THE EFFECT OF ENVIRONMENTAL FACTORS ON THE METABOLOMIC PROFILE AND THE BIOLOGICAL ACTIVITIES OF *HELICHRYSUM AUREONITENS*

by

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DECLARATION

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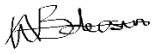
THE EFFECT OF ENVIRONMENTAL FACTORS ON THE CHEMICAL PROFILE AND THE ANTIMICROBIAL ACTIVITIES OF *HELICHRYSUM AUREONITENS*.

I declare that the above dissertation/thesis is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

I further declare that I have not previously submitted this work, or part of it, for examination at Unisa for another qualification or at any other higher education institution.



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PREFACE

Two publications from the PhD study

Adeosun WB., Olusola B., Prinsloo G. Effect of Different Climatic Regions and Seasonal Variation on the Antibacterial and Antifungal Activity, and Chemical Profile of *Helichrysum aureonitens* Sch. Bip. Metabolites, 12(8): 758. https://doi.org/10.3390/metabo12080758.

Adeosun WB., More GK., Steenkamp P, Prinsloo G. Influence of Seasonal and Geographic Variation on the Anti-HSV-1 Properties and Chlorogenic Acids Content of *Helichrysum aureonitens* Sch.Bip. Frontiers in Molecular Biosciences. https://doi.org/10.3389/fmolb.2022.961859.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL ABSTRACT

The use of plants for medicinal purposes precedes human written history. Accounts from many archaeological sources agree that the Sumerians were the first to compile plants' lists according to their herbal remedy potentials over 5, 000 years ago. The World Health Organisation (WHO) estimated that about 80% of the world population rely on herbal medicine as their primary source of healthcare. There is a heightened public interest in the use of plant sources as natural therapies in both developed and developing countries in the last decade, leading to a surge in the availability of herbal remedies in pharmaceutical stores and supermarkets.

The *Helichrysum* genus comprises flowering plants which are aromatic perennial shrubs belonging to the sunflower family. There are an estimated 600 species spread across Africa, Europe, Australasia, and Eurasia with almost half of the species found in South Africa. *Helichrysum aureonitens* is an important medicinal plant used for the treatment of many infections especially in the KwaZulu-Natal and Eastern Cape provinces.

Previous studies on *H. aureonitens* have established antimicrobial activities against Gram-positive bacteria (especially *Bacillus* species) and antiviral activity against viruses such as herpes simplex virus type 1 (HSV-1), coxsackie B virus type 1 (Cox B1), adenovirus type 31 (Ad31) and reovirus. Many phenolic compounds linked to the plants' activities include chlorophenol, 4-chloro-2-(hepta-1,3,5-triyn-1-yl)-phenol, galangin (3,5,7 trihydroxyflavone) and chlorogenic acids among others have been identified from the shoot and leaves of *H. aureonitens*.

Many studies have established the influence of environmental factors on plants' secondary metabolites. Only a few studies however exist on the influence of seasonal variation or different growing locations with dissimilar climates on the chemical profile and antimicrobial activities of any plant species from the *Helichrysum* genus. This study therefore focused on determining the effect that change in seasons and locality with different climate has on the chemical profile of *H. aureonitens* and how the shift in metabolites is linked to the plants' bioactivity. The antibacterial, antifungal, and antiviral activities of *H. aureonitens* extracts from different collection sites were evaluated against certain pathogens. The Gram-negative bacterium *Proteus vulgaris* and Gram-positive bacterium *Bacillus subtilis* were used for the study. The extracts' activities were also tested against five pathogenic fungal species including *Aspergillus flavus, Aspergillus nomius, Cladosporium cladosporioides, Fusarium oxysporum* and *Penicillum*

halotolerans. Lastly, the extracts were evaluated against the human virus, herpes simplex virus type 1 (HSV-1) to determine the antiviral activity.

Plant samples were collected during the spring (October) and autumn (May) seasons at Wakefield farms, Midlands in KwaZulu-Natal (wetter location with lower temperature) and Telperion farm in Mpumalanga (drier location with higher temperature) regions of South Africa. Collections were made in both wet and dry sites selected at each of the two localities. Temperature and rainfall data were also collected for Cedara and Witbank between August 2017 and June 2018 representing the closest stations of South African Weather Services to the two collection locations.

Hydroalcoholic extracts (30:70) of most *H. aureonitens* plants collected from both geographical locations and seasons showed good activities against the gram-negative bacterium *P. vulgaris* with Minimum Inhibitory Concentration (MIC) ranging between 62.5-125 µg/mL. Activities of extracts collected from spring however showed better activities than the autumn harvests. No activity was however recorded against the gram-positive bacterium *B. subtilis.*

Acetone extracts of *H. aureonitens* showed strong antifungal properties against four of the five fungal species tested. No activity was recorded against *A. nomius* by any of the extracts. All the other fungal species were however significantly inhibited by varied extract concentrations of *H. aureonitens* with MIC values between 0.39 – 3.125 µg/mL. The observed activities were specific to individual species. Generally, plant samples harvested from Telperion (drier location) had better activity against *A. flavus, C. cladosporioides, F. oxysporum*, and *P. halotolerans* compared to Wakefield (wet location). With the exception of *F. oxysporum*, rainfall had an inconsequential impact on the antifungal properties of the plant against the other tested species.

A cytopathic effect (CPE) reduction approach was used to evaluate the anti-HSV activity of the twenty-six samples of *H. aureonitens* leaves and stems extracts. Cytotoxicity evaluation of the extracts was carried out using MTT assay prior to antiviral determination. It was observed that seventeen (mostly spring collections) of the twenty-six extracts examined were found to have considerable anti-HSV activity, as measured by a reduction in tissue culture infectious dose ($TCID_{50}$) of less than 10^5.

Through the application of multivariate statistical analysis using a Nuclear Magnetic Resonance spectrometer (NMR), ^{1}H-NMR spectra of *H. aureonitens* leaf extracts were generated to compare samples collected in different seasons and locations as well as wet and dry sites. It was observed that changes in season played a significant role in the production of aromatics with the largest concentration found in the spring season and wet sites across both locations. Comparatively, the aromatics level is also favoured in the wetter geographical location which is Wakefield farm. Caffeoylquinic acids (CQA) which is the main Chlorogenic acids reported in nature are a class of compounds reported to be abundant in the *Helichrysum* genus. Ultra-Performance Liquid Chromatography Quadrupole Time of Flight Spectroscopy (UPLC-qTOF-MS), was used as an analytical platform to determine the identity of three derivatives of CQA and their isomers-monocaffeoylquinic acids (MCQA), dicaffeoylquinic acid (DCQA) and tricaffeoylquinic acid (TCQA) in the chemical profile of *H. aureonitens*. The DCQA derivative was the most abundant, with higher concentrations of all its isomers in both locations and seasons.

The study highlights the impact of seasonal variation and different geographical locations on the chemical profile and antimicrobial properties of *H. aureonitens*.

CHAPTER 1: GENERAL INTRODUCTION

1.1 BACKGROUND

The 4th industrial revolution is a harbinger of heightened anthropological activities. Increase in technological innovations coupled with rapid exodus to urban areas in many parts of the world making more manpower available has increased massive industrialization which inadvertently has resulted in a significant change in the climate as compared to the pre-industrial era (O'Neill B et al, 2010; Stephenson et al, 2010). A few of the outcomes of these activities have significantly affected our relationship with nature and this is observed in the change in such physical phenomena as: rising temperatures, increase in the amount of CO_2, more regular heavier rainstorms, rising sea levels, and melting glaciers to mention but a few (Majeed and Tauqir, 2020; Sarkodie et al, 2020; Wadanambi et al, 2020;).

The World Resources Institute in their December 2019 commentary on climate change provided support for some of the notable trends that have contributed significantly to climate change in the last decade. These include an increase by 10% of CO_2 emissions from fossil fuels, increase in global average temperature (about 1.1 °C above pre-industrial levels), atmospheric carbon dioxide concentration exceeding 400 ppm (part per million) in contrast with the pre-industrial era of only 280 ppm atmospheric CO_2, rise in sea levels beyond 4,064 cm and sea ice decline by 13% relative to the 1981-2010 average (Levin, 2019).

Because of the persistent mass of dense ice that glaciers form, it makes them a reliable tool for climate scientists to study changes occurring in climate over a very long period of time, often up to thousands of years. In addition, glaciers also reveal hints about global warming, according to scientists (Raper and Braithwaite, 2006; Radić and Hock, 2011). Between Ice Ages, there can be answers to questions such as how much our atmosphere has warmed up or the direct impact of human activities on the climate because of the sensitive nature of glaciers to temperature swings and consequently their associated impact on the native flora of a particular region (Morellon M et al., 2011; Kouli K, 2012)

Changes in glacial mass balance are reported by the World Glacial Monitoring Services (WGMS) in millimeters of water equivalence (WE). The water equivalence of all lost or acquired glacial ice transformed to water and spread evenly across the glacier surface area is the depth of that water layer (Blunden and Arndt, 2019).

The following graph presents the contrast between glacial mass balance of WGMS 37 reference glaciers for 52 years (1968-2020) with the total mass loss over the same period of time as expressed by the mean and cumulative mean specific annual balance of WE.

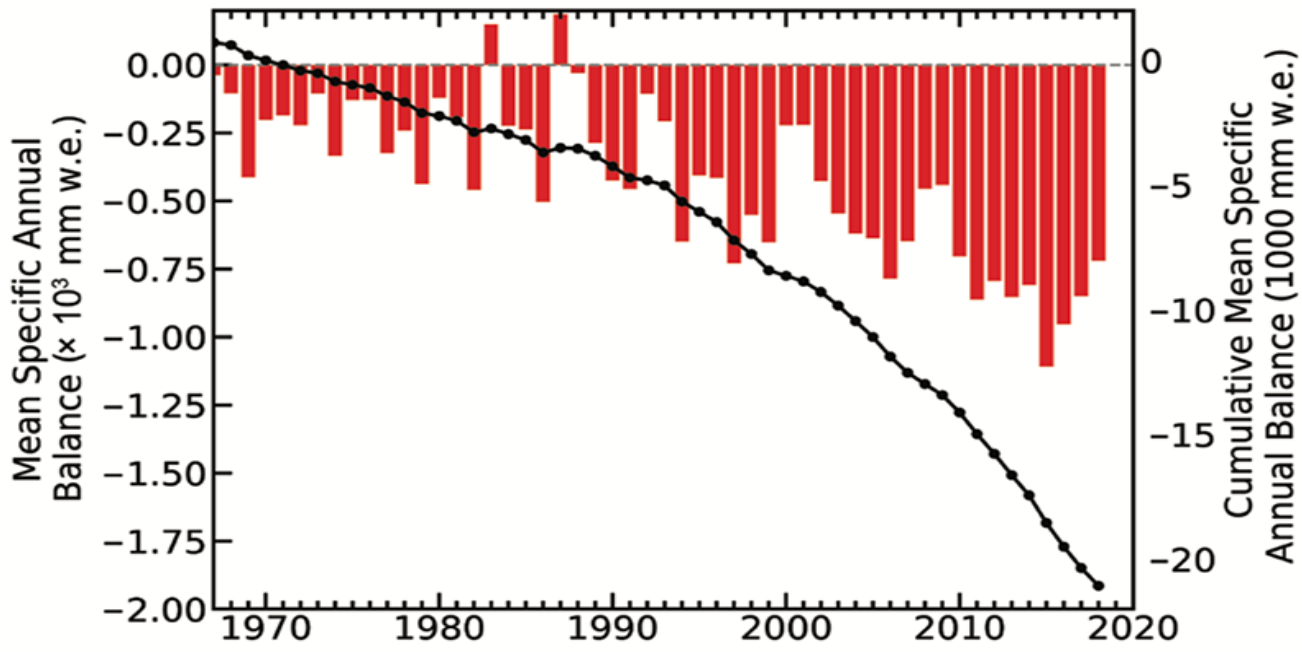

Figure 1.1: Glacial mass balance of the WGMS 37 reference glaciers between 1968 and 2020 (red bars), with the total glacial mass loss over same time period (black line) (Blunden and Arndt, 2019)

Another significant trend at the dawn of industrialization is the surge in the increase of CO_2. CO_2 being a key greenhouse gas is at the fore front in driving the global climate change. Because of its central importance to plants' metabolism, a steady rise in CO_2 may have a profound effect on the physiology, growth, and chemistry of plants (Ziska, 2008). Plants have been shown to be affected by increased atmospheric CO_2 concentrations in a variety of ways, including changes in their elemental composition. Plants often display greater carbon concentrations in their tissues when CO_2 concentrations rise, with correspondingly lower concentrations of other elements such as nitrogen, phosphorus, and other trace elements (Cotrufo et al., 1998; Gifford et al., 2000; Loladze, 2002).

Himanen et al (2008) reported the differences in the observation of constitutive and herbivore-inducible glucosinolate concentrations in oilseed rape (*Brassica napus*) leaves when observed under increased atmospheric CO_2. There is an inverse correlation between the concentration of

total glucosinolates produced and CO_2 levels. As CO_2 levels increased, production of total glucosinolates decreased, indicating a likely loss in constitutive glucosinolates defense in oilseed rape compared to its production under ambient CO_2 levels. Furthermore, when oilseed rape leaves were exposed to chronic O_3, a decrease in production of total glucosinolates was observed (Gielen et al., 2006).

Generally in most cases, unfavorable biotic and abiotic factors increase plants' stress levels which results in changes in the metabolic profile to mitigate the effect of the stress. Mansour-Gueddes et al (2020) evaluated the effect of climate change on the table olive plant *Olea europaea* L. Fresh leaves of the plants were collected in three different climatic locations in Tunisia: the northern region with sub-humid climate, the central region with higher semi-arid climate and the southern region having a lower arid climate. Result shows that there was a significant difference in the chemical profile of the leaves from the different regions. The leaves from the southern region showed more abundance of polyphenols, flavonoids, o-diphenols and tannins in comparison with the leaves harvested from the northern region. Carotenoids, a class of naturally occurring pigments were more abundant in the southern region than the northern region. There was however a decrease in chlorophyll a and b content of the leaves from the northern to the southern region.

In another study that investigated the effect of environmental factors on both the content and antioxidant activity of the active substances present in *Potentilla fruticosa* L. from different regions of China, Liu et al (2016) reported that the findings revealed that altitude had a substantial and negative correlation to tannin content. The flavonoids concentration, rutin content, and antioxidant activity were all significantly and positively correlated to annual sunshine duration and altitude. Also, the content of total phenolics was considerably and negatively correlated to annual mean temperature, while the content of total phenolics was strongly and positively correlated to altitude.

Temperature, being one of the key environmental variables, has a substantial impact on the composition of plants' metabolites. Many studies have reported that an increase in temperature almost always enhances the production of all secondary metabolites in plant species (Sharkey and Yeh 2001; Hanson and Sharkey 2001; Guo et al., 2020; Jamloki et al., 2021). For example, with regards to deciduous and coniferous plant species, the relationship between terpenoid output and temperature has been studied in recent years. In warm temperatures, *Quercus rubra* and *Q. alba* had twice the capacity for isoprene emission when compared to cold conditions

(Hanson and Sharkey 2001). Except for α-terpinolene, which declined dramatically with rising temperature, other terpenes in *Daucus carota* root showed increasing levels with increasing temperature (Rosenfeld et al., 2002). These results lend credence to the significant effect that changes in climate have on the chemical profile of plants.

The genus *Helichrysum* is an important medicinal plant in Africa and other parts of the world, with nearly half of the species found in South Africa (Hilliard, 1983; Lourens et al., 2008). Variety of species of *Helichrysum* are used for different medicinal purposes owing to the wide range of secondary metabolites that are produced by different plants of this genus (Lourens et al., 2008). The important role of the environment in influencing the chemical profile and thus the biological activities of plants have been well documented (Liu et al., 2016). Many studies have established the antimicrobial activities of scores of *Helichrysum* plants (Hutchings and Van Staden, 1994: Guarino and Sciarrillo, 2003; Stupar et al., 2004: Kutluk et al., 2018).

Since metabolomics involves the scientific study of metabolites produced by living systems using techniques such as NMR spectroscopy coupled with multivariate data analysis (Lankatillake et al., 2019), an attempt to understand how changes in seasons and different growing locations influence both the chemical profile and biological activities of *H. aureonitens* can therefore be accomplished through plant metabolomics and multivariate analysis.

1.2 RESEARCH PROBLEM

Owing to the dearth of information regarding the link between the influence of environmental conditions and the secondary metabolites, climate change will inadvertently affect the growth, development and metabolic processes in plants. The changes in the chemical profile, and subsequently associated medicinal properties of the plants, should however be considered, and quantified in the production and use of traditional medicine (TM). *Helichrysum aureonitens*, an important medicinal plant used in TM preparations, has been shown to vary widely in chemical profile from different sample collections. Using *H. aureonitens* as a model plant, this study aims at determining the possible effect of environmental conditions in two different

seasons and two different climatic locations on the secondary metabolite profile and biological activity of *H. aureonitens.*

1.3 JUSTIFICATION FOR THE STUDY

A study of the association between the climatic conditions and metabolites distribution within different samples of *H. aureonitens* will:

1. Provide insight into the chemical characterization of this species.

2. Assess plant-environment interaction at the molecular level by providing understanding into the plant's responses to environmental effects.

3. Contribute to the field of environmental metabolomics and medicinal plant research and provide some insights into the plant's response to climate change.

1.4 AIM AND OBJECTIVES

To determine the effect of seasonal variation and different geographical locations on the metabolite profile and biological activity of an important medicinal plant *H. aureonitens*.

The following are the objectives of the study:

1. To examine the antifungal, antibacterial and antiviral effects of plants collected from two areas and different seasons using plant and human pathogens including herpes simplex virus (HSV).
2. To compare the metabolites profile of *H. aureonitens* growing in two different geographical locations and two different seasons of the year.
3. To compare the metabolite profile of *H. aureonitens* growing in different sites in the same location.
4. Identify the changes in the plant (metabolite and bioactivity) due to environmental effects (seasonal changes and peculiar climate of each geographical location).

1.5 RESEARCH QUESTION

How do changes in the environment affect the metabolite content of plants, thus potentially effecting their biological activity?

1.6 HYPOTHESES

Temperature and water availability influence the metabolite profile of *H. aureonitens* both quantitatively and qualitatively.

Climatic conditions such as temperature and water availability results in metabolite changes, subsequently affecting bioactivity.

1.7 THESIS LAYOUT SUMMARY

The thesis begins with the introductory chapter and literature review, followed by four experimental chapters-two chapters on investigation of bioactivity, one on metabolomics analysis, followed by LC-MS studies. The last chapter being a summary of the novel contributions of the study and recommendations for future research.

Chapter 1: This chapter gives a general introduction and background to the study.

Chapter 2: Review of literature on the evolution of analytical equipment and the field of metabolomics, as well as the effect of environment on the chemical profile and biological activities of plants and the genus *Helichrysum*.

Chapter 3: The chapter reports on and discusses the investigation of seasonal variation and differences in geographical location on the antibacterial and antifungal properties of *H. aureonitens.* The plant's activity was tested against the bacteria *Proteus vulgaris* and *Bacillus*

subtilis and fungi *Aspergillus flavus, Aspergillus nomius, Cladosporium cladosporioides, Fusarium oxysporum,* and *Penicillum halotolerans.*

Chapter 4: The chapter reports on and discusses the investigation of seasonal variation and differences in geographical location on the antiviral property of *H. aureonitens* on herpes simplex virus (HSV-1) using the MTT assay.

Chapter 5: This chapter reports on the metabolomics study of the chemical profile of *H. aureonitens* using ^{1}H Nuclear Magnetic Resonance (NMR) and Ultra-performance liquid chromatography.

Chapter 6: As the final research chapter, it reports on and discusses significant differences in chlorogenic acids contents of *H. aureonitens* growing in two different regions and seasons in South Africa using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectroscopy (UPLC-qTOF-MS).

Chapter 7: Discussions and conclusions are presented clearly and concisely in this chapter to provide a logical summary of the thesis. It also provides the highlights and novel contributions of this study as well as future research identified to further understand and interpret environmental factors on medicinal plants.

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CHAPTER 2: LITERATURE REVIEW

2.1 HISTORICAL REVIEW OF MEDICINAL PLANT USE AND EVOLUTION OF HEALTH CARE PRACTICES

Before the advent of orthodox medicine, the potential of plants to treat ailments and diseases were discovered and deployed to use by humans since prehistoric times (Shi et al., 2010). Man's pursuit of healing with plants was a result of a long fight against illnesses and diseases leading him to seek healing in different plant parts such as bark, fruiting bodies, roots, and leaves (Fabricant et al., 2001). Traditional medicine (TM) is an aspect of cultures within societies that developed over generations to take care of medical needs of the people. It is defined by the World Health Organization (WHO) as "the sum 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 illness" (WHO, 2008). Medicinal plants have been pivotal to the operations of TM from time immemorial, a practice which continues till today. At the 2011 summit on 'The World Traditional Medicines Situation' in Geneva, Switzerland, WHO reported that some 65%-80% individuals from developing nations around the world use medicinal plants as remedies (WHO, 2011). Furthermore, a later report of the same organization, (WHO global report on traditional and complementary medicine, 2019) had posited that many countries were attempting to extend coverage of basic health services to include traditional and complimentary medicine especially at a time when expectations for meeting health needs were increasing and expenditures were growing, coupled with the static or shrinking budgets of many nations. Given the specific health challenges that the twenty-first century presents, traditional and complementary medicine is now experiencing a resurgence in popularity (WHO, 2019).

Varying accounts have reported that there are between 250,000-400,000 flowering plants species across the globe (Thorne, 2002; Scotland and Wortley, 2003;). This presents a huge resource and potential for the possible exploration of plant extracts as a source for drug development from natural products. Many studies have been carried out to research the pharmacologically active compounds trapped in botanical resources, some of which have been

used in the treatment of fatal diseases such as malaria, tuberculosis, and asthma to mention but a few (Mintah et al., 2019).

Prior to the dawn of science-based medicine, the discovery and use of specific plants found to be effective against a number of diseases came from experience and not established through research (Stojanoski 1999; Gao et al, 2007). These discoveries and indigenous knowledge of different cultures around the world regarding medicinal plants were orally transmitted across generations before the invention of writing (Abebe and Ayehu, 1993). The earliest attempt from history at documenting medicinal plants is found with the Sumerian civilization where a good number of recipes for different drug preparations were written on clay tablets (Sumner, 2000). Another important document that has survived since 1550 BC (although believed to have been a copy of much more older texts) after it was first put together is the Ebers Papyrus. It is a detailed documentation of the Egyptian medical material called papyrus, containing the description of many plant medicines used as remedies against several kinds of diseases (Stern, 1875; Bryan, 1930).

On the African continent long before the establishment of modern medicine, TM was the predominant means to meet medical needs (Romero-Daza, 2002). Fast forward to the 21st century, more people still depend on TM for their primary health care as opposed to conventional western medicine. This is due in part to cultural beliefs, and its easy accessibility and affordability as compared to western medicine (Xue et al., 2007; Clarke et al., 2015). The therapeutic qualities, a feeling of spiritual satisfaction and the trust many people repose in the system are also a few of the reasons for the preference. There have been a few discussions regarding the more common subscription to TM by Africans; many believe that it has a close connection with cultural and economic reasons (Sato, 2012).

South Africa has a robust history of traditional healing. There is ample evidence that South Africa is home to some 30,000 flowering plant species, a figure that accounts for roughly 10% of the global presence of higher plants (Louw et al., 2002; Van Wyk and Gericke, 2000). With a health care system that is pluralistic, typical of developed and other emerging economies around the world, a highly sophisticated health care system based on scientific discoveries have coexisted with a variety of other non-conventional health therapies whose fundamental practices are based on traditions and beliefs in South Africa. Medicinal plants have played a pivotal role within this unconventional traditional health care system of South Africa since time

immemorial (Campbell-Hall et al., 2010). Below are examples of some common plants used for their medicinal properties in South Africa.

Table 2.1: Examples of common plants being used within South Africa for their medicinal properties.

Plant species	**Common name(s)**	**Plant parts used**	**Uses**	**References**
Harpagophytum procumbens (Pedaliaceae)	Devil's claw	Mostly the tuber	Treatment of: Rheumatism, Arthritis, Diabetes, Gastrointestinal disturbances and Menstrual difficulties	Van Wyk *et al*, 1997; Barnes, 2009
Hypoxis hemerocallidea (Hypoxidaceae)	African potato, Star flower or yellow star.	Rootstalk	Treatment of: Impotency, Cancer, cardiac arrest Ulcer, Headaches Common cold, and Flu	Barnes, 2009; Drewes et al, 2008; Van Wyk et al, 1997
Merwilla natalensis (Hyacinthaceae)	Blouberglelie	Bulbs	Treatment of: Gastrointestinal ailments, Sprains and fractures, Tumours (cancerous), Menstrual pains,	Sparg et al., 2002

			Infertility. It is also used to assist in taking delivery of babies during childbirth in women.	
Agathosma betulina (Rutaceae)	Buchu	Leaf	Treatment of: Stomach aches, Kidney and urinary tract infections, Haematuria, Cholera, Bruises	Van Wyk et al., 1997; Lis-Balchin et al., 2001; Watt and Breyer-Brandwijk, 1962; Simpson, 1961.
Aloe ferox (Asphodelaceae)	Cape Aloe or bitter Aloe	Leaf, stem, and root	Treatment of: Skin and hair, burns, insect bites, sunburn, hypertension and stress, arthritis, conjunctivitis, toothaches, stomach pains	Crouch et al., 2006; Pujol, 1990.
Aspalathus linearis (Fabaceae)	Rooibos, long-life tea	Twigs and leaves	Used as an antiaging, antioxidant, antispasmodic and for	Van Wyk and Gericke 2000; Van Wy ketal et al., 1997; Watt and Breyer-

			antieczema activities. Also used for the relieving nausea and heartburn in pregnant women. Rich in iron, it's also used as colic relief and as substitute for milk for infants	Brandwijk, 1962; Joubert and De Beer 2011; Joubert et al., 2008; Gruenwald, 2009.
Pelargonium sidoides (Geraniaceae)	African geranium	Fleshy red rhizomes or tubers	Treatment of: Gastrointestinal tract disorders, diarrhea, dysentery, stomach upsets in infants	Moolla et al., 2007; Kolodziej et al., 2011; Brendler and Van Wyk, 2008; Matsiliza and Parker 2001.
Sclerocarya birrea (Anacardiaceae)	Morula or cedar tree	Fruit, leaves and bark	Treatment of: Rheumatism, dysentery, insect bites, malaria, proctitis, ear, nose, and throat infections.	Gurib-Fakim et al., 2010; Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996.
Siphonochilus aethiopicus (Zingiberaceae)	Wild ginger or African ginger	Rhizome and root	Treatment of: Dysmenorrhea, flu, cold, asthma, hysteria, malaria.	Van Wyk, 2008; Diederichs et al., 2002; Van

				Wyk et al., 1997.

2.2 THE ADVENT OF ANALYTICAL EQUIPMENT FOR THE DETERMINATION OF THE CHEMICAL PROFILE AND ACTIVE BIOLOGICAL INGREDIENTS WITHIN PLANTS

Historically, a trial-and-error approach was relied upon by humans for the use of medicinal plants regarding treatment of diseases since meaningful advancement in the science of drugs only began at the dawn of the last century (Stojanoski, 1999; Biljana 2012). One major reason the use of medicinal plants fell out of favour with science-based medicine in the past was the lack of substantial evidence regarding the chemical profile of these plants and detailed scientific data on the purported efficacy of such plants. Isolation and identification of the active ingredients within the plants were difficult and thereby their biological activities were shrouded by lack of enough knowledge. The inadequacy of this information makes dosage recommendation difficult, therefore generally leading to the repudiation of medicinal plants as either an alternative or complementary source to orthodox medicine by scientists owing to safety concerns (Clement et al., 2005; Barros et al., 2014; Abdullahi, 2011).

A more recent example that corroborates this assertion is the production of Covid-Organics (CVO) at the onset of the outbreak of the communicable respiratory disease- the coronavirus disease 2019 (COVID-19) in April 2020. Andry Rajoelina, President of Madagascar had asserted just before sipping from the bottle that CVO, an Artemisia-based drink produced from the *Artemisia* genus, which is made by extracting artemisinin (Weathers et al., 2011) for curing malaria can prevent and cure COVID-19. This somewhat hasty and overgeneralized statement compelled the WHO to issue a statement of caution against the dissemination of false information and alleged miracle cure that CVO brings. This is against the backdrop of a clear and unambiguous warning by the WHO against the use of untested remedies as alternative cure for sicknesses in spite of the organization's support for TM (WHO Africa, 2020). Dr. Luisa Dologuélé of the Maison de l'artemisia in Paris, France raised a valid concern when she said, the specific composition of the Malagasy beverage is unknown, and it is supplied without dosage instructions so people are unsure about how much and for how long they should drink

(Marbot, 2020). The whole production process did not follow properly approved scientific procedure and also lacked publicly available clinical study data that can establish its credibility, its efficacy and safety could therefore not be established. Months after, COVID-19 cases became astronomical in the Madagascar island, further lending credence to the non-effectiveness of the purported curative power of the drink (Kapepula et al., 2020; Nie et al., 2021).

