysporum* (MT560381.1)	99.60	MZ598577

5.4. Discussion

One of the omnipresent features of plants is harmless endophytes within their living tissues **(NAIR and PADMAVATHY, 2014)**. The population of endophytes within their hosts depends on the hosts' species, age, habitat and physiological status, the kind of plant tissues, sampling season, inoculum density, and prevailing environmental conditions **(TAN and ZOU, 2001)**. In this case, a total of 12 culturable fungal and 14 culturable bacterial endophytes were isolated from the leaves and roots of the medicinal plant *E. obtusifolius*. Globally, researchers have reported the isolation of several fungal and bacterial endophytes from the roots and leaves of medicinal plants **(ABOOBAKER et al., 2019; ALSULTAN et al., 2019; YADAV et al., 2016)**. Medicinal plants synthesise a wide range of phytochemicals which are the basis of their remarkable pharmacological activities. The quantity and quality of these phytochemicals vary widely depending on the edaphic and climatic factors, the species of the plant, and their relationships with microbes **(ZHAO et al., 2011)**. Medicinal plants are well-known hosts of endophytes **(FAETH and FAGAN, 2002)**. These endophytic species have been implicated in synthesising bioactive compounds to enhance their hosts' survival **(FIRÁKOVÁ et al., 2007)** and stimulate their hosts to accumulate valuable secondary metabolites under certain conditions **(JIA et al., 2016)**.

Flavonoids and phenolic compounds are two of the main bioactive components of medicinal plants, and they have also been detected and isolated from endophytic species **(HARPER et al., 2003; HUANG et al., 2007; PALANICHAMY et al., 2018)**. Flavonoids and phenolics are confirmed antioxidant candidates that are highly efficient in scavenging ROS and preventing tissue damage **(HUANG et al., 2007)**. Endophytic species isolated from medicinal plants have been reported to demonstrate potent pharmacological activity such as antitumor, antidiabetic, antimalarial and antioxidant activities owing to their richness in secondary metabolites **(XIAO et**

al., 2014). In this study, the total phenolic and flavonoid contents of the ethyl acetate extracts of the isolated endophytic species varied significantly. The total phenolic and flavonoid contents also correlated positively to the DPPH free-radical scavenging activity of the extracts. This result agrees with the earlier findings of **NAIR et al. (2017)** and **YADAV et al. (2014)**, who reported a positive correlation between endophytic extracts and their antioxidant properties. DPPH free radical scavenging assay is a commonly used assay to evaluate the antioxidant capacity of natural products **(GUNASEKARAN et al., 2017)**. This DPPH assay is accurate and reliable because neither metals nor enzyme inhibition influences its outcome, however it does not completely elucidate the actual reactivity of the antioxidant when considered alone **(YADAV et al., 2014)**.

Water is an indispensable resource for all forms of life. The scarcity of water usually influences the growth pattern **(MALAKAR et al., 2014)**, function **(LIU et al., 2010)**, and productivity **(ZAK et al., 2016)** of microorganisms. Some microorganisms under drought stress rapidly adjust their osmotic conditions to tolerate their environment's low water potential **(SCHIMEL, 2018)**. This study showed that all the selected endophytes resisted water stress at 20% PEG, whereas 82% of selected endophytes exhibited tolerance at 40% PEG. These results concur with **LI et al. (2017b)** and **RIPA et al. (2019)** observations, where all isolated bacterial and fungal endophytes grew in a media culture containing 20% PEG. Several findings have confirmed the drought resistance capacity of endophytes **(JAYAKUMAR et al., 2020; SADEGHI et al., 2020)**. Our results, however, partially disagree with the observation of **(RIPA et al. (2019)**, who reported that no fungal endophytes were able to survive at the highest PEG concentration (40%). Many plant species in dry or semi-arid conditions establish symbiotic relationships with drought tolerant endophytes such as bacilli which can survive under limited water availability and might benefit their host plant under drought conditions **(ROLLI et al., 2015)**.

Through their phytohormone regulation, osmolyte accumulation, synthesis of ACC deaminase, antioxidants and several biomolecules (exopolysaccharides), endophytic species tolerate changes in the osmotic potential of their immediate environments **(CHUKWUNEME et al., 2020; KONNOVA et al., 2001; VURUKONDA et al., 2016)**. Drought stress, like other stressors, enhance the production of the stress hormone ethylene in plants **(SAPRE et al., 2019)**. The inoculation of drought-tolerant and ACC deaminase (ACCD) producing endophytic species into drought stress susceptible plants species improved the tolerance of the stressed plants by reducing ethylene concentrations within the cells **(CHUKWUNEME et al., 2020; GLICK, 2004)**. In this study, seven of the eleven selected endophytes showed ACCD activities. Researchers have widely reported that many endophytic bacteria **(MAHESHWARI et al., 2020)** and a few endophytic fungi **(RAUF et al., 2021)** species possess the ACCD gene, which enables them to split the ethylene precursor ACC into ammonia and α-ketobutyrate, thereby improving the fitness of host plants to salinity, drought, heavy metals and pathogenic attack **(DUBEY et al., 2021)**.

Many studies have reported microbial endophytes stimulating plant growth individually or synergistically **(KHAN et al., 2015; VURUKONDA et al., 2018)**. Endophytes promote plants' growth and development by synthesising degrading enzymes, ammonia, HCN, phytohormones, siderophores, or improving the availability of nutrients such as phosphorus to their hosts **(HASSAN, 2017)**. In this current study, RB-2 and LF-5 produced the highest IAA among the bacterial and fungal isolates, respectively, signifying their plant growth-promoting abilities. IAA is the only naturally occurring auxin, and it has been widely reported as a regulator of several developmental processes in plants, including tropisms, organogenesis, cell expansion, division and differentiation, root and pigment formation, mineral nutrition and responses of the plant to stress **(SCAGLIOLA et al., 2016)**. Indole acetic acid-producing endophytic species promotes the

growth of plants through the synthesis of IAA. The stimulation of growth by IAA producing endophytes relies on the concentration of the IAA produced by the endophyte and the genetic makeup of both the plants and the endophytes involved **(AHMAD et al., 2005; SARWAR and FRANKENBERGER, 1994)**. Phosphorus is a vital element involved in various physiological and metabolic processes in plants. They are found in free and combined forms in many plant parts and their immediate environment **(CHAND et al., 2020)**. Phosphate solubilisation and transportation within plants are essential traits of plant growth-promoting microorganisms. Inorganic phosphate solubilising microbes synthesise various enzymes and organic acids, release protons during ammonia assimilation, reduce pH, and chelate cations to release organic and soluble phosphorus to plants **(OTEINO et al., 2015)**. From our results, ten of the selected isolates produced clear halo zones around their colonies, suggesting their phosphate solubilising capacity.

Furthermore, plant-endophyte interactions confer abiotic and biotic stress resistance through the production of siderophore, ammonia, HCN and volatile compounds **(VANDANA et al., 2021)**. Siderophores are microbial iron scavenging low molecular secondary metabolites that ensure sufficient iron supply to the microbes and their host during iron shortages **(KUNDAN and PANT, 2015; MAHESHWARI et al., 2019)**. Interestingly, from the result of this study, all eleven selected isolates tested positive for siderophore production. This result is similar to the observation of **CHOWDHURY et al. (2017)**, who reported that 75% of their isolates produced siderophores. Thus, our isolated endophytes could enhance plants' stress tolerance by stimulating the uptake of iron complexes by roots **(VESSEY, 2003)** and starving phytopathogens of iron, thereby limiting their proliferation **(GLICK, 2012; KAJULA et al., 2010)**. Ammonia production capability of endophytes confers resistance on plants against pathogens and directly promotes plant growth through a continuous nitrogen supply **(HASSAN, 2017)**. All isolates in this study produced

ammonia, indicating their plant growth-promoting capacities. Ten of the selected isolates showed HCN activity, suggesting their possible role as biocontrol agents to inhibit phytopathogens and weed proliferation **(KUNDAN and PANT, 2015)**.

Some studies have reported numerous inter-species relationships between endophytes *in planta* **(XIAO-MI et al., 2015)**. These interactions are believed to be governed by competition for space and resources and the nutrient derivation and plant tissues colonisation efficiency of the competitors **(KIA et al., 2019)**. In this study, there was no visible *in vitro* antagonistic interaction between RB-2 and LF-5, suggestive of a possible mutual or related spatial habitation. In sharp contrast to our observation, *P. polymyxa* has been reported as a fierce antagonist of many pathogenic *Fusaria* sp. **(KHAN et al., 2020b; TIMMUSK et al., 2019)**. Endophytic communities within plants often co-exist, and no species is entirely exterminated in the complex interactions. However, weak endophytic competitors may be deprived of specific nutrients and resources **(KIA et al., 2019)**. The use of ITS and 16S rRNA gene nucleotide sequences are rich, dynamic, and reliable species-specific methods commonly employed to identify fungi and bacteria, respectively **(CLARRIDGE III, 2004; KOUADRIA et al., 2018)**; thus, they were used to characterise the two isolates accurately. Many studies have reported the isolation and characterisation of bacterial and fungal endophytes from plants in the Mint (Lamiaceae) family. Specifically, *F. oxysporum* endophytic strains have been isolated and identified from *Monarda citriodora* **(KATOCH and PULL, 2017)**, *Leucas aspera* and *Ocimum sanctum* **(BANERJEE et al., 2009)**. In contrast, *P. polymyxa,* to the best of our knowledge, has not been isolated from the Mint family but has been obtained from other medicinal plants, including *Lonicera japonica* **(ZHAO et al., 2015)**, *Lilium lancifolium* **(KHAN et al., 2020a)**, *Ephedra foliate* **(GHIASVAND et al., 2019)**, and *Panax ginseng* **(GAO et al., 2015)**. As far as we know, this is the first report about the isolation,

characterisation, and *in vitro* plant growth-promoting activity of *F. oxysporum* and *P. polymyxa* obtained from *E. obtusifolius*.

5.5. Conclusion

This study revealed that *E. obtusifolius*, naturally found in semi-arid areas, hosts a diverse group of fungal and bacterial endophytes. A total of 26 culturable endophytes (twelve fungi and fourteen bacteria) were isolated from the organs of *E. obtusifolius*. These endophytic species displayed varying *in vitro* plant-growth-promoting and drought stress tolerance capacities. In this study, two of the most promising water stress-tolerant and plant growth-enhancing endophytic species (*F. oxysporum* and *P. polymyxa*) were subsequently identified using molecular tools. The species did not display any form of hostility in the *in vitro* dual culture experiment. Thus, data from this study indicated that the inoculation (individually or in combination) of *F. oxysporum* and *P. polymyxa* into plants could promote plant growth and enhance the tolerance of plants to water stress. Therefore, the characterisation and identification *F. oxysporum* and *P. polymyxa* from *E. obtusifolius*, for the first time as far as we know, elucidates further the nature of endophytes residing in the endosphere of medicinal plants and their possible potentials as synthetic fertiliser alternatives and efficient environment-friendly bio-inoculants for sustainable cultivation of indigenous plants.

CHAPTER 6: Endophytes enhance the growth, secondary metabolite contents, and alpha-glucosidase inhibitory activities of *Endostemon obtusifolius* (E. Mey. ex Benth.) N. E. Br. under drought stress

This chapter was written following the format of the Plant Growth and Regulation journal.

6.1. Introduction

Over the past decades, the harvesting of medicinal herbs and their products (secondary metabolites) for research, trade, therapeutic, and industrial benefits has increased tremendously, thereby pilling pressure on the wild populations **(ALAMGIR, 2017)**. Under these circumstances, biodiversity has been lost, and many habitats have been degraded **(LUBBE and VERPOORTE, 2011)**. Thus, it becomes important to investigate the optimum cultivation conditions and strategies to enhance the *in-planta* biosynthesis of these valuable bioactive compounds, achieve high-quality product uniformity, meet the escalating demands for these active principles and restore the environment **(ALAMGIR, 2017)**. Nonetheless, adverse climatic conditions, diseases and pests, crop failure, and the high cost of cultivation are some of the problems hindering extensive cultivation of medicinal plants globally **(ALAMGIR, 2017)**. Nowadays, one of the most popular and economically viable strategies adopted towards the enhancement of valuable plant secondary metabolite synthesis is the deliberate subjection of the plants to different types of abiotic stress together with the application of exogenous substances and beneficial microorganisms (biotic elicitors) such as endophytic symbionts **(SEKAR and KANDAVEL, 2015; THAKUR et al., 2019; ZHANG et al., 2019)**.

Drought is a major global environmental threat affecting plant metabolism, growth and yield **(OSMOLOVSKAYA et al., 2018)**. Water deficit effects can manifest at different levels of a plant organisation, and it may impair processes such as water absorption, mineral nutrient uptake,

photosynthesis, gaseous exchange, cell division, and cellular respiration **(KAPOOR et al., 2020)**. As a consequence of drought, excessive reactive oxygen species (ROS) accumulates in plant tissues. Excess generation of ROS is lethal to cells as it peroxidises membrane lipids, damages other macromolecules such as proteins, and nucleic acids, inhibits enzymatic activities and ultimately leads to the death of cells **(KHAN et al., 2018)**. In response to drought and oxidative stress, plants activate a series of protective mechanisms, including osmolyte (proline and soluble sugar) synthesis and accumulation, enhanced activities of antioxidant enzymes [superoxide dismutase (SOD)], and non-enzymatic antioxidants (phenolic compounds and ascorbic acid) to scavenge the toxic free radicals **(KAPOOR et al., 2020)**. Moreover, studies have shown that some of the resistance of plants to drought and other stresses are conferred by their symbiont endophytes **(LATA et al., 2018; RHO et al., 2018)**.

Endophytes (fungi or bacteria) are typically mutualistic microbes that colonise plant tissues intercellularly and intracellularly without manifesting any sign of infection or adverse effects **(KHARE et al., 2018; NAIR and PADMAVATHY, 2014)**. Under drought conditions, endophytes, in addition to their host's innate responses, promote growth, suppress diseases, assist in nutrient assimilation, induce drought tolerance via the synthesis of certain enzymes, exopolysaccharides, growth regulators, and volatile compounds **(KHAN et al., 2017; LU et al., 2021)**. Additionally, they may mediate stress-responsive genes, elicit anatomical and physiological responses, and increase the *in planta* concentrations of antioxidants and osmolytes **(KHARE et al., 2018)**.

Endostemon obtusifolius (E. Mey. ex Benth.) N. E. Br. is an indigenous perennial shrub of southern Africa belonging to the Lamiaceae (mint) family. *E. obtusifolius* is an aromatic plant, and its leaves are used traditionally as a culinary and, by extension, as a medicinal plant **(SADASHIVA et al.,**

2014). Although *E. obtusifolius* remains understudied and under-utilised, it has been reported as an inexpensive source of neuroprotective, analgesic, anesthetic, and antioxidant compounds **(SADASHIVA et al., 2014, SADASHIVA et al., 2013)**. Plant secondary metabolites with antioxidant properties are abundantly distributed in the Mint family **(TAMOKOU et al., 2017)**, and these antioxidants play a protective role in the pathogenesis of degenerative diseases, including renal dysfunction, diabetes, obesity, high blood pressure, and atherosclerosis **(ONG et al., 2018)**. *E. obtusifolius* is well distributed in the semi-arid regions of South Africa; thus, it is perpetually exposed to some level of water deficit. Previous studies on *E. obtusifolius* have focused mainly on its pharmacological values. However, its morphological, biochemical, and physiological responses, as well as its pharmacological status under drought stress with or without endophyte inoculation have not been reported. Consequently, the present study was conducted to explore the effects of individual and co-inoculations of the previously **(Chapter 5)** cultured and identified fungal endophyte (*Fusarium oxysporum*) and bacterial endophyte (*Paenibacillus polymyxa*) on the growth, tolerance, and pharmacological values of *E. obtusifolius* under varying watering regimes.

6.2. Materials and Methods

6.2.1. Cultivation of *E. obtusifolius* and growth conditions

E. obtusifolius stem cuttings used in this study were raised following **ANITH et al., (2018)** modified procedure. Stem cuttings of approximately 10 cm (height) and 3 cm (diameter) with 3-4 nodes from young and disease-free lateral branches of a solitary mother *E. obtusifolius* plant were harvested and defoliated except for a flag leaf. Thereafter, stem cuttings were carefully placed in sterile distilled water until they were treated. The basal portions of the stem cuttings were dipped in rooting hormone (Seradix B No.3, Bayer Crop Science, Germany) for 1 min and planted in

disinfected trays (27 cm × 18 cm) containing sterile vermiculite and maintained in a mist house with a day and night temperatures of 28/15 ºC, relative humidity of 80-90%, and 10 s misting at 15 min intervals. For three weeks, the stem cuttings were maintained in the mist house, and they were subsequently transplanted into 7.5 cm diameter pots containing sterile palm peat and vermiculite (1:1) and kept in a greenhouse with 30-40% relative humidity and an average photosynthetic photon flux density (PPFD) of 450 µmol $m^{-2}s^{-1}$. After a further three weeks, well-rooted stem cuttings were transplanted into 15 cm diameter pots containing 1:3 sterile vermiculite and twice autoclaved garden soil [with physiochemical properties including 11% moisture, 5 mg/kg of P, 1.7 mg/kg of K, 5 mg/kg of alkali hydrolysable N, 9 mg/kg of Ca, 40% field capacity (FC) and pH of 7.80] under the same greenhouse conditions as stated above. Finally, the stem cuttings were irrigated as required with half-strength Hoagland's solution **(HOAGLAND and ANON 1938)** and monitored for an additional four weeks.

6.2.2. Preparation and application of microbial inoculants

The cultured and previously identified **(Section 5.2.9.** of **Chapter 5)** fungal endophyte (*F. oxysporum*) was maintained on PDA plates for two weeks at 28 ºC in an incubator. Thereafter, the plates were flooded with 10 ml of sterilised distilled water containing 0.05% (v/v) Tween-20. The fungal colonies were then gently scratched off the media surface using a sterile spatula into a clean and autoclaved beaker. The conidial suspension was made by filtering the harvested mycelial mass through a double layer of sterilised cheesecloth to remove the agar and mycelia debris. The final spore number was evaluated and adjusted to 7×10^5 using a hemocytometer **(SADEGHI et al., 2020)**.

For the bacterial endophyte, previously cultured and identified **(Section 5.2.9.** of **Chapter 5)** *P. polymyxa* inoculum was prepared by growing the isolates in Schott bottles containing 250 ml of MHB and incubating at 28 °C on a shaking incubator at 180 rpm for two days. Subsequently, the cells in the broth were harvested by centrifugation (Avanti ® J-E Centrifuge Beckman Coulter, Ireland) at 5,000 g for 10 m at 4 °C, washed, and resuspended in sterile distilled water. The cell density (amount per ml) was adjusted to a 10^7 CFU/ml final concentration using sterile distilled water **(HUSSAIN et al., 2018c)**.

A modified soil-drenching inoculation method of **RAMAKUWELA et al. (2020)** was employed, and 20 ml of the bacterial suspension (1×10^7 CFU) or 80 ml of the conidial suspension poured around the root zone was taken as single inoculation. Co-inoculation was done by applying 20 ml of the bacterial suspension and 80 ml of the conidial suspension around the same region, whereas; the uninoculated group was pseudo-inoculated with 100 ml of distilled water. To confirm the establishment of the inoculated endophytes in the stem cuttings, five weeks after inoculation, the roots of each sub-groups were carefully harvested without disturbing the integrity of the root architecture. Isolation procedures, as explained earlier in **Chapter 5**, were followed, and the inoculated isolates were re-identified on plates using morphological characters.

6.2.3. Experimental design and treatments

The individual and combined effects of *P. polymyxa* and *F. oxysporum* inoculations on drought-stressed *E. obtusifolius* stem cuttings was investigated by setting up a factorial experiment with two combined factors: (1) inoculation of *P. polymyxa* (PI), *F. oxysporum* (FI), co-inoculation (P+FI), and uninoculated control (UI); (2) soil water content- well-watered (WW) or 100% FC, mild stress (MS) or 50% FC, and severe stress (SS) or 25% FC. Each of the twelve treatments had

ten replicates, and the plants (ten-week-old stem cuttings) were then arranged in a completely randomised fashion. Six weeks after inoculation and 16 weeks post stem cuttings, the plants were subjected to drought stress, as highlighted above, for 8 weeks.