The discovery and development of analytical methods, chiefly Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy (MS) in the last decades has opened up a wide range of possibilities in plant science research, which have seen the introduction of an entirely new field of study called metabolomics. Metabolomics is concerned primarily with the study of the isolation, identification, and quantification of the metabolome in biological systems and therefore is pivotal to the operations of other inter-disciplinary fields such as ethnobotany, phytochemistry and ethnopharmacology (Sumner et al., 2003; Fiehn et al., 2007;Verpoorte et al., 2007; Allwood et al., 2008). A combination of these sub-fields has further made the chemical and pharmacological studies of medicinal plants, in particular, to be on the rise. More antimicrobial studies and testing for biological activities are being carried out on medicinal plants, increasing our awareness of the chemical profile of such plants. The advantages of the results of these numerous research is firstly seen in the thorough understanding of contents within the raw materials derived from natural plant products. Furthermore, the results broaden our understanding on the effects of the plants' contents and how they can be explored for drug development and also the provision of solid foundation from a knowledge base upon which policies and regulations for the use of medicinal plants can be being developed (Goodacre et al., 2004; Wang et al, 2005; WHO, 2005; Shyur and Yang, 2008).

The promotion of the integration of TM and complementary and alternative medicine (CAM) into the national healthcare program of member nations is one of the major goals of the WHO, this goal has led to the introduction of national policies with regards to the use of TM and complementary and alternative medicine in many countries (WHO, 2005).

2.3 EFFECT OF THE ENVIRONMENT ON THE CHEMICAL PROFILE OF PLANTS

Plants are largely autotrophs by nature meaning they derive organic substances from simple inorganic matters such as CO_2, light, and water. Since plant-environment interaction is inevitable, growth rate, general development, and the metabolomic profile of plants are all quantitively affected by external factors. These conditions can also cause rapid changes in the quality of secondary metabolite synthesis (Laughlin, 1993; Pérez-Estrada et al., 2000). The metabolic process which ultimately results in either the synthesis or breakdown of organic compounds within the plant also has a direct correlation with the prevailing environmental factors (Broun et al., 2006; Yanqun et al., 2020). There are optimum environmental conditions that aid proper growth of plants. The most important ones are adequate light, CO_2 water, oxygen, and suitable temperature that enables effective physiological activities (Hatfield et al., 2008; Hatfield et al., 2011). These ideal environmental conditions vary between individual plant species depending on a few factors among which are the genetic make-up of the plant, the soil condition, and the microbial community in which the plant is growing and the long-term climatic conditions that is prevalent in a region which ultimately determines the type of vegetation that it supports and the geographical distribution of that environment. A deviation from these conditions is certain to limit the plants' optimum growth (Davis et al., 2005; Pregitzer et al., 2010; Alsos et al., 2012; Liu et al., 2012).

As sessile organisms, plants are devoid of motion and are therefore forced to interact with the prevailing conditions of the environment to which they belong. Plants have developed various coping mechanisms most of which are reactionary adaptations to mitigate external environmental stressors (Yamaguchi-Shinozaki and Shinozaki, 2006; Ahuja et al., 2010; Skirycz and Inze, 2010). Both biotic and abiotic stress sources engender complex biochemical reactions in plants, making their metabolism to change in response to the prevailing conditions.

Below is a diagram of key abiotic stress factors and possible severities that can affect plant development (Figure 2.1).

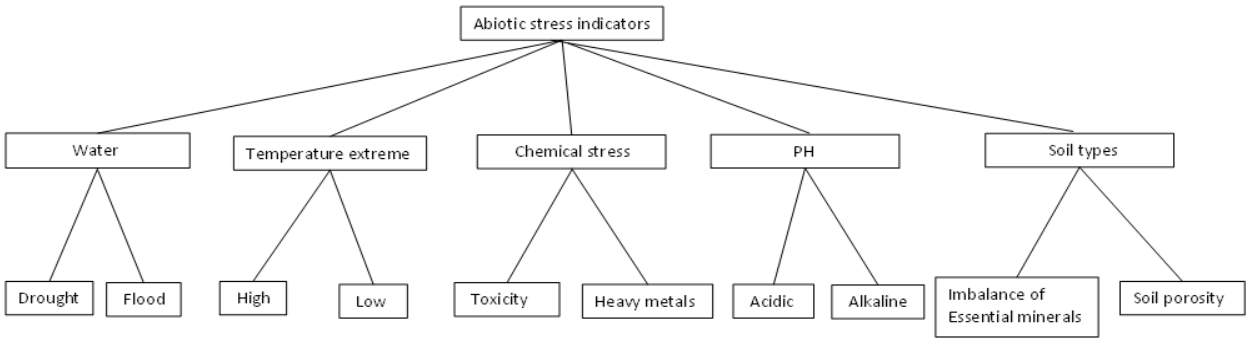


Figure 2.1: Some abiotic stress indicators and possible extremes that affect plants' development (Bailey-Serres and Voesenek, 2008; Anjum et al., 2017)

These abiotic stress factors result in metabolic variations, which undoubtedly results in a shift in the chemical profile of plants (Bray et al., 2000; Ahmad and Prasad, 2012; Dos Reis et al., 2012). A handful of studies proved that plant parts respond differently to changes in environmental factors as observed in the chemical profile composition of each plant part when subjected to varying environmental conditions (Albert et al., 2014; Sampaio et al., 2016). Unfavorable abiotic factors such as high or low temperature, high salinity, drought, alkalinity, heavy metals etc. cause plant stress which oftentimes lead to the production of closely regulated secondary metabolites in an attempt for the plant to localize the response to either a particular tissue, specific developmental stage, or response to certain stimulating factors to ultimately mitigate the effect of the adverse condition (Osbourne et al., 2003).

Prinsloo and Nogemane (2018) however observed that not in all cases are secondary metabolites increased in response to plants' stress conditions, seasonal variation, and water availability, and can equally result in the decrease in production of certain secondary metabolites. Plants' stresses as a result of abiotic factors interfere with the normal metabolism and the effect of this include changes in the plants' molecular, biochemical, physiological, and morphological appearances such as reduction of plant height, leaf sizes and general biomass production (Bray et al., 2000; Zhu, 2002; Mahajan and Tuteja, 2005). Metabolism at this point is intensely involved in cell signaling, regulation of physiological activities within the plant

and defense responses (Sanders et al., 1999; Knight, 2000; Zhou, 2019). The concomitant modular activities triggered by the plant during adverse environmental conditions are all geared towards maintaining an equilibrium with regards to their internal physiological state (Junghans et al., 2006; Osakabe et al., 2012).

2.3.1 Effect of excessive solar radiation on plants' secondary metabolites

Plants depend on sunlight as energy source for photosynthetic activities; solar radiation is therefore pivotal to the production of needed nutrients for growth and development in plants. Their ability to fix photosynthetic carbon and buildup biomass is critical to their survival, and as a result, they've acquired highly sensitive and specific abilities to detect the presence of ultraviolet (UV) light and diversities of light spectra in sun rays (Kazan and Manners, 2011). Elevated doses of light spectra and UV radiations are unsafe and therefore inimical to plants' survival; this makes the need for the optimization of absorption of relevant light wavelengths paramount. Plants have therefore evolved biochemical strategies to defend themselves from potentially harmful light intensities. Ultraviolet B-light (UV-B), a spectrum of wavelength between 280-315 nm has been repeatedly reported to cause damage to photosynthetic machinery and photosystem II (PSII), resulting in alterations in photoinhibition, photoreception and rate at which photosynthesis takes place in photosynthesizing organisms (Kataria and Jajoo, 2014; Hui et al., 2015). Furthermore, UV-B light is known to have sufficient energy to cause free radical overproduction and the development of oxidative stress (Pospíšil et al., 2014; Mattila et al., 2015) and also have the tendency to harm plants during photosynthesis while additionally interfering with gene transcription and translation (Jansen et al., 1998). Light signals are incorporated into the plant's complex signaling network system upon penetration, this results in the conversion of light inputs into products that influence plant growth and development, mainly oxygen, which they utilize for aerobic respiration. Glucose is also produced and gets oxidized by oxygen to produce needed energy for the plant in the cell's metabolic machinery system. The disruptive operation of UV-B radiation influences the outputs and/or intermediates compounds in this complicated metabolic interaction which are largely a wide range of secondary metabolites. These include alkaloids, phenolic compounds and terpenoids (Rozema et al., 1997; Kazan and Manners, 2011).

Nocchi et al., (2020) in their study on the effect of UV-B radiation on the secondary metabolites production, antioxidant activity, photosynthesis, and herbivory interactions in *Nymphoides*

humboldtiana reported that, because of the radiation's capacity to modify plants' physiological components, the constitutive chemical defenses that were induced during the photosynthetic activity of *N. humboldtiana* was increased significantly which also saw to the increase in production of flavonoids and concomitant increase in the antioxidant activity of the plant's extract. Generally, increased UV-B light, combined with high photosynthetic efficiency and electron transport, encourages the creation of free radicals, which leads to oxidative stress (Pospíšil et al., 2014; Mattila et al., 2015). The counter response of plants to enhanced UV-B radiation is observed in the production of phenolic compounds which are most likely thought to act as antioxidants protecting plant cells against oxidative stress (Agati et al., 2013).

The following diagram (Figure 2.2) give a visual representation of the effect of UV stress on plant metabolism.

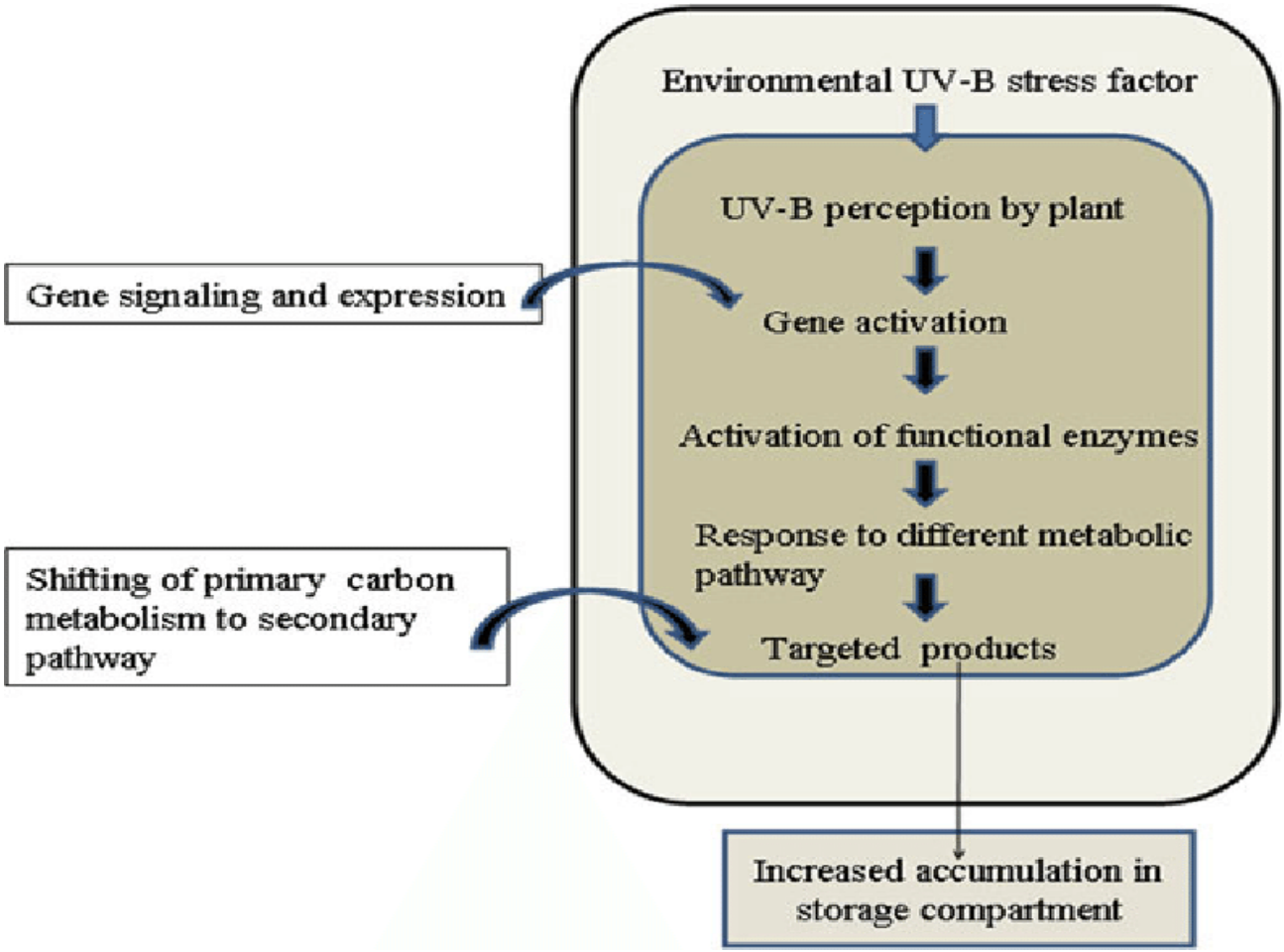


Figure 2.2: Effect of UV-B stress on plant metabolism (Kumari and Prasad, 2013). Abiotic stress triggers signal cascades that induce the transcription of defense-related genes. As a result of the metabolic shift to secondary metabolism, more secondary products are produced as adaptive responses.

2.3.2 Effect of water stress on plants' metabolites

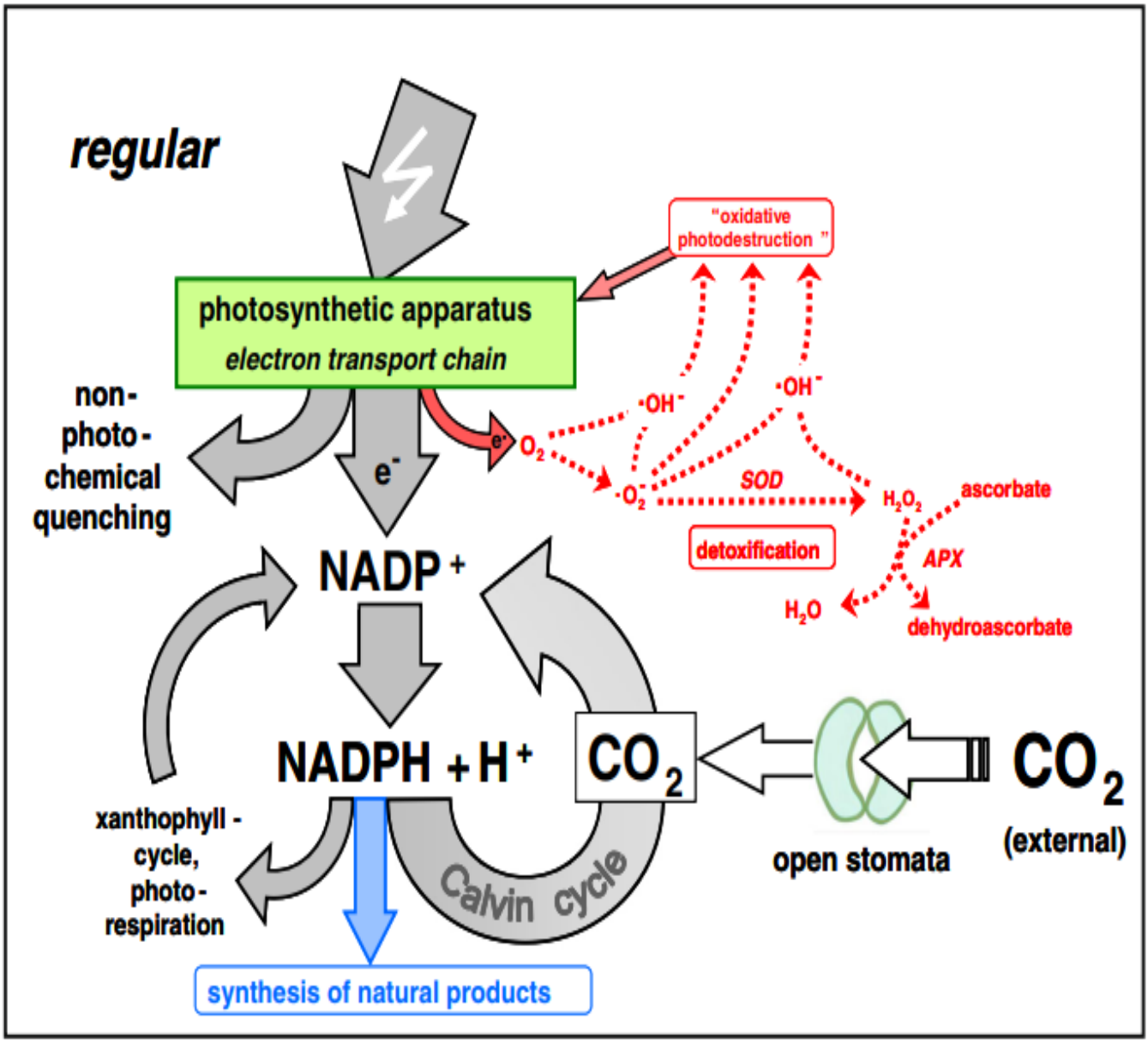
Water availability is pivotal to plants' survival since about 90% of their fresh weight is made up of water. Throughout plants' ontogenesis, the essentiality of water is observed in the way it regulates plant germination and metabolism as well as growth and development (Muscolo et al., 2011). Water shortage affects plants physiologically, morphologically, and molecularly. Drought is often responsible for imbalances in cellular reactive oxygen species (ROS) and reactive nitrogen species (RNS). The increased generation of these species disrupt cell redox regulation (Laxa et al, 2019; Sharma et al., 2020). An attempt is made by plants to make morpho-anatomical, biochemical, and physiological adjustments in plants during this period which are primarily aimed at limiting water loss through transpiration while also attempting to raise plant water content. One of the first consistent responses during these adjustments to maintain metabolic stability is seen in the adaptation of stomata aperture which triggers a cascade of physiological/biochemical changes to maintain an equilibrium in the photosynthetic process thereby strengthening the plant's defense against drought-induced stress. Some of the defence plants put up as a result of stomatal closure is the stimulation of antioxidant systems which can generate a range of antioxidant enzymes such as ascorbate peroxidase and superoxide dismutase capable of attenuating the ROS produced (Gill and Tuteja, 2010). For instance, catalase is known to break down H_2O_2 generated in plants into water and oxygen, lowering or repairing damage done by ROS (Chance and Maehly, 1955). The antioxidant systems also help to keep organelles stable by limiting damage that may have been done to the chloroplast membrane, which helps to keep the photosystem II (PSII system) stable (Lima et al., 2018).

Plants stressed by lack of water show a reduction in photosynthesis, which is invariably linked to reduced growth and an increased incidence of early senescence (Pic et al., 2002). Other significant effects of water deficiency on plants can be observed in serious decline in crop productivity due to alteration in plants' physiology and heightened production of secondary

metabolites due to decrease in biomass formation and the conversion of assimilated CO_2 to C-based secondary metabolites to avoid sugar-promoted photosynthetic feedback (Selmar and Kleinwächter, 2013).

Water deficit is positively correlated to secondary metabolites. Many studies report the considerable increase in secondary metabolites that plants experienced as a result of water shortage (Azhar et al., 2011; Zhang et al., 2017; Molero et al., 2019).

Figure 2.3 gives a visual comparison of the regular metabolism experienced by plants in the presence of sufficient supply of water in contrast to the metabolism of plants when they are drought-stressed.


regular
"oxidative photodestruction "
photosynthetic apparatus
electron transport chain
non-photo-chemical quenching
e-
e-
O2
·OH
·OH
·O2-
SOD
H2O2
ascorbate
detoxification
APX
H2O
dehydroascorbate
NADP+
NADPH + H+
CO2
CO2
(external)
open stomata
xanthophyll - cycle, photo-respiration
Calvin cycle
synthesis of natural products

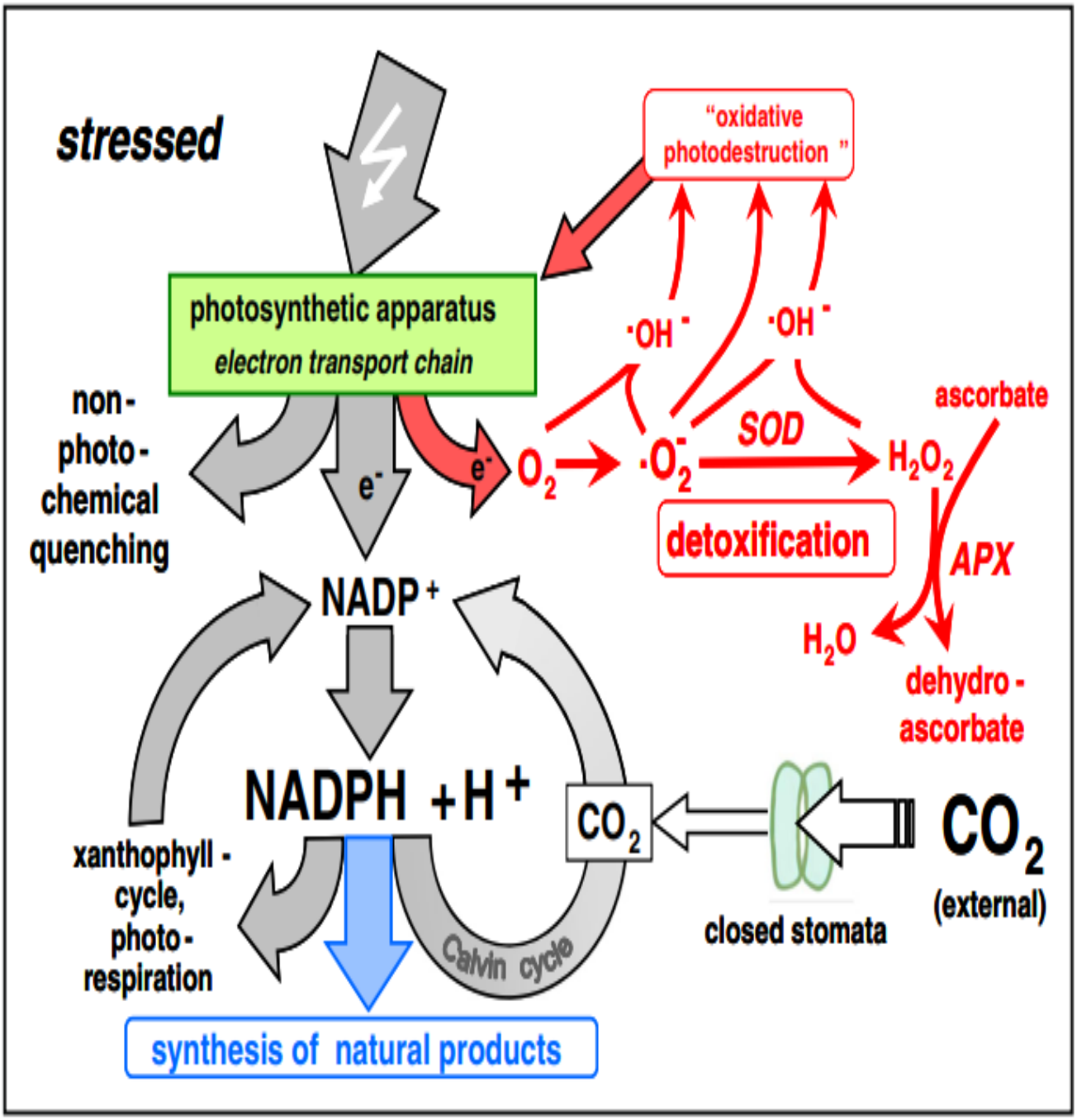


Figure 2.3: Drought stress-induced increase in secondary metabolite biosynthesis: a model system (Selmar and Kleinwächter, 2013; Kleinwächter and Selmar, 2014).

The amount of light received by the photosynthetic device is substantially greater than the amount of energy necessary to fix CO_2. As a result, massive amounts of energy must be expended. Non-photochemical quenching and effective re-oxidation are used to dissipate the

energy. $NADPH+H^+$ is produced, for example, by the xanthophyll cycle and photorespiration (Figure 2.3). The excess of reduction power does not result in large amounts of radicals in plentiful supply of water. Energy fluxes are dramatically changed in drought-stressed plants on the other hand. The internal concentration of CO_2 is substantially lower due to the increased diffusion resistance generated by stomata closure. As a result, the Calvin cycle consumes significantly less $NADPH+H^+$ for CO_2 fixation and reduction. Consequently, a substantially higher proportion of energy must be dispersed. Although feedback mechanisms improve the protective processes (non-photochemical quenching, photorespiration, and the xanthophyll cycle), many electrons are transported to molecular oxygen (Mehler reaction). The superoxide radicals that are produced then produce a variety of other ROS after which they are detoxified, and thus the generation of significant levels of ROS is prevented on account of the stress-related activation of superoxide dismutase (SOD) and ascorbate peroxidase (APX). With respect to the law of mass action, a significant rise in the reduction potential (ratio of $NADPH+H^+$ to NADP+) promotes the synthesis of highly reduced secondary metabolites, which are molecules with a greater degree of reduction than carbohydrates (Selmar and Kleinwächter, 2013; Kleinwächter and Selmar, 2014).

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2.4 EFFECT OF ENVIRONMENTAL FACTORS ON THE BIOLOGICAL ACTIVITIES OF PLANTS

A plant's interaction with the environment in a long-term evolutionary process is a predominant factor in the determination of active substances present within the plant. Quite a handful of studies have reported the influence of environment on the active substances within plants. For instance, terpene concentration was positively correlated to increased CO_2 levels over atmospheric CO_2 when *Rosmarinus officinalis L.* plants were grown under CO_2 concentrations of 350 and 700 µmol/mol (atmospheric CO_2, and elevated CO_2) (Peñuelas and Llusià, 1997). Also, Sampaio et al (2011) reported that there is a significant influence of environmental factors chiefly temperature, and foliar micronutrients (Zn, Mn, Cu and Fe) on the production of phenolic compounds (especially flavonoids, ellagic acids and tannins) in the leaves of *Lafoensia pacari.*

The types, contents, and biological activities of the active substances within a specific plant are appreciably influenced by the prevailing environmental factors around the locality the plant

grows in. The same plant species growing in different locations have been widely reported to produce different results with regards to their biological activities (Wink, 1988; Pavarini et al., 2012; Sampaio et al., 2016). In 2016, Liu et al., (2016) investigated the impact that environmental factors have on a mountainous shrubby medicinal plant called *Potentilla fruticosa.* The result showed that there is a negative and significant correlation between altitude and tannin content. Annual sunshine and altitude were positively and significantly correlated to the rutin and flavonoids content and antioxidant activity. Annual mean temperature was also reported to be significantly and negatively correlated to the total phenolics content, and altitude was significantly and positively correlated to the total phenolics content.

2.5 BOTANICAL DESCRIPTION OF THE GENUS *HELICHRYSUM*

Helichrysum, a genus generally referred to as everlasting flowers belong to the largest family of flowering plants called the sunflower family from the Inuleae tribe and subtribe Gnaphalieae. The name *Helichrysum* is derived from a combination of two Greek lexicons-helios and chrysos. Helios means sun and chrysos means gold, referring to the many clusters of the small yellow flowers (Pooley, 2003). *Helichrysum* species have developed adaptation strategies that make them withstand a long period of drought and as such are xerophytes in nature (Polunin, 1981; Caser et al., 2012; Papafotiou et al., 2013). The complete inflorescence of *Helichrysum* species contains the common polyphenolic compound-flavonoids, chiefly responsible for their anti-inflammatory, diuretic, detoxification and choleretic activities, a property which have made them to have been widely known in herbal medicine (Süzgec et al., 2005). Generally, the genus *Helichrysum* contains an important repository of secondary metabolites, especially in their essential oils (Manitto et al., 1972; Roussis et al., 2000; Angioni et al., 2003; Appendino et al., 2007). The genus is also known for its aromatic identity, and most of the plants in this genus have been shown to be high in essential oils, which contain a variety of chemicals (Akaberi et al., 2019).

The genus has between 500 and 600 species (Hilliard, 1983). Being mostly an African plant, majority of the species of the *Helichrysum* genus exist in Africa while South Africa alone is home to an estimated 246 species across different regions within the country. *Helichrysum* exhibits a great deal of morphological diversity and as such are classified into 30 different groups (Hilliard, 1983). A number of *Helichrysum* species have been widely used as key ingredients in traditional medicine in South Africa since the 18th century (Scott and Hewett, 2008). Traditional uses have ranged from the treatment of diarrhea and vomiting in children

(Jacot, 1971; Hutchings et al., 1996), to the treatment of chest problems or respiratory tract infections (Smith, 1895; Githens, 1949; Mathekga, 2001), small pox, coughs and colds, open wound (Arnold et al., 2002; Githens, 1949), and many other diseases of microorganism origin. Some other traditional uses include application as body perfumes and treatment of circumcision wounds (Watt and Breyer-Brandwijk 1962; Dlamini 1981; Dilika et al., 1997; Mathekga, 2001). In general, plants in the *Helichrysum* genus have been used in folklore medicine beyond the shores of Africa in the treatment of a variety of diseases which include hepatic disorders, diabetes mellitus, gall bladder problems, menstrual pain, bladder infections, insomnia, arthritis, icterus, stomach upset, various allergies, infections, colds, cough, skin infections, inflammation, asthma, and wound healing (Czinner et al., 2000; Erogluet al., 2010; Rigano et al., 2014; Viegas et al., 2014), with the scented leaves and flowers being the most commonly used parts.