Figure 6.1: A cross-section of endophytes inoculated and uninoculated *Endostemon obtusifolius* stem cuttings under varying water regimes.

6.2.4. Determination of dry shoot and root weights

The dry shoot weight (DSW) and root weight (DRW) of plants (triplicates) in each of the twelve treatment groups were harvested, washed under running tap water, and divided into root and shoot systems. Then, the plants were oven-dried at 70 ºC for 48 h until constant weights and measured.

6.2.5. Relative water content determination

The relative water content (RWC) of the leaves of the different treatments was estimated as per **ZHANG et al. (2019)** method with slight modification. Matured and fully developed leaves were

selected, and their fresh weight (FW) was immediately recorded. The weighed leaves were immediately immersed in pill vials containing 25 ml distilled water and incubated for 24 h at low temperature (~4 °C) in the dark. After drying the surface water with paper towels, each leaf's turgid weight (TW) was also recorded. The samples were then oven-dried at 70 °C for 48 h, and the dry weight (DW) of each sample leaf was measured. The plants' water status was evaluated by estimating RWC using the formula:

$$RWC(\%) = [(FW- DW) / (TW - DW)] \times 100$$

6.2.6. Electrolyte leakage estimation

Electrolyte leakage (EL) of the treatments was estimated from leaf discs obtained from leaf samples in each treatment to measure cell membrane stability. Briefly, equal-sized leaf discs (0.5 cm) from each treatment were immersed in pill vials containing 10 ml of distilled water for 4 h at room temperature. Afterwards the electrical conductivity of the medium (EC1) was recorded using an electrical conductivity meter. Thereafter, the pill vials were capped and autoclaved at 121 °C for 15 min to disrupt the cell membrane and fully release the electrolytes. After cooling at room temperature, the final electrical conductivity (EC2) was measured. EL percentage was calculated using the formula:

$$EL (\%) = (EC1/EC2) \times 100$$ **(KUMAR et al., 2018)**.

6.2.7. Measurement of antioxidant enzyme activity and lipid peroxidation level in leaves

6.2.7.1. Determination of superoxide dismutase activity

The superoxide dismutase (SOD) activity of the treatments was quantified spectrophotometrically at 560 nm using the nitroblue tetrazolium (NBT) method described by **YE et al. (2021)**, with slight adjustments. In brief, each weighed leaf sample (0.2 g) from each treatment was homogenised in a 2 ml extraction solution (100 mM phosphate buffer (pH 7.8) with 0.1 mM EDTA), and the homogenate was centrifuged at 25,000 *g* for 20 min at 4 °C. After that, in test tubes, 100 µl of the enzyme extract was added to a 2 ml reaction mixture (containing 130 mM methionine, 750 µM NBT, 20 µM riboflavin, and 1 mM $EDTANa_2$), and the photo-reduction was initiated under light (fluorescent lamp 4000 lx) and incubated for half an hour at room temperature. Similar tubes with the same reaction contents but without enzyme extracts served as a background control, while a dark incubated tube containing reaction mixture without enzyme extract was used as a blank. One unit of SOD activity was expressed as the amount of enzyme that suppressed the photoreduction rate of NBT by 50% compared to tubes without enzyme extracts and expressed as SOD units per gram fresh weight (U g^{-1} FW). Protein concentration was measured using bovine serum albumin as a standard **(BRADFORD, 1976)**.

6.2.7.2. Malondialdehyde content

The concentration of malondialdehyde (MDA) in the leaves was used to estimate the level of lipid peroxidation following a modified protocol of **TYAGI et al. (2017)**. Pre-weighed leaf samples were homogenised in 2 ml of 0.1% trichloroacetic acid (TCA) and centrifuged at 11,000 *g* for 10 min. Thereafter, 1 ml of 0.25% thiobarbituric acid (TBA) in 10% TCA was added to 1 ml of

the supernatant. The reaction mixture was heated at 95 °C on a water bath for 3o minutes and immediately cooled on ice. The absorbance of the reaction mixture was measured at 532 nm and 600 nm, and MDA content was calculated using the extinction coefficient of MDA (ε = 155 mM^{-1} cm^{-1}) and expressed in nanomoles per gram fresh weight (nmol g^{-1} FW).

6.2.8. Quantification of photosynthetic pigments

After the drought treatments, total chlorophyll (*a* + *b*) and carotenoids of fully expanded leaves of *E. obtusifolius* plants (n=3) were determined according to the described methods of **LICHTENTHALER (1987)**. In brief, 0.1 g of freshly harvested leaf materials were homogenised into 5 ml acetone (ice-cold). The resultant mixture was then centrifuged (Hettich Universal, Tuttlingen, Germany) at 1,000 g for 10 min under room temperature. The chlorophyll content was estimated by measuring the absorbance of the triplicate samples at 470, 645, and 662 nm using a UV-visible spectrophotometer. Total chlorophyll (*a* + *b*) and carotenoid contents (mg/g of FW) were calculated using the formulae:

Chlorophyll *a* = $11.23A_{662} - 2.04A_{645}$

Chlorophyll *b* = $20.13A_{645} - 4.19A_{662}$

Total Chlorophyll *a* + *b* = $7.05A_{662} + 18.09A_{645}$

Total carotenoids = $(1000A_{470} - 1.90Chla - 63.14Chlb)/214$

6.2.9. Measurement of proline content

The proline content of the sample leaves from each treatment was determined following the **BIBI et al. (2019)** protocol. One hundred milligrams of fresh leaf samples were homogenised in 5 ml of

3% sulfosalicylic acid and centrifuged at 11,000 *g* for 15 min at 4 °C. Then 200 µl of the supernatant was mixed with 250 µl of 3% sulfosalicylic acid, 500 µl glacial acetic acid, and 250 µl of 2.5% acidic ninhydrin in a test tube. The reaction mixture was then boiled at 100 °C for 60 min and cooled on ice. The cooled reaction mixture was then extracted with 1 ml toluene, vortexed, and the chromophore containing organic phase was allowed to separate. The chromophore-containing toluene absorbance was measured at 520 nm against toluene (blank), and the proline concentration was calculated from a proline standard calibration curve and expressed in mg g^{-1}FW.

6.2.10. Measurement of photochemical efficiency of photosystem II (F_v/F_m)

The effects of endophytes' inoculation and drought stress on the chlorophyll fluorescence of *E. obtusifolius* plants was assessed after 7 weeks of the drought treatments using a portable chlorophyll fluorometer (OS-30p; Opti-Sciences, Inc., NH, USA). The chlorophyll fluorescence measurements were carried out in the dark on the upper leaf (adaxial) surface on the youngest but mature leaf samples after an initial 45 min dark adaptation of the plant samples. After the dark treatments, minimal fluorescence (F_0) was recorded by applying weak modulated light (0.4 µmol $m^{-2}s^{-1}$), and the maximal fluorescence (F_m) was subsequently measured after illuminating a saturating flashlight (8000 µmol $m^{-2}s^{-1}$) for 0.8 s. Measurements were taken from the same leaves and the variable fluorescence (F_v) was computed by an in-built programme to determine the maximal photochemical efficiency of PSII **(KHAN et al., 2016)**.

6.2.11. Determination of total soluble sugar

After the treatments, the total soluble sugar in *E. obtusifolius* leaves was estimated following a modified protocol of **TYAGI et al. (2017)**. Two hundred milligrams of freshly harvested leaves

across treatments in test tubes were hydrolysed in 5 ml of 2.5 N HCl, boiled on a water bath at 95 °C for 3 h and then cooled to room temperature. The hydrolysed mixtures were then neutralised by adding sodium carbonate granules. Thereafter, appropriate volumes of distilled water were added to the 5 ml mark of the test tubes and centrifuged at 10,062 g for 15 min at 4 ºC. One hundred microliters of the supernatants were drawn out and added to 4 ml of anthrone reagent, followed by 0.9 ml of distilled water in test tubes. Finally, the solutions were boiled again for 8 min on a water bath and cooled immediately under running tap water. The developed dark green reaction mixtures' absorbance was recorded at 630 nm. The amount of total soluble sugar was estimated on a glucose concentration standard curve and expressed in µg g^{-1}FW.

6.2.12. Histochemical detection of hydrogen peroxide

Endogenously produced H_2O_2 was visualised using the 3′3-diaminobenzidine (DAB) staining method protocol described by **YANG et al. (2013)**. Leaf samples from each treatment were submerged in 1 mg/ml DAB solution at room temperature, pH 3.8 for 8 h under illumination. After the 8-h incubation, the leaves were recovered and boiled in 95% (v/v) ethanol for 10 min to decolourise the leaves (remove pigments), save the deep brown polymerisation product of DAB reaction with H_2O_2. After cooling, the bleached leaves were preserved in 4:1 (v/v) ethanol-glycerol solution and kept at 4 ºC until photographed. Intense brown colouration indicates a higher concentration of H_2O_2 in the leaves.

6.2.13. Estimation of phytochemical content

The total phenolics and flavonoid contents of the ethyl acetate leaf extracts from each treatment were quantified as described in **Section 3.2.6.** of **Chapter 3**.

6.2.14. 1-1- Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The radical scavenging activities of the ethyl acetate leaf extracts exposure was carried out as described in **Section 3.1.4.1.** of **Chapter 3**.

6.2.15. Ferric-reducing antioxidant power (FRAP) assay

The iron reducing power of each treatment after the drought stress exposure was carried out as described in **Section 3.1.4.2.** of **Chapter 3**.

6.2.16. α-glucosidase

The hypoglyceamic activity of the *E. obtusifolius* leaves in each treatment was done as described in **Section 3.2.5.2.** of **Chapter 3**.

6.2.17. Data analysis

Data obtained from this study were analysed statistically based on the experimental design with a two-way analysis of variance using GraphPad Prism 7 (GraphPad Software, Inc. California USA). Mean comparison was done with the Bonferroni post hoc test at a 5% significance level ($P \leq 0.05$).

6.3. Results

6.3.1. Shoot and root dry weights

The main factors (watering levels and endophyte inoculation) and their synergistic effects significantly ($P \leq 0.05$) influenced the root and shoot biomass of *E. obtusifolius* plants **(Table 6.1a**, **Figure 6.2)**. Overall, the dry shoot and root weights decreased with increased water deficit levels, whereas endophyte-inoculated *E. obtusifolius* plants had higher biomass than endophyte-free plants **(Table 6.2)**. The highest dry shoot and root weights (1.27 g and 0.55 g, respectively) in the uninoculated plants were found under the MS water regime. Moreover, at WW and SS (drought stress levels) conditions, the P+FI treatment was found to be most effective in the accumulation shoot biomass (3.91 g and 1.37 g, respectively), while the highest dry shoot weight (1.84 g) at MS was found in the FI treatment **(Table 6.2)**. **Table 6.2** shows that the P+FI treatment recorded the highest DRW values across the varying water regimes (WW = 1.75 g, MS = 1.27 g, and SS = 0.81 g).

6.3.2. Photosynthetic pigments and chlorophyll fluorescence

The concentration of photosynthetic pigments in *E. obtusifolius* plants were affected significantly by increasing water stress, individual inoculation, and co-inoculations with *P. polymyxa* and *F. oxysporum* **(Table 6.1)**. The total chlorophyll content of *E. obtusifolius* was sharply enhanced as the severity of water stress increased, and the highest total chlorophyll content (706.78 µg/g FW) in the uninoculated plants were recorded under the severe stress condition. The fungal inoculated plants had the highest quantity of total chlorophyll under WW with a 34% increase compared to the uninoculated plants **(Table 6.2)**. On the other hand, co-inoculation (P+FI treatment) under both

MS and SS conditions resulted in a 21% stimulation of total chlorophyll concentration. **Table 6.2** further revealed that in the uninoculated *E. obtusifolius* plants, carotenoid content diminished with increasing water deficit, although the highest concentration (77.35 µg/g FW) was found under mild stress conditions. The inoculation of *E. obtusifolius* with endophytes did not influence the carotenoid content under mild stress, whereas carotenoid concentrations were enhanced under both well-watered and severe stress conditions. The highest carotenoid content was found in the bacteria (*P. polymyxa*) inoculated plants under the SS condition, with a 130% increase relative to its uninoculated control **(Table 6.2)**.

The interactive effects and the main factors of this study noticeably influenced the photochemical efficiency (F_V/F_M) of *E. obtusifolius* plants **(Table 6.1)**. There was no difference between the F_V/F_M values of uninoculated plants under WW and MS conditions, whereas a 39% reduction was observed under the SS condition **(Table 6.2)**. Meanwhile, the co-inoculation treatment (P+FI) under all the watering regimes (WW, MS, and SS) improved the photochemical efficiency of *E. obtusifolius* plants by 1.2%, 6%, and 29%, respectively **(Table 6.2)**.

Table 6.1a: Two-way analysis of variance for the effects of drought stress, endophytes inoculation and their interaction on the dry shoot weight, root shoot weight total chlorophyll, carotenoid content, chlorophyll fluorescence, total soluble sugar, relative water content and proline content of *E*ndostemon *obtusifolius*.

Factor/ Interaction	Dry shoot weight		Dry root weight		Total chlorophyll		Carotenoids		Chlorophyll fluorescence		Total soluble sugar		Relative water content		Proline	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Drought (D)	371.01	0.00*	1173.66	0.00*	32607.66	0.00*	8.43	0.00*	4279.11	0.00*	574.64	0.00*	90.31	0.00*	13.67	0.00*
Endophyte (E)	187.26	0.00*	1044.33	0.00*	28729.05	0.00*	364.06	0.00*	1250.44	0.00*	4912.96	0.00*	15.71	0.00*	23.40	0.00*
D×E	85.26	0.00*	168.23	0.00*	5640.16	0.00*	361.35	0.00*	888.23	0.00*	2346.88	0.00*	3.16	0.02*	22.33	0.00*

ns = not significant at $p \leq 0.05$, * = significant at $p \leq 0.05$.

Table 6.1b: Two-way analysis of variance for the effects of drought stress, endophytes inoculation and their interaction on electrolyte leakage, SOD, MDA, flavonoids, total phenolic content, *in vitro* antioxidant activities and α-glucosidase activities of *E*ndostemon *obtusifolius*.

Factor/ Interaction	Electrolyte leakage		SOD		MDA		Flavonoids		Total phenolics		DPPH IC_{50}		FRAP slope		α-glucosidase IC_{50}	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Drought (D)	12.77	0.00*	102.81	0.00*	41.66	0.00*	7150.18	0.00*	34.94	0.00*	267.52	0.00*	286.70	0.00*	300.14	0.00*
Endophyte (E)	16.18	0.00*	40.68	0.00*	2.02	0.14ns	2650.03	0.00*	74.68	0.00*	54.96	0.00*	66.59	0.00*	129.75	0.00*
D×E	1.11	0.39ns	40.75	0.00*	0.72	0.64ns	1621.11	0.00*	22.93	0.00*	40.49	0.00*	72.57	0.00*	27.44	0.00*

ns = not significant at $p \leq 0.05$, * = significant at $p \leq 0.05$.

Table 6.2: Variations in dry shoot weight (DSW), dry root weight (DRW), total chlorophyll, carotenoid, chlorophyll fluorescence, and carbohydrate content in leaves of *E*ndostemon *obtusifolius* inoculated with or without endophyte inoculation under varying watering regimes.

Drought Treatment	Endophyte inoculant	DSW (g)	DRW (g)	Total chlorophyll (µg/g FW)	Carotenoids (µg/g FW)	Chlorophyll fluorescence (F_V/F_M)	Total soluble sugar (µg/g FW)
WW (100% FC)	UI	0.62 ± 0.27^{f}	0.37 ± 0.00^{g}	536.96 ± 27.19^{k}	61.72 ± 0.40^{d}	0.82 ± 0.00^{c}	44.27 ± 0.18^{i}
	PI	2.31 ± 0.02^{b}	1.40 ± 0.03^{b}	664.83 ± 19.56^{f}	83.22 ± 0.34^{b}	0.82 ± 0.00^{c}	46.34 ± 0.16^{h}
	FI	3.78 ± 0.02^{a}	1.63 ± 0.01^{a}	721.48 ± 16.35^{c}	83.24 ± 0.14^{b}	0.81 ± 0.00^{c}	69.47 ± 0.09^{c}
	P+FI	3.91 ± 0.02^{a}	1.75 ± 0.01^{a}	659.98 ± 19.62^{g}	51.85 ± 3.98^{e}	0.83 ± 0.00^{b}	72.25 ± 0.15^{b}
MS (50% FC)	UI	1.27 ± 0.02^{de}	0.55 ± 0.01^{f}	598.69 ± 36.89^{j}	77.35 ± 0.49^{c}	0.82 ± 0.00^{c}	57.50 ± 0.50^{e}
	PI	1.63 ± 0.02^{cd}	0.63 ± 0.01^{f}	603.01 ± 21.28^{i}	55.25 ± 0.69^{e}	0.82 ± 0.00^{c}	47.87 ± 0.18^{g}
	FI	1.84 ± 0.02^{c}	1.06 ± 0.02^{d}	672.45 ± 1.47^{e}	74.47 ± 0.26^{c}	0.84 ± 0.00^{c}	63.07 ± 0.15^{d}
	P+FI	1.75 ± 0.02^{c}	1.27 ± 0.01^{c}	724.24 ± 5.76^{c}	63.35 ± 0.23^{d}	0.87 ± 0.00^{a}	82.78 ± 0.00^{a}
SS (25%FC)	UI	0.81 ± 0.02^{f}	0.21 ± 0.03^{h}	706.78 ± 6.55^{d}	46.52 ± 0.77^{f}	0.59 ± 0.00^{f}	49.76 ± 0.50^{f}
	PI	1.07 ± 0.07^{ef}	0.78 ± 0.00^{e}	644.12 ± 4.77^{h}	106.34 ± 0.47^{a}	0.76 ± 0.00^{e}	69.74 ± 0.32^{c}
	FI	1.09 ± 0.04^{ef}	0.53 ± 0.02^{f}	817.18 ± 26.35^{b}	65.35 ± 0.03^{d}	0.79 ± 0.00^{d}	39.51 ± 0.24^{j}
	P+FI	1.37 ± 0.01^{cd}	0.81 ± 0.03^{e}	856.59 ± 5.85^{a}	61.97 ± 0.23^{d}	0.76 ± 0.00^{e}	68.57 ± 0.90^{c}

Means ± standard error (n=3) in each column followed by different letter(s) indicate a significant difference (at $p < 0.05$) between treatments as determined by two-way ANOVA and Bonferroni post hoc test. UI = uninoculated, PI = *P. polymyxa* inoculated, FI = *F. oxysporum* inoculated, and P+FI = *P. polymyxa* and *F. oxysporum* inoculated.

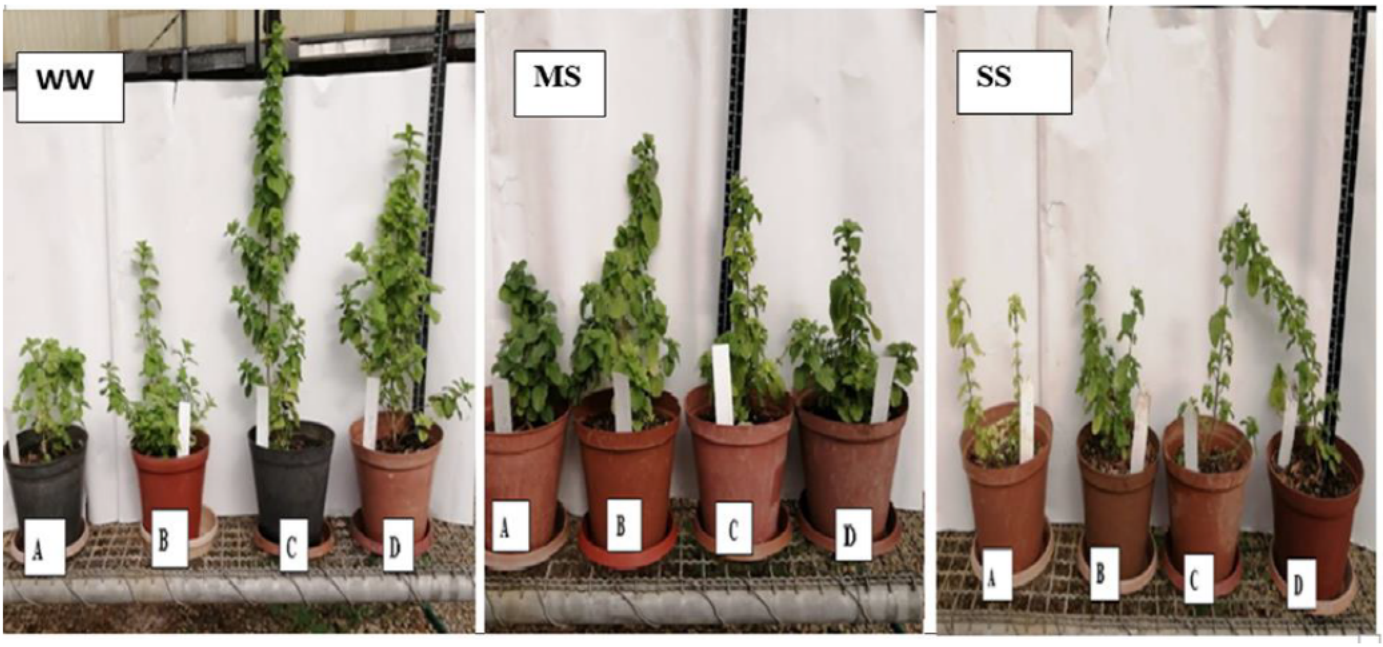

Figure 6.2a: The effects of drought stress levels on the growth of endophyte inoculated and uninoculated *Endostemon obtusifolius* stem cuttings (before harvest). WW = well-watered, MS = mild stress, SS = severe stress, A = uninoculated treatment, B = *P. polymyxa* inoculated, C = *F. oxysporum* inoculated, and D = *P. polymyxa* and *F. oxysporum* inoculated.

Figure 6.2b: The effects of drought stress levels on the growth of endophyte inoculated and uninoculated *Endostemon obtusifolius* stem cuttings (after harvest). WW = well-watered, MS = mild stress, SS = severe stress, A = uninoculated treatment, B = *P. polymyxa* inoculated, C = *F. oxysporum* inoculated, and D = *P. polymyxa* and *F. oxysporum* inoculated.

6.3.3. Relative water content and electrolyte leakage

The varying water regimes, the endophyte inoculation, and their interactive effects significantly affected the relative water content of *E. obtusifolius* leaves at $P \leq 0.05$ **(Table 6.1a)**. Drought stress reduced the relative water content of *E. obtusifolius* as its severity increased in this study. The highest relative water content value in the uninoculated plants was found under the WW condition, which was 11% and 55% higher than the *E. obtusifolius* RWC values under MS and SS conditions, respectively **(Figure 6.3)**. *F. oxysporum* inoculation was most helpful in improving the RWC of *E. obtusifolius* under the stress conditions as it yielded the highest RWC values under WW and MS conditions. Moreover, under SS conditions, the co-inoculation treatment significantly enhanced the relative water content of *E. obtusifolius* compared to the other treatments **(Figure 6.3)**.

The electrolyte leakage of *E. obtusifolius* was influenced by the factors (individually); however, their synergistic effect at $P \leq 0.05$ was not statistically significant **(Table 6.1b)**. In general, there was an increase in electrolyte leakage as the drought stress became more devastating. The highest electrolyte leakage was recorded in the uninoculated plants under the SS condition **(Figure 6.4)**. As further displayed in **Figure 6.4**, fungal inoculation under the well-watered treatment, led to the highest substantial reduction in electrolyte leakage.

6.3.4. Total soluble sugar and proline content

In this study, the two factors and their interactive effects substantially affected the proline content and total soluble sugar of *E. obtusifolius* **(Table 6.1a)**. The highest total soluble sugar concentration in the uninoculated plants was found in the MS treatment whereas the lowest concentration was found in plants under WW conditions **(Table 6.2)**. Overall, endophyte

inoculation promoted soluble sugar accumulation; notably, the co-inoculation treatments across the watering regimes (WW, MS, and SS) increased total soluble sugar by 64%, 44%, and 38%, respectively **(Table 6.2)**.

As presented in **Figure 6.5**, the highest proline content (13.24 mg g^{-1} FW) in the uninoculated plants was found in the mild water-deficit stress, whereas non-statistically different concentrations of proline were recorded at WW (9.34 mg g^{-1}FW) and SS (8.88 mg g^{-1} FW). This further implies that both WW and SS conditions are stressful conditions. Interestingly, microbial inoculation irrespective of the stress condition enhanced proline accumulation in this study. Co-inoculation treatments under mild and severe stress conditions markedly improved the proline contents of *E. obtusifolius* **(Figure 6.5)**.

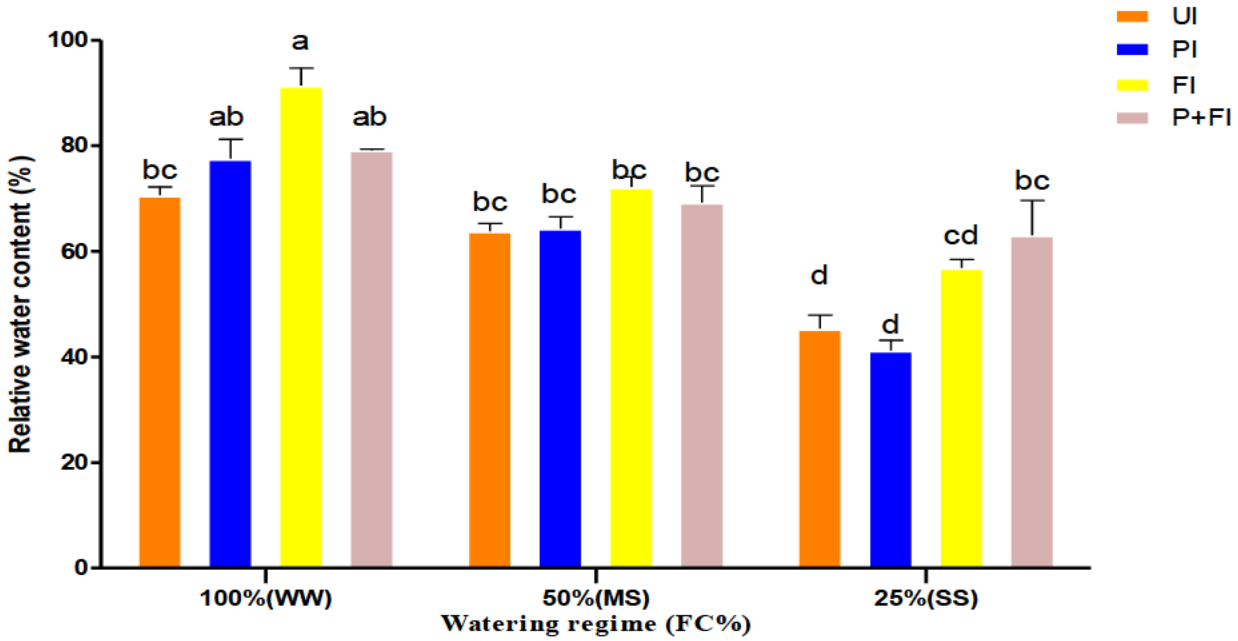

Figure 6.3: Impacts of varying water stress on the relative water content of *Endostemon obtusifolius* with or without endophyte inoculation. Means with the same letter(s) are not significantly different at $P < 0.05$ as determined by two-way ANOVA and Bonferroni post hoc test.

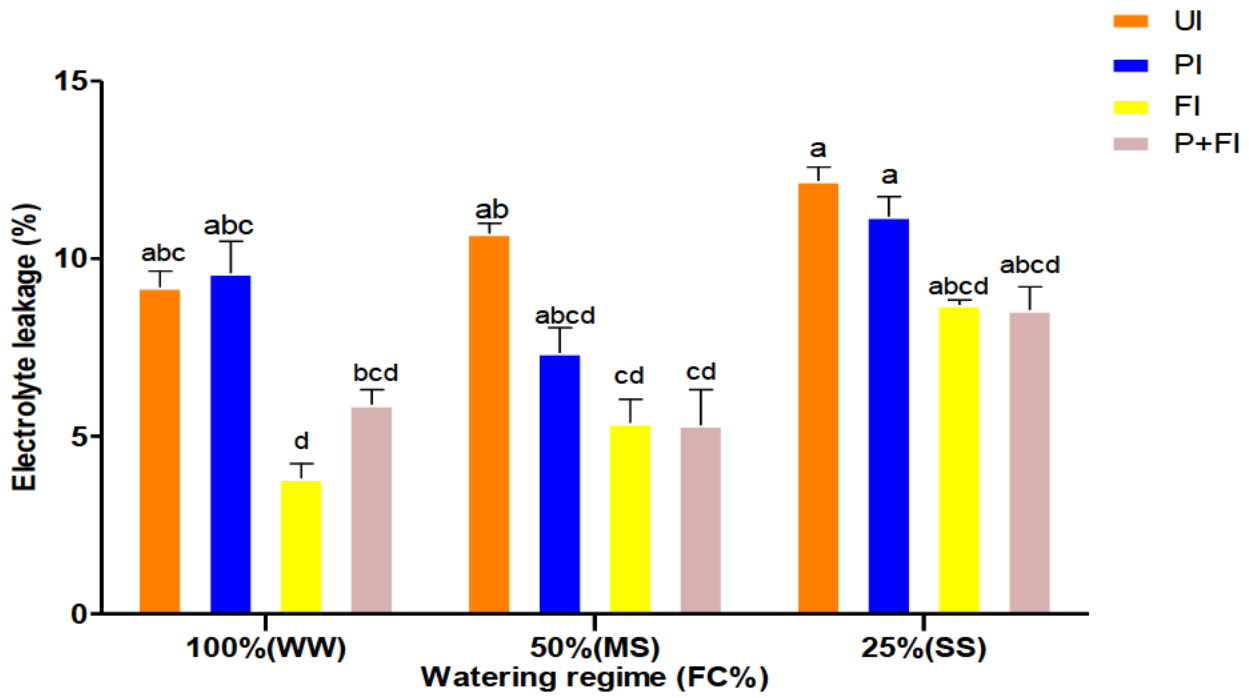

Figure 6.4: Impacts of varying water stress on the electrolyte leakage of *Endostemon obtusifolius* with or without endophyte inoculation. Means with the same letter(s) are not significantly different at $P < 0.05$ as determined by two-way ANOVA and Bonferroni post hoc test.

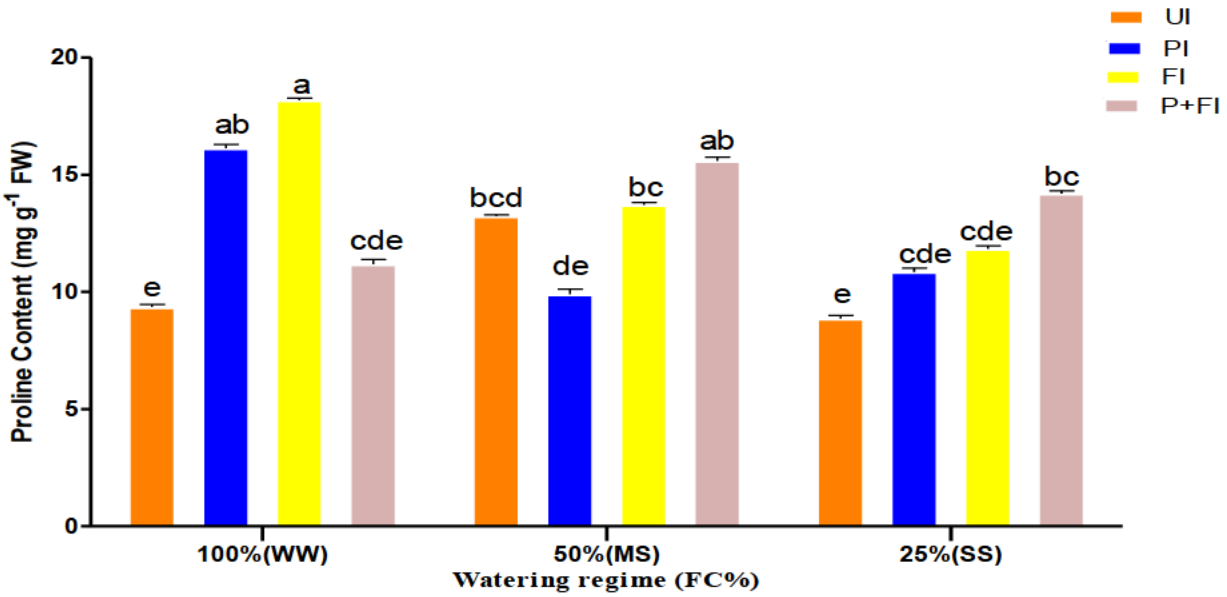

Figure 6.5: Effects of varying water stress on the proline contents of *Endostemon obtusifolius* with or without endophyte inoculation. Means with the same letter(s) are not significantly different at $P < 0.05$ as determined by two-way ANOVA and Bonferroni post hoc test.

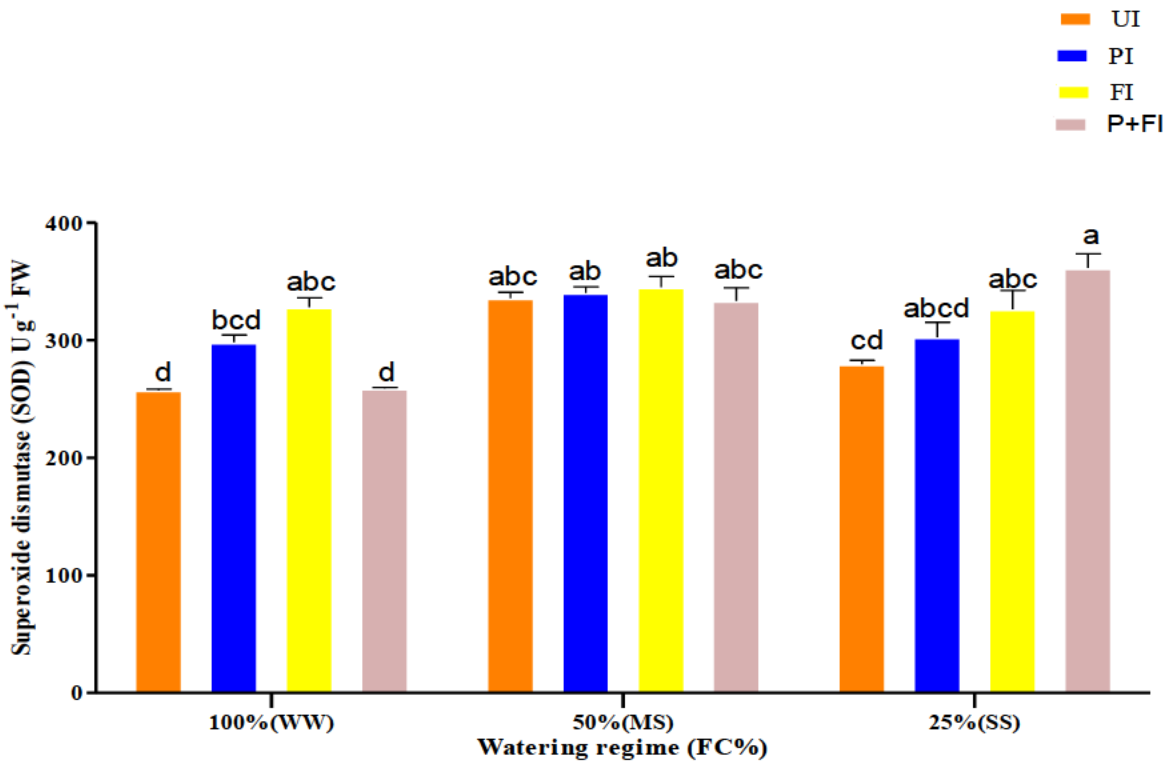

Figure 6.6: Superoxide dismutase activities in endophyte inoculated and uninoculated *Endostemon obtusifolius* stem cuttings under varying water regimes. Means with the same letter(s) are not significantly different at $P < 0.05$ as determined by two-way ANOVA and Bonferroni post hoc test

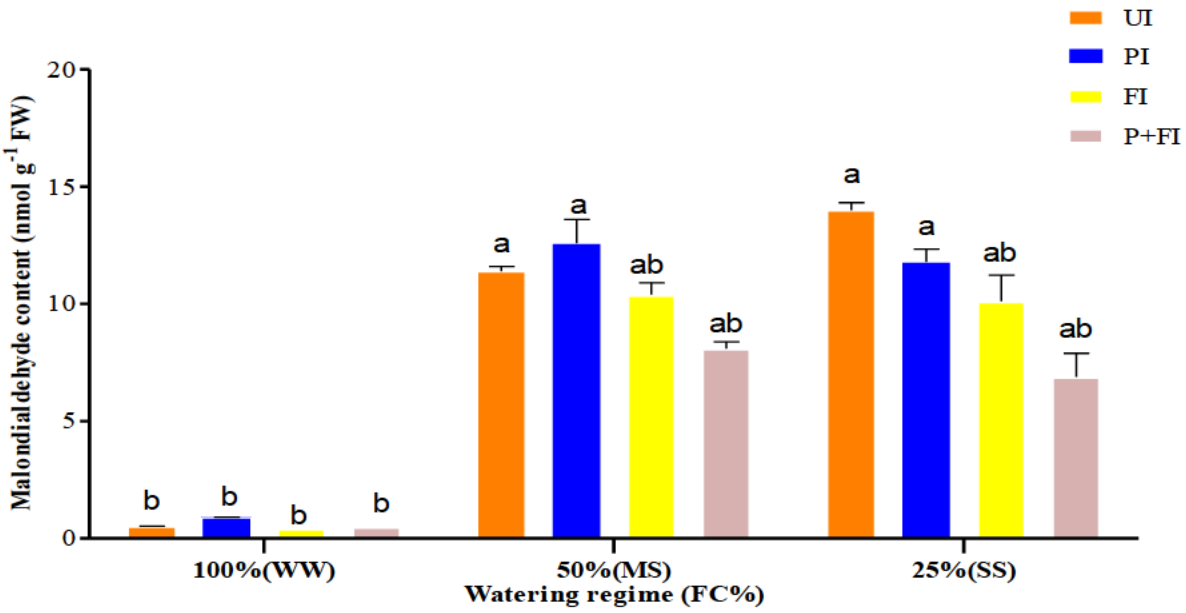

Figure 6.7: Effects of varying water regimes on the malondialdehyde contents of endophyte inoculated and uninoculated *Endostemon obtusifolius* stem cuttings. Means with the same letter(s) are not significantly different at $P < 0.05$ as determined by two-way ANOVA and Bonferroni post hoc tests at $P < 0.05$.

6.3.5. Superoxide dismutase activities, malondialdehyde content, and hydrogen peroxide visualisation

Superoxide dismutase (SOD) is one of the vital antioxidant enzymes involved in combating the resultant oxidative stress induced by drought stress. Drought stress, endophyte inoculation and their interactive effects caused significant (at $P \leq 0.05$) changes to SOD activities in *E. obtusifolius* **(Table 6.1b)**. Results indicated that the highest SOD activities in the uninoculated plants were recorded at mild stress conditions; it was, however, not statistically different from the SOD activities value of uninoculated plants under severe stress conditions **(Figure 6.6)**. Under SS conditions, co-inoculation of the endophytes markedly enhanced SOD activities, whereas the fungal treated plants displayed more SOD activities under WW and MS conditions **(Figure 6.6)**.

In this study, neither the varying water regimes nor the microbial inoculation significantly ($P \leq 0.05$) influenced the MDA content of *E. obtusifolius*, whereas their synergistic effects appreciably affected the extent of lipid peroxidation or cell damage in *E. obtusifolius* **(Table 6.1b)**. A significant accumulation of MDA was observed as the water deficit increased in this study in MS (22-fold) and SS (28-fold) conditions relative to the WW-treated plants **(Figure 6.7)**. Under the 25% FC, MDA content was lowered by 16%, 29% and 49% in the bacterial inoculated, fungal inoculated and co-inoculation plants, respectively **(Figure 6.7)**. Similarly, except for the bacterial treated group, all the inoculated plants reduced lipid peroxidation compared to the uninoculated plants under the MS water regime **(Figure 6.7)**.