Following in Table 2.2 is a summary of some medicinal uses of various *Helichrysum* species and the plant parts commonly used.

Table 2.2 General medicinal importance of some members of the *Helichrysum* genus.

***Helichrysum* Species**	**Common name(s)**	**Plant parts used**	**Medicinal uses**	**Cited literature**
H. odoratissimum (L.)	Impepho, Hotnotskooigoed	Flowers, leaves, root, twigs, whole plant	Treatment of: Respiratory problems, headaches, heart problems	Van Puyvelde et al., 1989; Swelankomo, 2004; Van Wyk et al, 2013
Helichrysum caespititium (DC.)	Speelwonderboom, boriba	Whole plant, roots	Treatment of: Headaches, chest colds, nausea. virility	Arnold et al., 2002; Dekker et al., 1983; Gelfand et al 1985; Swanepoel, 1997

H. adenocarpum DC	Pink everlasting	Root	Treatment of: diarrhea and vomiting in children	Arnold et al., 2002; Neuwinger, 1996; Philips, 1917
H. crispum (L.)	Silver bush everlasting	Leaves	Treatment of: coughs, tuberculosis, urinary tract infection	Arnold et al., 2002; Roberts, 1990
H. cymosum (L.)	Gold carpet, goue tapyt	Leaf, root	Treatment of: headaches, colds and coughs, also used as a purgative	Bhat and Jacobs, 1995; Van Vuuren et al., 2006; Van Wyk et al., 2000
Helichrysum foetidum (L.)	Stinking strawflower, muishondblaar, isicwe	Whole plant, leaf, root	Treatment of: influenza, circumcision and infected wounds, herps, eye problems	Watt and Breyer-Brandwijk,1962; Steenkamp et al., 2004, Hutchings et al., 1996, Roberts, 1990; Batten and Bokelmann, 1966; Gerstner, 1938
H. litorale	Not available	Whole plant	Treatment of: Ulcer, boils	Smitt, 1966; Swanepoel, 1997; Watt and Breyer-Brandwijk,1962

H. kraussii	Curry bush, Mupumhanhuka	Whole plant, leaf, root, dry flower, and seed	Treatment of: Keloid scars, cough, and pulmonary tuberculosis	Arnold et al., 2002; Watt, and Breyer-Brandwijk, 1962; Swanepoel, 1997

2.6 EFFECT OF ENVIRONMENTAL FACTORS ON THE CHEMICAL PROFILE AND BIOLOGICAL ACTIVITIES OF *HELICHRYSUM* SPECIES

A significant number of reviews have showed that abiotic factors such as soil types, climatic variations and many other environmental conditions influence biological activities of plants (Liu et al., 2016; Borges et al., 2017; Yang et al., 2018). Such factors also result in notable differences in the chemical profiles and variations observed in the distribution of secondary metabolites in plants generally and especially medicinal plants of the same species (Gobbo-Neto and Lopes, 2007; Ramakrishna and Ravishankar, 2011; Gouvea et al., 2012, Pavarini et al., 2012).

Both the quantity and quality of plants' secondary metabolite production have also been reportedly influenced by geographical locations (Simone et al., 2012, Wei et al., 2015).

In a study that compares the influence of environmental factors on the phytochemical analysis of volatile constituents and biological activities of methanolic extracts of *Helichrysum italicum* growing in 2 distinct regions of Italy-Calabria and Sardinia, Tundis et al., (2004) reported an average higher quantity of the compound *trans*-cariophyllene (used as a phytochemical marker in the experiment) in the Calabrian samples over the Sardinian samples. The study reveals that *trans-cariophyllene* content is affected by altitude: samples taken at 490 meters above sea level contained the most quantity of the compound, while those taken at 800 meters had the least. Significant variations were also observed in the activities of the plant species from the 2 different geographical locations. The changes in phytochemical composition and biological activity observed in the samples studied are related to chemical–physical qualities, soil composition, and the influence of other factors such as geographical coordinates, altitude, and solar exposure.

Years after, further studies done by Melito et al., (2013) on the chemical composition of *H. italicum* growing in different environmental conditions discovered that essential oils in *H. italicum* depends on the collection site and stage of development of the plant. 35 distinct compounds were analyzed by means of GC-MS in a collection of 294 young stems of the well-spaced plants in 50 different collection sites with their meteorological data. The profile of essential oils obtained from *H. italicum* accessions at two environmentally dissimilar collection sites (Corsican and Tuscan) produced two unique groups. Neryl acetate, neryl propionate, nerol, acyclic ketones and b-diketones oils are more dominant in Corsican while α-pinene and β-caryophillene were present in the Tuscan site.

2.7 ANTIMICROBIAL ACTIVITIES OF THE GENUS *HELICHRYSUM*

The *Helichrysum* genus is a large group of angiosperms that are utilized by native South Africans for their therapeutic worth. The many applications of *Helichrysum* in traditional medicine is a strong indicator of the antimicrobial properties present in the compounds of the plant. Antimicrobial activities of the species of the genus *Helichrysum* have been widely reported by a number of authors (Rios et al., 1988; Tomas-Barberan et al., 1988; Tomas-Lorente et al., 1989; Tomas-Barberan et al., 1990; Mathekga and Meyer; 1998, Mathekga et al., 2000, Heyman et al., 2015; Yazdi et al., 2019).

The genus had previously been discovered to be rich producers of compounds involved in plants' defence mechanism against fungi and bacteria such as alkaloids, tannins, phloroglucinols, flavonoids, sesquiterpenoids, and acetophenones (Dekker et al., 1983; Hilliard, 1983; Jakupovic et al., 1989).

2.7.1 Antibacterial activity of *Helichrysum* species

Helichrysum species native to South Africa are frequently used in the treatment of a variety of infections which are of bacterial origin (Watt and Breyer-Brandwijk, 1962; Hutchings and Van Staden, 1994). Many studies have reported strong antibacterial activities of *Helichrysum* extracts against Gram-positive bacteria compared to Gram-negative bacteria (Meyer and Dilika; 1996; Navarro et al., 1996; Salie et al., 1996; Mathekga and Meyer, 1998; Rios et al., 1990). Table 2.3 summarizes the significant antibacterial activities of some selected *Helichrysum* species against the Gram-positive *Bacillus* bacteria.

Table 2.3: Antibacterial activity of crude extracts of some selected *Helichrysum* species against *Bacillus* bacteria (Mathekga and Meyer, 1998).

***Helichrysum* species**	**Bacteria species MIC (mg/mL)**			
	B. cereus	*B. pumilus*	*B. subtilis*	*B. aureus*
H. caespititium	1.0	1.0	1.0	1.0
H. callicomum	1.0	1.0	1.0	1.0
H. decorum	1.0	0.10	0.10	0.10
H. kraussii	0.10	0.10	0.10	0.10
H. melanacme	0.10	0.10	0.10	0.10
H. psilolepis	0.10	0.10	0.10	0.10
H. candolleanum	0.10	0.10	0.10	0.10
H. simillimum	0.10	0.10	0.10	0.10
H. rugulosum	0.10	0.10	0.10	0.10
H. trilineatum	1.0	1.0	1.0	1.0
H. odoratissimum	0.10	0.10	0.10	0.10

2.7.2 Antifungal activity of *Helichrysum* species

The antifungal properties of *Helichrysum* species have been established by many studies (Angioni et al., 2003; Mastelic et al., 2005; Stupar et al., 2014; Kutluk et al., 2018). Methanolic extracts of different parts of *H. odoratissimum* were evaluated for their antifungal properties against *Candida albicans* using the agar dilution streak method (Boily and Van Puyvelde, 1986). Activities were observed by the flower extract against the fungus.

Table 2.4 shows some *Helichrysum* species containing antifungal properties.

Table 2.4: *Helichrysum* species and their reported antifungal activities against some fungal pathogens.

***Helichrysum* species**	**Plant parts used**	**Fungal isolates**	**Assay method**	**References**
H. odoratissimum	Flower	*Candida albicans*	Agar dilution streak method	Boily and Van Puyvelde, 1986
H. italicum	Glandular hairs on the leaves and flowers	*Epicoccum nigrum* and *Penicillium* sp.	Micro-atmosphere method	Stupar et al., 2014
H. odoratissimum	Aerial parts	*Aspergillus flavus*, *Aspergillus niger*, *Cladosporium cladosporioides*, *Cladosporium cucumerinum*, *Cladosporium sphaerospermum*, and *Phytophthora capsici*	Agar dilution method	Mathekga, 2001
H. foetidum	Leaves and flowers	*Cladosporium cucumerinu*	Bioautography on silica gel plates	Malolo et al., 2015
H. cameroonense	Leaves	*Penicillium oxalicum*	Dilution on a solid medium	Tchoumbougnang et al., 2010
H. rugulosum	Shoots (excluding flowers)	*Aspergillus flavus*, *Cladosporium cladosporioides*	Agar based disk diffusion method	Mathekga, 2014
H. fulgidum	Aerial parts	*Candida albicans*, *Candida tropicalis*, *Candida glabrata*	Agar dilution technique	Bougatsos et al., 2004

2.7.3 Antiviral activity of *Helichrysum* species

Although more biological activities of *Helichrysum* species against pathogens of bacterial and fungal sources have been reported in literature than any other microbe, the genus has also been reported active against some viral organisms. Notable among these is the Herpes simplex virus

type 1 (HSV-1). The species *H. litoreum* Guss-a medicinal plant native to the region of Campania in South Italy, exhibited significant antiviral activity at a concentration of 1.35 µg/ml (ww/v) against HSV-1 in human lung fibroblasts and were found to be free of cytopathic effect (Guarino and Sciarrillo, 2003). Lall et al., (2006) also reported the inhibitory activity of the crude ethanolic extract of *H. melanacme* against human influenza virus type A at a concentration of 0.01 mg/mL. Furthermore, combination of two isolated compounds from *H. melanacme*, prenylated chalcones and a pyranochalcone demonstrated promising activities against the influenza virus.

2.8 HELICHRYSUM AUREONITENS

2.8.1 Botanical description

Helichrysum aureonitens, whose common name is golden everlasting in English, goue sewejaartjie in Afrikaans, toane-ntja, toane-poli in Sotho and impepho-emphlope, indondokozane, inkondlwane in Xhosa is a tufted perennial herb with many slender unbranched stems, each about 300 mm tall, emerging from a crawling rootstock on the plant (Figure 2.4). There are white fuzzy hairs growing tightly compressed to the surface of the leaves and stems, making them to look pale grey. The leaves have a narrow oblong shape, with each bearing a little, hairless, sharp tip and having flat or revolute edges which are rolled to their under surfaces (Hilliard, 1983). It possesses imbricate, almost obtuse involucral bracts. Flowering occurs between September and February, and it bears one-seeded fruits with each fruit bearing various fine bristles (Ready, 2007).

Figure 2.4: *Helichrysum aureonitens* growing in the field, the leaves and flowers.

2.8.2 Distribution and Habitat

Helichrysum aureonitens is widely distributed in seven provinces around South Africa, including the Eastern Cape (spreading all through to Qonce (formerly King William's Town), Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, and North West. Its presence has also been confirmed in western Lesotho (Pooley, 2003). It is more prevalent in the summer rainfall region of South Africa. The soil type most suited for its growth is the moist to well-drained sandy loam soils in the presence of full sun (Ready, 2007). Due to the difficulty in growing, there is a need for fire stimulation of *H. aureonitens* seeds if they must germinate (Ready, 2007).

2.8.3 Medicinal uses and antimicrobial activities of H. aureonitens

Through folkloric information, *H. aureonitens* plant parts have been reportedly used in the treatment of variety of infections for centuries mostly by the people of KwaZulu-Natal and Eastern Cape province (Phillips, 1917; Afolayan et al., 1995; Yani et al., 2004). The shoots and occasionally the stems are commonly used for headaches, influenza, fever, wounds, coughs and dysmenorrhea (Watt and Breyer-Brandwijk, 1962; Hutchings and Van Staden, 1994; Hutchings et al., 1996). The leaves and stem of *H. aureonitens* are also regularly applied for the treatment of enuresis in children and skin infections (especially those associated with HSV) (Watt and Breyer-Brandwijk, 1962; Guillarmod, 1971; Meyer and Afolayan, 1995; Meyer et al., 1996). In South Africa, extracts from the plant are used for treating *Herpes zoster* infection (shingles) (Meyer et al., 1996; Afolayan and Meyer, 1997). The plant is also reportedly used by diviners for the induction of trances and for invoking the goodwill of ancestors (Pooley, 1998).

The antimicrobial activities of *H. aureonitens* have been widely reported. The antifungal activities of the aerial part of the plant have been reported against fungal species such as *Aspergillus flavus, Cladosporium sphaerospermum, Aspergillus tamarii, Penicillium digitatum* and *Penicillium italicum* (Afolayan and Meyer, 1997; Mathekga, 2014). The antibacterial effects of dichloromethane extracts of *H. aureonitens* on some selected bacteria species were also investigated by Meyer and Afolayan, (1995) and their results showed the effectiveness of the plant's shoot metabolites against Gram-positive bacteria *Bacillus cereus* (0.5 mg/ml), *Micrococcus kristinae* (0.5 mg/ml), and *Bacillus pumilus* (1.0mg/ml). In their publications Meyer et al., (1996, 1997) reported that at concentrations of 1.35 mg/ml and 6 µg/ml, the

aqueous extract of *H. aureonitens* and galangin (a compound found in high concentration in the species) respectively showed significant antiviral activity against HSV-1 *in vitro*.

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CHAPTER THREE

EFFECT OF DIFFERENT CLIMATIC REGIONS AND SEASONAL VARIATION ON THE ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF *HELICHRYSUM AUREONITENS* Sch. Bip.

ABSTRACT

Native South Africans make use of *Helichrysum. aureonitens* Sch. Bip. extracts for the treatment of a variety of infections and therefore it is an important plant in traditional medicinal preparations. The plant is widely distributed and grows in various areas exposed to different environmental conditions. Traditional health practitioners argue that the curative potency of a medicinal plant is dependent on the harvest season. This study investigated the effect of seasonal variation and geographical location on the antibacterial and antifungal activity of *H. aureonitens.* Above ground whole plant materials were collected in two different seasons-early spring, with high rainfall and high temperature (October) and late autumn, with low rainfall and lower temperature (May). Plant materials were collected from two sites for each season, at Wakefield farm (KwaZulu-Natal), representing a colder, wetter environment and Telperion (Mpumalanga), representing a drier and warmer environment. Leaves of *H. aureonitens* were tested against bacteria *Proteus vulgaris (P. vulgaris)* and *Bacillus subtilis (B. subtilis)* as well as fungi *Aspergillus flavus (A. flavus), Aspergillus nomius (A. nomius), Cladosporium cladosporioides (C. cladosporioides), Fusarium oxysporum (F. oxysporum)* and *Penicillum halotolerans (P. halotolerans)*. Extracts from the October harvest showed significant activities against the gram-negative bacteria *P. vulgaris* compared to the May harvest at an MIC value of 62.5 µg/mL. Similar activity was observed between the extracts from the wet season across the two geographically different locations. There was generally very good antifungal activity observed for all the species except for *A. nomius* with MIC values ranging from 0.39-1.56 µg/mL. Extracts of plant materials harvested in the wetter region had a significantly higher activity against *A. flavus* and *F. oxysporum* in both seasons than the drier region. Telperion harvested plants exhibited better activity against *F. oxysporum* during autumn.

3.1 INTRODUCTION

A substantial number of the African population depend on medicinal plants as their primary healthcare source. In South Africa, an estimated figure of 60%-80% of the rural black population still rely on traditional healers to treat their health problems with medicinal plant infusions (herbal teas) and concoctions delivered by traditional healers, rather than or in addition to mainstream synthetic pharmaceuticals (Matotoka & Masoko, 2017; Street & Prinsloo, 2013; van Wyk et al., 1997). Over 600 species of the genus *Helichrysum* are present in countries like South Africa, Turkey, Madagascar, Eurasia and Australasia, and are reputable for their use in traditional medicine wherever they occur (Galbany-Casals et al., 2014). For example, colds and coughs are treated with various concoctions of *H. odoratissimum*, *H. cymosum*, and *H. kraussii*, while *H. nudifolium* leaves are used as treatment for wounds and against respiratory infections (Brenan et al., 1964; Hutchings & van Staden, 1994; Lourens et al., 2008). The plant *H. aureonitens* is also reported by oral tradition to have a wide usage against variety of diseases such as skin infections and disorders like enuresis. (Marais & Guillarmod, 1972; Meyer et al., 1996).

Several studies have reported the antimicrobial activities of *Helichrysum* extracts (Afolayan & Meyer, 1995, 1997; Demir et al., 2009; Lourens et al., 2004; Mathekga et al., 2000). Results of antibacterial activities of *Helichrysum* from various extractants reported so far show that the species is more active against gram-positive bacteria. Acetone extracts of three *Helichrysum* species, including *H. hypoleucum, H. odoratissimum* and *H. rugulosum* were found to significantly inhibit the growth of five gram-positive bacteria species-*Bacillus cereus, B, pumilus, B. subtilis, Micrococcus kristinae* and *Staphylococcus aureus* while none of the extracts showed any activity against gram-negative bacteria *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Serratia marcescens* (Mathekga & Meyer, 1998). Seven species of *Helichrysum*, including: *H. araxinum, H. armenium, H. arenarium, H. pallasii, H. stoechas, H. sanguineum*, and *H. graveolens* were found to show antifungal activities (Kutluk et al., 2018).

The climate is an important seasonal factor that has a direct impact on plant ecosystem processes and structures such as photosynthesis, nutrient cycling, transpiration, together with production of both primary and secondary metabolites (Goyal et al., 2012). Plants are exposed to a variety of temperature levels (including extremes) when the seasons change. This has an impact on their phytochemical compositions and the volatile chemicals are reportedly the compounds that are the most affected (Usano-Alemany et al., 2014). The ability to determine

the effect that seasonal variation has on plant phytochemical compositions provides useful information on the best time of the year or season to harvest each plant species for maximum active component concentration (Kale, 2010). Literature is replete with the effect of seasonal variation and climatic factors on the biological activities of plants. No information is available regarding the effect of environmental factors on both the biological activity and chemical properties of *H. aureonitens.* This study therefore investigated the effect of seasonal variation and different climatic locations on the biological activity of *H. aureonitens* against two bacterial species: gram-negative bacteria *Proteus vulgaris* and the gram-positive bacteria *Bacillus subtilis* and five pathogenic fungi: *Aspergillus flavus, Aspergillus nomius, Cladosporium cladosporioides, Fusarium oxysporum,* and *Penicillum halotolerans,* all belonging to the division Ascomycota.

3.2 MATERIALS AND METHODS

3.2.1 Plant material collection

Collections of whole plant materials were made at two different locations and at two different seasons of the year which are spring (late October 2017) and autumn (early May 2018). The experiment was designed with the aim of comparing a "treatment" group with the "control" group in both locations. The wet sites (areas with higher moisture content due to proximity with water bodies) were chosen as the "control" groups while the dry sites were considered the "treatments" in each of the locations in the experiment. Telperion nature reserve is situated in Mpumalanga (25.7039° S, 28.9814° E) and Wakefield farm, in the KwaZulu-Natal Midlands region (29°30'0" S and 29°54'0" E) representing a warmer and drier climate, and a cooler and wetter climate, respectively. Three batches of representative plant samples were collected at different sites for each location and were transferred into brown paper bags (10 cm x 20 cm) and transported to the laboratory. Plant materials were identified, and representative voucher specimens were deposited in the UNISA Science Campus horticulture centre's herbarium with the names WAHA-01, WAHA-02, WAHA-03 and WAHA-04. (WA-Wilson Adeosun. HA-*Helichrysum aureonitens*)

Helichrysum aureonitens possesses tough fibrous tissues and as a result require longer time to completely dry. Since one of the main objectives of the study is the determination of the chemical profile of the plant, any method that will interfere with the drying process and as such cause loss or reduction of bioactive compounds was therefore avoided. In a review that

investigated the efficiency of drying conditions for essential oil productions from aromatic plants, (Özgüven et al., 2019) found that better results were obtained with natural drying methods. Mirhosseini et al., (2015) similarly discovered that due to the lower temperatures employed in shade drying of *Stachys lavandulifolia*-(an important plant used as herbal tea in Iranian folk medicine), the evaporation of fragrant composition is lower comparatively to both oven and sun drying. This makes the amount of essential oils present to be more in the shade-dried samples. In view of this, the plant samples in this study were shade-dried at ambient temperature (around 25° C) in the laboratory and being monitored weekly until properly dried by the fourth month. Furthermore, stems and leaves of each plant were separated and stored in transparent cellophane bags at ambient temperature of about 25° C until further analysis.

3.2.2 Preparation of plant extracts and antimicrobial testing

3.2.2.1 Preparation of plant extracts

Plant materials for the antibacterial assay were ground and pulverized into powdery form by use of a kitchen blender and pestle and mortar and were extracted using methanol/water (70:30) according to Ramlan et al (2017). The mixtures were shaken for 5 minutes on a shaker (Thermo fisher Scientific, United States), centrifuged at 3000 rpm for 10 minutes (Eppendorf 5424, Germany), and further decanted and filtered by discarding the plant rests. Extracts were concentrated to dryness by evaporation in a drying chamber (Airvolution dryer, South Africa), the yield determined, introduced into glass vials and stored in a refrigerator at 4° C until further use.

Plant leaf materials for the antifungal assay were prepared according to the paragraph above. A ratio of 10:1 acetone to plant materials (50 mg of plant material to 500 mL of acetone) was used for the extraction in a centrifuge tube according to (Eloff, 1998b). Samples were put on a shaker overnight at 130 rpm (Thermo Fisher scientific, USA) following which samples were centrifuged at 3000 rpm for 10 minutes (Eppendorf microcentrifuge 5427R, Germany) and the supernatant transferred to a glass vial. A stream of air at room temperature (25° C) was used to remove the remaining solvent from the extracts already placed in pre-weighed glass tubes and stored at room temperature.

3.2.2.2 Antimicrobial testing

3.2.2.2.1 Antibacterial testing

The Gram-positive bacteria *B. subtilis* (ATCC 33420) and the Gram-negative bacteria *P. vulgaris* (ATCC 84270) (Anatech, KwikStick ®) were used for this study. The organisms were maintained in nutrient agar slants (Biolab) and later recovered for testing by growing them in a nutrient broth slant (Sigma-Aldrich, United States).

The experiment was performed in duplicate. Three biological replicates were used to determine the initial minimum inhibitory concentration (MIC). Thereafter the experiment was repeated, and the MIC confirmed (Eloff, 1998a). The MIC was determined using the 96 well plate microdilution method at the tested concentrations 250, 125, 62.5, and 31.25 µg/mL. Bacteria with nutrient broth and extract was used as negative control while gentamicin (1000 µg/mL) (Sigma-Aldrich, United States) was used as positive control. The extracts were then dried and re-dissolved in 30% acetone (which is used as the vehicle control). This was included in the experiment to ensure that the observed activity against the bacteria does not originate from the applied solvent. Plates were incubated with natural air circulation at 37° C (Memmert incubator IN55, USA) for 24 hrs followed by the addition of 40 µL of 2 mg/mL p-iodonitrotetrazolium (INT) chloride to each well (Sigma-Aldrich, Germany) to determine the MIC. A change in colour to pink indicated bacterial growth.

3.2.2.2.2 Antifungal testing

The fungal isolates used in the study were *A. flavus* (MRC 3951), *A. nomius* (PPRI 3753), *C. cladosporioides* (PPRI 10367), *F. oxysporum* (MRC 1907), and *P. halotolerans* (PPRI 25804) collected from the Agricultural Research Council (ARC), Roodeplaat, Pretoria. The isolates were maintained on potato dextrose agar (PDA) in petri dishes and stored at 4° C. The fungal cultures were subcultured into plates of newly prepared Potato dextrose broth (PDB) growth medium from potato dextrose agar (PDA) slants and incubated at 30° C for 3 days. A full loop of each actively growing fungal species was introduced into 50 mL freshly prepared PDB and re-incubated for another 3-4 days until some turbidities were observed indicating active growth.

The broth microdilution method was used to determine the antifungal activity of the plant extracts. 100 µL of plant extracts were introduced into the first row of the 96 well plates followed by another 100 µL of autoclaved distilled water. The total of 200 µL in each first well

'A' was serially diluted until well 'H'. Positive and negative controls were made with amphotericin B (1 mg/mL) and 30% acetone, respectively. After that, 40 µL of 2 mg/mL INT chloride was added to each well (Sigma-Aldrich, Germany). The plates were sealed with parafilm and incubated for 24 and 48 hours at 30°C with 100% humidity. Three biological replicates were used to determine the initial MIC. Thereafter the experiment was repeated, and the MIC confirmed (Zgoda & Porter, 2001). The lowest concentration of plant extract that suppressed fungal growth as observed by the colour change after 48 hours of incubation was established to be the MIC and thus recorded.

3.3 RAINFALL AND TEMPERATURE DATA OF COLLECTION LOCATIONS

Both rainfall and temperature data were supplied by the South African Weather Services (Table 3.1). Cedara is the closest weather station to Wakefield farms. It is about 15km from Wakefield farms and assumed to have similar weather conditions. Data for the closest station to Telperion is Witbank. Table 3.1 shows the average minimum and maximum temperatures between August 2017 to June 2018 as well as the average daily rainfall data across the two locations.

Table 3.1: Average minimum and maximum temperatures and daily rainfall data between August 2017 and June 2018 for Cedara and Witbank. (Months when plants were collected are highlighted).

	Wakefield (Cedara Data)			**Telperion (Witbank Data**)		
Month	**Avg daily Max T (°C)**	**Avg daily Min T (°C)**	**Total monthly Rainfall (mm)**	**Avg daily Max T (°C)**	**Avg daily Min T (°C)**	**Total monthly Rainfall (mm)**
August	20.8	5.2	3.6	21.3	5.6	3.8
Sept	24.5	8.9	9.6	26.8	9.8	27.2
Oct	22.3	9	110	24.5	10.7	83.8
Nov	23.7	10.7	105.8	27	11.9	109.2
Dec	23.8	12.6	79	26.5	14.1	153.2

Jan	27.3	14.2	79.4	27.9	13.9	71.8
Feb	26.5	15.3	170.2	26.3	15	75.8
March	25.7	13.7	124.2	26.0	13.4	148.4
April	23.9	12.2	52.4	23.9	12.0	26.0
May	21	6.7	31.8	21.4	7.0	24.6
June	20.2	3.4	1	20.1	4.5	0.2

3.4 RESULTS

3.4.1 Antibacterial activity of *H. aureonitens* extracts

Moderate antibacterial activity was observed against *P. vulgaris*, although no activity was found at the highest concentration tested for *B. subtilis* (Table 3.2).

Table 3.2: Antibacterial activity of the hydroalcoholic extracts from leaves and stems of *H. aureonitens* showing the MIC values (µg/mL) obtained from different sites at two different climatic locations and at two different seasons against the gram-negative bacteria *P. vulgaris* and the gram-positive bacteria *B. subtilis*.

Plant part	Season	Location	*P. vulgaris*	*B. subtilis*
Leaf (Site 1)	spring	Telperion	62.5	> 250
Stem (Site 1)	spring	Telperion	62.5	> 250
Leaf (Site 2)	spring	Telperion	62.5	> 250
Stem (Site 2)	spring	Telperion	62.5	> 250
Leaf (Site 1)	spring	Wakefield	62.5	> 250
Stem (Site 1)	spring	Wakefield	62.5	> 250
Leaf (Site 2)	spring	Wakefield	62.5	> 250
Stem (Site 2)	spring	Wakefield	62.5	> 250
Leaf (Site 1)	autumn	Telperion	62.5	> 250
Stem (Site 1)	autumn	Telperion	62.5	> 250
Leaf (Site 2)	autumn	Telperion	62.5	> 250

Stem (Site 2)	autumn	Telperion	62.5	> 250
Leaf (Site 3)	autumn	Telperion	> 250	> 250
Stem (Site 3)	autumn	Telperion	250	> 250
Leaf (Site 1)	autumn	Wakefield	250	> 250
Stem (Site 1)	autumn	Wakefield	125	> 250
Leaf (Site 2)	autumn	Wakefield	125	> 250
Stem (Site 2)	autumn	Wakefield	125	> 250
Leaf (Site 3)	autumn	Wakefield	125	> 250
Stem (Site 3)	autumn	Wakefield	125	> 250
Gentamicin				1000

3.4.2 Antifungal activity of *H. aureonitens* extracts

In general, very good antifungal activity was observed with values ranging from 0.39-1.56 µg/mL in both seasons and across sites, except for *A. nomius* where no activity was observed for the highest concentrations tested (Table 3.3).