Histochemical visualisation of hydrogen peroxide accumulation in the sample leaves of *E. obtusifolius* by DAB staining in this study showed that DAB polymerisation products appeared to increase significantly under stress, and the accumulation was more in uninoculated plants under 100% and 25% FC indicating a reduction in H_2O_2 accumulation in the inoculated plants. However,

there was little or no visual differences between inoculated and uninoculated plants under 50% FC **(Figure 6.8)**.

6.3.6. Phytochemical contents, radical scavenging activities and ferric reducing antioxidant power

Drought stress induces the accumulation of reactive oxygen species (ROS); thus, the total phenolic, flavonoid contents, radical scavenging activities, and ferric reducing power of inoculated and uninoculated *E. obtusifolius* plants under varying watering regimes were investigated. The radical scavenging capabilities, ferric reducing power, total phenolic contents (TPC) and flavonoids of *E. obtusifolius* were significantly affected at $P \leq 0.05$ by the watering regimes, microbial inoculation, and their interactive effects **(Table 6.1b)**. An overall increase in TPC and flavonoids were registered in inoculated *E. obtusifolius* plants **(Table 6.3)**. Under the varying watering regimes, endophyte inoculation accounted for 20 - 67%, 3 - 13%, and 28 - 35% in WW, MS, and SS conditions, respectively **(Table 6.3)**. Following the same trend, the highest flavonoid contents were found in the P+FI treatments within each watering regime **(Table 6.3)**. Generally, the quantity of TPC and flavonoids in the leaves of *E. obtusifolius* under drought stress were enhanced as the drought stress was increased.

The DPPH free radical scavenging activities of the ethyl acetate extracts of leaf samples, as shown by the IC_{50} values presented in **Table 6.3**, were lower in the inoculated plants across the watering regimes. Co-inoculated plants in all the watering levels exhibited slightly higher free radical scavenging abilities than the single inoculated plants. Nonetheless, the differences between uninoculated plants' antioxidant potentials (IC_{50} values) are negligible **(Table 6.3)**. This result demonstrated that the antioxidant potency of *E. obtusifolius* was elevated by microbial treatments

and co-inoculation in particular. Likewise, the slope values of FRAP increased in the inoculated *E. obtusifolius* compared to the uninoculated plants. Although the BHT (2.42) exhibited the highest antioxidant power, it was not significantly different from the same slope values (2.01) recorded for fungal and bacterial treated plants under the 100% FC **(Table 6.3)**.

6.3.7. α-Glucosidase inhibitory activity

The medicinal value (α-glucosidase retardation activity) of *E. obtusifolius* leaves was remarkably altered by the varying water levels, endophyte inoculation, and synergetic effects **(Table 6.1b)**. **Table 6.3** shows that as the watering level increases, the hypoglyceamic properties of uninoculated *E. obtusifolius* plants improved as the IC_{50} values reduced considerably by 57% and 87%, respectively, in MS and WW regimes **(Table 6.3)**. Similarly, under the watering regimes, microbial inoculations drastically lowered the α-glucosidase inhibition IC_{50} values of *E. obtusifolius*. Interestingly, the highest inhibitory effect was recorded in FI treatments under the WW (34.16 µg/ml) and SS (183.50 µg/ml) conditions, whereas the P+FI treatment showed the lowest IC_{50} (43.88 µg/ml) values under MS conditions **(Table 6.3)**.

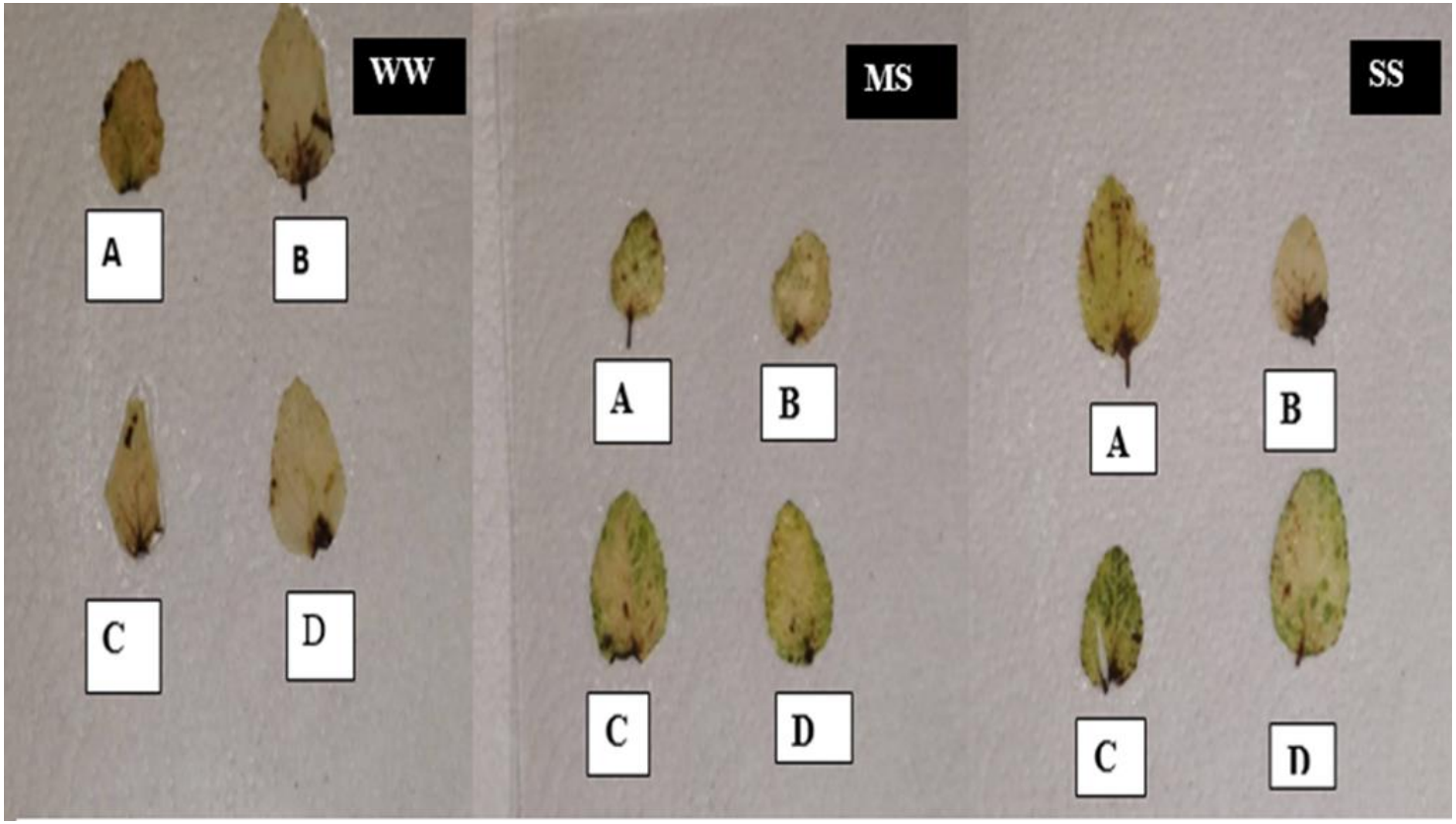

Figure 6.4: Pictorial depiction of hydrogen peroxide accumulation in the leaves of endophyte inoculated and uninoculated *Endostemon obtusifolius* stem cuttings under varying watering regimes. WW = well-watered, MS = mild stress, SS = severe stress, A = uninoculated treatment, B = *P. polymyxa* inoculated, C = *F. oxysporum* inoculated, and D = *P. polymyxa* and *F. oxysporum* inoculated.

Table 6.3: Changes in the total phenolic content, flavonoid content, IC_{50} values against α-glucosidase, ferric-reducing antioxidant power (FRAP), and IC_{50} values against DPPH in leaves of *E. obtusifolius* with or without endophyte inoculation under varying watering regimes.

Drought Treatment	Endophyte inoculant	Total phenolic content (mg GAE g^{-1} FW)	Flavonoid content (mg CE g^{-1} FW)	IC_{50} of DPPH (µg/ml)	Slope values of FRAP	IC_{50} of α-glucosidase (µg/ml)
WW (100% FC)	UI	6.35 ± 0.15^{f}	513.64 ± 1.06^{h}	35.52 ± 0.47^{ab}	1.73 ± 0.08^{bc}	63.45 ± 5.51^{c}
	PI	7.65 ± 0.04^{de}	577.03 ± 1.75^{g}	34.43 ± 0.22^{c}	2.04 ± 0.15^{ab}	48.12 ± 1.09^{c}
	FI	8.44 ± 0.40^{bcd}	685.36 ± 3.82^{f}	32.24 ± 0.12^{g}	2.04 ± 0.02^{ab}	34.16 ± 4.03^{c}
	P+FI	$10..58\pm0.28^{a}$	885.57 ± 2.63^{a}	27.29 ± 0.2^{h}	2.01 ± 0.04^{ab}	43.67 ± 2.52^{c}
MS (50% FC)	UI	8.27 ± 0.31^{cd}	816.31 ± 1.74^{d}	35.34 ± 0.12^{ab}	1.70 ± 0.15^{bc}	201.07 ± 5.76^{b}
	PI	8.57 ± 0.19^{bcd}	820.98 ± 2.781^{d}	34.00 ± 0.00^{cd}	1.76 ± 0.08^{bc}	248.25 ± 4.46^{b}
	FI	$8.53\pm0.11b^{cd}$	810.14 ± 2.51^{d}	33.62 ± 0.07^{de}	1.63 ± 0.03^{bc}	83.36 ± 1.92^{c}
	P+FI	9.34 ± 0.04^{b}	849.46 ± 2.23^{c}	26.09 ± 0.34^{i}	1.71 ± 0.10^{bc}	43.88 ± 0.55^{c}
Severe Stress (25%FC)	UI	6.55 ± 0.19^{f}	739.93 ± 2.63^{e}	36.00 ± 0.07^{a}	1.78 ± 0.09^{bc}	463.81 ± 52.84^{a}
	PI	8.39 ± 0.05^{bcd}	859.09 ± 2.00^{bc}	35.28 ± 0.03^{b}	1.59 ± 0.01^{c}	410.50 ± 11.00^{a}
	FI	8.72 ± 0.06^{bc}	847.06 ± 2.00^{c}	33.21 ± 0.04^{ef}	2.01 ± 0.04^{b}	183.50 ± 13.73^{b}
	P+FI	8.84 ± 0.07^{bc}	869.12 ± 0.00^{b}	32.64 ± 0.18^{fg}	1.79 ± 0.04^{bc}	230.48 ± 2.67^{b}
BHT/Acarbose	-	-	-	33.35 ± 0.05^{def}	2.42 ± 0.05^{a}	51.06 ± 2.72^{c}

Means ± standard error (n=3) in each column followed by different letter(s) indicate a significant difference (at $P < 0.05$) between treatments as determined by two-way ANOVA and Bonferroni post hoc tesst. UI = uninoculated, PI = *P. polymyxa* inoculated, FI = *F. oxysporum* inoculated, and P+FI = *P. polymyxa* and *F. oxysporum* inoculated.

6.4. Discussion

The importance of valuable indigenous plants in managing various ailments in developing countries cannot be over-emphasized. Thus, increasing the quality and quantity of pharmacologically active compounds in medicinal plants continue to receive attention from researchers to exploit the full economic benefits of these plants **(LI et al., 2020; YE et al., 2021)**. Over the years, the effects of abiotic factors on plants' secondary metabolite accumulation have inundated scientific reports **(LI et al., 2020; VERMA and SHUKLA, 2015; YANG et al., 2018)**, and interestingly, many endophytes have also been reported to promote the accumulation of bioactive compounds in medicinal plants **(CHEN et al., 2021; YANG et al., 2019)**. Drought is perceived as one of the foremost environmental stress factors in arid and semi-arid regions, influencing many medicinal plants' curative properties, growth, and development **(FARAHANI et al., 2009)**. Water deficit generally decreases water absorption in plants, which can be debilitating and evoke a series of morphological, physiological, and biochemical changes that affect plant health and efficiency **(CASER et al., 2019)**. However, plant tolerance to drought stress depends on plant species and genotype, the severity of the stress and the developmental stage of the plants **(KHAN et al., 2018)**. Evolutionarily, medicinal plants have adopted several strategies to cope with drought stress, and the integration of microbial endophytes for biomass and bioactive compounds accumulation is a valuable and promising approach in the cultivation of medicinal plants **(YANG et al., 2019)**.

This study indicated that *P. polymyxa* and *F. oxysporum* can successfully colonise the root tissues of *E. obtusifolius* plantlets. The attachment and subsequent ingress of plants by endophytes depend on their rhizospheric presence, their motility rate towards their prospective host, and their ability to break down plant cell walls **(MENGISTU, 2020)**. Additionally, the individual and co-

inoculations of *P. polymyxa* and *F. oxysporum* in this study improved the growth of plants under drought stress and non-stress conditions compared to uninoculated plants, resulting in higher dry shoot and root biomass. A similar result was recorded in some indigenous plants, including *Glycyrrhiza uralensis* **(XIE et al., 2019)**, *Astragalus mongholicus* **(SUN et al., 2019)**, and *Piper nigrum* **(ANITH et al., 2018)**. The two endophytes employed in this study were isolated from a mature *E. obtusifolius* mother plant. Thus, it appears that *E. obtusifolius* has perhaps, over time, established a symbiotic relationship with these beneficial endophytes for its survival under the prevailing challenging conditions. Endophytes generally promote plant growth under normal and stressful conditions by improving the uptake of nutrients and water, and through the regulation of growth and stress phytohormones **(AFZAL et al., 2019)**.

Furthermore, data from this experiment shows that the total chlorophyll content of *E. obtusifolius* increased with the severity of drought stress, and the inoculation of endophytes further improved the chlorophyll content. On the other hand, although increased with microbial inoculations, the carotenoid content was proportional to drought severity. Similarly, some endophytes were implicated in photosynthetic pigments content increase in *Helianthus tuberosus* **(SUEBRASRI et al., 2020)** and *Citrus reticulata* **(SADEGHI et al., 2020)**. It is logical to assume that endophytes, like other symbiotic microbes, up-regulate the expression of chlorophyll biosynthetic genes and down-regulate chlorophyllase activity, thereby resulting in a more remarkable pigment production **(AL-ARJANI et al., 2020)**. The higher carotenoid contents observed in some of the endophyte-infected *E. obtusifolius* was also reported by **SADEGHI et al. (2020)** in *C. reticulata*, and these carotenoids may also serve as additional protection (antioxidant) to the photosynthetic system preventing mitigating against ROS accumulation and their devastating effects **(AL-ARJANI et al., 2020)**. Drought stress affects chlorophyll contents differently in plants depending on plant

tolerance and severity of the stress factor. Generally, drought stress has been widely reported to reduce photosynthetic pigments in plants **(LI et al., 2011)**. However, drought-induced higher chlorophyll concentration was previously reported in *Solanum tuberosum* **(RAMÍREZ et al., 2014)**, clones of *Eucalyptus grandii* × *E. robusta* **(MICHELOZZI et al., 1995)**, and *Nicotiana tabacum* **(GUBIŠ et al., 2007)**. Alteration in chlorophyll degradation during routine senescence occasioned by the "stay-green effect" may be responsible for the higher chlorophyll content in plants under drought stress **(RAMÍREZ et al., 2014)**, and this may further lead to a rise in ROS accumulation as the chlorophyll continue to trap and transfer energy onto oxygen molecules **(HÖRTENSTEINER, 2009)**.

Plants in the severe drought stress treatment in this experiment showed a decline in the F_v/F_m ratios, indicating photo-inhibition due to protein damage and subsequent disruptions in the photochemical activities at the PSII reaction centers **(BANKS, 2017)**. Conventionally, F_v/F_m ratio is used as a stress marker. Thus, plants with F_v/F_m ratio values less than 0.7 imply that the plant is not tolerant to the prevailing conditions **(BU et al., 2012)**. Endophyte treatments (individual and co-inoculation) in this study improved the photochemical efficiency of PSII in both stressed and unstressed *E. obtusifolius* plants. Some endophytes have been reported to improve the photochemical efficiency of PSII via the stabilisation of grana structure, synthesis of protective metabolites and the enhancement of nitrogen and potassium absorption **(ALKAHTANI et al., 2020)**. Earlier, **SARAVI et al. (2021)** and **SADEGHI et al. (2020)** reported the positive influence of endophytes on the maximum quantum yield in *Stevia rebaudiana* and *C. reticulata,* respectively. In the present study, the leaf RWC decreased under drought stress but increased with endophyte treatments, as shown by other researchers **(HUSSIN et al., 2017; ZHANG et al., 2019)**. RWC often reflects a plants current physiological status, and higher RWC values in endophyte-

infected *E. obtusifolius* could be linked directly to enhanced water and nutrient transportation, stomatal regulation, and efficient hydraulic conductivity in plants **(ZHANG et al., 2019)**.

Water deficit exposed *E. obtusifolius* in this study produced higher ROS as indicated by an increase in hydrogen peroxide and MDA contents, thereby leading to a rise in the electrolyte leakage of the plants and subsequent deterioration of their cellular membrane integrity. This observation could be due to the formation of cytotoxic lipid alkenals, hydroxy-alkenals, and aldehydes such as malondialdehyde in chloroplast and mitochondria membranes due to peroxidation under drought stress **(ABIDEEN et al., 2020)**. Our findings are in concordance with those of **ARPANAHI et al. (2020)** and **EMAMI BISTGANI et al. (2017)**, who demonstrated that drought stress led to membrane damage in *Thymus vulgaris* and *Thymus daenensis*, respectively. Remarkably, endophyte inoculated *E. obtusifolius* in this experiment exhibited lower oxidative stress marker levels under all the watering regimes. These findings might be due to the role of the endophytes in combating and regulating ROS generation, thereby maintaining membrane functions as reported previously by **KHAN et al. (2016)** in the growth enhancement and stress tolerance of a *Populus deltoides* x *P. nigra* clone. The up regulation of SOD activities mediated by progressive drought stress and its amplification by endophyte inoculations were recorded in this study. This outcome is in line with the findings of **XIE et al. (2019)** and **ZHANG et al. (2019)**, who indicated that higher SOD activities were reported in drought-stressed and *Bacillus pumilus* inoculated *G. uralensis*. Superoxide dismutase usually converts O_2^- to hydrogen peroxide, which is further reduced to water and oxygen by the activities of other enzymatic and non-enzymatic antioxidants **(DAS and ROYCHOUDHURY, 2014)**. Simultaneously, the total phenolics and flavonoid concentrations of *E. obtusifolius* in this study increased in drought-stressed and endophyte inoculated treated plants. The increase in these metabolites may be an additional layer of defense

employed by the plants to mitigate against the negative consequences of drought stress directly as antioxidants or indirectly as photo-protection **(BETTAIEB et al., 2011)**. In several studies, indigenous plants inoculated with microbial endophytes have been reported to contain higher phenolics and flavonoid content **(GOLPARYAN et al., 2018; JHA, 2019; MONA et al., 2017)**.

In addition to the up regulation of the antioxidants system in plants as drought stress intensified, the accumulation of osmolytes, including proline and total soluble sugar, was observed in this study under rising water deficit and enhanced with endophyte inoculation. Accumulation of proline content in drought-stressed plants is in line with the findings of **EGAMBERDIEVA et al., (2017)** and **YANG et al., (2014)**. The accumulation of proline under water shortage is a common and basic stress response **(AL-ARJANI et al., 2020)**. Proline is a known amino acid that assists in maintaining the integrity and fluidity of cellular and molecular structures **(ABD-ALLAH et al., 2015)**, osmotic homeostasis **(AL-ARJANI et al., 2020)**, photosynthetic apparatus **(ABD-ALLAH et al., 2015)**, and scavenges free radicals **(CHIAPPERO et al., 2019)**. Similarly, certain plants may also maintain their turgor pressure under drought stress by accumulating osmolytes other than proline, such as soluble sugars **(EMAMI BISTGANI et al., 2017)**, as observed in this study. Endophyte inoculation appears to have accelerated the activities of specific pathways involved in synthesizing these stress-responsive osmolytes, including proline and total soluble sugar. As observed in this study, it was observed that endophyte inoculation increased the proline and total soluble sugar content of *Atractylodes lancea* **(DASTOGEER et al., 2018; YANG et al., 2014)**.