Table 3.3: The MIC values (µg/mL) of the leaves and stems of *H. aureonitens* extracts obtained from different sites at two different climatic locations and at two different seasons against the fungi *Aspergillus flavus* (*A. flavus*), *Aspergillus nomius* (*A. nomius*), *Cladosporium cladosporioides* (*C. clados*), *Fusarium oxysporum* (*F. oxy*), and *Penicillium halotolerans* (*P. halo*).

Plant Part	Season	Location	*A. flavus*	*A. nomius*	*C. clados*	*F. oxy*	*P. halo*
Leaf (Site 1)	Spring	Telperion	0.39	>250	0.78	0.78	6.25
Stem (Site 1)	Spring	Telperion	0.39	>250	0.78	0.78	6.25
Leaf (Site 2)	Spring	Telperion	0.39	>250	0.39	0.78	6.25
Stem (Site 2)	Spring	Telperion	0.39	>250	0.78	1.56	3.125
Leaf (Site 1)	Spring	Wakefield	0.78	>250	0.78	3.125	3.125
Stem (Site 1)	Spring	Wakefield	0.78	>250	0.78	3.125	6.25
Leaf (Site 2)	Spring	Wakefield	0.78	>250	1.56	3.125	6.25

Stem (Site 2)	Spring	Wakefield	0.78	>250	0.78	6.25	3.125
Leaf (Site 3)	Spring	Wakefield	1.56	>250	0.78	6.25	3.125
Stem (Site 3)	Spring	Wakefield	1.56	>250	0.78	3.125	3.125
Leaf (Site 4)	Spring	Wakefield	1.56	>250	0.78	3.125	3.125
Stem (Site 4)	Spring	Wakefield	1.56	>250	1.56	6.25	3.125
Leaf (Site 1)	Autumn	Telperion	0.39	>250	0.78	3.125	1.56
Stem (Site 1)	Autumn	Telperion	0.39	>250	0.78	3.125	1.56
Leaf (Site 2)	Autumn	Telperion	0.39	>250	0.78	3.125	1.56
Stem (Site 2)	Autumn	Telperion	0.39	>250	0.78	3.125	1.56
Leaf (Site 3)	Autumn	Telperion	0.39	>250	0.39	3.125	1.56
Stem (Site 3)	Autumn	Telperion	0.39	>250	0.78	6.25	1.56
Leaf (Site 1)	Autumn	Wakefield	0.78	>250	1.56	1.56	1.56
Stem (Site 1)	Autumn	Wakefield	0.78	>250	0.78	1.56	1.56
Leaf (Site 2)	Autumn	Wakefield	0.78	>250	0.39	1.56	1.56
Stem (Site 2)	Autumn	Wakefield	0.78	>250	1.56	1.56	1.56
Leaf (Site 3)	Autumn	Wakefield	1.56	>250	0.78	1.56	3.125
Stem (Site 3)	Autumn	Wakefield	1.56	>250	1.56	1.56	3.125
Leaf (Site 4)	Autumn	Wakefield	3.125	>250	1.56	6.25	3.125
Stem (Site 4)	Autumn	Wakefield	6.25	>250	1.56	6.25	3.125
Amphotericin B							1000

3.5 DISCUSSION

3.5.1 Antibacterial activity

Hydroalcoholic extracts from the leaves and stems of *H. aureonitens* showed activity against the gram-negative bacteria *P. vulgaris* varying from 62.5 to 250 µg/mL. However, the extracts showed no activity against the gram-positive bacteria *B. subtilis* at the tested concentrations (Table 3.2). The results also showed that the extracts have better activity during the wetter season (spring) in most of the sites and at both locations with 62.5 µg/mL.

There is a clear difference noticed in the activities of extracts between the two locations during late autumn. The extracts from the wetter location (Wakefield) exhibited better activity at 125 µg/mL with inhibition against the gram-negative bacteria *P. vulgaris* in comparison to the drier

location (Telperion) at 250 µg/mL. Previous studies have shown that the production and accumulation of primary and secondary metabolites fluctuate significantly between specimens of the same plant species grown in different environments (Bennett & Wallsgrove, 1994; Pavarini et al., 2012; Ramakrishna & Ravishankar, 2011).

There is also no remarkable difference noticed in the activity of the leaves in comparison with the stems in both locations and during the two seasons with leaves activity at 62.5 µg/mL and stems at 62.5 µg/mL at both locations during spring and 250 µg/mL activity for both leaves and stems at Telperion during autumn, contrasting with 125 µg/mL for both leaves and stems activity at Wakefield during autumn.

The antibacterial activity results obtained from *H. aureonitens* demonstrated more potency against *P. vulgaris* which is a gram-negative bacterium (62.5 and 125 µg/mL) than *B. subtilis*, a gram-positive bacterium (> 250 µg/mL). The result is however consistent with the view that most *Bacillus* species are in general less resistant (Turnbull & Kramer, 1996). Given that gram-negative organisms are more resistant to antimicrobial compounds from plant sources than gram-positive organisms (King & Stickler, 1992), the inhibition of the growth of *P. vulgaris* at a relatively low MIC concentration of between 62.5-125 µg/mL of the plant species tested is noteworthy and might indicate the differentiation of antibacterial compounds at the different sites that increase with water availability.

3.5.2 Antifungal activity

With the exception of *A. nomius*, all of the fungal species were inhibited by varied extract concentration of *H. aureonitens* with MIC values ranging from 0.39 – 1.56 µg/mL. Plant extracts from spring and autumn showed the same activity for *A. flavus* and *C. cladosporioides* for both seasons although the MIC values for A. *flavus* were better at Telperion than for Wakefield. With regards to the activity against *F. oxysporum* however, there is a good activity demonstrated by the extracts of plants harvested during the wet season (spring). At Telperion, the observed activity was demonstrated at a concentration of 0.78 µg/mL while the activity observed at Wakefield was at a concentration of 3.125 µg/mL. During autumn, the activity demonstrated at Telperion was at a concentration of 3.125 µg/mL, while a better activity of 1.56 µg/mL was recorded at Wakefield. The drier location in autumn again showed reduced activity at 6.25 µg/mL as compared to the wetter locations. Plants harvested during the autumn season (comparatively drier season) exhibited better activity at 1.56 µg/mL and 3.125 µg/mL

at Telperion and Wakefield, respectively, against *P. halotolerans* compared to plants harvested during spring season with activity of 6.25 µg/mL and 3.125 µg/mL at Telperion and Wakefield respectively.

A closer look at the activity of the extracts against each fungal species in the two locations exhibited differing activities. Extracts maintain fairly the same activities against *A. flavus* in both locations. Activity against *C. cladosporioides* were the same for each location in spring at 0.78 µg/mL. This is similar to the activity observed against *A. flavus* at a concentration of 0.39 µg/mL at Telperion and a concentration ranging between 0.78-1.56 µg/mL at Wakefield. In each of the sites however, there are no observed significant difference in the activities.

In the spring season, there was a better activity at the drier location (Telperion) than Wakefield (wetter) against *F. oxysporum* at 0.78 g/mL and 3.125 g/mL, respectively. Extracts harvested from the autumn season however demonstrated opposite activity to what was observed during spring against *F. oxysporum* in the two locations. Extracts from Wakefield (wetter region) however exhibited better activity against *F. oxysporum* with values of 1.56 g/mL for Telperion (drier region) at 3.125 g/mL during autumn.

The acetone extract of *H. aureonitens* gave variable activities against the tested fungi. Based on reports of past studies on *Helichrysum* species and a handful of medicinal plants, acetone extracts gave considerable activity against the tested fungi while rather low activities were recorded comparatively against same fungi when hydroalcoholic solvents were used (Afolayan et al., 2002; Afolayan & Meyer, 1997; Akinyede et al., 2021; Mathekga & Meyer, 1998). Furthermore, there is no clear distinction between the activities recorded for Wakefield extracts or the wetter sites as variable results were obtained for all the fungal species. However in general, very good activity ranging from 0.39 – 1.56 µg/mL were achieved for the fungal species with acetone as extractant, indicating that *H. aureonitens* contains strong antifungal compounds. Since no study has reported the effect of seasonal variation on the antifungal properties of *Helichrysum* species, influence of seasonality on the antifungal property of other medicinal plants were compared. In support of the findings of this study, de Macêdo et al (2018) reported that based on their study on the effect of seasonality on the antifungal properties of *Psidium salutare*, a medicinal plant native to Brazil, they could not establish the influence of any of the three seasonal collection periods on the antifungal properties of the plant. Similarly, no effect of season on the antifungal activities of the seaweeds was also observed by Stirk et al (2007), in their study on the seasonal variation in the antifungal activity of seven seaweeds

(*Caulerpa racemosa* var. *laetevirens, Codium capitatum, Halimeda cuneata, Ulva fasciata, Amphiroa bowerbankii*, *Amphiroa ephedraea* and *Dictyota humifusa*) from South Africa.

3.6 CONCLUSION

This study reports for the first time the comparison of antibacterial and antifungal activity of samples collected in different climatic regions and different seasons of any *Helichrysum* species. The study showed that plant materials collected in spring (higher rainfall) show better activity than the samples collected in autumn for wet sites, as the dry sites showed comparable lower activity in spring and autumn. The acetone extracts showed generally very good antifungal activity at 0.39-1.56 µg/mL. Significant variations were observed with no activity of any of the plant samples against *A. nomius* and *A. flavus*. *C. cladosporioides* showed similar activity for the samples collected during spring and autumn season, although better activity was observed for Telperion. Only *P. halotolerans* showed better activity in autumn when compared to the spring samples for both sites. In contrast to the antibacterial activity, seasonal variation showed very little or no impact on antifungal activity which could be attributed to the use of a different solvent.

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CHAPTER FOUR

INFLUENCE OF SEASONAL AND GEOGRAPHICAL VARIATION ON THE ANTI-HSV-1 PROPERTIES OF *HELICHRYSUM AUREONITENS Sch.Bip.*

ABSTRACT

Disease causing microorganisms are a part of human lives from time immemorial. Cellular activities are disrupted when they gain entrance into humans mostly through contact with contaminated surfaces or unhealthy practices. In the last 70 years, antimicrobial drugs have been prescribed by doctors for the treatment of infectious diseases, some of which can lead to serious fatality. Adaptation of microbes to these drugs which have led to resistance is now a major hazard to human health all around the world. Viruses are most likely the most numerous biological entities on earth. Unlike other microbes, they cannot survive in the absence of a host, they can only thrive and multiply in living organisms. Viral infections are ubiquitous, and they play a defining role in the health of living organisms. Their pathogenicity ranges from benign to highly morbific activities. Scores of pharmacological studies have been conducted in the past decades on medicinal plants, being a potential source for the development of novel medicines and having a prospect in the enhancement of orthodox antimicrobial drug research towards improvement of treatment quality. However, secondary metabolites which are responsible for plant bioactivities are often produced in response to biotic and abiotic stresses. Chief of the abiotic stress include variations in season. *Helichrysum aureonitens* is a medicinal plant noted for its traditional use and previously reported to have antiviral effects against Herpes simplex virus (HSV-1) type 1 in previous studies. Further investigation was conducted in this study to determine the influence of seasonal variation and locality on the antiviral property of *H. aureonitens.* Using the cytopathic effect (CPE) reduction approach, twenty-six samples of the plants' leaves and stems collected during spring and autumn at Telperion nature reserve in Mpumalanga and Wakefield farm, Midlands in KwaZulu-Natal region of South Africa were evaluated for anti-HSV activity. The MTT assay was used for antiviral screening which was preceded by the cytotoxicity evaluation of the extracts. Seventeen of the twenty-six extracts examined were found to have considerable anti-HSV activity, as measured by a reduction in tissue culture infectious dose ($TCID_{50}$) of less than 10^5. This study further lends credence to the influence of seasonal variations on the antimicrobial properties of plants.

Keywords: Seasonal variation; Antiviral; *Helichrysum aureonitens*: Herpes simplex virus; Medicinal plants

4.1 INTRODUCTION

With over 350, 000 species of flowering plants on earth today (Cheek et al., 2020), flora species present a rich untapped reserve for the production of new medications against microbes. Medicinal plants account for 10% of all vascular plants (Fonnegra, 2007). Indigenous medical systems rely heavily on traditional medicinal plants. According to the World Health Organization (WHO), more than 80% of the member countries report the use of traditional and complimentary medicine as their primary source of health treatment (WHO, 2019).

Viral infections constitute a leading cause of death amidst infectious diseases battling humans all over the world (Howard & Fletcher, 2012). The most virulent viral infections resulting in the highest number of morbidity and mortality are AIDS (acquired immunodeficiency syndrome), SARS (severe acute respiratory syndrome), influenza, and Ebola (Ben-Shabat et al., 2020). Unlike other microbes, viruses cannot multiply outside of a host cell, their survival in nature is dependent on the maintenance of repeated infections, or transmission chains. The pathogenic mechanisms followed by a typical viral illness include virus implantation at the point of entry, local replication which involves spread and infection of neighbouring cells to the point of entry, dissemination to organs of interest which are the disease sites, and finally spread to locations where the virus is discharged into the environment (Samuel Baron, 1996).

Humans play host to Herpes simplex virus (HSV), a big DNA virus belonging to the family Herpesviridae. The virus exists in two categories: HSV-1 known as oral herpes and HSV-2 otherwise referred to as genital herpes. WHO reported that close to 67% of the world's population 50 years below are infected with HSV-1 (World Health Organisation, 2016). It is a highly communicable disease that is endemic worldwide, it can lead to a variety of infections ranging from moderate to severe, including cold sores, corneal blindness, encephalitis, and keratitis especially in immunocompromised persons. HSV infections increase the likelihood of contracting HIV infection and thus contributing to HIV epidemy (Jiang et al., 2016). The disease development begins with the infection of mucosal tissue severely and progresses to sensory neurons, where it creates a latent infection (Cliffe et al., 2013).

Orthodox antiviral medicines such as interferon and ribavirin available for treatment of viral related infections are only potent against most viruses *in vitro* while they are often ineffective when administered to patients (Ben-Shabat et al., 2020). Furthermore, many of the medications

lack specificity when it comes to treatment of particular viral infection (Jiang et al., 2016). Various medicinal plants have been reported to have potent antiviral effects at various stages of viral development (Abd-Elazem et al., 2002; Karimi et al., 2016; Serkedjieva et al., 1990). Many studies have shown that plant species of the genus *Helichrysum* are known to possess antiviral activities (Dhakad et al., 2017; Guarino & Sciarrillo, 2003; Kutluk et al., 2018; Sindambiwe et al., 1999; van Vuuren, 2008). Meyer et al., (1997) in their study of evaluation of the antiviral potentials of *H. aureonitens* have established a remarkable activity of the plant on HSV-type 1 as evidenced by the absence of a cytopathic effect on human lung fibroblasts.

Generally in plants, variation in seasons among other factors is an important contributor to both the quality and quantity of active compounds responsible for the biological activities of plants (Gouveia & Castilho, 2011; Harbone J.B, 1978; Mahajan & Tuteja, 2005). Results showed that variations in season influence distribution of biochemical compounds in plants leading to differing biological activities. Furthermore, species of the same plants growing in different sites at the same location may possess different biological activities in what seems to be influenced by a microclimate with a local set of atmospheric conditions that are particular to different sites. In this study, an attempt was made to investigate the influence of seasonal variation and different sites in the same locality.

There is a need to investigate the present therapy methods (particularly resistant virus strains therapies) with a view to enhance and augment them with the discovery of novel antiviral agents from plant sources for the treatment of resistant viral infections, especially due to the rising prevalence of viral infections.

4.2 MATERIALS AND METHODS

4.2.1 South Africa's Seasons

Unlike what is obtainable in other sub-Saharan African countries where there are only dry and wet seasons, South Africa's seasons are divided into four, namely: autumn/fall, winter, spring and summer with the transitional seasons of autumn and spring being very short. The exact dates for the beginning and ending of each season remain a subject of controversy both from the scientific and lay man standpoint, and there is therefore no official starting dates set for each of the seasons. For the sake of conformity to established conventions however, calendar dates for the four seasons in the southern hemisphere are: autumn-1st March to 31 May, winter-

1st June to 31st August, spring-1st September-30th November and summer-1st December-28/29 February. During summer, most of the country apart from the Western Cape province receives most of the rain and vegetations are green.

4.2.2 Plant material collection

As described in section 3.2.1

4.2.3 Plant extraction preparation

Plant extraction was carried out as reported by Eloff (Eloff, 1998). A 10:1 ratio of acetone to plant materials (50g of plant material to 500m of acetone) was introduced into the centrifuge tube (1998). Samples were shaken overnight at 130 rpm in a shaker (Thermo Fisher Scientific, USA), then centrifuged for 10 minutes at 3000 rpm in an Eppendorf microcentrifuge (5427R, Germany), with the supernatant transferred to a glass vial. The residual solvent was removed from the extracts that had already been deposited in pre-weighed glass tubes and stored at room temperature using a stream of room temperature air.

4.2.4. Preparation of assays

4.2.4.1 Virus culture and assay

Herpes simplex virus type 1 (HSV-1; 15577 strain) purchased from Anatech Analytical Technology (South Africa) was used in the study. The strain is susceptible to the standard drug Acyclovir (Garber et al., 2021). Vero cells (African green monkey kidney) (Cellonex Separation Scientific, Roodepoort, South Africa) were used to grow the virus while Minimal Essential Medium (MEM) supplemented with 5% (v/v) fetal bovine serum (FBS), 2mM L-glutamine, non-essential amino acids (1×) and 100 g/mL streptomycin (Celtic Molecular Diagnostics SA (Pty) Ltd., Cape Town, South Africa) was used as a medium to propagate the Vero cells. This was followed by incubation at 37°C in an atmosphere of 5% CO_2 and observed daily for evidence of cytopathic effect as determined by changes in the morphology of the cell. Thereafter, the flasks were frozen at -70°C and then thawed to release cell-associated virus. The Reed and Muench method, which primarily helps in the determination of the concentration of a test substance that produces a desired effect of interest (in this case, tissue culture infection

dose-TCID) in 50% of the test units was used to estimate the $TCID_{50}$ values of each of the samples employed in this investigation (Reed & Muench, 1938).

4.2.4.2. Cytotoxicity Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric (MTT) assay as described by Mosmann, (1983) was used to measure cell viability. The process for determining the cytotoxic concentration of the plant extracts began with seeding the Vero cells cultured in Dulbecco's modified eagle's medium (DMEM; Gibco) at a density of 25 000 cells/well of 96 well flat bottom cell culture microtiter plates and incubated for 24 h to allow the cells to attach to the bottom of the 96 well plates. After 24 hours of incubation at 37°C in a humidified 5% CO_2 environment, cells were exposed to various doses of the extracts dissolved in 10 % Dimethyl sulfoxide (DMSO) (Sigma-Aldrich® Darmstadt, German) and further incubated for 24 h. The media was discarded and replaced with fresh 20 µL of MTT prepared in phosphate buffered saline (PBS) (Gibco), which was added and the plates were incubated for 4 h followed by the addition of 100 µL (DMSO) which was pipetted into each well. The plates were carefully rocked to disintegrate formazan crystals which are the by-products of the tetrazolium salt. The presence or absence of purple formazan colour as observed in the wells gives an indication of the cytotoxic effect of the extracts on cells. The optical density (OD) of the MTT was read at a wavelength of 570 nm and at a reference wavelength of 630 nm using an ELISA microplate reader (VarioSkan Flash, Thermo Fisher Scientific, Vantaa, Finland). Cytotoxicity results were expressed as extract concentration that are lethal to cell growth by 50% (LC_{50}), calculated using a linear regression equation. Vero cells monolayers treated with Acyclovir (Sigma-Aldrich® Darmstadt, German) were used as positive control, while untreated cells were used as negative control. Cytotoxicity experiments were done in triplicates.

4.2.4.3. Antiviral assay

A method by Barnard et al., (1992) was used with some modifications to determine the antiviral activity of the extracts. Extract concentrations that were not toxic to the cells were diluted in DMEM containing 5% FBS and 1% penicillin/streptomycin (PenStrep, Sigma-Aldrich® Darmstadt, German), to which equal volume of HSV (20 µl) at an infective titre of 10^2 TCID50/mL was added. The combined extract-virus solution was incubated at 37°C for a time

range of between 1 to 3 hours. Cell monolayers grown in 96-well plates that were confluent had their growth media removed. One-hundred (100) µL of the mixture of extract-virus was added to the cells at each (10 µg/mL) concentration and incubated for a time period that ranged between 1 to 5 days depending on when cytopathic effect (CPE) was observed. Untreated infected cells were used as negative control and Acyclovir treated infected cells were used as positive control. Furthermore, serially diluted solvent control (10 % DMSO) was included in experiments. The presence of CPE was confirmed by microscopic examination. Plant extracts that limit viral growth at dilution range above 10^5 indicate mild to weak activity, whereas those that reduce viral infectivity at dilutions ranging between 10^5 to 10^0 have strong activity. Experiments were conducted in triplicates and two independent experiments were conducted.

4.3 STATISTICAL ANALYSIS

Experiments were done in triplicate with two independent assay repeats, and results expressed as mean ± standard deviation (SD). The LC_{50} values, corresponding to the concentration required to inhibit 50% of cell viability, were calculated from a sigmoidal dose–response of a non-linear regression and R-square values representing the best fit of the model were assessed using One-way analysis of variance (ANOVA) as well as to determine the differences in means, and statistical processing of the data was performed using GraphPad Prism software (Version 8.0). Tukey's multiple comparison test was used to determine significant differences between the means of treated and untreated groups.

4.4 RESULTS

4.4.1 Cytotoxicity result

The MTT assay was used to assess the cytotoxicity of the extracts on the African green monkey kidney Vero cell line. Extract/Acyclovir concentration ranging from 8.0–1000 µg/mL were used to treat cells. The findings revealed dose-dependent toxicity, with higher cell viability at lower concentrations and a gradual decline in cell viability as concentrations increased. Table 4.1 lists the lethal concentrations that lowered viability of cells by 50% (LC_{50}). These concentrations lower than the LC_{50} (10 µg/mL) were further tested for antiviral activity. All plant extracts exhibited a varying degree of toxicity on Vero cells with LC_{50} values greater than 20 µg/mL for most samples. These values were determined to ensure that the LC_{50} values used in this study are safe when compared to the LC_{50} value for acyclovir (positive control).

Table 4.1: Cytotoxic effects of leaves and stem extracts of *H. aureonitens* at different sites at two diverse geographical locations and two seasons of the year.

Collection Location	Site	Plant Parts	Collection Season	Cytotoxicity LC_{50} (µg/mL)	Selectivity Index (SI)
Telperion	Site 1	leaves	Spring	17.8 ± 1.48	0.200
Telperion	Site 1	Stem	Spring	14.4 ± 0.56	0.917
Telperion	Site 2	leaves	Spring	16.0 ± 0.51	0.320
Telperion	Site 2	Stem	Spring	29.5 ± 0.25	1.050
Wakefield	Site 1	leaves	Spring	19.9 ± 2.73	0.215
Wakefield	Site 1	Stem	Spring	42.5 ± 4.92	0.662
Wakefield	Site 2	leaves	Spring	26.4 ± 4.37	0.301
Wakefield	Site 2	Stem	Spring	23.4 ± 4.89	0.835
Wakefield	Site 3	leaves	Spring	27.6 ± 0.51	0.554
Wakefield	Site 3	Stem	Spring	32.2 ± 0.44	0.994
Wakefield	Site 4	leaves	Spring	35.7 ± 1.21	0.472
Wakefield	Site 4	Stem	Spring	46.9 ± 0.43	0.640
Telperion	Site 1	leaves	Autumn	24.3 ± 6.29	1.538
Telperion	Site 1	Stem	Autumn	18.5 ± 0.45	1.170
Telperion	Site 2	leaves	Autumn	24.8 ± 1.30	0.001
Telperion	Site 2	Stem	Autumn	21.3 ± 0.21	0.687
Telperion	Site 3	leaves	Autumn	35.2 ± 0.94	0.488
Telperion	Site 3	Stem	Autumn	31.4 ± 3.43	1.468
Wakefield	Site 1	leaves	Autumn	24.2 ± 9.41	0.618

Wakefield	Site 1	Stem	Autumn	40.7 ± 3.02	0.806
Wakefield	Site 2	leaves	Autumn	24.9 ± 2.68	0.294
Wakefield	Site 2	Stem	Autumn	43.6 ± 5.68	0.509
Wakefield	Site 3	leaves	Autumn	25.6 ± 3.12	0.317
Wakefield	Site 3	Stem	Autumn	25.9 ± 3.43	0.302
Wakefield	Site 4	leaves	Autumn	27.9 ± 10.56	1.570
Wakefield	Site 4	Stem	Autumn	20.4 ± 8.39	1.284

LC_{50} figure represents extract concentration that are lethal to 50% of Vero cells.

Positive control, acyclovir LC_{50} = 10 µg/mL

Selectivity Index (SI) = LC_{50} (µg/mL)/$TCID_{50}$. The higher the SI value, the safer the extracts are.

4.4.2 Anti-HSV result

Plant extract concentration of 10 µg/mL was used to determine the antiviral activity, based on the non-toxic nature of the extracts as shown by the LC_{50} values against Vero cells. Further to this was the simultaneous inoculation of the combination of extract-virus inoculum to the cells in their individual wells. The tested concentration was observed to significantly decrease the viral titre as observed by the inhibition of the CPE in Vero cells caused by viral infection. Because the extracts reduce viral load by 2 logs when compared to the virus control, the HSV titre log benchmark was therefore set at 10^5 $TCID_{50}$ to classify activity where all extracts that reduce the viral burden to a figure below log 10^5 were considered very active as seen in figure 4.1.

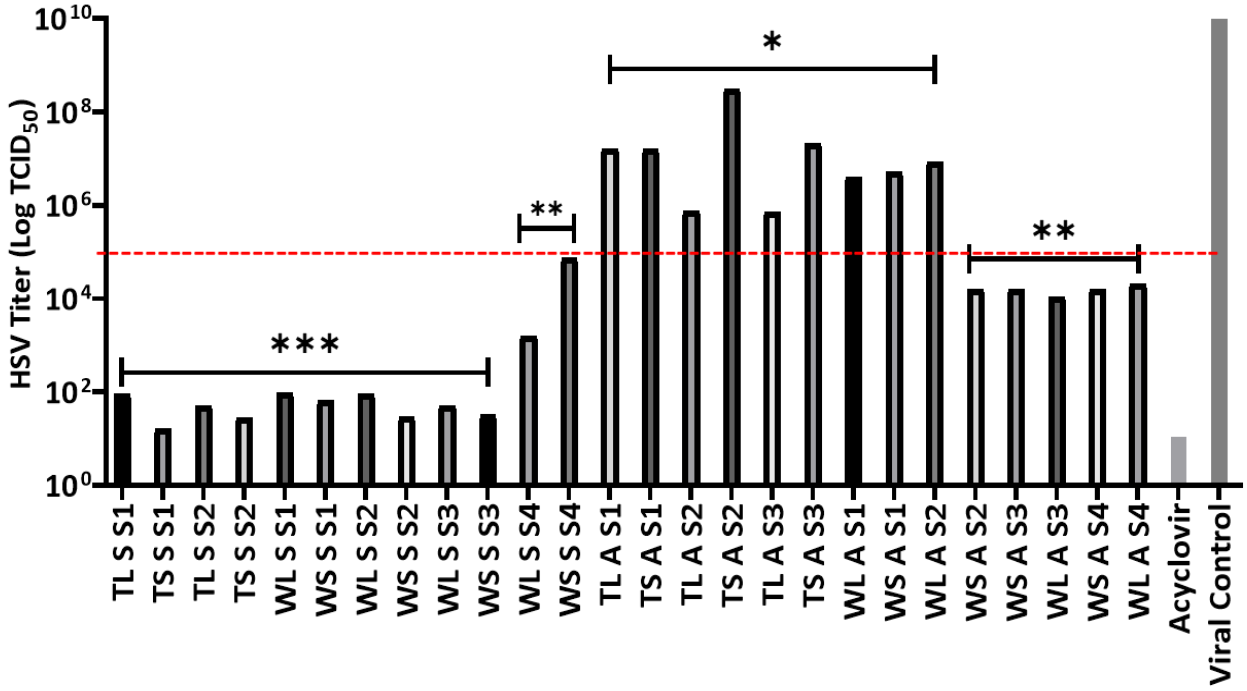

Figure 4.1: Effects of *H. aureonitens* extracts (leaves and stems) from different sites at two different locations and at two different seasons against HSV tissue culture infections dose ($TCID_{50}$) in Vero cell culture. Mean HSV-1 titers (±SD) determined using one-way ANOVA where * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. TL = Telperion leaves, TS = Telperion stems, WL = Wakefield leaves, WS = Wakefield stems, S = spring, A = autumn, S1-S4 = collection site.

4.5 DISCUSSION

In this study, anti-HSV activity of twenty-six extracts of *H. aureonitens* from different sites at two different locations and at two different seasons were evaluated, and the result showed varying degrees of pharmacological potency against the virus. The choice of Vero cells as the study's primary cell line was due to its reputation as the most susceptible cell line to virus-mediated cell death and virus proliferation. They have been widely used in toxicology, virology, and pharmacology studies, as well as vaccine development and diagnostic reagent production (Shen et al., 2019; Wu et al., 2017).

The cytotoxicity result presented in Table 2 showed a general high level of toxicity of *H. aureonitens* extract across both seasons and different climatic regions with LC_{50} values ranging between 14.48-46.99 µg/mL. Heyman and Meyer (2012) reported a similarly high level of

toxicity of the following *Helichrysum* species, *H. acutatum* (25.16 µg/mL), *H. appendiculatum* (29.01 µg/mL), *H. panduratum (*3.40 µg/mL) and *H. psilolepis* (27.07 µg/mL). *Helichrysum aureonitens* and *H. nudifolium* both have even higher cytotoxic values of < 3.13 µg/mL each, very close to the positive control zearalenone with the cytotoxic value of 1.33 µg/mL. The results, although close to the current study, still have some variation with report of higher toxicity compared to the current result. This can be due to reasons such as use of different extraction solvents, variations in seasons or difference in climatic regions. Heyman & Meyer, (2012) extracted with chloroform while the current study used acetone. Umar et al., (2013), in their study that compared antimicrobial properties of extracts from dried stems of *Opuntia dillenii* and rhizomes of *Zingiber officinale* using non-polar (petroleum ether and chloroform) and polar solvents (methanol and water), and discovered that ether and chloroform extracts of *Opuntia dillenii* demonstrated better antibacterial efficacy against *Escherichia coli* (gram negative) when compared to methanolic and water extracts indicating that the polarity of the solvent used during extraction significantly influenced the antimicrobial activity of the plants. Eloff (Eloff, 1998) also compared various extractants in common use and reported different results based on individual extractants used in the isolation of antimicrobial components of plants.

The extracts at 10 µg/mL investigated in this study significantly reduced the HSV infection in Vero cells. Figure 1 shows that seventeen of the twenty-six extracts tested had significant anti-HSV activity, as evaluated by a tissue culture infectious dose ($TCID_{50}$) reduction of less than 10^5. The observed strong activities of the seventeen extracts as seen in the reduction in the HSV titre values is significantly better when compared to the positive control. Twelve of the seventeen were extracts from plants collected during the spring season while the remaining five were collected during autumn. Significantly higher antiviral activities of *H. aureonitens* plant extracts were recorded in the spring season in both locations, and the extracts' activities were more than double in comparison to the activity of extracts of plants collected in autumn as shown by the significant reduction in the viral load depicted by the HSV titre values represented in Figure 1. This supports many studies that have reported the influence of seasonal variation on the antimicrobial activities of plants (Ncube et al., 2011; Ramírez-Briones et al., 2019). Further to the ongoing, it is clear that different climatic regions exert influence on the potency of *H. aureonitens* against the HSV-1. This is confirmed by a comparison between the activity of extracts from Telperion and Wakefield during the autumn season. While extracts' activities in spring was better and comparatively the same (except for the dry site of Wakefield) in both

locations, extracts from autumn demonstrated better activity in the dry sites at Wakefield as seen by the reduced HSV titer (figure 1), although much lower than the extracts collected in spring. A few studies conducted on the antiviral properties of *Helichrysum* species reported varying activities. At concentrations ranging from 12 to 47 µg/mL, galangin, isolated from *H. aureonitens,* demonstrated considerable antiviral activity against a DNA virus, HSV-1, and an RNA virus, Coxsackie B Type-1, while showing no activity against Adenovirus Type-31 (Meyer et al., 1997). Also, ethanolic extracts of *H. arenarium* and *H. armenium* showed significant antiviral activities against HSV-1 and PI-3 at concentrations of 2–32 and 4–64 µg/mL, respectively. Selective index (SI) is a ratio that measures the cytotoxicity and antiviral activity of a sample and its bioactive concentration. A higher SI ratio is an indication of how effective plant extracts would theoretically be in vivo against a virus. More than 75% of the plant extracts in this study are <1 which means most of the extracts are quite toxic. Those greater than 1 are also not far to 1. This means extracts are therefore more toxic than active.

4.6 CONCLUSION

The result of the study supports the outcome of many studies on the influence of seasonal variation on the antimicrobial activity of medicinal plants. It is therefore concluded that the antiviral activity of *H. aureonitens* is contingent upon a number of factors which include seasonal variation and the specific climatic region in which the plant is growing. From the result of this study, the spring season favours a much better antiviral activity of the plant in both wet and dry locations and collections are therefore advised to be made in spring season for effective antiviral activity.

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CHAPTER FIVE

METABOLOMICS STUDY ON THE CHEMICAL PROFILE OF HELICHRYSUM AUREONITENS IN DIFFERENT SEASONS AND DIFFFERENT SITES IN DIFFERENT CLIMATIC REGIONS

ABSTRACT

The many uses of *Helichrysum aureonitens* include topical skin infection treatment, particularly herpes zoster and other herpes-like illnesses. It is further used in the treatment of nocturnal enuresis in kids and also traditionally believed that the incense from burning the whole plant helps in the evocation of ancestor's goodwill. The flavonoid galangin (3, 5, 7 – trihydroxyflavone), a polyphenolic compound found in a variety of medicinal plants has been repeatedly reported as an active compound in *H. aureonitens.* Also, the polyphenol chlorogenic acid (CGA) which is a derivative of quinic acid is a very abundant compound found in many *Helichrysum* species including *H. aureonitens.* Since many studies have associated part of the differences observed in the chemical profile of medicinal plants of the same species to harvests made in regions with contrasting climate and different seasons of the year, this study therefore investigated the influence of seasonal variation and difference in local climate of two dissimilar regions in South Africa on the metabolomic profile of *H. aureonitens.* ^{1}H-NMR generated spectra data were subjected to a multivariate statistical analysis using Soft Independent Modeling of Class Analogy (SIMCA) software, with the goal of evaluating and monitoring their seasonal fluctuations. The result of the study showed that aromatics content of *H. aureonitens* is positively linked to high rainfall and low temperature. This study confirms the significant effect of seasons and peculiar climates of different localities on the secondary metabolite profile of *H. aureonitens*.

Key words: *Helichrysum aureonitens*; medicinal plants; Nuclear Magnetic Resonance (NMR), seasons, chemical profile

5.1 INTRODUCTION

South Africa is home to over 30,000 plant species, accounting for 10% of all plant species on the planet (Van Wyk, 2008). Ethnobotanical plant research, whether for pharmacological purposes, cosmeceutical or nutraceutical applications has been in the spotlight in recent years, as evidenced by the number of research outputs in many studies that focus primarily on providing scientific basis for traditional use based on indigenous knowledge systems.

Oftentimes, plants respond to both biotic and abiotic stress conditions by producing secondary metabolites. The production of secondary metabolites however does not follow a predictable pattern with regards to how much or what exactly is produced (Prinsloo & Nogemane, 2018; Ramakrishna & Ravishankar, 2011; Street & Prinsloo, 2013; Tanko et al., 2005). Seasonal dynamics of plant phenology, geographic differences between plant populations, environmental variables of the specific species' growing location, especially when they exhibit genetic homogeneity or changes in biotic and abiotic factors in the environment are all factors that can affect the synthesis and accumulation of primary and secondary metabolites (Sampaio et al., 2016). An important abiotic factor that is responsible for both the quality and quantity of phytochemical profile of plants is changes in season (Dhami & Mishra, 2015; Ncube et al., 2011). According to Lemos et al., (2015), the chemical compositions of plants are altered by seasonal changes in temperature and rainfall as well as soil humidity, and also specific stages of plants' metabolism.

Helichrysum aureonitens is a perennial plant that belongs to the family Asteraceae and grows in the summer rainfall parts of South Africa. Its medicinal properties against many diseases makes it one of the choice plants that are of special interest to traditional healers (Pooley, 2003). Many studies have reported its antifungal, antibacterial and antiviral activity (Afolayan & Meyer, 1997; J. J. M. Meyer et al., 1996; J. J. M. J. M. Meyer & Afolayan, 1995). The range of compounds found in plants are influenced by a number of factors which include plant species, specific plant parts, harvest period, prevailing climatic condition of the environment where the plants is growing, and the extraction method used (Ebani & Mancianti, 2020; Managa et al., 2021b, 2021a; Van Wyk & Prinsloo, 2021). Many studies have revealed the positive correlation of season/location on the chemical profile of *Helichrysum* species (Caser et al., 2016; Gouveia & Castilho, 2012; Melito et al., 2016). In a study where the chemical profile of *H. italicum* from two dissimilar habitats were analysed, the volatile fractions of the two groups differed significantly, indicating a link between secondary metabolite production and habitat type (Melito et al., 2016). Based on the fact that no study is yet to report the effect

of environmental factors on the chemical profile of *H. aureonitens*, the aim of the present work was therefore to determine the link between the influence of environmental conditions on the secondary metabolite distribution in *H. helichrysum* growing in different seasons and at different locations.

Metabolomics is a broad term that refers to a comprehensive qualitative and quantitative examination of all metabolites found in a biological system under specified conditions (Allwood et al., 2008; Fiehn et al., 2000; Sumner Judith, 2003; Verpoorte et al., 2002). Metabolomics is employed in a wide variety of applications, including plant breeding, crop quality evaluation, food evaluation, toxicity evaluation, nutrition assessment and medical diagnostics, diagnosis of ailments, the development of pharmaceutical drugs, integrated systems biology and as technological tool in advancing analytical chemistry (Moco et al., 2007). The use of a combination of analytical techniques, such as NMR and LC-MS, aids in the detection of changes in chemical profiles with the purpose of determining which chemicals are accountable for the changes observed in biological function (Dunn, 2008; Dunn & Ellis, 2005). Since spectra generated from NMR contains detailed qualitative and quantitative information about an analysed sample (Reo, 2002), NMR based metabolomics therefore promises to be a reliable method for studying the link between seasonal variation, geographical distribution and chemical profile of *H. aureonitens*.

5.2 MATERIALS AND METHODS

5.2.1 Plant material collection

As reported in section 3.2.1

5.2.2 Metabolomic assessment

Fifty mg of powdered leaf material was weighed and stored in 2 mL Eppendorf tubes and extracted following an established direct extraction method. Plant material was extracted with 0.75 mL deuterated methanol (CD_3OD) and 0.75 mL, deuterium water (D_2O) (pH 6.38), with potassium dihydrogen phosphate (KH_2PO_4) and 0.1 % (w/w) TSP (Trimethylsilylpropionic acid sodium salt) added. The samples were vortexed for 1 minute at room temperature to combine the reagents. After ultrasonically (Branson 2800, USA) breaking down the cell walls

for 15 minutes, the mixture was centrifuged for 20 minutes to separate the supernatant from the pellet. Each tube's supernatant was then transferred to a 5 mm NMR tube for analysis on a 600 MHz NMR spectrometer (Varian Inc., Palo Alto, CA, USA) with 32 scans recorded.

5.2.2.1 NMR Spectra data acquisition and pre-processing

Spectral data obtained from the NMR were pre-processed with MestReNova software (9.0.1, Mestrelab Research, Spain) and subjected to phase correction, baseline correction, referencing and normalization. TSP was included as a standard in all samples and the quality control of the analysis was done by confirming five sharp peaks for methanol. TSP was also used to reference and normalise all samples. Additionally, MestReNova was also used for bucketing NMR spectra. The spectral intensities were then reduced to integrated sections of identical width (0.04 ppm each), corresponding to the 0.04–10.00 ppm range in a process often referred to as binning. After that, the resulting ASCII files were imported into Microsoft Excel 2013. SIMCA-P software (version 15.0, Umetrics, Umea, Sweden) was used to conduct the PCA and OPLS-DA multivariate data analyses while further transformation of the data was carried out with pareto scaling. The final data does not include the residual water peak (4.60–5.00 ppm) and the methanol peak (3.28–3.36 ppm) (Mediani et al., 2012).

5.2.2.2 Multivariate data analysis

Metabolomics studies yield high-dimensional and complicated data sets that are challenging to analyse and interpret merely by visual examination or any standard quantitative univariate analysis. Multivariate data analysis (MVDA) tools - mathematical modeling approaches – are thus utilized to extract useful information from these large empirical data sets (Tugizimana et al., 2013). SIMCA-P (15.0, Umetrics, Umea, Sweden) software was used to analyse multivariate data from MestReNova files using principal component analysis (PCA) and orthogonal partial least square discriminatory analysis (OPLS-DA). Scatter plots of PCA scores, which is an unsupervised analysis were generated and used to compare samples across the different sites in each location and different seasons. This allows an easy identification of groups, patterns and conspicuous outliers between and within treatments, allowing groupings, trends, and notable outliers to be identified.

PCA is a mathematically rigorous approach based on projections that gives a global and qualitative visual representation of sample similarity or dissimilarity (Boccard et al., 2007).

PCA, an unsupervised method, was used to assess the data initially, followed by orthogonal partial least square discriminatory analysis, a supervised model (OPLS-DA). To examine the contribution of each processed variable to the overall data, a scores plot, and a contribution plot were employed and the NMR values from the plots were then utilized to annotate the data using existing databases and published literature.

5.2.2.3 Compounds annotation

The Chenomx NMR suite was used to detect and quantify metabolites employing TSP as a reference and the integrated metabolite spectrum library. Data from existing literature was used to verify the annotated chemicals.

5.3 RESULTS

Significant amount of metabolic variation was observed in the study by comparing the H-NMR metabolomics data. The figures below compare the data generated from the analysis at two separate levels for ease of reporting: (i) determining the effect of seasonal variation and different growing locations on the chemical profile of *H. aureonitens* collected from Telperion and Wakefield during spring and autumn season (ii) determining the effect of seasonal variation (spring and autumn) on the chemical profile of *H. aureonitens* collected in wet and dry sites from both Telperion and Wakefield.

5.3.1 Chemical profile of *H. aureonitens* collected from Telperion and Wakefield during spring and autumn season

A total of 57 samples of methanolic extracts of *H. aureonitens* leaves and stems collected during spring and autumn from two geographically different locations (Telperion and Wakefield) were subjected to ^{1}H-NMR-based metabolomics to investigate how seasonal variation and different growing locations influence the plant's chemical profile. The maximum and minimum recorded temperature for the first collection in spring season (October) in both locations are 22.3 / 9 °C and 24.5 / 10.7 ° C for Wakefield and Telperion respectively. The total monthly rainfall for the locations in October are 110 mm and 83.8 mm for Wakefield and Telperion respectively (Table 3.1). The maximum and minimum recorded temperature for the second collection in autumn season (May) in both locations are 21 / 6.7 °C and 21.4 / 7.0 °C for Wakefield and Telperion respectively. The total monthly rainfall for both locations in May

are 31.8 mm and 24.6 mm for Wakefield and Telperion respectively (Table 3.1). PCA and OPLS-DA models were used to analyse the data which helps in determining if there are sufficiently observed differences in the metabolic fingerprints of the extracts to distinguish between seasons and locations. A single sample is represented by each point on the PCA and/or OPLS-DA score scatter plot. Samples having similar chemical profiles cluster together. The PCA scatter plot of all the samples across the two seasons (spring and autumn) and different locations pulled together showed slight clustering for the two seasons (green and blue circles), but no clustering for each locality per season as seen in figure 5.1, indicative of larger variation in the chemical profile per season than per collection site.

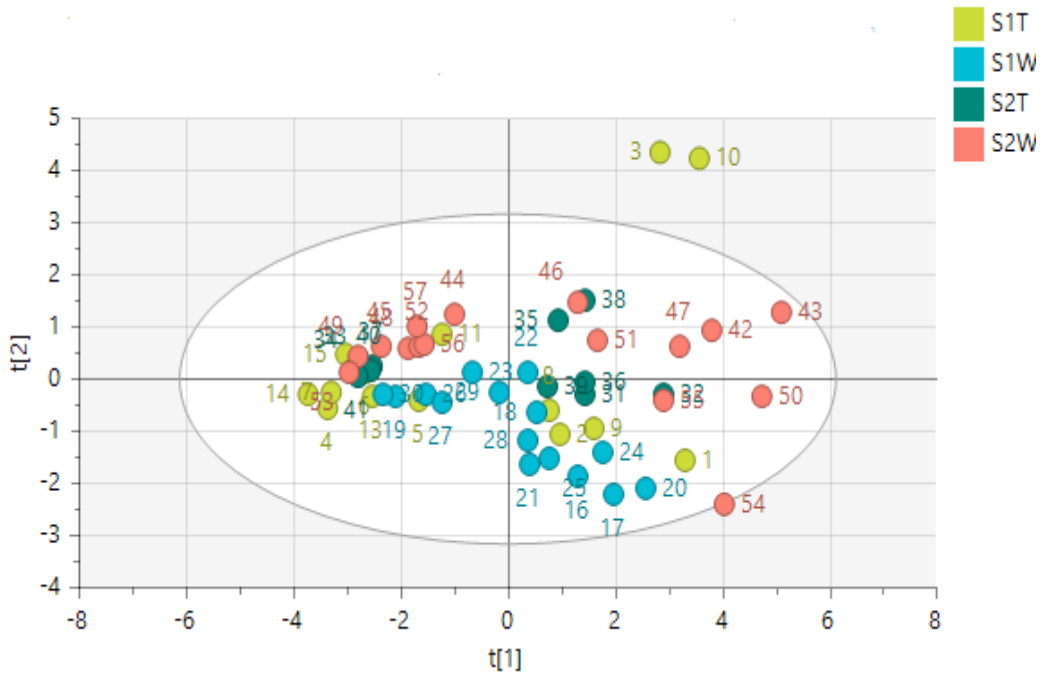

Figure 5.1: PCA score scatter plot showing component 1, x-axis (PC1 = 54.6%) and component 2 y-axis (PC2 = 14.6%) of *H. aureonitens* leaves and stems during spring and autumn at Telperion and Wakefield. S1T= spring, Telperion. S1W= spring, Wakefield. S2T= autumn, Telperion. S2W= autumn, Wakefield.

An OPLS-DA model was further constructed to facilitate clustering and identification of the metabolites responsible for distinguishing between differences in seasons and geographical

locations (Figure 5.2A). A clear separation was then observed between samples across seasons and locations. The model displayed a goodness of fit and predictability as presented by an R^2X of 0.905, an R^2Y of 0.727 and a Q^2 of 0.384. Further to that, the response permutation test (with n = 100) was constructed in order to validate the predictive capability of the computed OPLS-DA models. This statistical test compares the R^2 and Q^2 values of the true model to the permutated model. The test is carried out by randomly assigning the two groups, and then fitting the OPLS-DA models to each permuted class variable. The permutated models' R^2 and Q^2 values are then calculated and compared to the true models' values. The results show that the measured models have significantly higher R^2 and Q^2 values (Figure 5.2B), implying that the calculated true OPLS-DA models for each dataset are statistically better to the 100 permuted models.

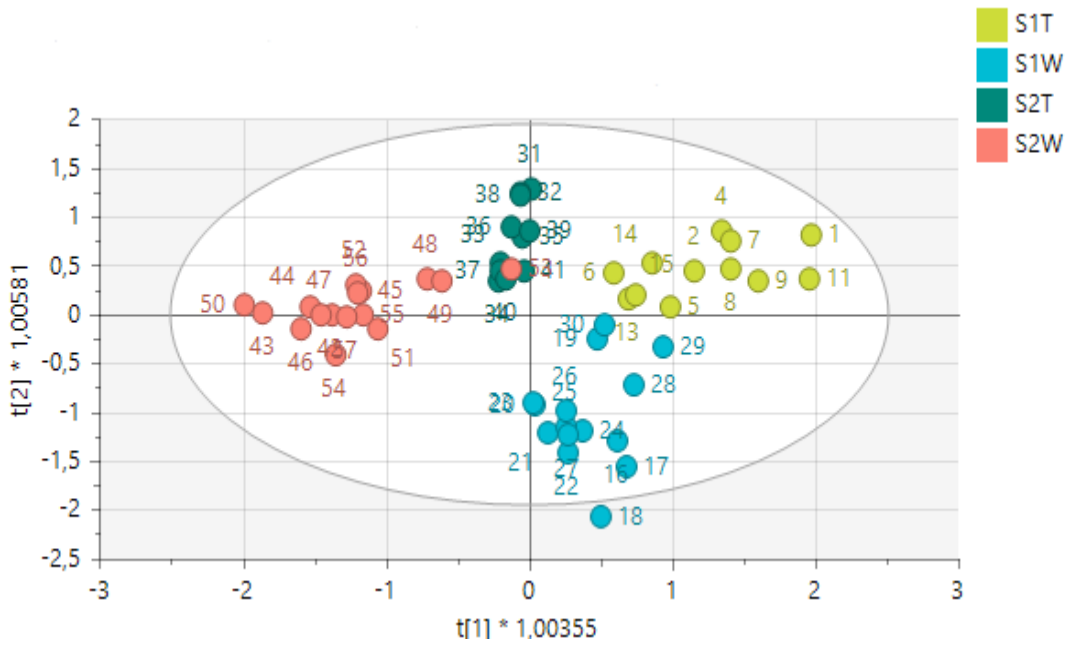

Figure 5.2A. An OPLS-DA Score scatter plot showing the predictive (x-axis) and orthogonal (y-axis) components of *H. aureonitens* leaves and stems during spring and autumn at Telperion and Wakefield. R^2X = 0.905, R^2Y = 0.727 and Q^2 = 0.384. S1T=spring, Telperion. S1W=spring, Wakefield. S2T=autumn, Telperion. S2W=autumn, Wakefield.

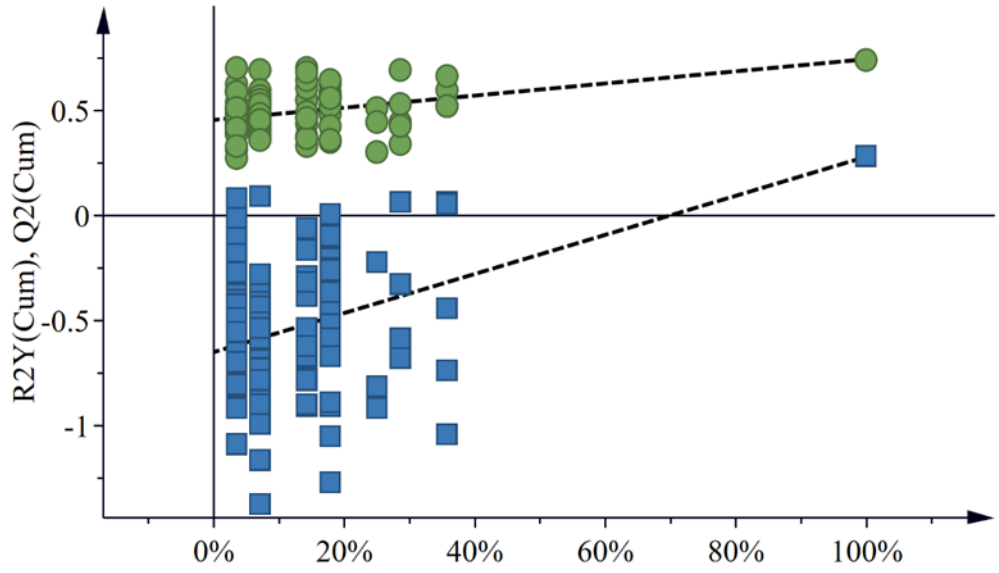

Figure 5.2B. The response permutation test (n=100) for the OPLS-DA model corresponding to y-axis intercepts (Table 3.1): R^2 = (0.0, 0.46) and Y^2 = (0.0, -0.65)

Figures 5.3-5.6 show representative ^{1}H-NMR spectra profiles of *H. aureonitens* leaves collected from wet and dry sites at both Telperion and Wakefield during spring and autumn compared for similarity or contrast. The ^{1}H-NMR spectra revealed varying peak signals across wet and dry sites from the two locations when compared, indicating significant changes in chemical composition of *H. aureonitens* leaves.

In Figure 5.3, the spectra from two wet sites in Wakefield showed conspicuous margin with taller peaks comparatively to spectra from the two set sites at Telperion which is a drier location than Wakefield.

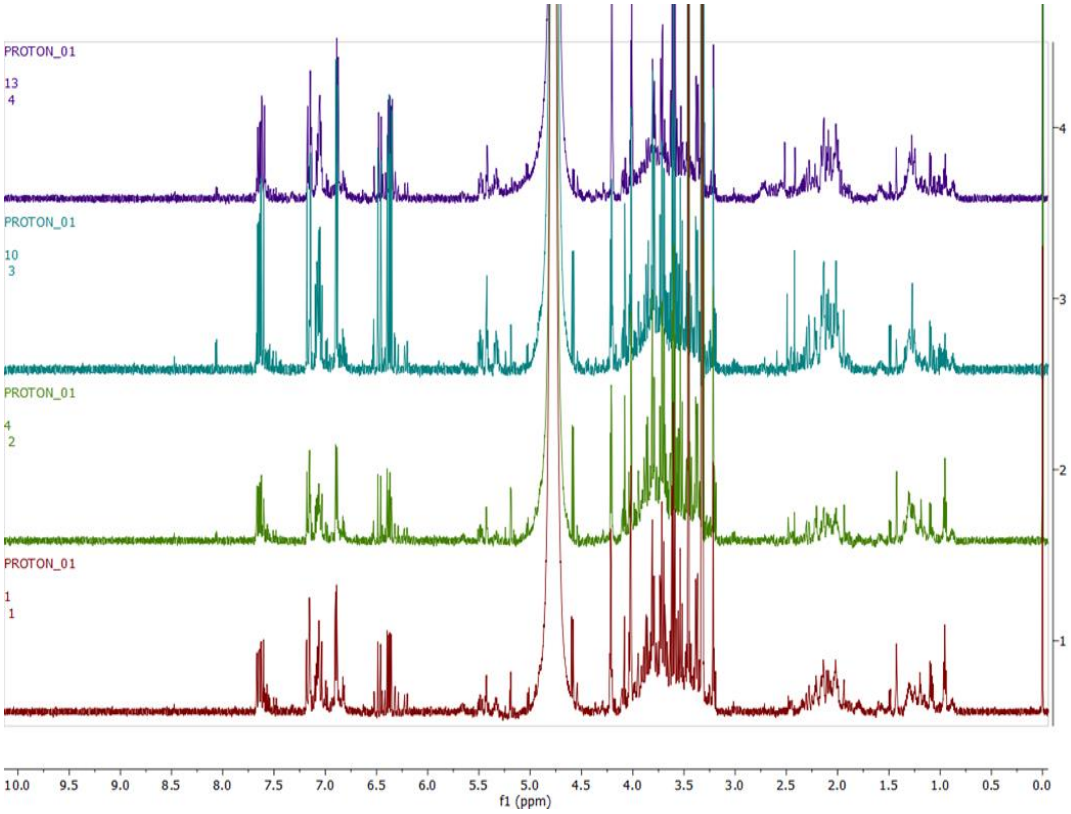

Figure 5.3. 600 MHz 1H-NMR spectra of *H. aureonitens* leaf extracts showing comparison between samples harvested from wet sites in the spring season across the two locations. Red- spring season, site 1 (wet) Telperion. Green- spring season, site 2 (wet) Telperion. Light Blue- spring season, site 1 (wet) Wakefield. Purple- spring season, site 2 (wet) Wakefield.

In Figure 5.4, spectra from the extracts collected from two dry sites from the two locations during spring season is compared. The dry site in Wakefield comparatively has taller peaks than the one from Telperion, indicating the presence of a higher concentration of compounds comparatively in the aromatic region. In the aliphatic region, higher peaks are visible for the Telperion samples.