The free radical scavenging activities, and the ferric reducing antioxidant power of the ethyl acetate leaf extracts of *E. obtusifolius* were not significantly different under the varying watering regimes. Endophyte inoculation on the other hand, enhanced the antioxidant capacity of the plants. Our findings agree with **REZAYIAN et al. (2018)**, who showed that drought stress levels did not affect

the antioxidant capacity of *Brassica napus* but disagree with that of **HASAN et al. (2018)**, who showed that *Moringa* species improved in their antioxidant capacities as drought stress intensified. Similarly, **KILAM et al. (2015)** and **GOLPARYAN et al. (2018)** demonstrated that the antioxidant capacities of *Stevia rebaudiana* and *L. citriodora* increased with endophyte inoculation. In the present study, the *in vitro* α-glucosidase inhibitory potential of *E. obtusifolius* improved significantly as watering level increases. Endophyte inoculation also enhanced the *in vitro* hypoglyceamic activities of the plants. Reports on the effects of drought stress and endophyte inoculations on the *in vitro* α-glucosidase inhibitory activities are limited in the literature. Nonetheless, drought stress **(IFIE et al., 2018)** and endophytic bacteria inoculation **(PUJIYANTO and FERNIAH, 2017)** improved the antidiabetic activities of *Hibiscus sabdariffa* and *M. charantia*, respectively. Our findings suggest that certain bioactive compounds of *E. obtusifolius* are negatively affected by water stress while others remain intact.

6.5. Conclusion

The findings presented in this study demonstrate that although *E. obtusifolius* is drought tolerant, water shortage affected the plant in different ways. Drought stress negatively affected the growth, root and shoot biomass, carotenoid content, chlorophyll fluorescence, and relative water content. Under increasing water deficit, *E. obtusifolius* generated more ROS, which caused the peroxidation of cellular molecules such as lipids and negatively affected the cellular membrane integrity. Additionally, the antioxidant capacities of the ethyl acetate leaf extracts of *E. obtusifolius* were unchanged; however, its α-glucosidase inhibitory properties of *E. obtusifolius* was significantly affected by drought. The individual and co-inoculation of *P. polymyxa* and *F. oxysporum* into *E. obtusifolius* mitigated against the devastating impacts of water deficit via the up-regulation of

antioxidant systems, down-regulation of ROS production in cells, and improved osmolyte accumulation. The pharmacological potential of *E. obtusifolius* leaves in this study also improved with endophyte inoculation. This study acknowledges the role of endophyte inoculation in managing drought-stressed medicinal plants to enhance their productivity and efficacy under greenhouse conditions. Thus, to further ascertain our claims, well designed and controlled field trials should be conducted before deliberate application of water deficit during the cultivation of endophyte inoculated *E. obtusifolius* for its medicinal values.

CHAPTER 7: Antidiabetic compound profiling and GC-based metabolomics from drought stressed and endophyte elicited *Endostemon obtusifolius*

This chapter was written following the short communication format of the Natural Product Research Journal.

7.1. Introduction

Plant adaptation to the ever-changing environmental factors or environmental stress is widely accepted as a way to enhance plant cultivation and efficiency **(ZENG et al., 2021)**. Under individual or combination stress (abiotic or biotic), multilayered responses are induced, including the accumulation of plant secondary metabolites. The synthesis of secondary metabolites is a key strategy in the adaptation of plants to environmental stress such as drought **(MAHAJAN et al., 2020)**. Plant secondary metabolite accumulation under stress conditions can be observed as antioxidants, cell wall strengthening agents, pest and microbial antagonists as well as cellular component protectors **(YADAV et al., 2021)**. Specifically, under drought stress, plant secondary metabolites modulates resistance and sugar production by influencing key biochemical pathways including the Krebs cycle and the glycolytic pathway **(QU et al., 2019)**. In addition, plant secondary metabolites are also useful to humans as food additives, cosmetics and pharmaceutical agents **(TIWARI and RANA, 2015)**.

It is widely thought that plant secondary metabolites can be increased by the application of several elicitation strategies. However, this notion is not entirely accurate as studies have shown neutral, positive, and negative effects of stress (such as drought) on the concentrations of plant secondary metabolites **(PRINSLOO and NOGEMANE, 2018)**. Species or cultivar differences, the magnitude and duration of the stress, and other factors such as the plants interaction with microbial species such as endophytes may complicate the resultant metabolic responses of plants to different

stress **(DASTOGEER et al., 2020; PRINSLOO and NOGEMANE, 2018)**. For instance, **BAGHBANI-ARANI et al. (2017)** reported an increase in the content of carotenoids and trigonelline in *Trigonella foenum-graecum* under drought stress. **MAHDAVI-DAMGHANI et al. (2010)**, however reported a marked reduction in the alkaloid production in *Papaver somniferum*. *Glycyrrhiza uralensis* inoculated with dark septate endophytic fungi (*Alternaria chlamydospore* and *Preussia terricola*) synthesised more active ingredients (glycyrrhizic acid and glycyrrhizin) compared to the uninoculated plants **(HE et al., 2021)**.

Therefore, to explore endophyte-induced metabolome changes in the leaves of *Endostemon obtusifolius* under varying water deficit treatments, a Gas Chromatography-Mass Spectroscopy (GC-MS)-based untargeted metabolomics approach was adopted. Metabolomic tools have recently gained the attention of researchers and it is widely used to establish metabolic correlation or changes in plants under various environmental conditions **(FERNANDEZ et al., 2016)**.

7.2. Materials and Methods

7.2.1 Experimental design and treatments

This experiment was designed as described in **Section 6.2.3.** of **Chapter 6**.

7.2.2 Plant material harvest and sample preparation

After the drought exposure, the leaves of inoculated and uninoculated *E. obtusifolius* plants were harvested, quenched under liquid nitrogen, pulverised into a fine powder, and the water content of the samples were eliminated using a freeze-dryer **(ISHA et al., 2020)**. The freeze-dried leaf powder (2 g) of each treatment was extracted with 20 ml of ethyl acetate, placed on a mechanical shaker for 30 min and sonicated in ice for another half an hour. Thereafter, the crude extracts were

filtered through Whatman No. 1 filter paper under vacuum, and the resulting extracts were concentrated at 40 °C using a rotary evaporator. The extracts were stored in a -80 °C freezer until they were needed. The extracts were later retrieved from the freezer for the GC-MS analysis and resuspended in ethyl acetate.

7.2.3. Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis

The GC-MS analysis of the ethyl acetate extracts of each treatment was done as detailed in **Section 4.2.9.** of **Chapter 4**.

7.2.4. Data processing and multivariate statistical analysis

Raw chromatogram datasets obtained from the GC-MS analysis were initially converted into Network Common Data Form (NetCDF) format (.cdf) using the GCMS solution software from a Shimadzu QP-2010 SE Gas Chromatography. The NC data were subsequently processed for peak recognition, filtering, and alignment using the XCMS R package **(SMITH et al., 2006)**. Multivariate statistical analysis [Principal Component Analysis (PCA)] was carried out to visualise the metabolic differences between the 12 experimental groups after scaling datasets based on their variance.

The alterations in the metabolite contents between the experimental treatments were evaluated using the unsupervised PCA using the factoextra R package **(KASSAMBARA and MUNDT, 2020)**. The heatmap was constructed using the pheatmap R package with normalised values of eight METLIN (https://metlin.scripps.edu/) identified metabolites obtained from the machine learning recursive feature elimination of the total metabolites recognized from the spectra on XCMS after grouping and peak filling.

7.3. Results and Discussion

The total ion chromatograms of *E. obtusifolius* leaves under different elicitation treatments is presented in **Figure 7.1-7.12**. The number of compounds in the ethyl acetate crude extracts of *E. obtusifolius* under different treatments as revealed by the chromatograms can be ordered as follows: *Paenibacillus polymyxa* and *Fusarium oxysporum* inoculated under mild stress (P+FIMS) > *P. polymyxa* inoculated under well-watered regime (PIWW) > *P. polymyxa* inoculated under mild stress (PIMS) > *P. polymyxa* and *F. oxysporum* inoculated under well-watered regime (P+FIWW) > *F. oxysporum* inoculated under mild stress (FIMS) > uninoculated treatment under well-watered regime (UIWW) > *F. oxysporum* inoculated under well-watered regime (FIWW) > uninoculated treatment under mild stress (UIMS) > *P. polymyxa* and *F. oxysporum* inoculated under severe stress (P+FISS) > *F. oxysporum* inoculated under severe stress (FISS) > *P. polymyxa* inoculated under severe stress (PISS) > uninoculated treatment under severe stress (UISS). The differences in the metabolite profiles observed in the plants under different elicitation treatments can be attributed to the variations in their metabolite content. Further, it was observed that under severe stress, the lowest quantity of metabolites were accumulated by *E. obtusifolius*. However, endophyte inoculated *E. obtusifolius* (especially the combined inoculation) across the watering regimes accumulated more secondary metabolites when compared with the uninoculated *E. obtusifolius*. This observed trend concurs with the findings of **CHENG et al. (2018)** and **SINGH et al. (2021)**, who demonstrated that drought stress and endophytes inoculation influenced the accumulation of secondary metabolites in plants, respectively.

The effects of the elicitation treatments on the abundance of 10 identified and previously reported antidiabetic compounds were evaluated and presented in **Table 7.1**. The highest quantity of α-

pinene (1.36%), a monoterpene, was recorded by the co-inoculated well-watered *E. obtusifolius*, whereas no traces of α-pinene was observed in *E. obtusifolius* grown under FIMS, UISS, PISS and FISS conditions **(Table 7.1)**. Our results are in tandem with the findings of **CASER et al. (2019)**, who reported an increase in the percentage composition of α-pinene in *S. dolomitica* as water deficit was reduced, and **KHALVANDI et al. (2021)**, who demonstrated that endophyte inoculation enhanced the percentage composition of α-pinene in *Mentha piperita*. Phytol acetate, a diterpenoid, was downregulated as drought stress became severe but increased in the FIWW and P+FIWW plants **(Table 7.1)**. Begum and his colleagues recently reported that drought stress negatively affected the phytol acetate content of *Nicotiana tabacum,* and the inoculation of plant growth-promoting microbes improved phytol acetate content quantity in *N. tabacum* **(BEGUM et al., 2021)**. Essentially, the inoculation of indigenous plants with plant growth-promoting microbes improves dry matter contents, water and minerals uptake of plants which ultimately increase the concentration of certain secondary metabolites **(KHALEDIYAN et al., 2021)**. An increase in the concentration of secondary metabolites in plants inoculated with endophytes may also be a defense mechanism of the plant in response to microbial colonisation **(MUCCIARELLI et al., 2003)**. Moreover, it is generally acknowledged that fluctuation in environmental factors influence plant metabolism **(YANG et al., 2018)**. Accordingly, studies have reported contrasting data on the effects of water shortage on bioactive compounds accumulation in Mint plants **(GARCÍA-CAPARRÓS et al., 2019)**. The variation in the active principles composition of plants subjected to drought could be a consequence of the frequently reported reduction in the biomass production of drought-stressed plant **(RAMEZANI et al., 2020)**. Alternatively, the increase in the biosynthesis of pharmacologically active compounds under drought stress might be due to an oversupply of $NADPH + H^+$ occasioned by stomata closure and a decline in carbon dioxide

concentration in the mesophyll cells. The increase in the biosynthesis of these compounds might also be explained from the upregulation in the corresponding enzymes activity point of view **(RAMEZANI et al., 2020)**.

The principal component analysis score biplot based on the GC-MS spectra results showed that the treatments in this experiment were separated into five groups **(Figure 7.13)**. Dimension or Principal component 1 (DIM1 or PC1) accounts for 74% of the observed variance in the dataset, while DIM 2 or PC2 showed 8% variation in the analysed dataset **(Figure 7.13)**. Furthermore, it is apparent from the PCA biplot that the co-inoculated set of plants under the well-watered and moderate stress are positively correlated and farther away from other clusters. Additionally, the fungal inoculated plants under moderate stress considerably differ from the other segregations **(Figure 7.13)**. The observed separations between the treatments could be linked to the changes in the leaf metabolome of the treatments (varying water regimes with or without endophyte inoculation). A similar result on drought-stressed endophyte inoculated *N. benthamiana* was reported by **DASTOGEER et al. (2017)**. The synthesis of secondary metabolites by endophytic species is well documented **(OGBE et al., 2020),** but a comprehensive molecular understanding of the effects of plant-endophyte interaction on the changes in plant metabolome under stress is still poorly known **(DASTOGEER et al., 2017)**. Perhaps, endophytes adjust their host metabolism to combat stress by rapidly stimulating certain biochemical reactions leading to the synthesis and accumulation of specific secondary metabolites **(YANG et al., 2014)**.

The changes in *E. obtusifolius* leaf metabolome under different elicitation treatments become apparent from the heatmap's hierarchical grouping generated from 8 identified metabolites presented in **Table 7.2**. The normalised z-score representing the quantities of each metabolite under the treatments are shown in a heatmap presented in **Figure 7.14**. Two major treatment

groupings, 1 (FIMS, PIMS, P+FIWW, P+FIMS, UIWW, FIWW and PIWW) and 2 (UIMS, PISS, UISS, FISS and P+FISS), were identified. In general, the strongest and weakest responses were found in P+FISS and FIMS, respectively. The non-protein amino acids and organic acids such as 2-butyl sulfanyl-4,5-dihydro-1H-imidazole and phosphor-fluoridothioic O, O-acid were mainly increased in the endophyte inoculated plants under the different watering regimes. Non-protein amino acids and organic acids protect the cellular integrity of plants under different abiotic stress **(KHAN et al., 2020c; RODRIGUES-CORRÊA and FETT-NETO, 2019)**. Similarly, Vílchez and his colleagues reported the accumulation of organic acids and non-protein amino acids in *Capsicum annuum* inoculated with a plant growth-promoting microbe *Microbcterium* sp. **(VÍLCHEZ et al., 2018)**. From our result, it could be deduced that changes in the metabolome of inoculated *E. obtusifolius* is dependent on the inoculated endophyte species and the severity of the drought stress. Alterations in the metabolome of endophyte infected and non-infected plants under specific environmental conditions could hypothetically be linked with the different genetic and biochemical pathways involved in conferring drought stress tolerance on the plants **(KHAN et al., 2019)**.

Table 7.1: Previously identified hypoglyceamic compounds found in ethyl acetate crude extracts of *Endostemon obtusifolius* leaves and their percentage area.

S/N	Compound	Similarity index (%)	Reference	Area (%)											
				UIWW	PIWW	FIWW	P+FIWW	UIMS	PIMS	FIMS	P+FIMS	UISS	PISS	FISS	P+FISS
1.	α-Pinene	94	**ÖZBEK and YILMAZ (2017)**	0.44	0.34	0.21	1.36	0.27	0.30	0.00	0.36	0.00	0.00	0.00	1.15
2.	Benzoic acid	97	**DEBNATH et al. (2020)**	7.52	9.76	8.70	9.86	19.75	15.33	11.90	15.59	12.14	11.97	8.52	11.34
3.	Caryophyllene	96	**ZACCAI et al. (2020)**	0.93	1.10	0.74	1.76	1.65	1.95	0.00	1.40	1.95	1.34	0.94	1.91
4.	Humulene	95	**ZACCAI et al. (2020)**	0.40	0.42	0.34	0.59	0.65	0.14	0.00	0.57	1.17	0.64	0.35	0.60
5.	Santalol	81	**BOMMAREDDY et al. (2019)**	0.00	0.61	0.83	0.55	0.79	0.79	0.00	0.49	0.00	0.00	0.00	0.00
6.	D-Limonene	94	**BACANLI et al., (2017)**	0.00	0.00	0.00	0.17	0.00	0.10	0.00	0.16	0.00	0.00	0.00	0.00
7.	Cubenol	57	**YA'NI et al. (2018)**	0.00	0.00	0.00	0.52	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00
8.	Hexadecane	93	**MAHMOOD et al. (2020)**	0.58	0.38	0.36	0.49	0.64	0.52	0.00	0.09	0.58	0.55	0.60	1.03
9.	Phytol acetate	91	**WANG et al. (2017)**	1.45	1.38	1.77	1.50	1.17	0.78	0.71	0.93	0.00	0.00	0.84	0.64
10.	Phenol, 2,4-bis(1,1-dimethylethyl)-	96	**BENOITE, and VIGASINI (2021)**	2.24	3.01	1.86	2.64	3.24	2.59	0.00	3.22	3.59	2.69	1.83	2.86

Table 7.2: Details of the identified differential metabolites.

S/N	Metabolite	Metabolite Identity	Formula	Molecular Weight	CAS number
1.	M88T221	2,4-Dimethylbenzaldehyde	$C_9H_{10}O$	134.17	15764-16-6
2.	M112T506	Nom-protein amino acids	-	-	-
3.	M68T510	Alpha kosin	$C_{25}H_{32}O_8$	460.5	568-50-3
4.	M111T508	Non-protein amino acids	-	-	-
5.	M167T994	Non-protein amino acids	-	-	-
6.	M326T861	2-butylsulfanyl-4,5-dihydro-1H-imidazole	$C_7H_{14}N_2S$	158.27	62059-38-5
7.	M167T994	4H-1-Benzopyran-4-one, 7-chloro-2,3-dihydro-3- hydroxy-2-phenyl-	$C_{15}H_{11}ClO_3$	274.70	644973-51-3
8.	M78T1455	Phosphorofluoridothioic O,O-acid	FH_2O_2PS	116.05	14465-90-8

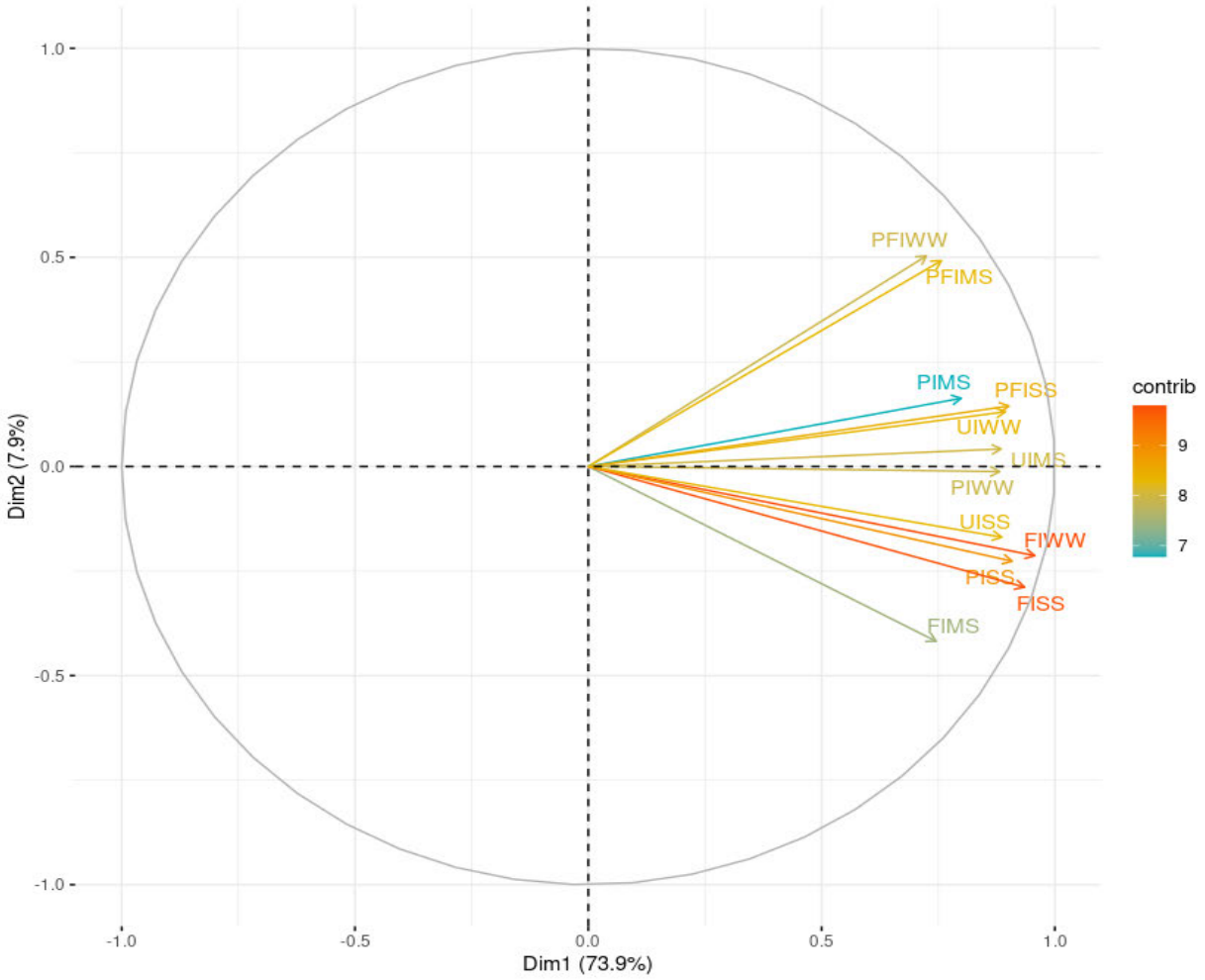

Figure 7.1: Biplot representation of the PCA conducted on the crude ethyl acetate extracts of endophyte inoculated and uninoculated *Endostemon obtusifolius* under varying water regimes.