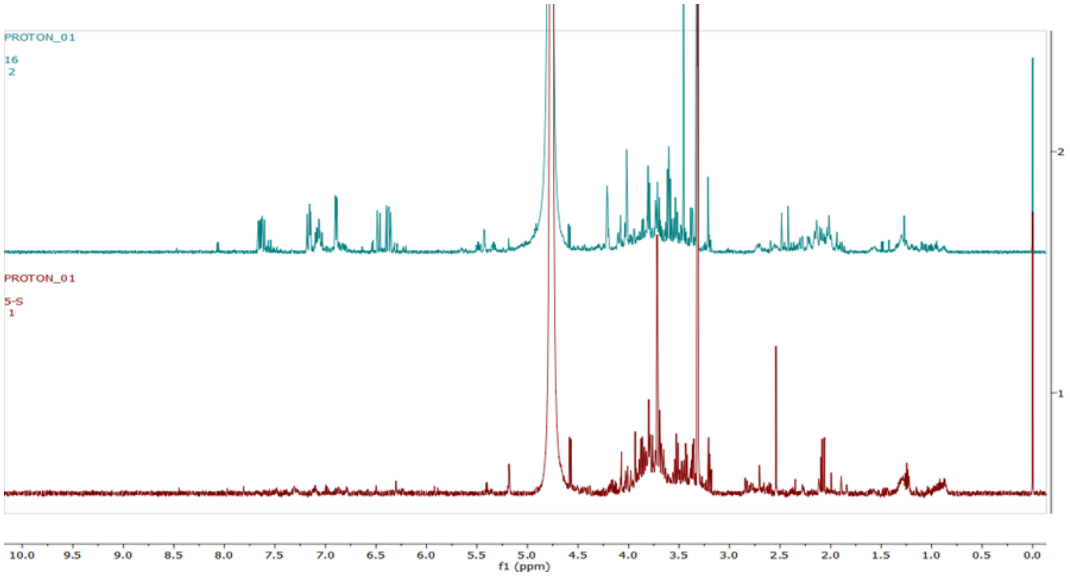

Figure 5.4: 600 MHz ^{1}H-NMR spectra of *H. aureonitens* leaf extracts showing comparison between samples harvested from dry sites in the spring season across the two locations. Red- spring season, site 3 (dry) Telperion. Light Blue- spring season, site 3 (dry) Wakefield.

Figure 5.5 compares the spectra from the two wet sites in both Telperion and Wakefield during the autumn season. The spectra do not show conspicuous differences with visible observation, with all the samples showing peaks similar in height to the dry sites in spring. The spectra from site 1 (wet) at Telperion and the spectra from site 2 (wet) at Wakefield are very similar by visual inspection with no remarkable difference in the two sites. A slight difference is however observed in the spectra from site 2 (wet) at Telperion as compared with site 1 (wet) at Wakefield.

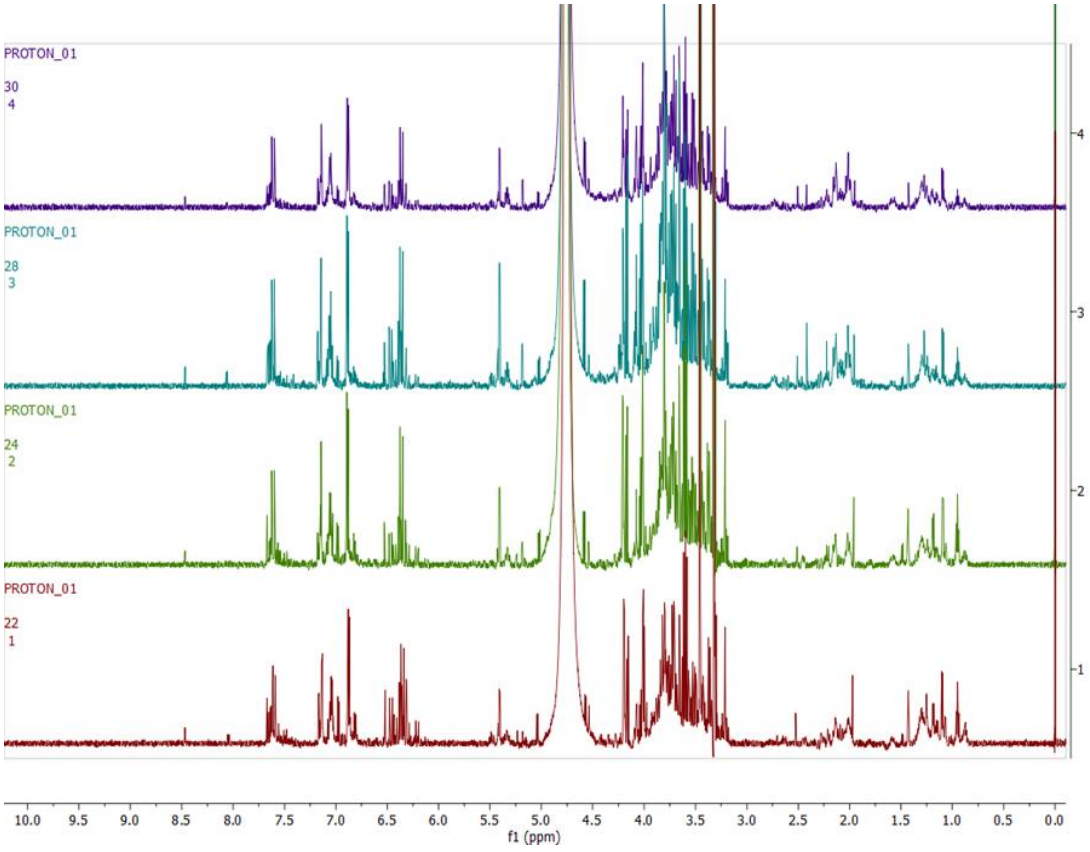

Figure 5.5. 600 MHz 1H-NMR spectra of *H. aureonitens* leaf extracts showing comparison between samples harvested from wet sites in the autumn season across the two locations. Red- autumn season, site 1 (wet) Telperion. Green- autumn season, site 2 (wet) Telperion. Light Blue-autumn season, site 1 (wet) Wakefield. Purple-autumn season, site 2 (wet) Wakefield.

In Figure 5.6, 1H-NMR spectra from the two dry sites from both locations for the autumn season is presented. Similar to the observation in the wet sites from both locations in the autumn season, the spectra do not show conspicuous margin with visible observation, with similar height of the peaks as compared to the wet sites in autumn. The spectrum from Site 3 (dry) at Wakefield only show slightly taller peaks when compared to equivalent dry site from Telperion.

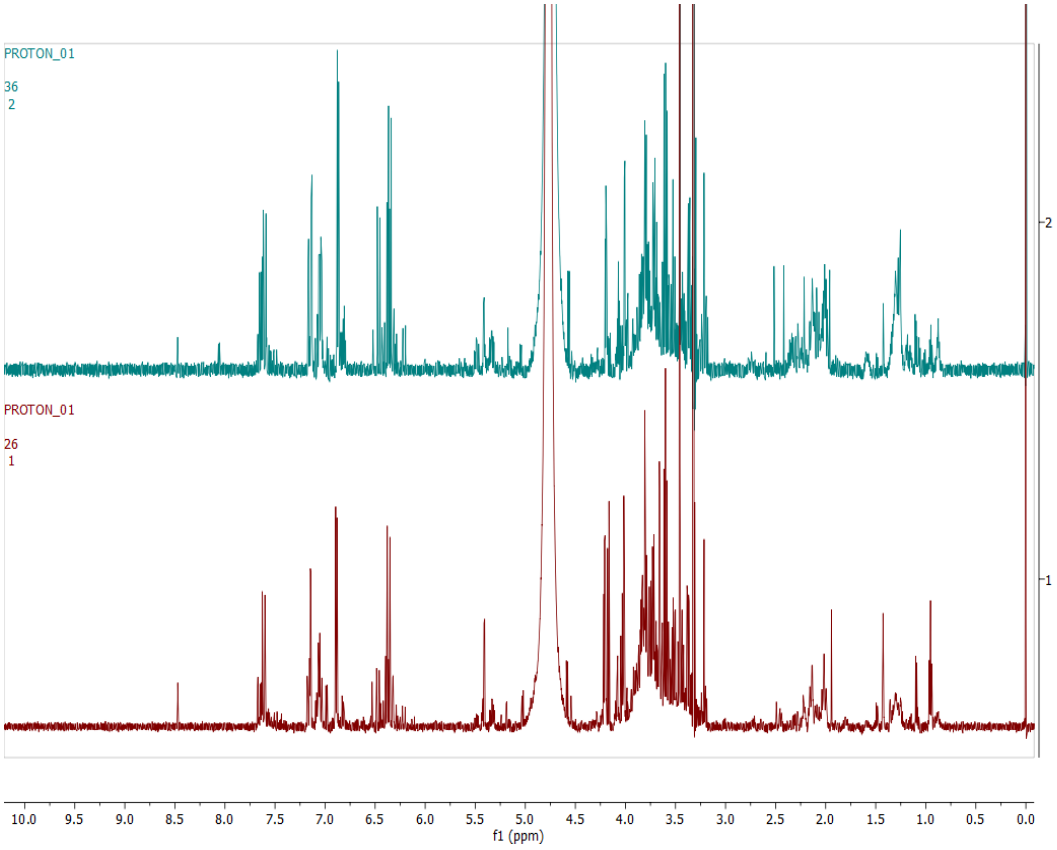

Figure 5.6. 600 MHz ^{1}H-NMR spectra of *H. helichrysum* leaf extracts showing comparison between samples harvested from dry sites in the autumn season across the two locations. Red- autumn season, site 3 (dry) Telperion. Light Blue- autumn season, site 3 (dry) Wakefield.

5.3.2. Chemical profile *of H. aureonitens* extracts collected in wet and dry sites from both Telperion and Wakefield during spring and autumn seasons.

Figure 5.7 shows the supervised multivariate analysis model (OPLS-DA) of hydroalcoholic extracts of *H. aureonitens* leaf samples collected from the wet and dry sites at Telperion during both spring and autumn seasons. The dry and wet sites in spring separate into different clusters, while the dry and wet sites show less variation during autumn collection with samples clustering together.

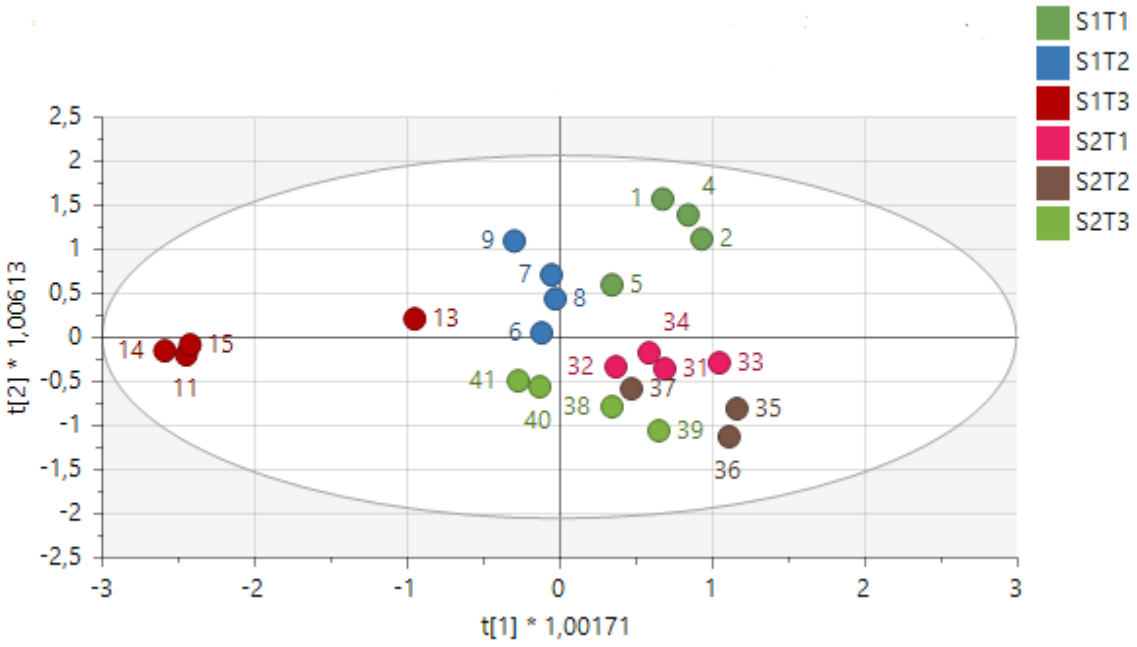

Figure 5.7: OPLS-DA score scatter plot of *H. aureonitens* leaf extracts collected from the wet and dry sites at Telperion during both spring and autumn seasons. $R^2X = 0.948$, $R^2Y = 0.715$ and $Q^2 = 0.024$. Green (S1T1) = spring site 1(wet); blue (S1T2) = spring site 2 (wet); red (S1T3) = spring site 3 (dry); pink (S2T1) = autumn site 1 (wet); brown (S2T2) = autumn site 2 (wet); olive (S2T3) = spring site 3 (dry).

Figure 5.8 shows representative ^{1}H-NMR spectra of *H. aureonitens* plant extracts demonstrating the chemical profile of wet sites compared to the dry site during spring season in Telperion. The peaks in the ^{1}H-NMR spectra are remarkably different by visual observation. The peaks in the aromatic region (6.5-8.0 ppm) of the wet site was much more pronounced as compared to the spectra of the extracts from the dry site which are almost not detectable. In contrast the peaks in the aliphatic region are more pronounced in the samples from the drier sites.

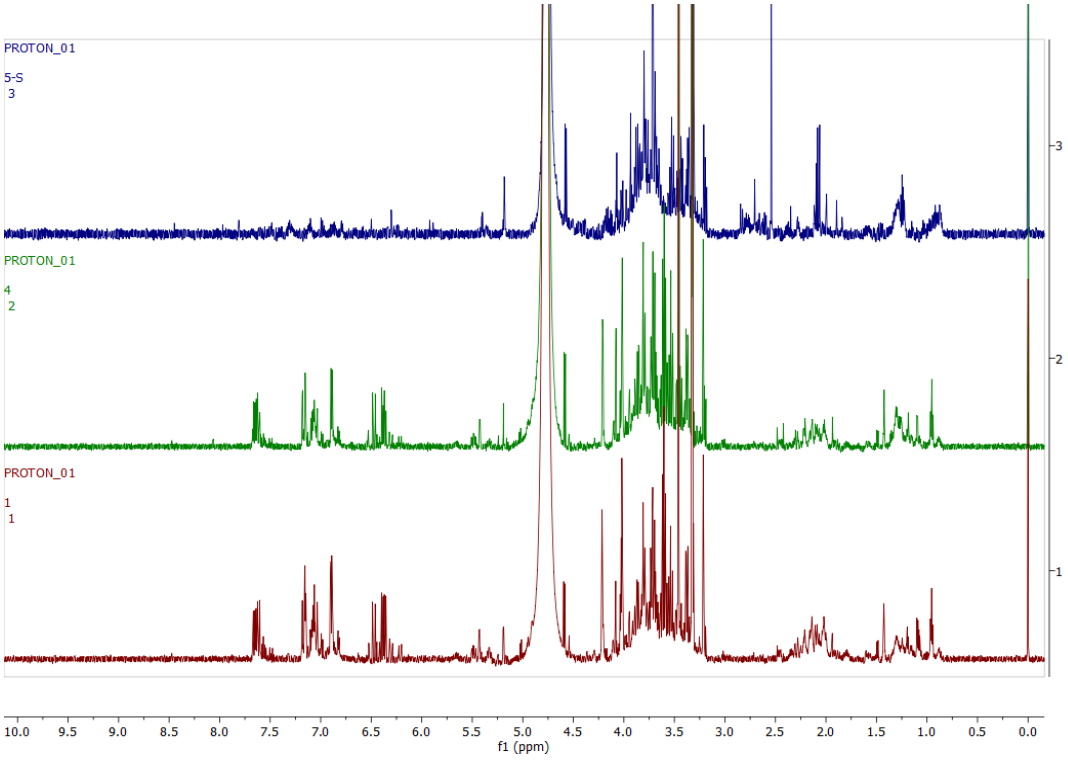

Figure 5.8: The 600 MHz ^{1}H-NMR spectra of *H. aureonitens* leaf extracts collected from the wet and dry sites at Telperion during spring season. Red = site 1 (wet), Green = site 2 (wet), Blue = site 3 (dry).

Figure 5.9 shows the ^{1}H-NMR spectra of the leaves collected from the wet and dry sites at Telperion during autumn season. A visual observation of the stacked spectra shows that the peaks in all the samples are very similar for both the aromatic and aliphatic regions.

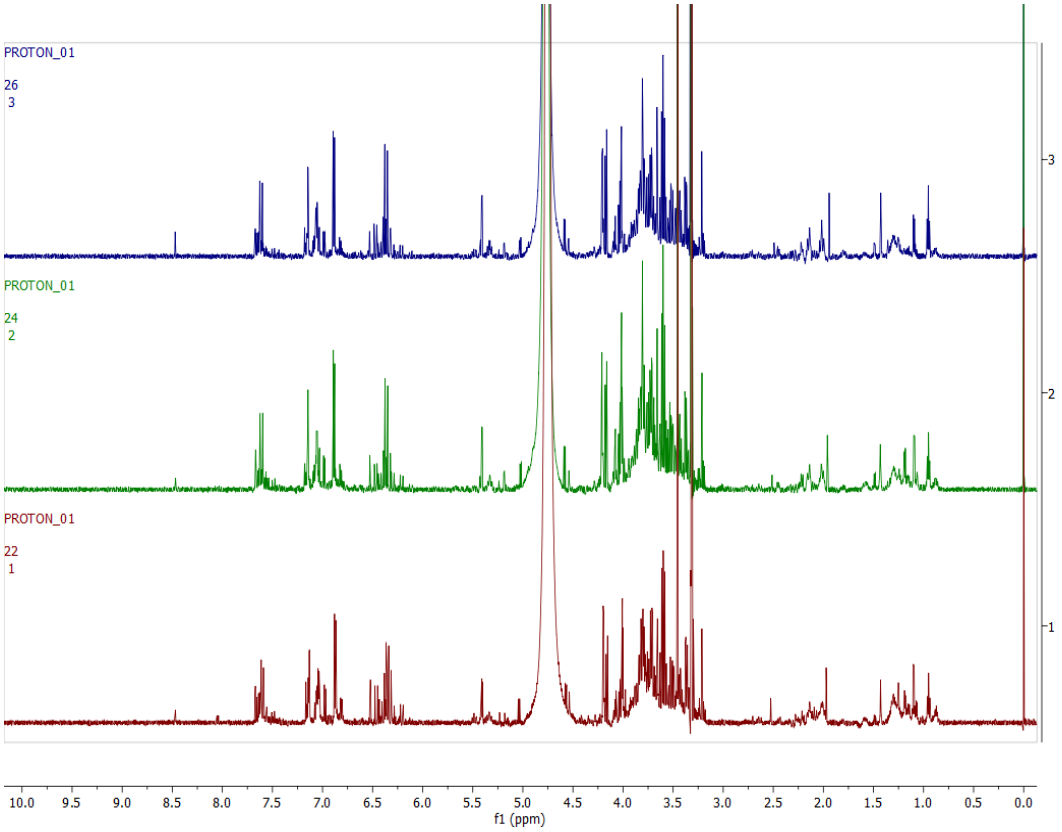

Figure 5.9: The 600 MHz ^{1}H-NMR spectra of *H. aureonitens* leaf extracts collected from the wet and dry sites at Telperion during autumn season. Red = site 1 (wet), Green = site 2 (wet), Blue = site 3 (dry).

Figure 5.10 shows representative ^{1}H-NMR spectra of *H. aureonitens* plant extracts demonstrating the chemical profile of wet sites compared to the dry site during spring season in Wakefield. The peaks in the aromatic region (6.5-8.0 ppm) of site 1 (wet) is significantly different from the peaks in site 4 (dry) by visual observation. Peaks in site 2 (wet but not as wet as site 1) are slightly similar to peaks in site 3 (dry but not as dry as site 4). The two sites with contrasting moisture i.e., sites 1 and 4 show noteworthy difference indicating presence of more bioactive compounds in the wet site.

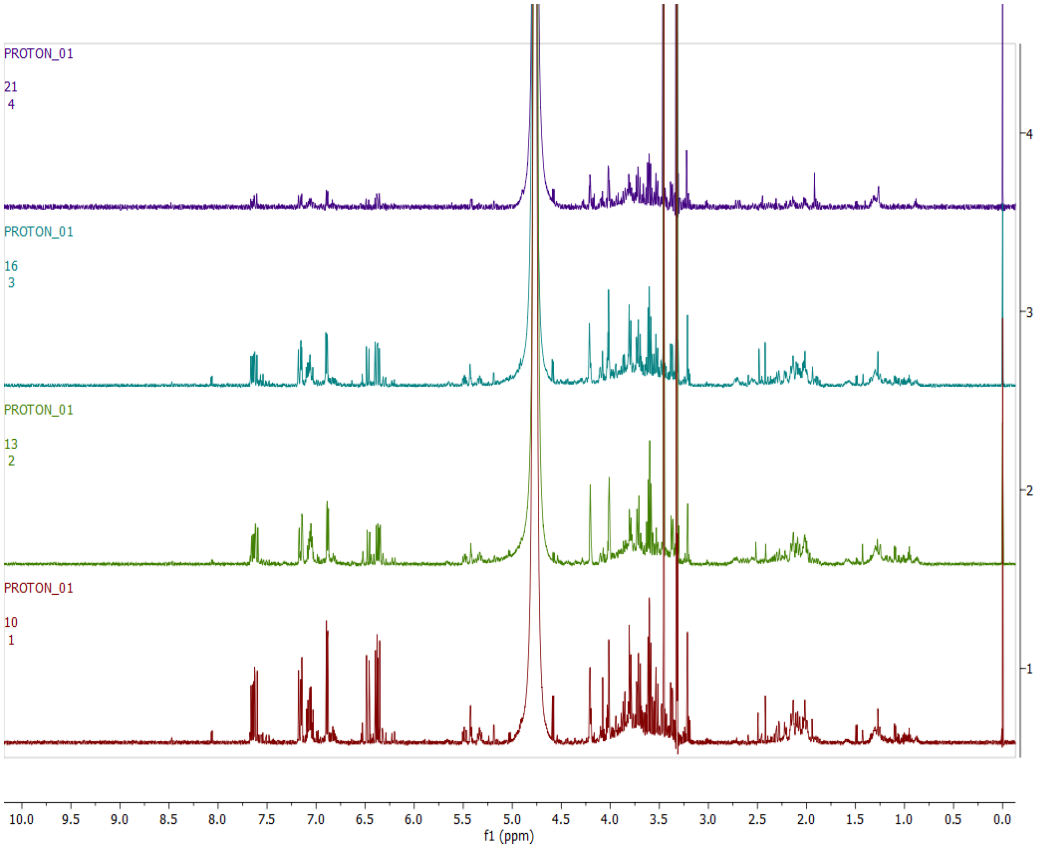

Figure 5.10 The 600 MHz ^{1}H-NMR spectra of *H. aureonitens* leaf extracts collected from the wet and dry sites at Wakefield during spring season. Red =site 1 (wet), Green = site 2 (wet), Blue = site 3 (wet), Purple = site 4 (dry).

Figure 5.11 shows representative ^{1}H-NMR spectra of *H. aureonitens* plant extracts demonstrating the chemical profile of wet sites compared to the dry site during autumn season in Wakefield. The peaks from all four spectra from all the sites are close, similar to the observation at the Telperion location during autumn season.

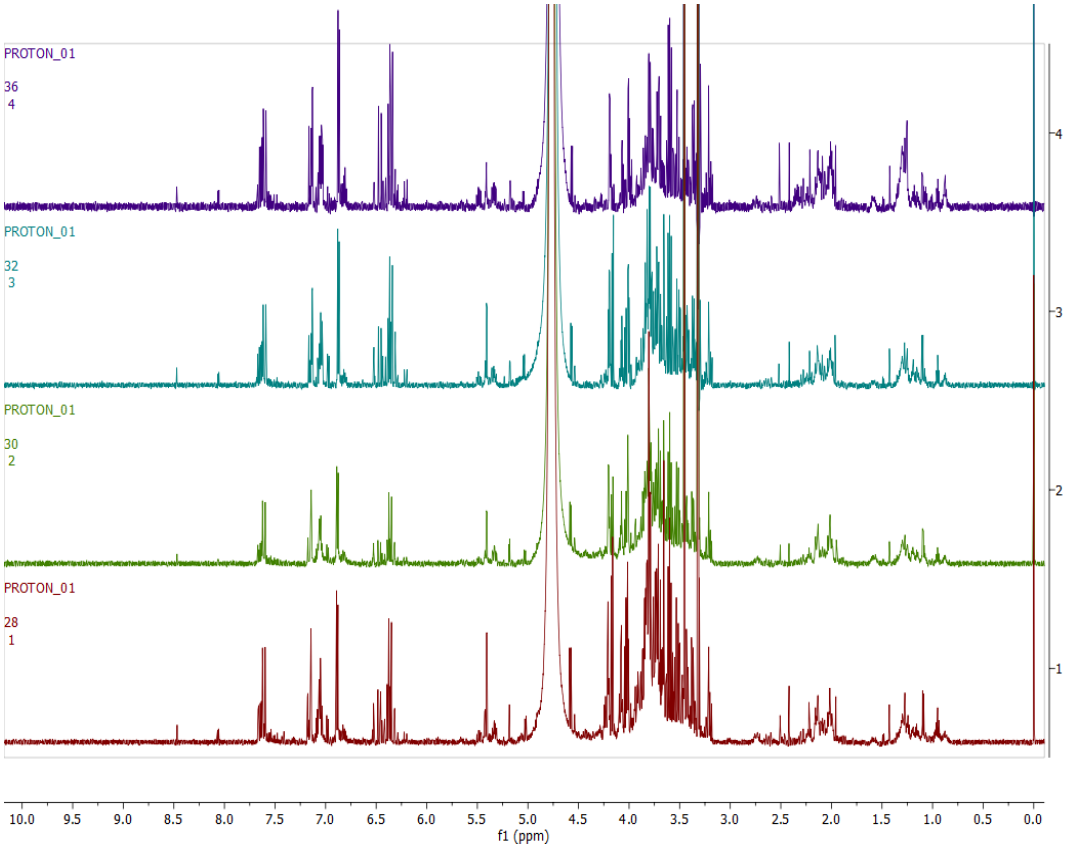

Figure 5.11 The 600 MHz ^{1}H-NMR spectra of *H. aureonitens* leaf extracts collected from the wet and dry sites at Wakefield during autumn season. Red = site 1 (wet), Green = site 2 (wet), Blue = site 3 (dry), Purple = Site 4 (dry).

The aromatic region showed significant differences in height of the peaks for the wet and dry sites in spring. These areas were therefore considered for annotation of compounds which are presented in Table 5.3. The annotation was done by comparing the compound peaks of each compound to the representative compound peaks in Chenomx profiler and the Human Metabolome Database (HMDB). The annotation of the compounds and their representative compound peaks are presented in Figure 5.12

Table 5.3: ^{1}H NMR peaks (ppm) of annotated compounds that contributed to the observed separation of extracts collected from Wakefield in both seasons as it differs from Telperion collections across both seasons.

Compound	**^{1}H-NMR Chemical shift**	**Referenced ppm**	**Literature**
Galangin	8.06, 7.58, 7.49, 6.54, 6.28	8.25, 7.49 – 7.57, 6.55, 6.28	(Afolayan & Meyer, 1997)
Chlorogenic acid	7.56, 7.06, 7.03, 6.83, 6.22, 5.17, 3.95, 2.14, 1.97	7.66, 7.18, 7.0, 6.9, 6.4, 5.3, 3.86, 1.74 – 2.04	(López-Martínez et al., 2015)
Kaempferol	8.06, 6.94, 6.44, 6.20	8.03, 6.93, 6.43, 6.18,	(Kim et al., 2015)
Quercetin	7.66, 7.54, 6.88, 6.20	7.67, 7.53, 6.88, 6.40, 6.18	(Kim et al., 2015)

Figure 5.12 shows peaks of annotated metabolites in table 5.3 above. Representative peaks of galangin, chlorogenic acid, kaempferol and quercetin are indicated in different colours.