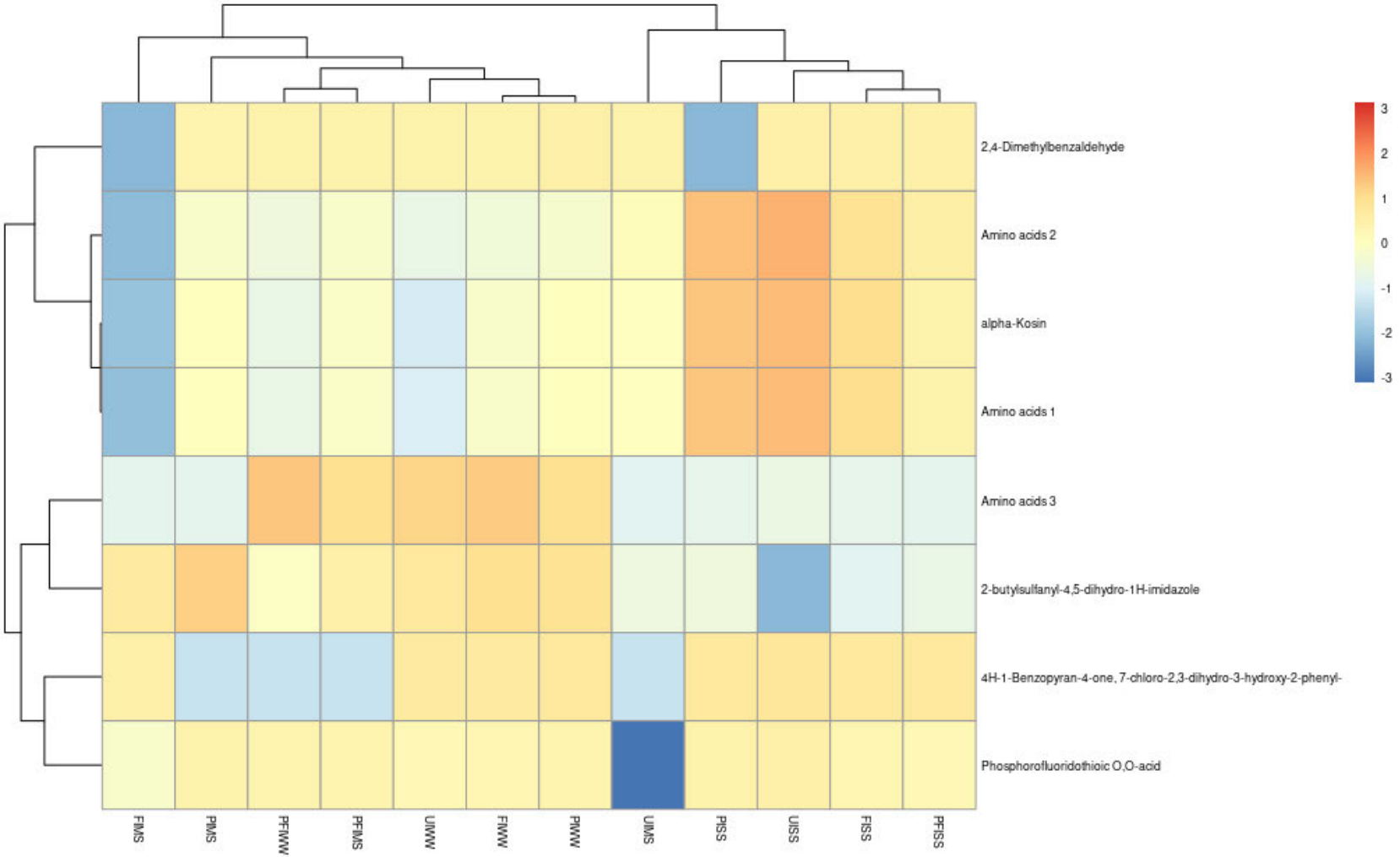

Figure 7.2: Heatmap visualisation of 8 metabolites identified from the machine learning recursive elimination feature. Cell colors indicate normalized compound concentrations, compounds in columns and with samples in rows. The color scale at the right indicates the relative metabolite concentrations with high concentrations in red and low concentrations in blue.

7.4. Conclusion

In brief, this Chapter revealed that the varying water regimes, individual and co-inoculations of *E. obtusifolius* with *P. polyxma* and *F. oxysporum* affected the concentrations of some antidiabetic compounds (including caryophyllene, alpha-pinene, phytol acetate, humulene) identified in the GC-MS analysis of the ethyl acetate crude extracts of *E. obtusifolius*. The GC-MS based metabolomics adopted in this study further revealed that the varying watering regimes and the inoculation of *E. obtusifolius* with *P. polyxma* and *F. oxysporum* individually and in combination influenced the metabolome of *E. obtusifolius* leaves. Specifically, organic acids and non-protein amino acid contents were mostly increased across watering regimes in plants infected with the endophytes. The use of endophytes in the cultivation and elicitation of medicinal plants is an eco-friendly and sustainable approach in optimizing the inherent potentials of medicinal plants and their products.

CHAPTER 8: General Conclusions

In light of the prevalence and staggering statistics on diabetes, primarily type 2 diabetes (T2DM), it has been recently estimated that by 2045 about 700 million adults will be affected globally **(COLE and FLOREZ, 2020)**. Diabetes is not solely a disease, but a group of metabolic disorders characterised by hyperglycaemia, a resultant abnormality in the actions or secretion of insulin. Diabetes has serious health outcomes, and its morbidity and mortality surge with complications to vital tissues and organs, including the heart, kidneys, retina, nerves, limbs and blood vessels. The un-abating consumption of junk food, unhealthy lifestyles, urbanisation and westernisation has led to an increase in the prevalence of T2DM. Moreover, increasing evidence suggests that oxidative stress, occasioned by abnormal accumulation of reactive oxygen species (ROS), has a pathological trajectory in the development of T2DM. Oxidative stress increases pancreatic β-cell disorders and insulin resistance in body cells. Various standard antidiabetic therapies, including insulin, dietary manoeuvres, and oral hypoglyceamic agents, have been widely prescribed; however, due to the cost, effectiveness, and undesirable side effects of oral hypoglyceamic agents, medicinal plants and their bioactive compounds are an effective alternative in the treatment of T2DM.

As a result of their widely reported efficiency in managing metabolic disorders such as T2DM in humans, valuable medicinal plants are consistently sourced from their wild populations leading to habitat degradation and biodiversity loss. Indeed, medicinal plants synthesize phyto-active compounds in minute concentrations, and changes in seasons and environmental factors continually affect their availability, as well as the quality and quantity of their active principles. Thus, with the rising demands for natural products from medicinal plants, effective cultivation strategies, including elicitation, are used to optimise biomass production, bioactive compounds

accumulation, and pharmacological properties of indigenous plants. Drought is one the most efficient abiotic elicitors affecting the growth, development and accumulation of bioactive compounds in medicinal plants. Similarly, endophytic microbes (biotic elicitors) play vital roles in accumulating secondary metabolites in medicinal plants and shielding them from the negative consequences of abiotic stress. Hence, this study was designed to explore some indigenous plants' antioxidant and hypoglyceamic properties and subsequently identify potential antidiabetic compounds from one of the plants *Syzygium cordatum*. In addition, this study aimed to isolate drought tolerant and plant-growth-promoting endophytes and study their roles in affecting morphology, physiology and the accumulation of secondary metabolites by *Endostemon obtusifolius* plants under varying water stress.

The leaves of eleven indigenous plants (*Catha edulis, Celtis africana, Combretum kraussii, E. obtusifolius, Lippia javanica, Pachira aquatica, Pentanisia prunelloides, Psidium guajava, Ruta graveolens, S. cordatum,* and *Vernonia amygdalina*) were extracted with five different solvents (ethanol, 50% aqueous ethanol (v/v), ethyl acetate, distilled water, and boiled distilled water) and the *in vitro* antioxidant and antidiabetic properties of the resultant crude extracts were evaluated. All the tested plants showed good dose-dependent *in vitro* antioxidant and antidiabetic properties. Notably, *C. krausssii, L. javanica, P. guajava, P. prenulloides, E. obtusifolius, S. cordatum* and *C. edulis* contained a significant quantity of phytochemicals and displayed the highest *in vitro* antioxidant and hypoglyceamic capacities. This study established the *in vitro* antioxidant and antidiabetic potential of *E. obtusifolius,* a relatively unknown shrub. Further investigation is, however, needed to validate the antidiabetic claims of *E. obtusifolius* using *in vivo* experimental designs. This research would also help direct future studies on the use of indigenous plants in the development of lead antidiabetic molecules.

The availability of *S. cordatum* leaf materials and its impressive *in vitro* antioxidant and antidiabetic potency (from the previous study) accentuates the potential for the possible extraction and identification of potential hypoglyceamic agents. The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the petroleum ether, dichloromethane, and ethyl acetate fractions of *S. cordatum* leaf extracts identified 34 bioactive compounds. Of the 34 compounds, 21 returned binding energy scores of ≤ -7.5 kcal/mol. Some of the detected bioactive compounds including cubenol, bis(3,3,4,7-tetramethyl-1,3-2H-benzofuran-1-yl)-ether and andrographolide interacted with the amino acid residues involved in enhancing hydrolysis of disaccharides through hydrogen or hydrophobic bonds **(Table 4.4)**. Thus, these compounds appear more to be some of the effective blood sugar reducing bioactive agents in *S. cordatum*. The findings of this study further established the hypoglyceamic potential of cubenol, bis(3,3,4,7-tetramethyl-1,3-2H-benzofuran-1-yl)-ether and andrographolide. In the quest to develop these compounds as oral antidiabetic agents, further *in vivo* investigations are required to validate their digestive enzymes' inhibitory capacity.

Endophytes' interaction with medicinal plants has been reported to up-regulate the accumulation of bioactive compounds in some indigenous plants under normal and stress conditions. However, these claims have not been verified for most African indigenous plants. Based on the remarkable *in vitro* antidiabetic potentials exhibited by the crude extracts of *E. obtusifolius* in Chapter 3, 26 endophytes were isolated from its organs (roots and leaves). Two promising plant-growth-promoting and drought-tolerant endophytic microbes, *Paenibacillus polymyxa* (bacteria) and *Fusarium oxysporum* (fungi), were identified using molecular tools and evaluated for their greenhouse growth-promoting abilities on *E. obtusifolius* stem cuttings under varying water regimes. The effects of the elicitation strategy on the accumulation of secondary metabolites and

the α-glucosidase inhibitory properties of the plants were also evaluated. Drought stress adversely affected the α-glucosidase inhibitory activities, root and shoot biomass, fundamental physiological and biochemical parameters of *E. obtusifolius*. The phenolic content and the free radical scavenging of *E. obtusifolius* leaves were largely unaffected by the varying water stress. On the other hand, individual and co-inoculation of *E. obtusifolius* with *P. polymyxa* and *F. oxysporum* mitigated *E. obtusifolius* against the devastating impacts of water deficit via the up-regulation of antioxidant systems, down-regulation of ROS production in cells, and osmolyte accumulation. The free radical and α-glucosidase inhibitory properties of *E. obtusifolius* leaves also improved with the inoculation of the endophytes, albeit the co-inoculation treatments gave the best set of results. As *P. polymyxa* and *F. oxysporum* inoculation enhanced the biomass, biochemical status and therapeutic properties of *E. obtusifolius* under varying water regimes, they may be employed as plant growth promoters in the deliberate cultivation of *E. obtusifolius* and other plant species in semi-arid or arid regions.

The GC-MS analysis results of *E. obtusifolius* leaves (ethyl acetate crude extracts) under different elicitation treatments revealed that the concentrations of some known antidiabetic compounds including caryophyllene, alpha-pinene, phytol acetate, humulene, D-limonene and phytol acetate were positively influenced by the individual and co-inoculation of *P. polymyxa* and *F. oxysporum*. In addition, the GC-MS based metabolomics showed that the treatments differed from one another due to the differences in their final metabolomes. Eight metabolites comprising non-protein amino acids and organic acids were further identified as differential metabolites amongst the treatments. The use of metabolomics to fully understand the impacts of endophytes' infection in the metabolome of plants is an emerging research interest. Thus, further investigations are needed to clarify the overlapping biosynthesis pathways between host plants and their endophytic species.

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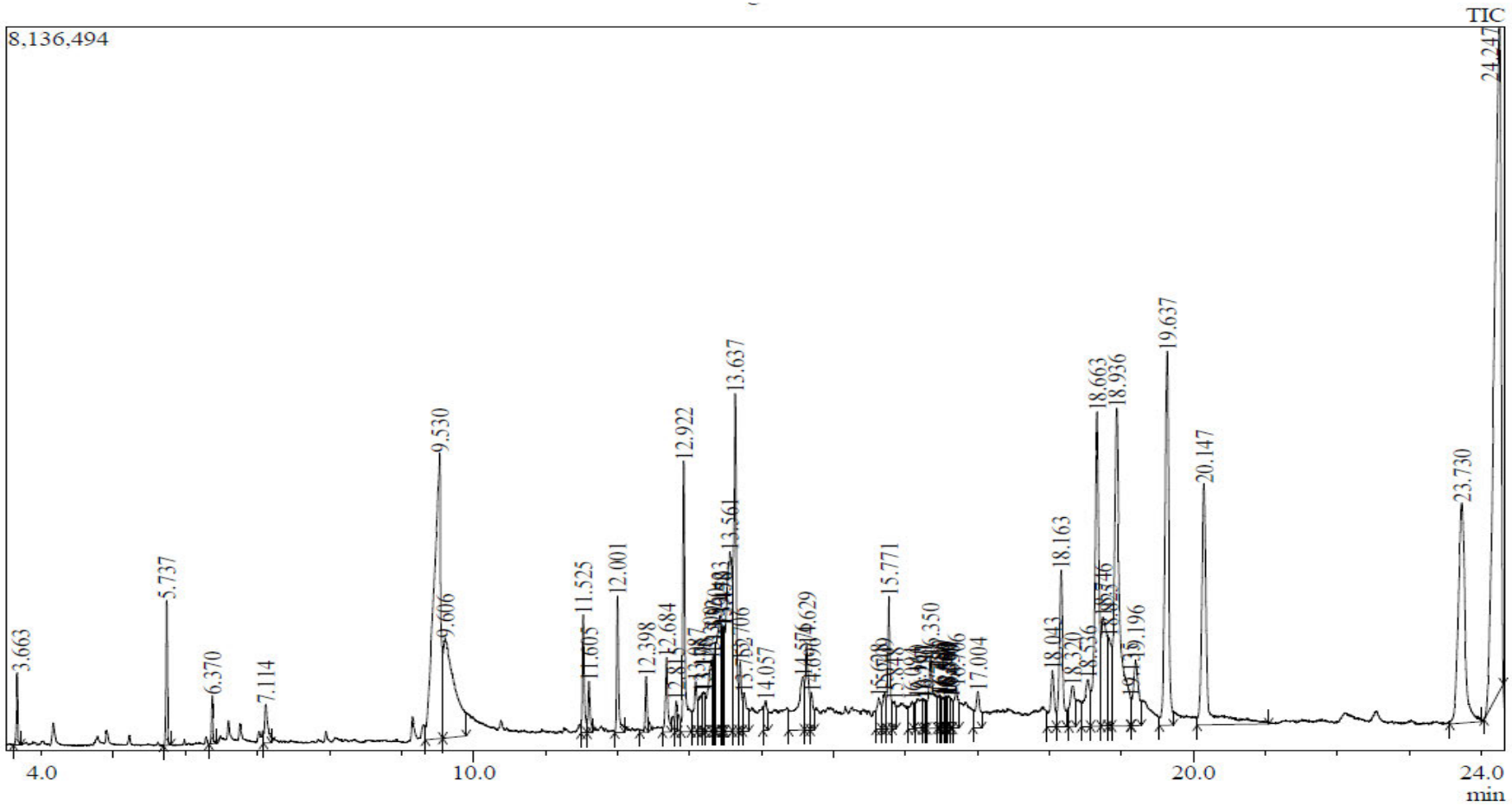
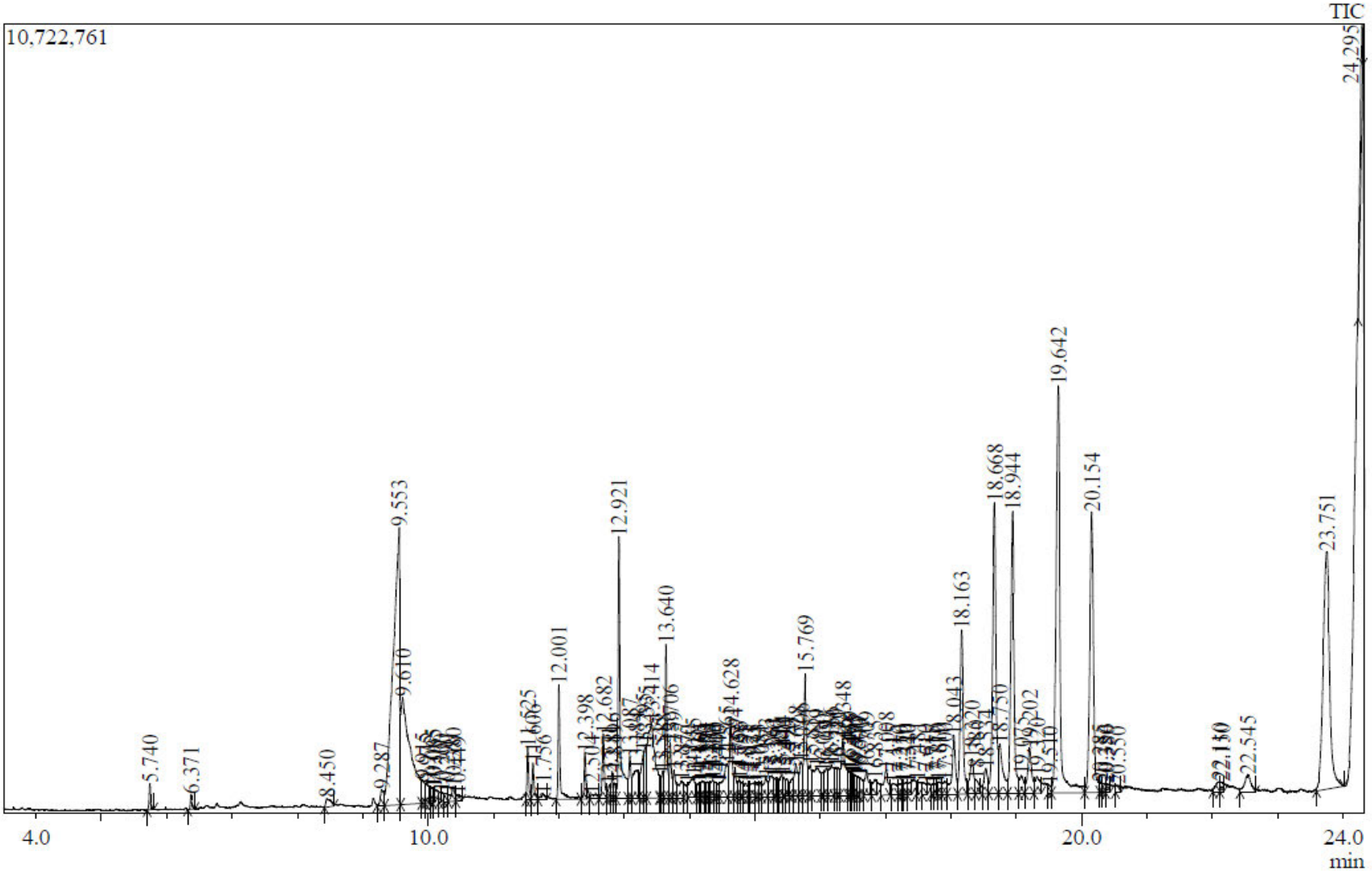
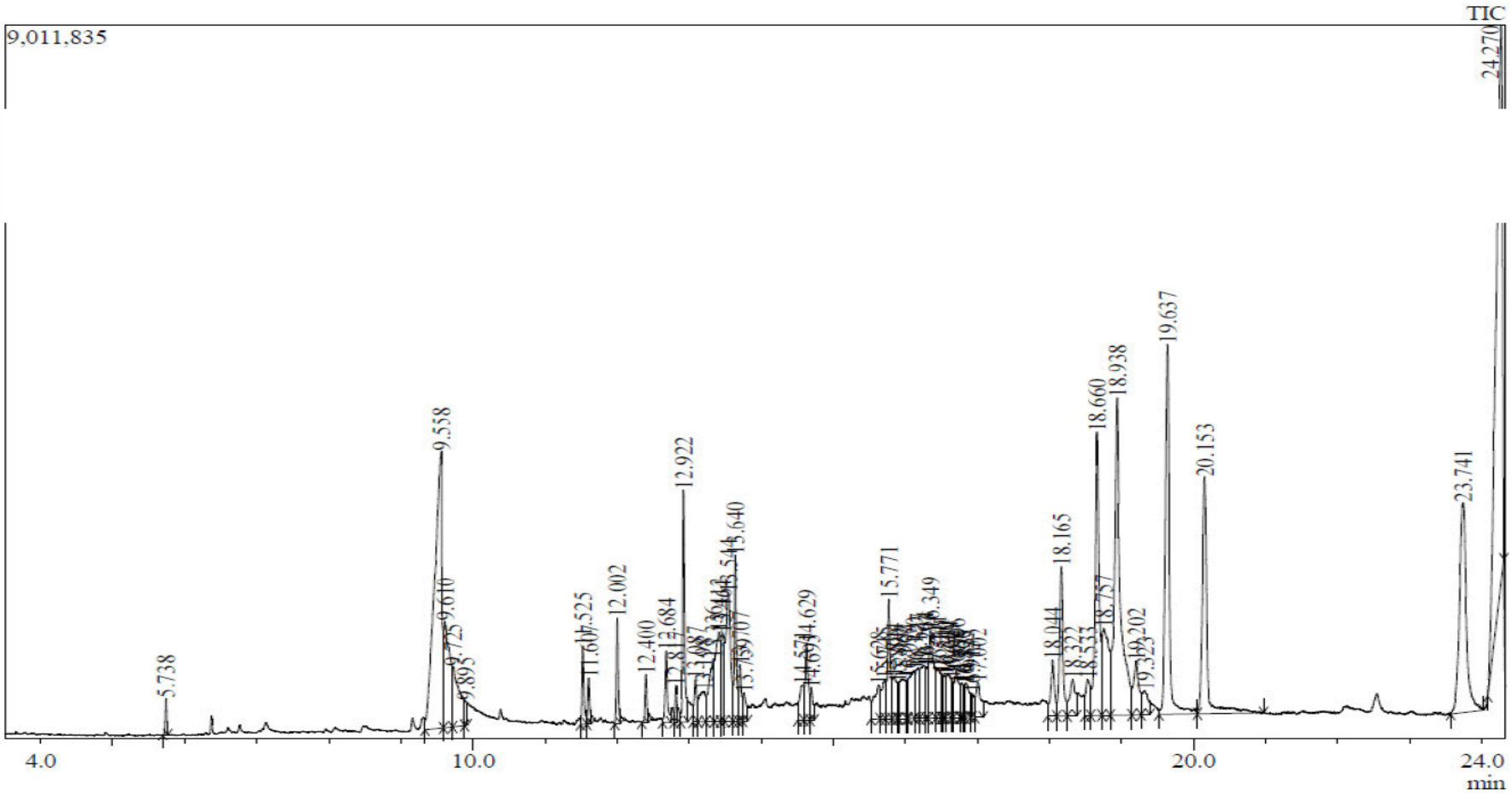
APPENDICES