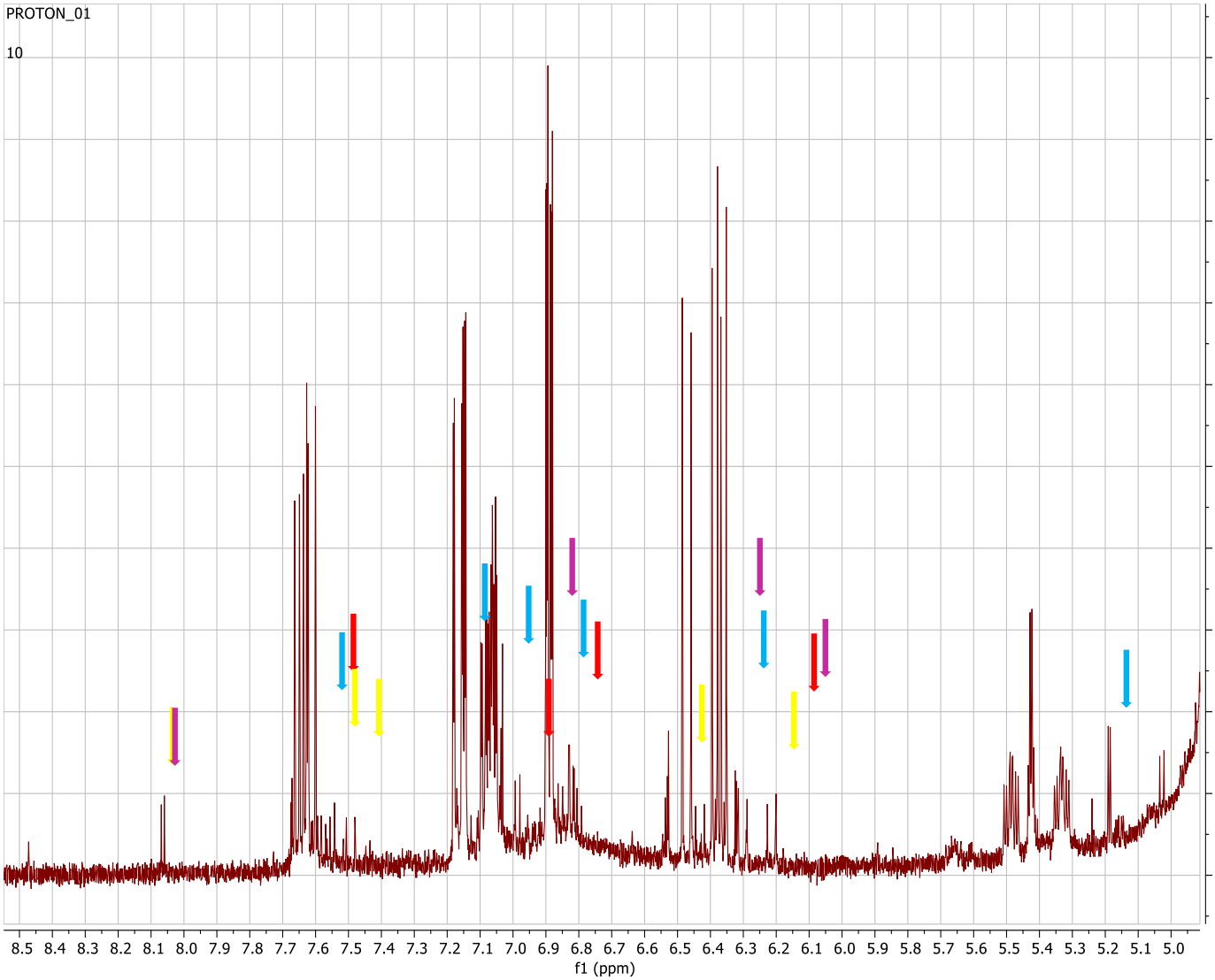

Figure 5.12 Peaks of the annotated compounds indicated for each of the compounds. Yellow = galangin (8.06, 7.58, 7.49, 6.54, 6.28 ppm), purple = kaempferol (8.06, 6.94, 6.44, 6.20 ppm), blue = chlorogenic acid (7.56, 7.06, 7.03, 6.83, 6.22, 5.33 ppm) and red = quercetin (7.66, 7.54, 6.88, 6.20 ppm).

5.4 DISCUSSION

Due to the economic importance and the many medicinal uses of *H. aureonitens*, determining the effect of varying climatic conditions provides insights into the harvesting periods/conditions or seasons that will yield the highest amounts of phytochemicals and the best possible geographical locations, which information is critical to the maximization of its medicinal potential. There is, however, a scarcity of data comparing seasonal metabolite

changes in *H. aureonitens*. This is also important given that climate change is generally creating drier and warmer locations, which might impact significantly the medicinal and biological activities of plants used for medicinal purposes.

A PCA was performed to determine whether there was an observable clustering pattern. The PCA showed loose clustering of the samples based on season (Figure 5.1). Further analysis was carried out using the supervised OPLS-DA model to obtain clear seasonal class groupings between samples across seasons and collection locations (Figure 5.2A). Figure 5.3 showed that the wet collections sites in spring at Telperion had much lower concentrations of the aromatics than the wet collection sites of Wakefield in the spring. Much lower concentrations of aromatics were observed for the dry sites in spring at both locations, although the concentrations of aromatics were significantly lower for Telperion (Figure 5.4). This could be explained by the slightly higher rainfall in August–October and lower temperature for Wakefield compared to Telperion (Table 3.1). When spectra from the wet sites in both Telperion and Wakefield during the autumn season were compared, no conspicuous margin was seen (Figure 5.5), indicating that the lower rainfall and similar lower temperatures resulted in lower concentrations of aromatic compounds at both sites (Table 3.1). The heights of the peaks of the aromatic compounds were similar to those for the dry conditions at Wakefield in spring.

Figure 5.7 clearly shows the separation of the dry location from the wetter locations in spring at Telperion. However, this was not observed for the autumn collection, as the dry site clustered with the wetter location, indicating a change in chemical profile as a result of water availability.

At Telperion in spring, the two wet collection sites showed much higher concentrations of aromatics than the dry collection site (Figure 5.8). This observation was the same for the dry collection site in autumn, which also showed similar lower concentrations of aromatics (Figure 5.9), thereby supporting the clustering observed in Figure 5.7. This, again, supports the notion that higher rainfall might be conducive to aromatic production in *H. aureonitens*.

Similarly, the dry collection sites at Wakefield showed much lower concentrations of the aromatics than the wet collection site in spring (Figure 5.10). Once again, low concentrations of aromatics for the wet and dry sites at Wakefield during autumn, as shown in Figure 5.11, are congruent with the observation at Telperion, where low concentrations of aromatics were also observed at both wet and dry sites.

Several studies have linked increased rainfall to increase in the production of chlorogenic acid (Liu et al.,2016; Rihan et al.,2017). In a recent study on the effects of increase in atmospheric CO2 and other climatic variations on phenolics in coffee trees, (Batista et al., 2021) reported that phenolic levels were positively correlated with the rainy season. The report further showed that chlorogenic acid concentration, in particular, was reduced during the dry season. In another study conducted on walnut leaf samples by (Amaral et al., 2008), the authors reported a phenomenal increase in phenolic compounds, which included 3-caffeoylquinic acid, when walnut leaf samples from nine different cultivars were investigated for their phenolic compounds. The considerable changes observed between three consecutive production years were attributed to climatic factors, mainly temperature and rainfall. Samples harvested from the year with the highest amount of rainfall (2002) showed a surge in the production of caffeoylquinic acid and other phenolic compounds.

5.5 CONCLUSION

An understanding of the distribution of secondary metabolites as influenced by seasons and different growing locations in *H. aureonitens* in this study adds to our understanding of the phytochemistry of this plant. The synthesis of primary and secondary metabolites in the leaves of *H. aureonitens* changed in response to seasonal variations as established by the NMR-based metabolomics approach. Changes in season particularly played a significant role in the production of aromatics in *H. aureonitens* with the highest concentration largely found in the wet collection sites in autumn and in all collection sites (wet and dry) during spring. A comparison between the aromatics content of *H. aureonitens* growing in two geographically diverse locations also showed that the levels of aromatics is favoured in the wetter geographical location-Wakefield, Midland KwaZulu-Natal. The study therefore concludes that aromatics is positively associated with rainy season and lower temperatures in *H. aureonitens*. Compounds such as chlorogenic acid, quercetin and kaempferol are compounds with numerous reports on their biological activity, and therefore important for the plants' use in medicinal preparations. Information on the environmental conditions that are different for various collection times and locations are therefore important, and changes to warmer and drier climate will decrease aromatic compounds production in *H. aureonitens*.

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CHAPTER SIX

LC-MS ANALYSIS OF THE CHLOROGENIC ACIDS CONTENT OF *HELICHRYSUM AUREONITENS* GROWING IN TWO DIFFERENT REGIONS AND SEASONS IN SOUTH AFRICA

ABSTRACT

Helichrysum aureonitens is a perennial plant and one of the 240 species of the genus *Helichrysum* that are native to southern Africa. It is an important medicinal plant for treating a wide range of infections and also has an extensive use in traditional medicine to invoke the goodwill of ancestors and inducing of trances by diviners. Secondary metabolite profiles in plants provide a unique opportunity to investigate seasonal variation and environmental responses. The phenolic content of medicinal plants which contains chlorogenic acids (CGA), has been linked to a variety of pharmacological effects. The *Helichrysum* genus is particularly known for its wide range of chlorogenic acid compounds, and earlier research has found CGA in a number of species. Since little is known with regards to the effect of environmental factors on the chemical profile of *H. aureonitens*, this study therefore uses ultra-performance liquid chromatography-quadrupole time-of-flight mass spectroscopy (UPLC-qTOF-MS) analysis, specifically focusing on the metabolic profile variations of the three derivatives of chlorogenic acids-caffeoylquinic acid (CQA), dicaffeoylquinic acid (DCQA) and tricaffeoylquinic acid (TCQA) in harvested *H. aureonitens* plants growing in two diverse geographical climates and two different seasons to reveal both the identity and abundance of the compounds. The results show that dicaffeoylquinic acid (DCQA) is the most abundant, with higher concentrations in both regions and seasons. 3-CQA was also shown to be the most prevalent isomer of caffeoylquinic acid in this investigation. This large data set provides the opportunity to explore environmental dynamics that are represented in the chemical profile of climatically diverse geographical locations.

Keywords: *Helichrysum aureonitens*; UPLC-qTOF-MS; seasonal variations; chlorogenic acids

6.1 INTRODUCTION

Helichrysum aureonitens, otherwise known as golden everlasting is widespread in KwaZulu-Natal, North West, Gauteng, Mpumalanga and Free State provinces, and the northern regions of the Eastern Cape in South Africa. Flowering occurs from September to February and woolly hairs are present on both sides of the leaves. The plant also has involucral bracts with a variety of colours ranging between brown and yellow. These plants like damp meadows, have a grey colour, and are widely distributed, yet they resemble other species, particularly *H. psilolepsis* (Pooley, 2003). Whole plant parts of *H. aureonitens* are used as incense in traditional medicine to induce trances. Because of its antifungal, antibacterial and antiviral properties, the leaves, and stems are also used to reduce urinary incontinence in children, to treat skin diseases like herpes simplex, and to treat other microbial infections (Afolayan & Meyer, 1997; Hutchings & van Staden, 1994; Mathekga & Meyer, 1998; Meyer et al., 1996; Pooley, 2003).

CGA have been reported to have antioxidant, anti-inflammatory, anti-HIV, anti-HBV, anti-diabetes, and carcinogenic properties and as such are regarded as greatly beneficial to the health of humans (Hemmerle et al., 1997; Kweon et al., 2001; Kwon et al., 2000; Wang et al., 2009). As common secondary metabolites in plants, CGA are found in abundance in coffee, tea, potatoes, and a variety of other vegetables and fruits. The main source of CGAs in the human diet is mainly coffee beans and commercial coffee products (Clifford et al., 2003).

The chemical profile of medicinal plants determines their activities (Soni et al., 2015). Nevertheless, different seasons of the year may determine the availability of some precursors that the plant requires for the production of active components, either directly or indirectly (Kim et al., 2015). Active metabolites in plants are the outcome of long-term interactions between plants and their environment, and their production and modifications have a significant relationship and association with the environment (Blanch et al., 2007). Some compounds can only be synthesized under certain conditions, or there can be an upsurge in the levels of some compounds in specific environmental conditions (Blanch et al., 2007). Medicinal plants' active compounds have been studied in relation to their growth environment. For instance, in *Eucommia ulmoides* Oliv., altitude and annual mean temperature were significantly and positively correlated with the contents of chlorogenic acid and flavonoids ($P < 0.05$); annual sunshine duration was significantly and positively correlated with the content of geniposidic acid ($P< 0.05$); and annual mean temperature was significantly and negatively correlated with the content of geniposidic acid ($P < 0.05$) (Dong et al., 2011).

LC-MS analysis has become a standard approach for exploring the quantity, quality, and chemical variety of plant metabolites as a result of recent technological and methodological breakthroughs in both liquid chromatography (LC) and mass spectrometry (MS). The goal of targeted LC-MS metabolite analysis is to detect and quantify the target metabolites of interest (Shimizu et al., 2018).

Although CGA has been reported in a number of *Helichrysum* species including *H. aureonitens* (Albayrak et al., 2010; Heyman et al., 2015; Vujić et al., 2020; Yazdi et al., 2019), no study has been conducted to determine the specific derivatives of CGA present in *H. aureonitens* leaves in response to seasonal variations and different geographical locations. In this study, LC-MS analysis was used to investigate the metabolic composition and variations of *H. aureonitens* leaves harvested during the spring and autumn season and growing in different sites in two dissimilar climatic regions, Wakefield farms, KwaZulu-Natal, Midlands and Telperion farms, Mpumalanga provinces of South Africa. The results showed that dicaffeoylquinic acid was the most abundant derivative of chlorogenic acid during both seasons and all the collection locations.

6.2 MATERIALS AND METHODS

6.2.1 Plant material collection

As reported in section 3.2.1

6.2.2. Chemical profile determination

An established method was used, proven to separate the different chlorogenic acids in plant extracts (Clifford et al., 2003). Reference standards for CQA, DCQA and TCQA of these compounds were used for identification. Dried leaves of *H. aureonitens* (50 mg) were pulverized and extracted with 1.5 mL of 80% methanol (LC-grade and ultrapure LC-grade water). Extracts were later homogenized and sonicated in an ultrasonic bath for 5 minutes followed by centrifugation of the homogenates for 15 minutes at 15,000 rpm. Each sample was filtered through a 0.22-micron nylon syringe filter (Sartorius Minisart RC 4), and the filtrate concentrated by evaporation to dryness. The dried extract was reconstituted in 300 µL of 50% methanol and pipetted into HPLC glass vials (2 mL). Before analysis, aliquots of extracts were produced in triplicates and kept at -20°C.

6.2.3. Ultra-performance liquid chromatography (UPLC) analysis

A Waters Classic UPLC, coupled in series to a Waters SYNAPT G1 HDMS mass spectrometer was used to generate full scan accurate mass data. Optimization of the chromatographic separation was done utilizing a Waters HSS T3 C18 column (150 mm x 2.1 mm, 1.8 µm) and the column temperature controlled at 60°C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (natural pH of 2.4) and acetonitrile (Eluent B) containing 10 mM formic acid. The initial conditions were 100% A at a flow rate of 0.4 mL/min and were maintained for 1 minute, followed by a linear gradient to 1% A at 15 minutes. These conditions were kept constant for 2 minutes and then changed to the initial conditions. The runtime was 20 minutes, and the injection volume was 1 µL. Samples were kept cool at 6°C in the Waters Sample Manager during the analysis.

6.2.4 TOF Mass Spectrometer analysis

The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray mode to enable detection of all ESI-compatible compounds. Leucine enkephalin (50 pg/mL) was used as a reference calibrant (Lock Mass) to obtain typical mass accuracies between 1 and 5 mDalton (mDa). The mass spectrometer was operated in both ESI positive and negative modes with a capillary voltage of 2.5 kV, the sampling cone at 30 V, and the extraction cone at 4.0 V. The scan time was 0.1 seconds covering the 50 to 1200 Dalton mass range with an interscan time of 0.02 second. The source temperature was 120 °C and the desolvation temperature was set at 450 °C. Nitrogen gas was used as the nebulization gas at a flow rate of 550 L/h and cone gas was added at 50 L/h. Argon was used as collision gas during fragmentation experiments. The software used to control the hyphenated system and do all data manipulation was MassLynx 4.1 (SCN 872). Compound identification was further enhanced by analysing all samples with low and high collision energy settings of the collision cell. To minimize compound fragmentation a low energy setting of 3 V was used, but to enhance fragmentation of molecules, five different collision energy profiles between 10 – 50 V were used (MS^e).

6.3 RESULTS

6.3.1. Chlorogenic acids profile

Chlorogenic acids (CGA) are a type of natural substance that can be found in a variety of plant species, and as such are of special interest in many research studies. Since the main CGA represented in nature is caffeoylquinic acid (CQA), it is therefore often used as a quality control indicator for a variety of natural products (Gil and Wianowska, 2017). On that account, and on the presence of these compounds in *Helichrysum* species as reported in previous studies (Gradinaru et al., 2014; Grinev et al., 2016), this study hence focused on evaluating specifically CQA distribution and its derivatives of other classes of compounds namely dicaffeoylquinic acids (DCQA) and tricaffeoylquinic acids (TCQA) in the chemical profile of *H. aureonitens*. Given that a variety of mechanisms may be involved in plant metabolite production, this study investigated whether CQA accumulation in *H. aureonitens* is affected by seasonal fluctuations and the species' growing site. The extracts were treated to ultra-performance liquid chromatography-quadrupole time-of-flight mass spectroscopy (UPLC-qTOF-MS) and Table 6.1 lists the compounds from the extracts that have been detected. Eleven isomers of CGA from three derivatives of CQA were detected in this study, four isomers of CQA (monocaffeoylquinic acids), six isomers of DCQA and one isomer of TCQA.

Table 6.1: Chlorogenic acids composition of *H. aureonitens*.

Classification	Number	Retention Time (Min)	Compounds	Calculated Empirical formula	Calculated Mass	Detected Mass	Mass Accuracy (mDa)	DBE count	MS/MS Fragmentation ions
CQA	1	2.79	5-CQA (Trans)	$C_{16}H_{18}O_9$	354.0951	353.0860	1.3	8	353.1; 191.1;
	2	3.47	3-CQA	$C_{16}H_{18}O_9$	354.0951	353.0865	0.8	8	353.1; 191.1; 179.0; 135.0
	3	5.73	5-CQA (Cis)	$C_{16}H_{18}O_9$	354.0951	353.0853	2.0	8	353.1; 191.1;

	4	5.96	4-CQA	$C_{16}H_{18}O_9$	354.0951	353.0859	1.4	8	353.1; 191.1; 173.0; 135.0
DCQA	1	6.15	3,4-DCQA (Trans)	$C_{25}H_{24}O_{12}$	516.1268	515.1207	1.7	14	515.1; 353.1; 173.1; 335.1
	2	7.27	3,5-DCQA (Trans)	$C_{25}H_{24}O_{12}$	516.1268	515.1203	1.3	14	515.1; 353.1; 191.1
	3	8.79	3,4-DCQA (Cis)	$C_{25}H_{24}O_{12}$	516.1268	515.1181	1.1	14	515.1; 353.1; 173.1; 335.1
	4	8.95	3,5-DCQA (Cis)	$C_{25}H_{24}O_{12}$	516.1268	515.1180	1.0	14	515.1; 353.1; 191.1
	5	9.08	4,5-DCQA (Trans)	$C_{25}H_{24}O_{12}$	516.1268	515.1177	1.3	14	515.1; 353.1; 335.1; 173.1
	6	9.22	4,5-DCQA (Cis)	$C_{25}H_{24}O_{12}$	516.1268	515.1200	1.0	14	515.1; 353.1; 335.1; 173.1
TCQA		10.14	TCQA 2	$C_{34}H_{30}O_{15}$	678.1585	677.1520	1.3	20	677.2; 515.1; 353.1; 335.1; 173.1

Reference sources: Clifford et al., 2003 and Ramabulana et al., 2020.

6.3.2. Integration values comparison

The following figures (Figures 6.1-6.5) show the comparison between integration values of isomers of CQA, DCQA and TCQA across locations and seasons, with the X-axis presenting each compound of the isomers, while the Y-axis gives the integration values for the compounds. The integration values represent the concentration in quantity of each isomer and derivatives of chlorogenic acids in this study. They are depicted by the numerical data labels on each of the bars in the graph.

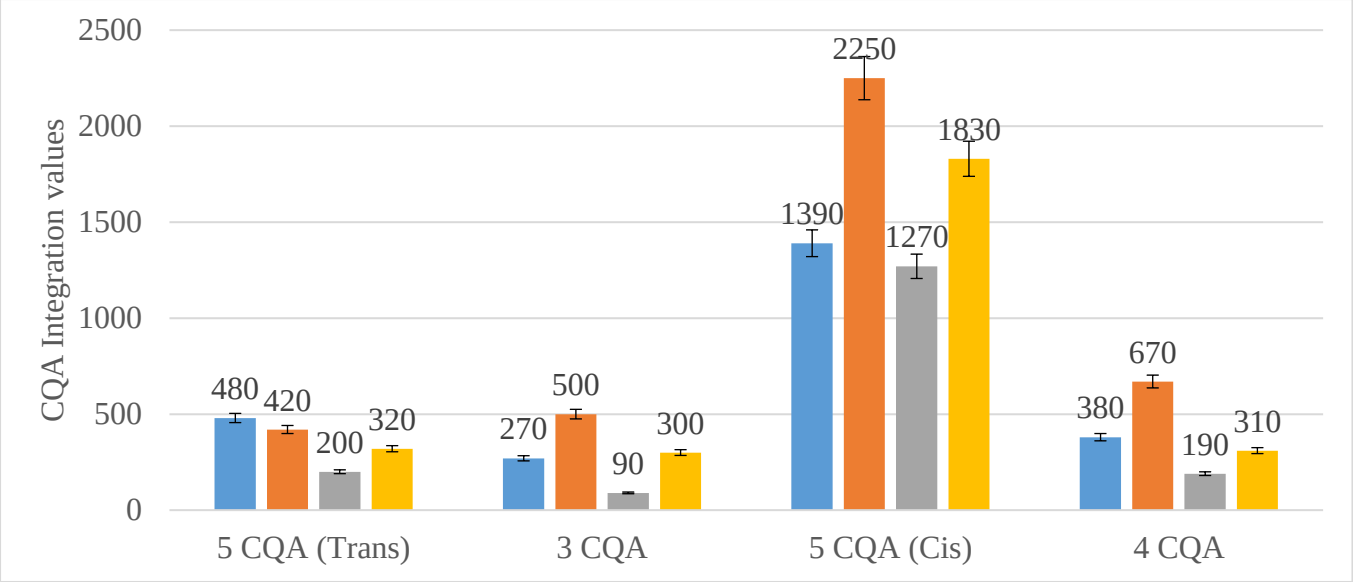

Figure 6.1: CQA isomers mean concentration comparison between wet sites in each of the two locations in both seasons. Blue-wet site spring (Telperion), Orange-wet site spring (Wakefield), Grey-wet site autumn (Telperion), Yellow-wet site autumn (Wakefield).

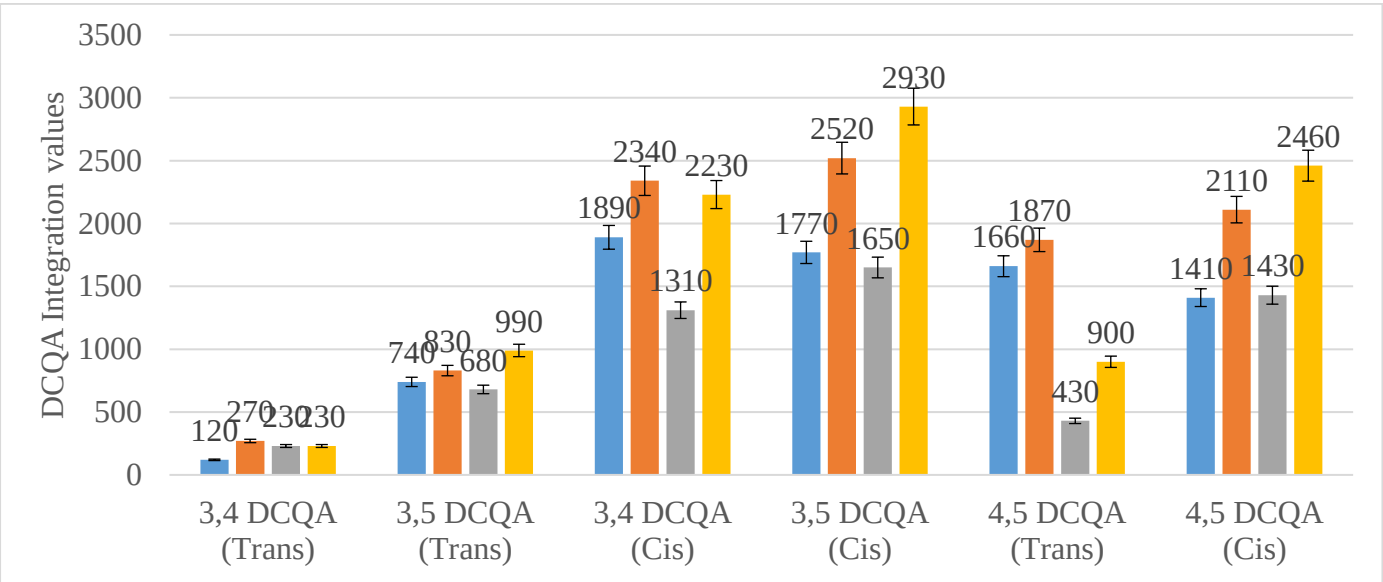

Figure 6.2: DCQA isomers mean concentration comparison between wet sites in each of the two locations in both seasons. Blue-wet site spring (Telperion), Orange-wet site spring (Wakefield), Grey-wet site autumn (Telperion), Yellow-wet site autumn (Wakefield).

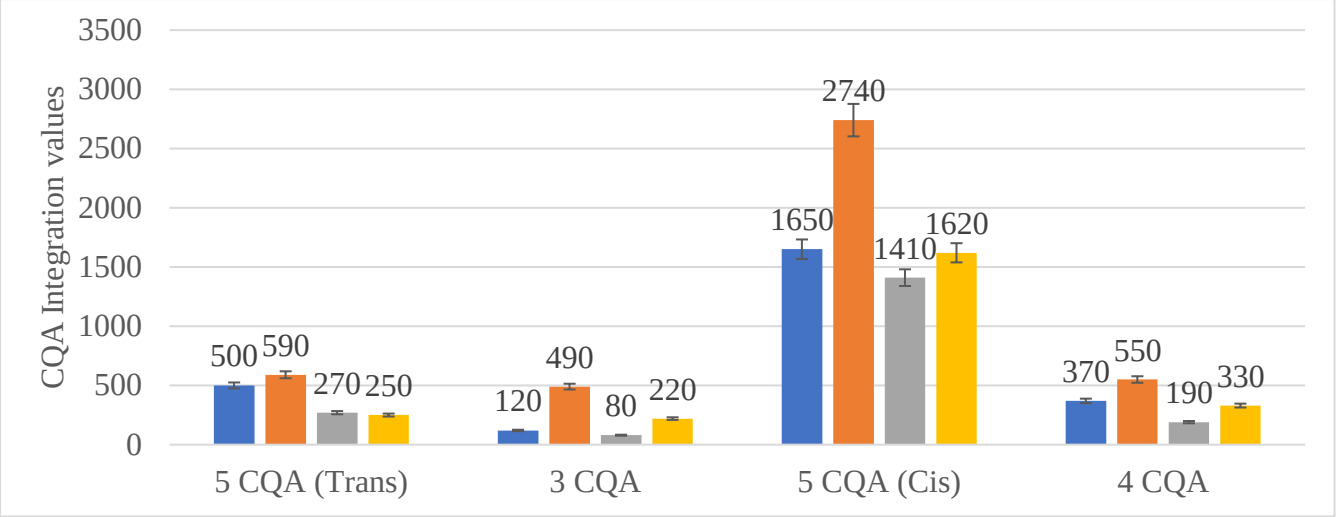

Figure 6.3: CQA isomers mean concentration comparison between wet and dry sites in each of the two locations in autumn. Blue-wet site autumn (Telperion), Orange-wet site autumn (Wakefield), Grey-dry site autumn (Telperion), Yellow-dry site autumn (Wakefield).