Appendix 7.1: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under UIWW obtained from GC-MS.

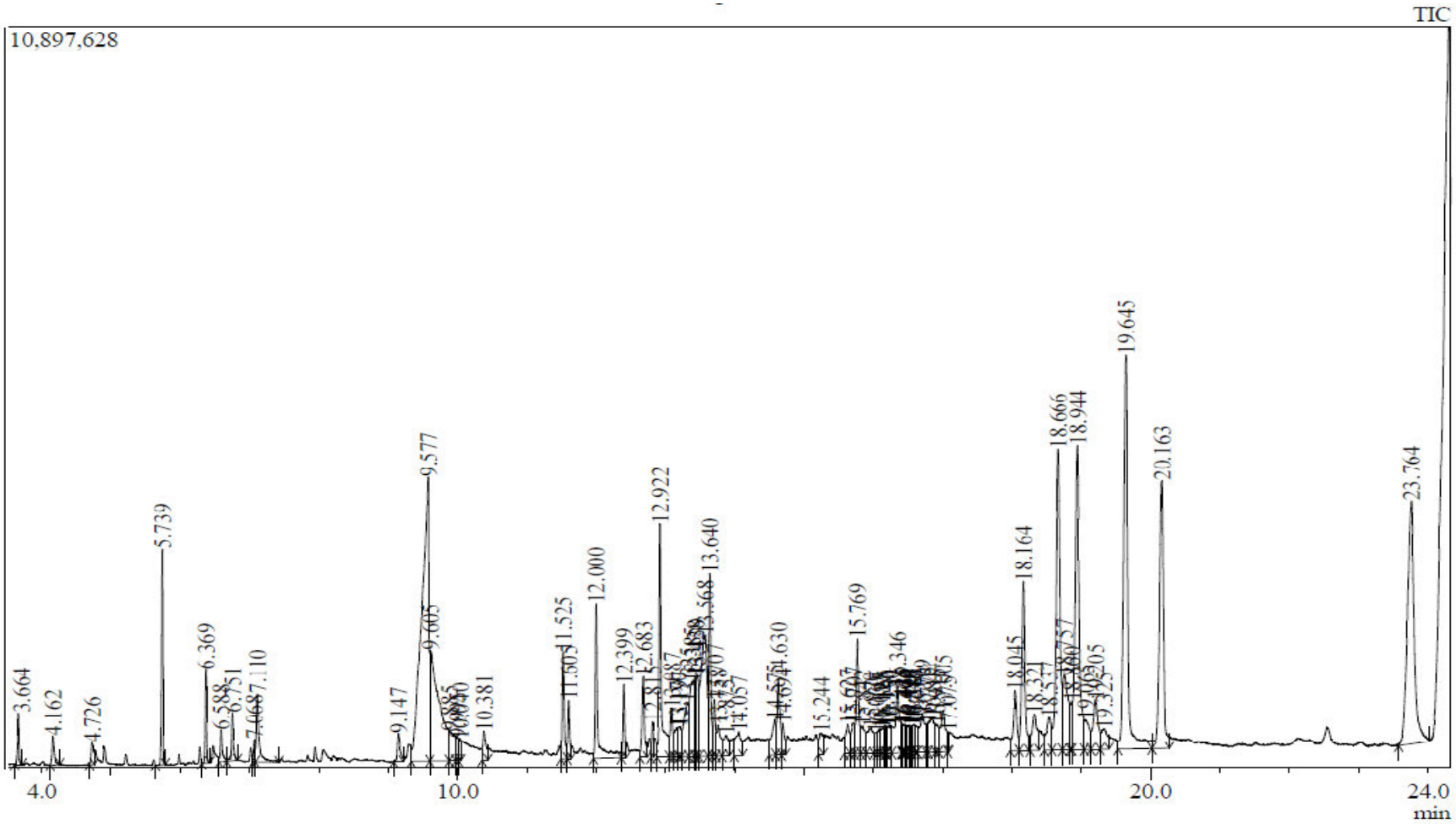


Appendix 7.2: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under PIWW obtained from GC-MS.

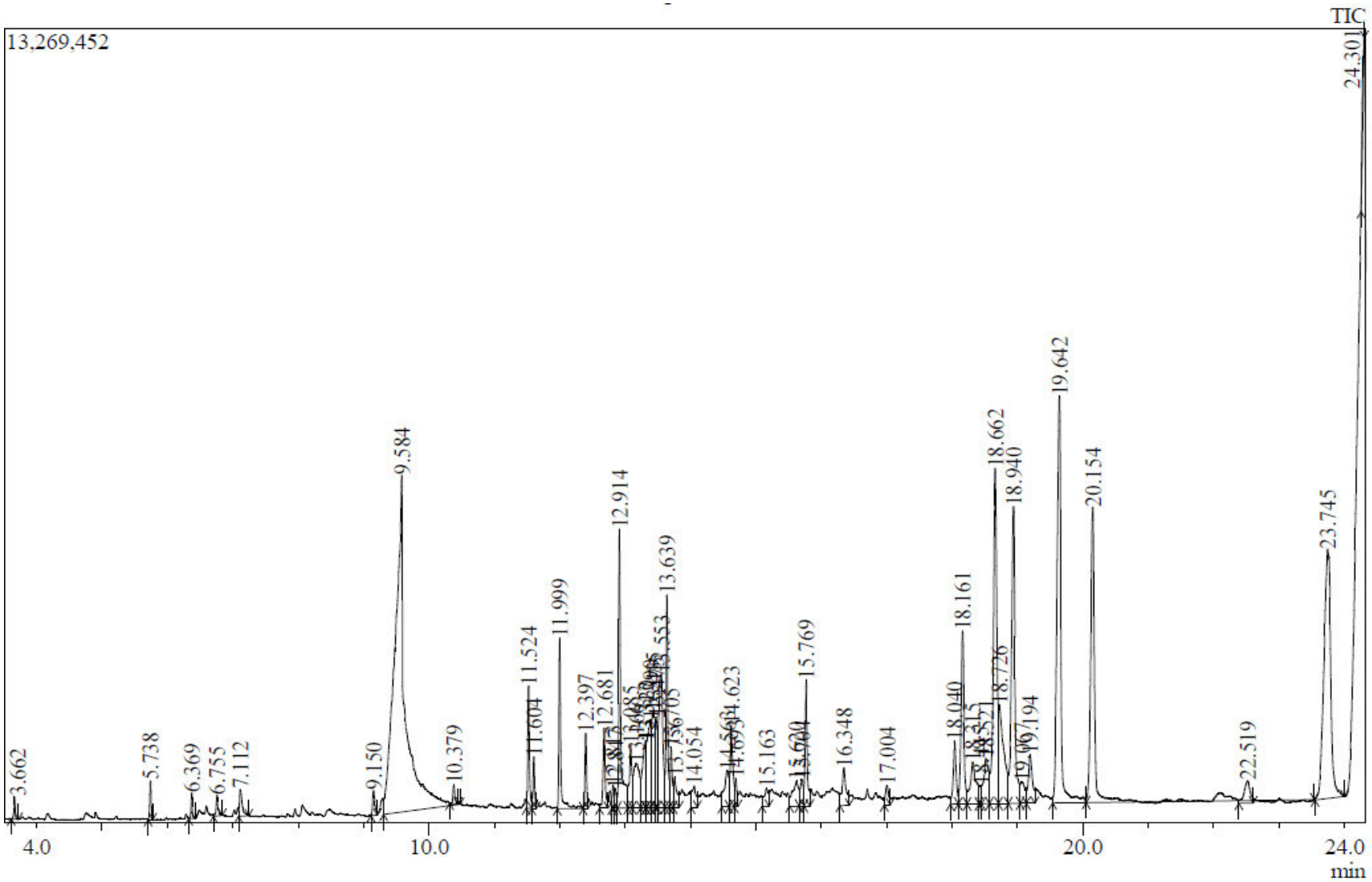


Appendix 7.3: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under FIWW obtained from GC-MS.

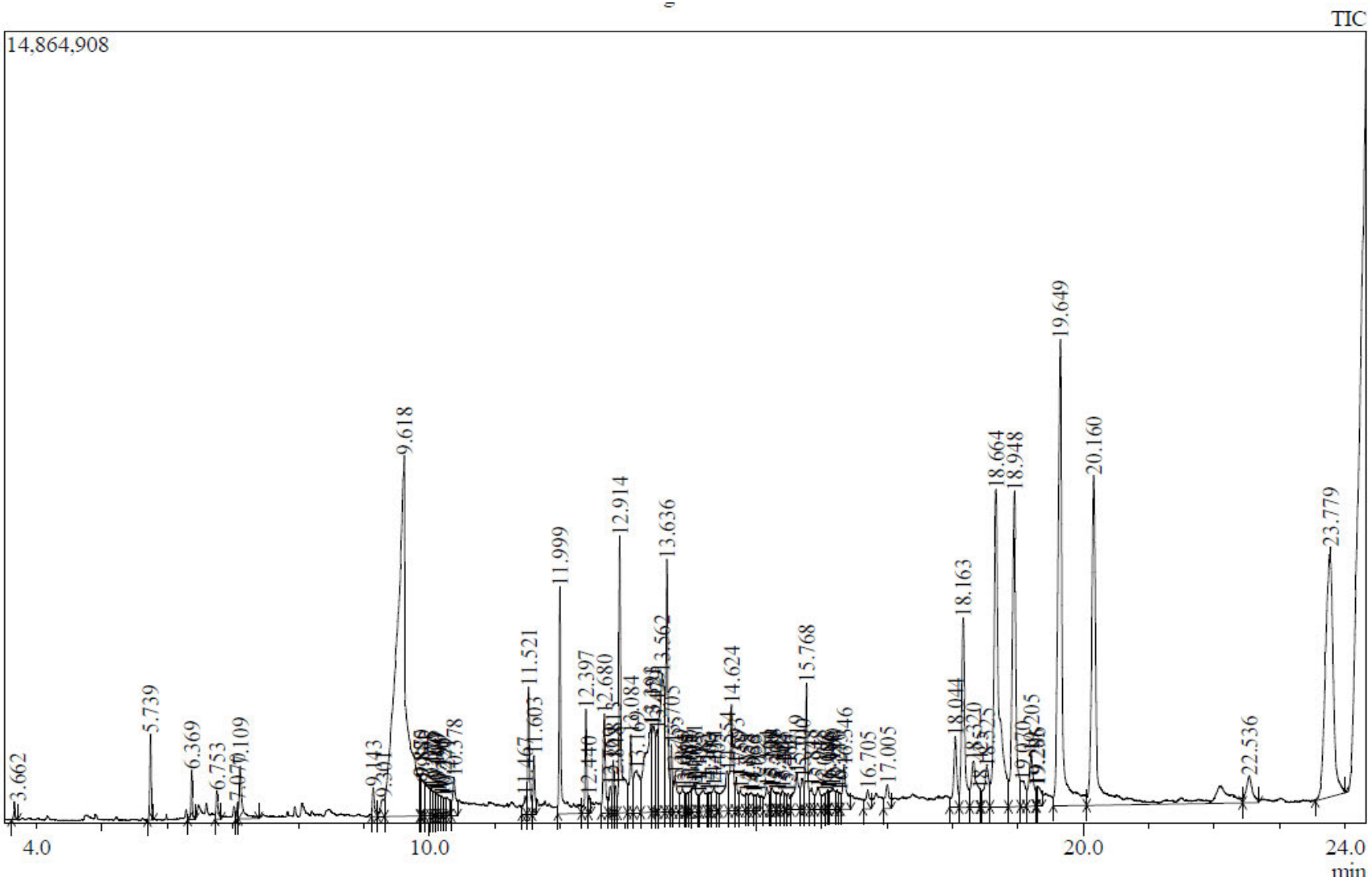
5378



Appendix 7.4: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under P+FIWW obtained from GC-MS.

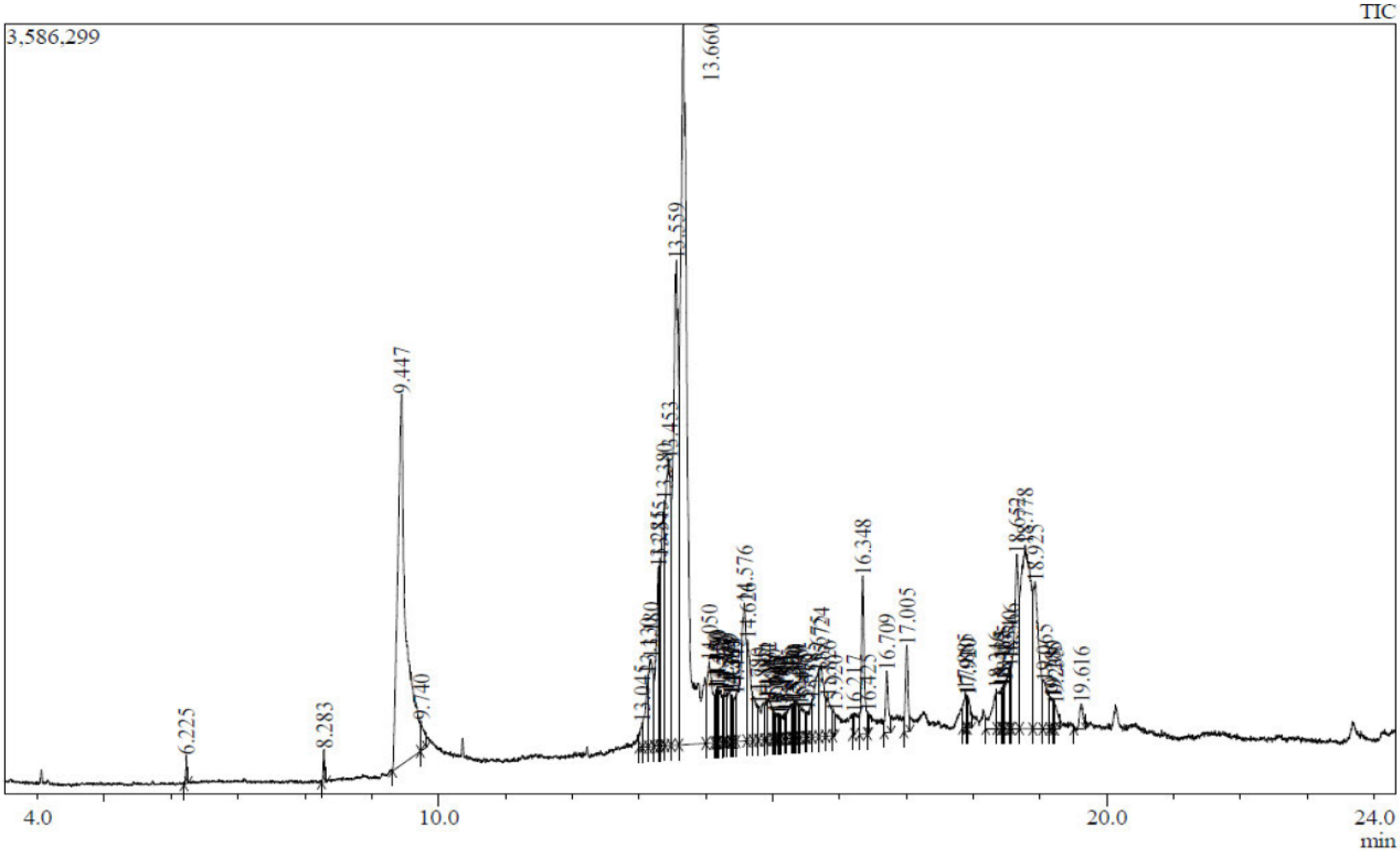


Appendix 7 5: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under UIMS obtained from GC-MS.

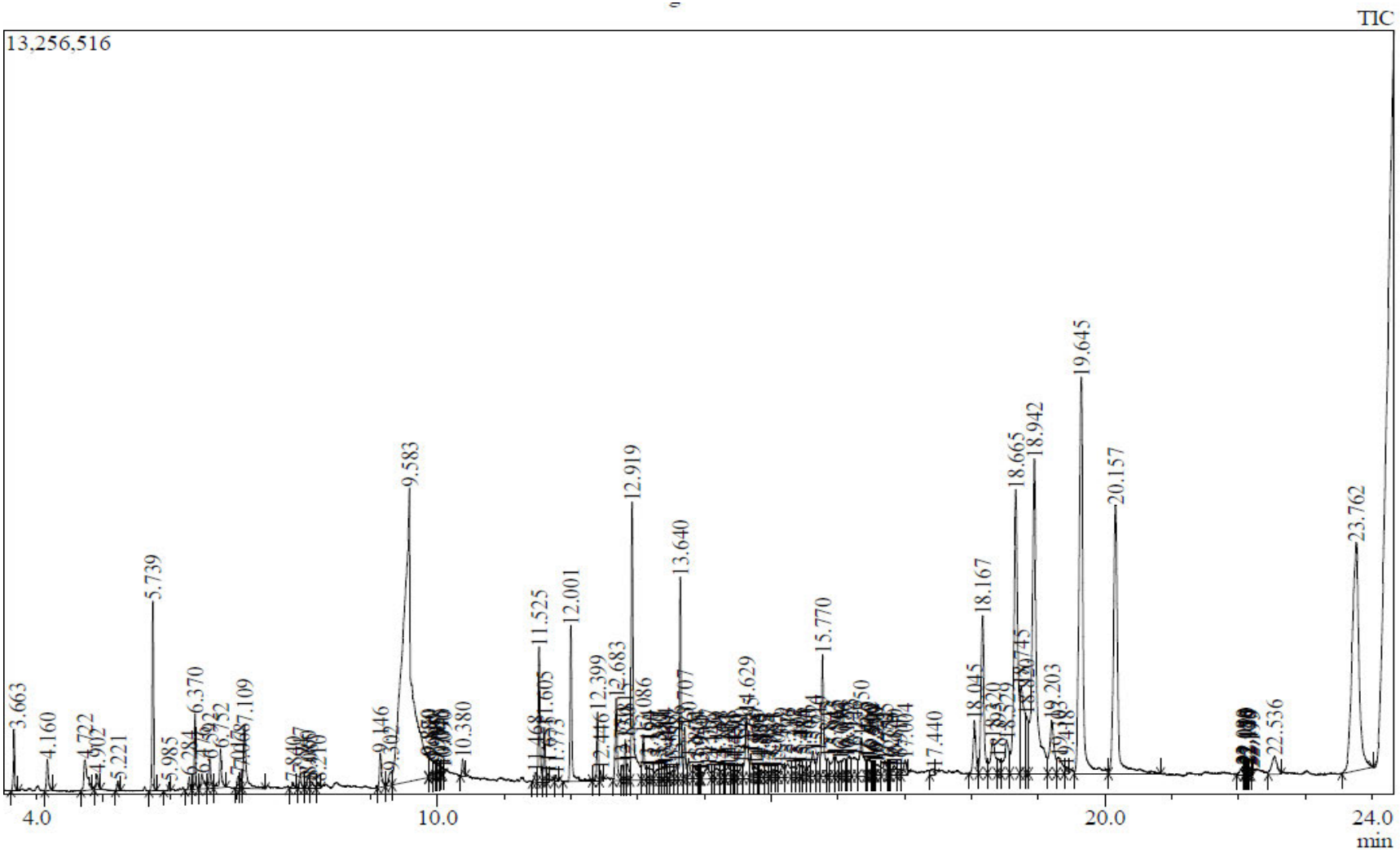


Appendix 7 6: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under PIMS obtained from GC-MS.

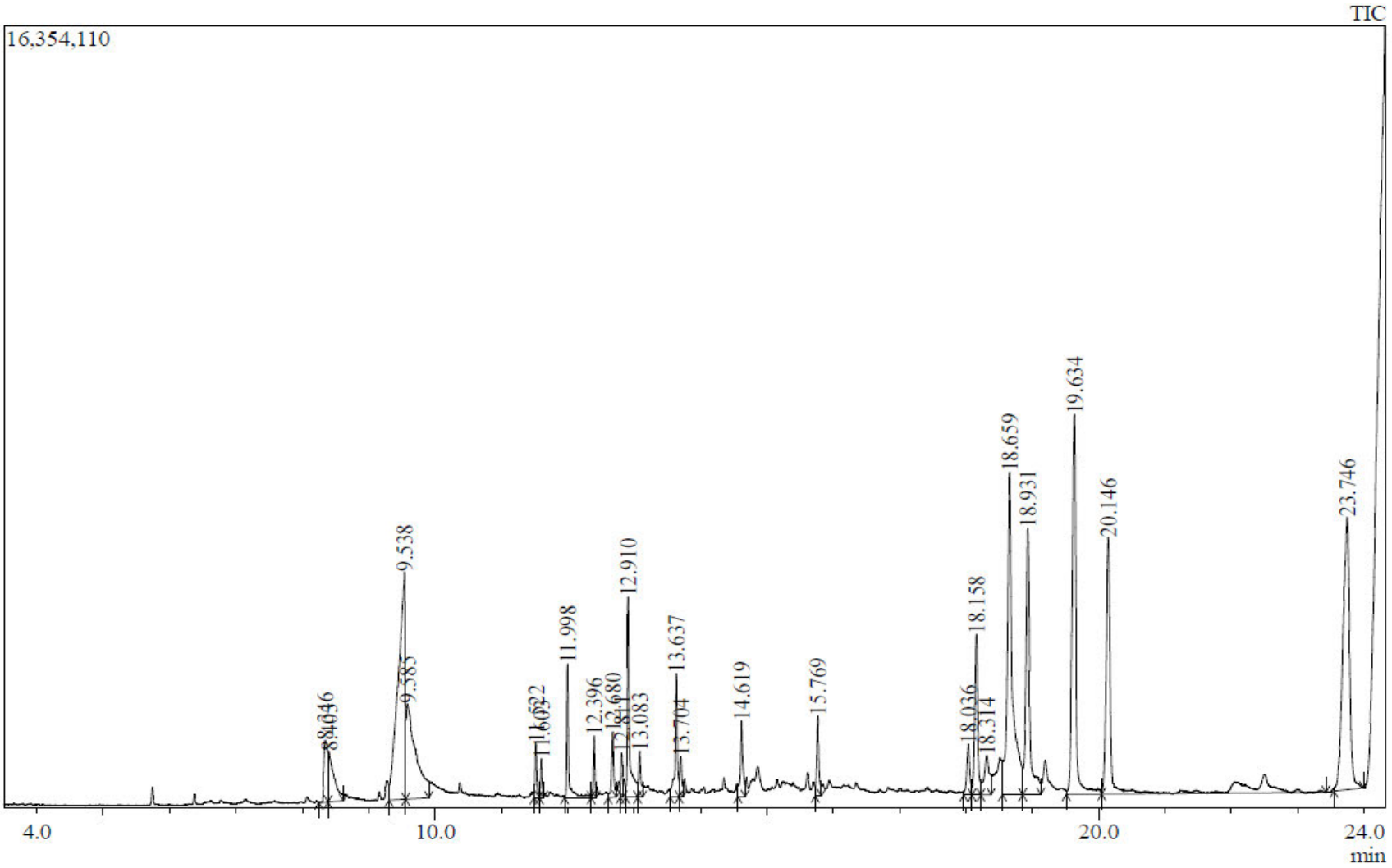
5388



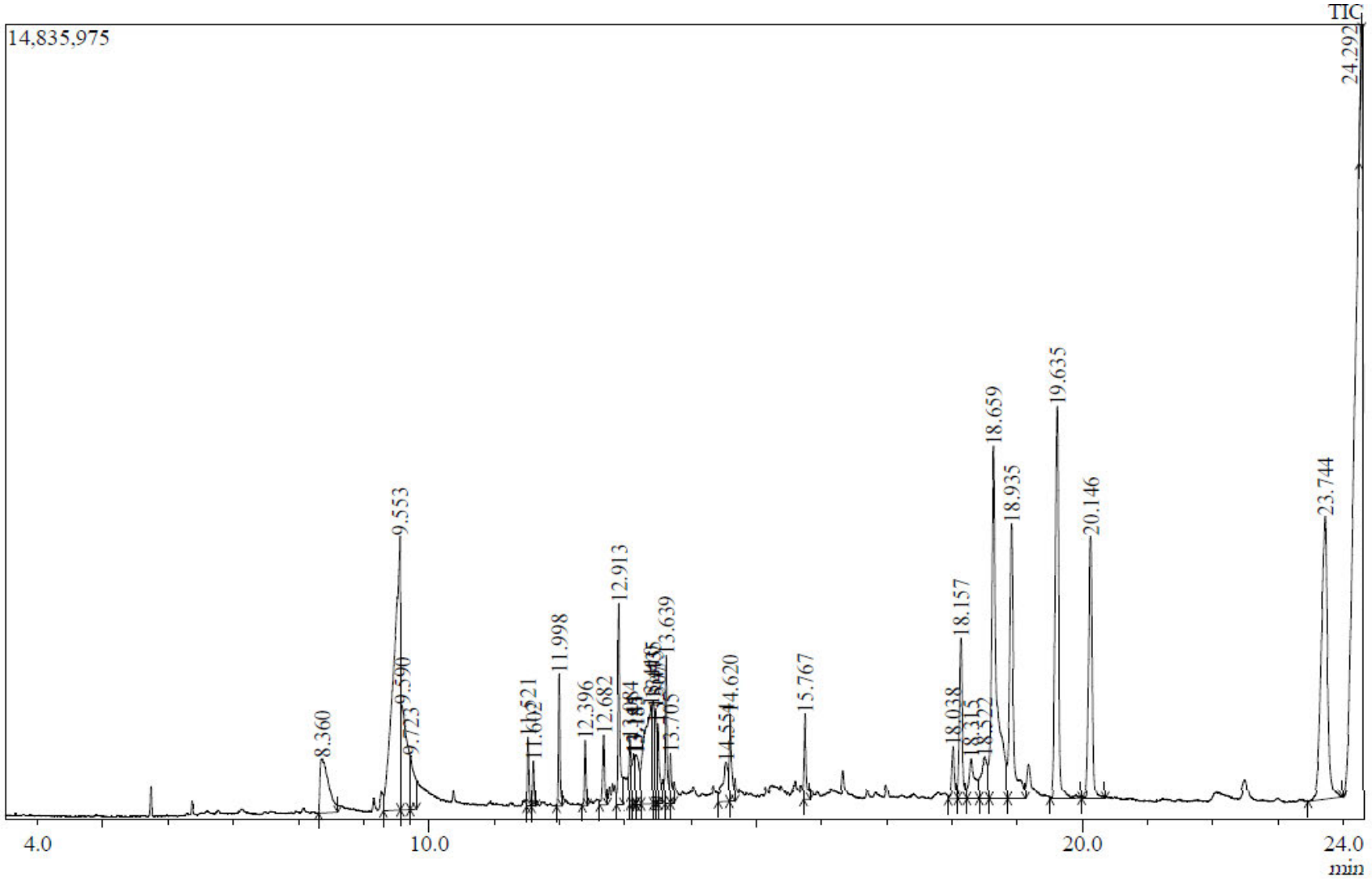
Appendix 7.7: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under FIMS obtained from GC-MS.



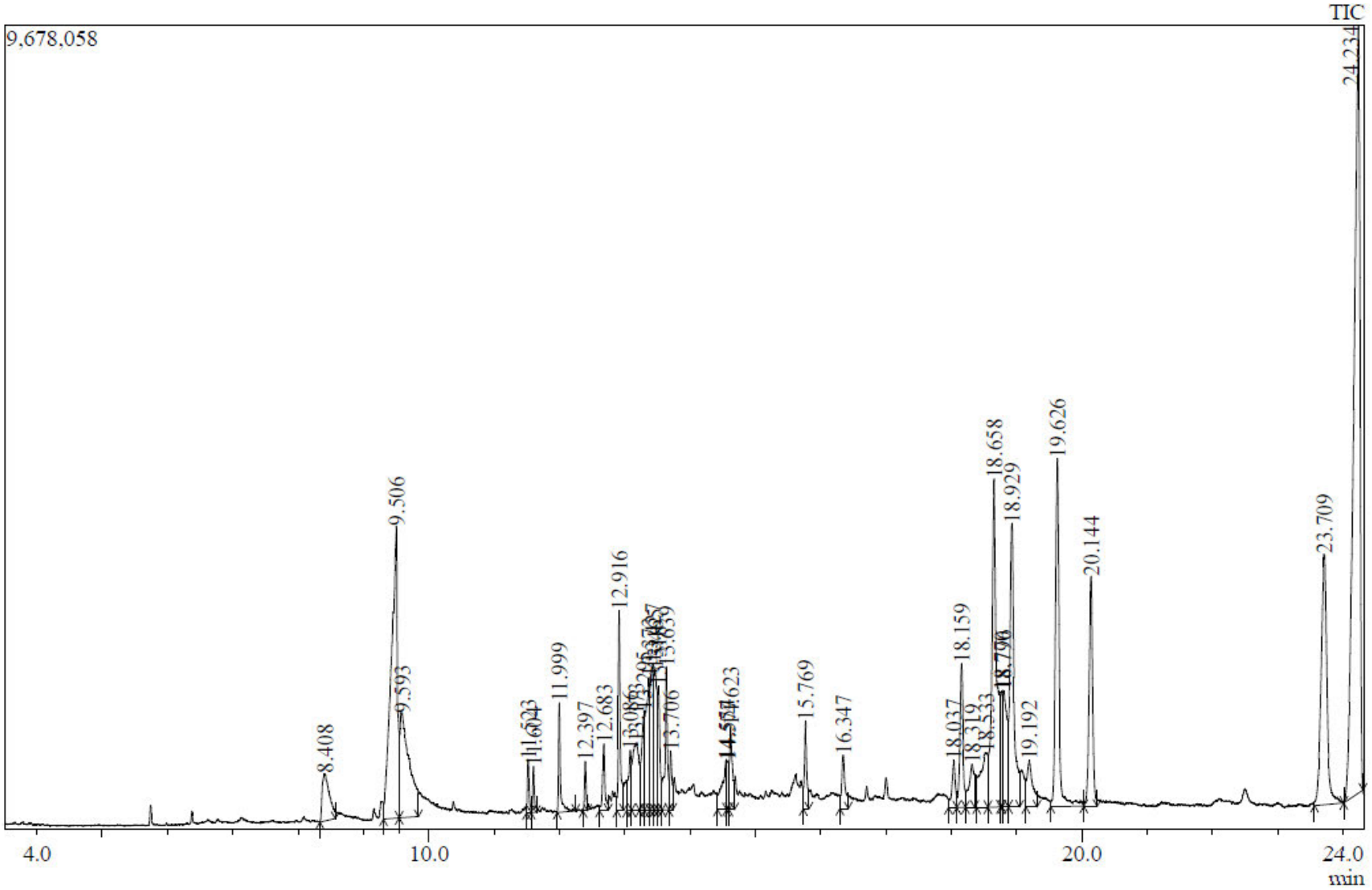
Appendix 7.8: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under P+FIMS obtained from GC-MS.



Appendix 7.9: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under UISS obtained from GC-MS.



Appendix 7.10: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under PISS obtained from GC-MS.



Appendix 7.11: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under FISS obtained from GC-MS.

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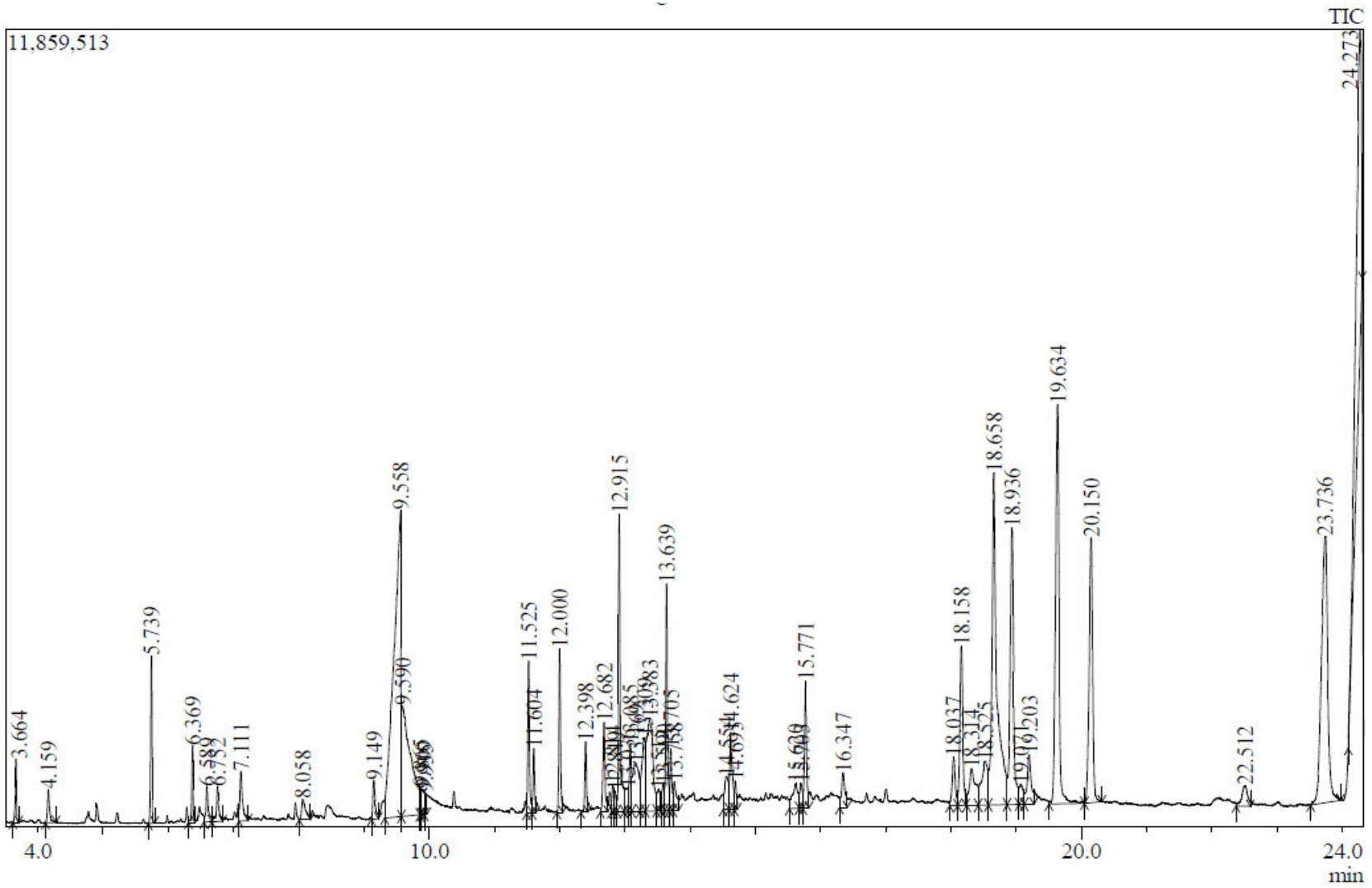
5398



Appendix 7.12: Chromatogram of ethyl acetate crude extract of *Endostemon obtusifolius* under P+FISS obtained from GC-MS.