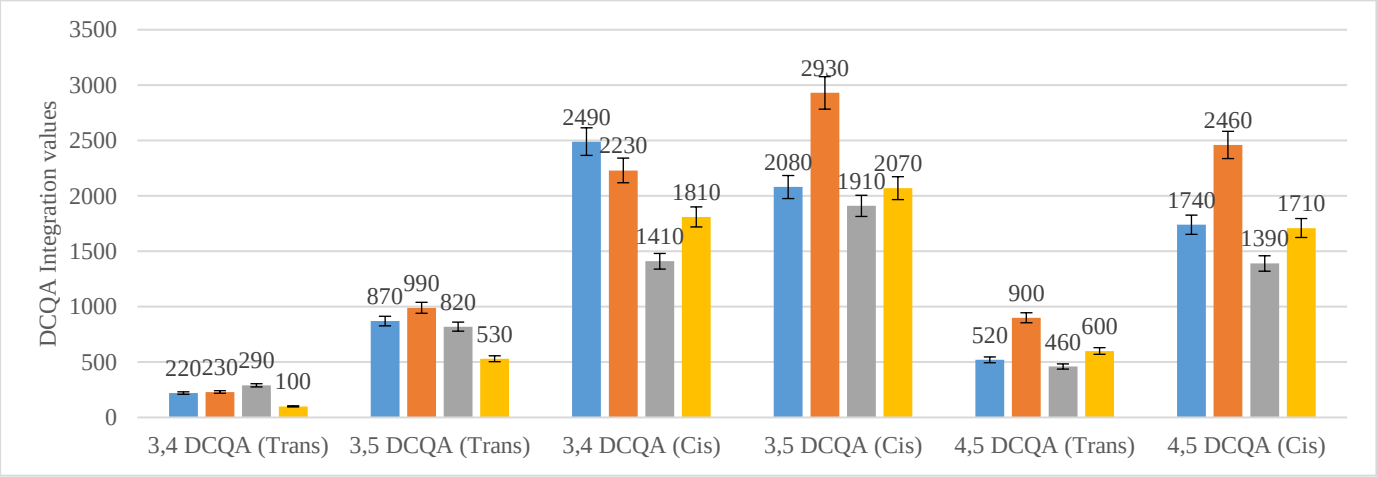

Figure 6.4: DCQA isomers mean concentration comparison between wet and dry sites in each of the two locations in autumn. Blue-wet site autumn (Telperion), Orange-Wet site autumn (Wakefield), Grey-dry site autumn (Telperion), Yellow-dry site autumn (Wakefield)

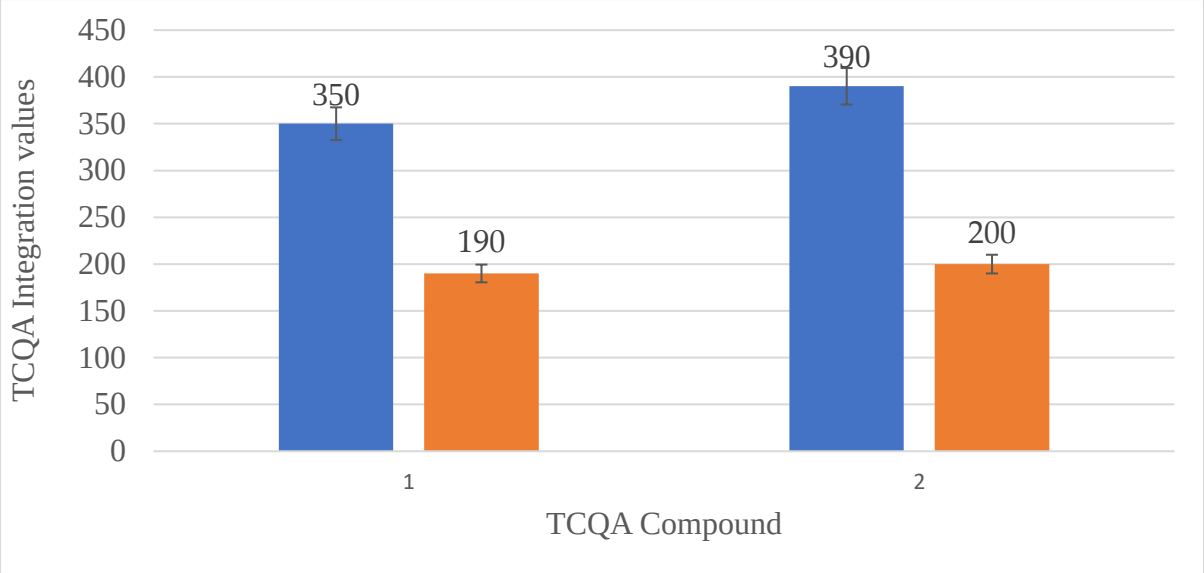

Figure 6.5: TCQA isomers mean concentration comparison between wet and dry sites from Telperion and Wakefield in autumn. 1= Blue-Wet site Telperion and Orange-Dry site Telperion, 2= Blue-Wet site Wakefield, and Orange-Dry site Wakefield.

6.4 DISCUSSION

The *Helichrysum* genus is generally known for its variety of chlorogenic acids compounds and previous studies have highlighted the presence of CGA in many of the species (Albayrak et al., 2010; Heyman et al., 2015; Babotă et al., 2018). Chlorogenic acids, more accurately referred to as 3-CQA as per IUPAC guideline is a caffeoylquinic acid and the most abundant isomer among the other caffeoylquinic acid isomers (3-, 4-, and 5-CQA) (Naveed et al., 2018). Other derivatives of CGA accessed in this study are dicaffeoylquinic acid (DCQA) and tricaffeoylquinic acid (TCQA). Eleven caffeoylquinic acid compounds, belonging to three derivatives were identified. The first derivative which is monocaffeoylquinic acid, CQA has four isomers, the second derivative, DCQA has six isomers while the last derivative TCQA has only one isomer. Although the presence of chlorogenic acid has been confirmed in *H. aureonitens* by a number of studies, no study has however delineated the specific derivatives of chlorogenic acid that are present in the species or the most abundant of these derivatives. This study determined the various caffeoylquinic acid compounds in the three derivatives groups of chlorogenic acid in the harvested plants across two seasons (spring and autumn) and in different sites from two geographically diverse locations. LC-MS analysis was used to

determine which of the caffeoylquinic acids was more abundant in response to seasonal changes. With the exception of the first isomer of monocaffeoylquinic acid, 5-CQA (Trans), the rest of the isomers of CQA all have higher concentrations comparatively at Wakefield as compared to Telperion during the spring season while all CQA isomers with no exception have larger concentrations than the Telperion sites in autumn (figure 6.1). A combined comparison between the integration values of all four isomers of caffeoylquinic acid between wet and dry sites from each of the two locations in the autumn season also clearly showed a higher concentration of CQA for all wet sites at both locations, with Wakefield having relatively higher concentrations than Telperion and an observed significant increase in 5-CQA (Cis) concentration (figure 6.3). Apart from an equal 3,4 DCQA integration value for both locations in the autumn season, all other DCQA integration values support higher concentration of DCQA across all the isomers in Wakefield for both seasons, with higher values recorded in spring except for 3,5 DCQA (Trans) and 4,5 DCQA (Cis) (figure 6.4). With the exception of the 3,4 DCQA (Trans) at Telperion, all other values again showed a higher concentration in the wetter sites than the drier sites across the two locations. These evidently show that chlorogenic acids isomers production is increased as conspicuously seen in figures 6.1, 6.2, 6.3, and 6.4 with increased moisture which triggers the production of both CQA and DCQA irrespective of the climatic regions. It is also observed that higher rainfall triggers the production of DCQA and CQA in wetter climatic regions than drier location (figure 6.4).

TCQA levels are fairly the same in the two locations during spring. The TCQA values between wet and dry sites in both locations during autumn are comparable to each other. The wet site recorded higher concentration of TCQA (about double) above the dry site in both Telperion and Wakefield (Figure 6.5).

Of the three derivatives of chlorogenic acids, the most abundant is dicaffeoylquinic acid (DCQA) which has a larger concentration in both locations and in both seasons. Many studies have reported the medicinal activities of different isomers of DCQA. Such activities include, antioxidant properties as observed in the activity of the isomer 1,3-DCQA extracted from the herbaceous plant *Inula viscosa* (Danino et al., 2009), analgesic bioactivity of the DCQA derivative 3,4-*O*-dicaffeoylquinic acid methyl ester isolated from the root of *Calea urticifolia* (Mijangos-Ramos et al., 2018), antidiabetic effects of three isomers of DCQA (3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, and 3,4- dicaffeoylquinic acid methyl ester) all isolated from the leaves of *Artemisia annua* L (El-Askary et al. 2022) and anti-influenza A virus activity of 3,4 DCQA (Takemura et al., 2022). The presence of dicaffeoylquinic acid

isomers in *Helichrysum* species has already been reported by Gouveia and Castilho, (2011) in their work on *H. obconicum* (an indigenous *Helichrysum* species from the Madeira Archipelago), where the fragmentation characterization is described extensively. In the further work of the same team (Gouveia and Castilho, 2012) their study on caffeoylquinic acids separation, quantification and identification in medicinal *Helichrysum* species (*H. devium*, *H. melaleucum*, *H. obconicum*) arrived at similar results where they reported that among the measured hydroxycinnamic acids, dicaffeoylquinic acids isomers were the most prevalent. The most abundant isomer of caffeoylquinic acid recorded in this study is 3-CQA. This is in alignment with reports from studies of other plants (Moeenfard et al., 2014; Alc Azar Maga~ Na et al., 2021).

6.5 CONCLUSION

Given that plant metabolite synthesis can be influenced by a variety of processes, LC-MS analysis confirmed that seasonal fluctuations and the species' growing site affect chlorogenic acid accumulation in *H. aureonitens.* The synthesis of different derivatives and isomers of each derivative in the leaves of *H. aureonitens* changes in response to seasonal variations as established with the UPLC-qTOF-MS analytical platform. Changes in season particularly played a significant role in both DCQA and CQA production in *H. aureonitens* with the highest concentration largely found in the wet season-spring. Even though there are slight differences in the concentration of compounds from both locations during autumn, the chemical profile remained largely the same. A comparison between the CQA content of *H. aureonitens* growing in two geographically diverse locations also showed that the levels of CQA is favoured in the wetter geographical location-Wakefield, Midland in KwaZulu-Natal. The study therefore concludes that chlorogenic acids isomers is increased with increased moisture levels which triggers the production of both CQA and DCQA irrespective of the climatic regions in *H. aureonitens.*

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CHAPTER SEVEN

7.1 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Helichrysum aureonitens is a very important medicinal plant used to treat involuntary urination in children and skin infections mostly associated with herpes simplex virus (Watt, and Breyer-Brandwijk, 1962; Guillamord 1971). It is widespread across South Africa and some neighbouring countries like Lesotho and Mozambique. The ethnopharmacological uses, biological activities and chemistry of *H. aureonitens* as well as other species in the *Helichrysum* genus has been widely studied and well documented. No information is however available to date on the influence of seasonal variation and water availability on the metabolic response of any *Helichrysum* species growing in different climatic locations. Using *H. aureonitens* as a model plant, the study therefore investigated the influence of different seasons and geographical locations on the bioactivity of *H. aureonitens* leaf extracts against the following pathogens: gram-negative bacteria *Proteus vulgaris*, gram-positive bacteria *Bacillus subtilis*, five pathogenic fungal species of the ascomycetes division including *Aspergillus flavus, Aspergillus nomius, Cladosporium cladosporioides, Fusarium oxysporum, Penicillum halotolerans*, and a human virus, the herpes simplex virus type 1 (HSV-1). The study further investigated the link between variation in seasons cum climatic locations and metabolites distribution in the extracts of harvested samples of *H. aureonitens* growing in different sites at each of the different climatic locations. The antimicrobial activities of the plant in different seasons and across dissimilar climatic regions were compared to establish a possible link between bioactivity and chemical profiles.

7.2 ANTIBACTERIAL GENERAL DISCUSSION AND CONCLUSION

The hydroalcoholic extract of *H. aureonitens* harvested from both locations and across the two seasons all showed good activity against the gram-negative bacteria *P. vulgaris* with MIC ranging between 62.5-125 µg/mL for most of the extracts. In comparing the two seasons, activities of extracts collected in the spring season showed better activity at 62.5 µg/mL than the autumn season at both locations.

No activity was however recorded against the gram-positive bacteria *B. subtilis* across locations and seasons with the tested concentrations 31.25, 62.5, 125 and 250 µg/mL (Table 3.1). A few previous studies have reported relatively low activity of medicinal plants against *B. subtilis.*

For instance, the result of the study conducted by Rabe and van Staden (1997) on pharmacological properties of the crude extracts from twenty-one South African medicinal plants showed that twelve out of the tested plants had some activity against *B. subtilis*, with more than half of them showing activity only at a MIC value greater than 250 µg/mL. This further support the resistance of *B. subtilis* to medicinal plants as shown in the current study. Aiyegoro et al, (2009) also reported the activity of another *Helichrysum* species, *Helichrysum longifolium* against a few gram-negative bacteria including *P. vulgaris* at an MIC value of around 100 µg/mL.

7.3. ANTIFUNGAL GENERAL DISCUSSIONS AND CONCLUSION

The result of the study established a strong presence of antifungal compounds in the chemical profile of *H. aureonitens*. Plant materials were tested against five pathogenic fungal species including: *Aspergillus flavus, Aspergillus nomius, Cladosporium cladosporioides, Fusarium oxysporum*, and *Penicillum halotolerans*. None of the plant extracts showed any activity against *A. nomius*. All the other fungal species were however significantly inhibited by varied extract concentrations of *H. aureonitens* with MIC values ranging between 0.39 – 3.125 µg/mL. The study highlights the effects that climatic differences in locations have on the antifungal properties of *H. aureonitens.* Activity was species specific in each of the seasons and locations. The same trend of antifungal activity was observed with collections from both seasons against the species *A. flavus* and *C. cladosporioides*. Collections from Telperion had an overall better activity than Wakefield against both species. This suggests that *H. aureonitens* collected in the drier location provides a chance at producing *H. aureonitens* with higher activity against *A. flavus* and *C. cladosporioides.* Plant collections in spring demonstrated better activity comparatively to autumn against *F. oxysporum*. This indicates that an increase in rainfall is positively associated to the antifungal property of *H. aureonitens* against *F. oxysporum.* Collections at Telperion also had better activity against *F. oxysporum* compared to Wakefield. However, collections made during the autumn season recorded better activity at Telperion than Wakefield against *P. halotolerans*. In summary, *H. aureonitens* plant samples collected from the drier location (Telperion) had better activity against the fungi *A. flavus, C. cladosporioides, F. oxysporum*, and *P. halotolerans* than the wet location (Wakefield), while rainfall had an insignificant effect on the antifungal property of the plant against the tested species except *F. oxysporum*. The antifungal property of *H. aureonitens* was also established against *A. flavus* and two other species of *Cladosporium*, *C. herbarum* and *C. sphaerospermum*

by Afolayan and Meyer (1997). The compound galangin extracted from the plant showed activity at MIC of about 10 µg/mL. Due to lack of available published materials on the effect of seasonal variation on the antifungal properties of *Helichrysum* species, the effect of seasonality on the antifungal property of other medicinal plants were compared. *Psidium salutare* (Kunth) O. Berg is a plant found in Brazil and commonly used both for its medicinal properties and food. In support of the findings of this study with regards to seasonal changes on the antifungal properties of *H. aureonitens* against *A. flavus, C. cladosporioides, F. oxysporum,* and *P. halotolerans*, Macedo et al., (2018) reported that based on their study on the effect of seasonality on the antifungal properties of *Psidium salutare*, they could not establish the influence of any of the three seasonal collection periods on the antifungal properties of the plant. Also in the study on the seasonal variation in the antifungal activity of seven seaweeds from South Africa conducted by Stirk et al., (2007), no effect of season on the antifungal activities of the seaweeds was observed.

7.4 ANTIVIRAL GENERAL DISCUSSIONS AND CONCLUSION

The extracts investigated in this study significantly reduced the HSV infection in Vero cells at 10 µg/ml. Figure 4.1 shows that seventeen of the twenty-six extracts tested had significant anti-HSV activity, as evaluated by a tissue culture infectious dose ($TCID_{50}$) reduction of less than log 10^5. The observed strong activities of the seventeen extracts (those extracts that reduce the viral burden of HSV titre log to a figure below log 10^5) are comparable to the positive control, acyclovir which exhibited an anti-HSV activity with the $TCID_{50}$ value of 10^1. Twelve of the seventeen were extracts from plants collected during the spring season while the remaining five were collected during autumn. Significantly higher antiviral activities of *H. aureonitens* plant extracts were recorded in the spring season in both locations and the extracts' activities showed a twofold increase when compared to the activities of extracts of plants collected in autumn as shown by the significant reduction in the viral load depicted by the HSV titre values represented on the graph. This supports many studies that have reported the influence of seasonal variation on the antimicrobial activities of plants (Ncube et al., 2011; Ramírez-Briones et al., 2019). In this study however, better activity was obtained when more water was available, and therefore favorable conditions, and not due to unfavorable conditions that can cause stress. Further to the ongoing, it is clear that different climatic regions exert influence on the potency of *H. aureonitens* against HSV-1. This is confirmed by a comparison between the activity of extracts from Telperion and Wakefield during the autumn season. While

extracts' activities in spring was the highest and comparatively the same (except for the dry site of Wakefield) in both locations, extracts from autumn demonstrated better activity in the dry sites at Wakefield as compared to the dry sites at Telperion as seen by the reduced HSV titer (figure 4.1). This again confirms that the higher water availability at Wakefield is more favourable to produce antiviral compounds, even in seasons with lower rainfall. A few studies conducted on the antiviral properties of *Helichrysum* species reported varying activities. At concentrations ranging from 12 to 47 μg/ml, galangin, isolated from *H. aureonitens*, demonstrated considerable antiviral activity against a DNA virus, HSV-1, and an RNA virus, Coxsackie B Type-1, while showing no activity against Adenovirus Type-31 (Meyer et al., 1997). Also, ethanolic extracts of *H. arenarium* and *H. armenium* DC showed significant antiviral activities against HSV-1 and PI-3 at concentrations of 2–32 and 4–64 μg/mL, respectively.

7.5 METABOLOMICS GENERAL DISCUSSIONS AND CONCLUSION

In the quest to determine the link between variation in seasons and the distribution of metabolites in different samples of the plants collected from wet and dry sites in two climatically different locations, a conventional NMR direct extraction process using a two-phase deuterated solvent solution consisting of methanol and water, followed by multivariate data analysis was used. NMR spectroscopy in combination with multivariate data analysis proved effective in assessing and clearly displaying chemical profile differences, as well as annotating metabolites that influenced clustering between samples harvested in the different seasons and different sites from the different growing locations.

The general observation as revealed by the ^{1}H-NMR spectra generated from the analytes displayed changes in the production of chemical constituents in the leaves of *H. aureonitens* in response to seasonal changes and geographical distribution. The spectra captured both secondary and primary metabolites, providing a holistic picture of the plant metabolome for the two seasons under consideration (spring and autumn), as well as the two geographically dissimilar locations where harvests were made.

The use of multivariate data analysis (OPLS-DA) revealed additional variations in the effect of different seasons and climatically different geographical locations on the plant. Different seasons and locations were used as basis to separate the sample groups into clusters. Because the OPLS-DA model displayed high goodness of fit R^2X (cum) = 0.96 and predictive ability

Q^2 (cum) = 0.84, the observed clustering therefore confirms the validity model for the current investigation. Contribution plots indicated NMR regions showing chemical shifts of metabolites that are most likely responsible for changes in OPLS-DA score plots of different seasons (spring and autumn) and geographical locations (Telperion, Mpumalanga and Wakefield farms, KwaZulu Natal Midlands). Using Chenomx and a literature search to link these chemical shifts to classes of secondary metabolites already found within the plant kingdom, it was possible to successfully annotate metabolites that differentiated extracts from different seasons and geographical locations. These differences were mainly observed in the aromatic regions.

Metabolites which are abundantly expressed, including flavonoids and other phenolic compounds such as chlorogenic acid, caffeic acids, apigenin and quercetin are positively correlated with extracts of plant samples in spring season in both locations. This is in tandem with a few other studies on how seasons influence the phytochemical distribution in plants. In their study on the seasonal variation in antimicrobial and phytochemical properties of medicinal bulbous plants (*Tulbaghia violacea, Hypoxis hemerocallidea, Drimia robusta* and *Merwilla plumbea*) frequently used in South Africa, Ncube et al., (2011) reported that levels of tannin, gallotannin and flavonoids were increased in spring relative to the other seasons of the year.

Following the annotation of these metabolites, the study further focused on chlorogenic acids, compounds found in abundance in many *Helichrysum* species (Albaryak et al., 2010; Babota et al., 2018; Yazdi et al., 2019) and linked to their medicinal properties. The identity of caffeoylquinic acids (CQAs) which is the main chlorogenic acids represented in nature and also most abundant compound found in spring from the result of this study was confirmed using UPLC-qTOF-MS analytical technique. Many studies have previously reported the potency of CQAs against HSV-1 (Ikeda et al., 2011; Mahmood et al., 1993), it is therefore most likely that CQAs are probable contributors to the antiviral activity recorded from this study. Specific evaluation of CQA distribution and its derivatives of other classes of compounds such as dicaffeoylquinic acid (DCQA) and tricaffeoylquinic acid (TCQA) in the chemical profile of *H. aureonitens* were carried out. Dicaffeoylquinic acid (DCQA) was found to be the most abundant, with higher concentrations in both regions and seasons. Generally, the result showed that while CQA was present in nearly all the analysed samples in both seasons and from both locations, the quantitative distribution of CQA was much higher in the spring season and at Wakefield farms. This firmly corroborates the findings of the antibacterial as well as the

antiviral studies. Several studies have linked increased rainfall to increase in the production of chlorogenic acid (Liu et al., 2016; Rihan et al., 2017). In a recent study on the effects of increase in atmospheric CO_2 and other climatic variations on phenolics in coffee trees, Batista et al., (2021) reported that phenolic levels were positively correlated with the rainy season. The report further showed that chlorogenic acid concentration (5-CQA, the only chlorogenic isomer in this study) in particular was reduced during the dry season. In the current study, the total monthly rainfall recorded in October was 110 mm and 83.8 mm for Wakefield and Telperion respectively, while the May record showed 31.8 and 24.6 respectively (Table 3.1). A comparison between the chlorogenic acid in the two locations for both seasons revealed a quantitative difference in the concentration of the isomer 3-CQA (Figure 6.1), this result is in consonance with the study conducted on walnut leaf samples by Amaral et al., (2008). The study reported a phenomenal increase in phenolic compounds which included 3-CQA when walnut leaf samples from nine different cultivars were investigated for their phenolic compounds. The considerable changes observed between three consecutive production years, were attributed to climatic factors, mainly temperature and rainfall. Samples harvested from the year with the highest amount of rainfall (2002) had a surge in the production of caffeoylquinic acid and other phenolic compounds. In another study that identified and quantified phenolic compounds in various eggplant cultivars that were cultivated in different seasons, a decline in the concentration of caffeoylquinic acid was reported from spring to summer season (García-Salas et al., 2014). The derivative DCQA in general is the most abundant, with higher concentrations of all its isomers in both regions and seasons.

In establishing a possible link between the metabolomics results and antimicrobial activities of plant extracts from the various collection sites across seasons and locations, it is observed that the compounds from the aromatic region showed significant taller peaks in both the wet and dry sites in both location during spring compared to autumn. This is an indication that higher rainfall supports the production of aromatic compounds in *H. aureonitens*. When both wet and dry sites are then compared in the spring season, it is observed that the peak heights in the aromatic region in wet sites are taller than the dry sites in both locations. Compound peaks found in this region such as galangin, chlorogenic acid, kaempferol and quercetin, were annotated to determine possible connection between them to both the clustering and activities of the extracts across sites and locations. Comparison between the ^{1}H-NMR spectra in Telperion for both the wet and dry sites in spring season showed a marked difference in peak heights as observed in the aromatic region (6.5-8.0 ppm) of the wet site compared to the spectra

of the extracts from the dry site which is barely seen. A visual observation of the stacked spectra of extracts from wet and dry sites in Telperion during autumn season however shows that the peaks in all the samples are very similar in the aromatic region. For Wakefield, there is also a noteworthy difference between the wet and dry sites in spring, with the wet sites having taller peaks indicating presence of higher concentration of bioactive compounds in the aromatic region in the wet site. The peaks from all four spectra from both wet and dry sites in Wakefield at autumn are close and very similar to the observation in Telperion.

A close association is observed between the extracts with more bioactive compounds in the aromatic region of the spectra and some antimicrobial activities. From the antiviral result, better activity of extracts harvested during the wet season (spring) is recorded in all the sites and across the two locations (figure 4.1). In comparing the wet sites and dry site in Wakefield however, a much better anti-HSV activity is observed in the extracts from the three wet sites as against the extracts from the dry site (WL S S4 and WS S S4) as seen in figure 4.1. This establishes a direct proportionality between aromatic compounds and anti-HSV activity.

Similar to anti-HSV, extracts with higher aromatics showed related activity against the fungi *A. flavus* (Table 3.2). It is observed that extracts from the wet sites showed better activity than the dry sites in both seasons at Wakefield, indicating a possible effect of aromatics on the activity of the extracts against the fungus. Comparatively, there is a better activity observed against *A. flavus* at Telperion than Wakefield although not too wide a distinction. Generally, it is concluded from the metabolomics result that a direct link cannot be established between aromatic compounds from *H. aureonitens* extracts and its activity against the tested fungi. It is however important to indicate that acetone and not a hydroalcoholic extract was prepared for the antifungal assay, which would extract a different chemical profile of compounds. The consideration for the preferred use of acetone stems from the reported low aqueous solubility of flavonoids such as galangin, quercetin and kaempferol (Gao et al., 2011; Deng et al., 2019; Bacanli et al, 2018). A synergistic association of aromatic compounds and other antifungal compounds not covered in this study might be responsible for the activity. The good antifungal activity is however possibly due to acetone used as solvent as it has been reported to be a superior extractant in antifungal assays. Also, it is clear there are other factors apart from variation in seasons responsible for the observed antifungal activity of the plant species as the effect of seasonal variations on its activity is not conspicuous.

One of the key findings in this study, which is the establishment of the fact that increase in water levels increase chlorogenic acids production in *Helichrysum* species, and as corroborated by many other studies is an indication that increase in water availability leading to increase in chlorogenic acids production is a general trend not only in *Helichrysum* species but in other chlorogenic acids producing plant species. In the study conducted by Mahmood et al., (2017) on the effect of drought stress on phytochemically active compounds of the German chamomile (*Matricaia chamomilla* L.) plant, results showed that there was a remarkable decrease in chlorogenic acid production under drought conditions. But the converse is also true, chlorogenic acid was present in higher concentration in well-watered condition. Liu et al., (2016) also reported a higher production of chlorogenic acids when the effects of irrigation on yield of the nutritional quality of Arabica coffee (*Coffea arabica*) was studied. With the many predictions of higher temperatures and lesser rainfall due to climate change, it is expected that various plant species where chlorogenic acids contribute to their medicinal activities may be impacted.

The metabolite variations reported in this study could provide further insights into the comparison between phytochemical characterization of the *Helichrysum* genus within the limit of the conditions in consideration and also serve as a springboard for future research that want to investigate the relationship between entire metabolome functions of the plant in different climatic conditions. In general, an understanding of how the entire plant metabolome responds in relation to variations in season and different climatic regions could be important in molecular studies aiming to influence the metabolome for increased metabolite production.

The primary research question that this study sought to answer was to investigate the possible effect that environmental conditions in two different seasons and two different climatic locations have on the metabolite profile of *H. aureonitens.* The aim of the study was therefore accomplished in that through experimental assays and metabolomic/LC-MS studies, the effect of seasonal variation and different geographical locations was clearly demonstrated on both the biological activities and metabolite profile of *H. aureonitens.* Additionally, this is the first study to report the influence of seasonal variations and different growing localities with dissimilar climate on the metabolite profile of any species of the genus *Helichrysum.*

7.6 RECOMMENDATION AND FUTURE WORK

The following observations are noteworthy at the end of this research which can make significant contributions to future studies.

1. Even though established and proven methods were used in this study, it resulted in the use of different solvents for the different assays. The solvent acetone was used for the antifungal bioassay, whereas hydroalcoholic extracts were prepared for the metabolomic and LC-MS analyses. Standardisation of the same solvents might even align the different assays more accurately, especially for the antifungal assays where acetone was used as a solvent. Chlorogenic acids and its derivatives reportedly dissolve in a range of solvents such as methanol, ethanol, acetone, water etc. (De Azevedo et al., 2008; Gil et al., 2017), while quercetin has an extremely low water solubility (Riva et al., 2019).
2. The result of this study shows slightly different report to similar studies on the antimicrobial activities of some selected *Helichrysum* species when the solvent ethanol is used (Kutluk et al., 2018). This contributes to the continuous discussion on the use of solvents and the effect it has on extracting compounds and determining the biological activity of plants. The results of many studies have established the effect of different extraction solvents on the phytochemical components of plants and the subsequent differences observed in the bioactivities of such compounds (Iloki-Assanga et al., 2015; Ngo et al., 2017; Thouri et al., 2017).
3. Soil types account for varying levels of metabolites distribution as reported by a few studies conducted on other plants (Egamberdieva et al., 2017: Cappelli & Mariani 2021). It is recommended that future studies investigate the role of soil mineral and composition on the chemical profile and antimicrobial activities of *H. aureonitens.* In this study the effect of soil was not considered as both wet and dry sites were investigated per location. It was therefore assumed that the same soil is present per location, and that soil should not contribute to the differences in the chemical profile and associated biological activity. The soil from Telperion and Wakefield however would certainly be different, and in conjunction with the higher rainfall and lower temperature, might also contribute to the higher aromatic compound profile of the samples collected from Wakefield. However, in accordance with the findings at Telperion that the wetter sites had higher aromatic profiles, it would seem that the soil is not a determining factor, as the wetter sites at Wakefield also showed higher activity.

In accordance with other reports, it would therefore seem as if water availability and possibly the related temperature conditions influence the aromatic profiles of the *H. aureonitens*.

4. The environment is known to be a determining factor in the chemical profile of plants. Climate change is a global phenomenon of environmental changes, and it is therefore expected that it will significantly affect the medicinal compound profile and activity of plants. Dryer and warmer climates affect aromatic compound production, especially the chlorogenic acids as clearly demonstrated in this study. Wetter environments significantly increased the chlorogenic acid concentrations, also notably the associated antiviral activity against HSV-1. This is also supported by various other studies, indicating an increase in chlorogenic acid concentration where water is more abundant. This observation therefore seems to span wider than just *H. aureonitens* and warrant further intensified studies into the possible effects of climate change on the chemical profile of plants and the associated biological activity.
5. *Helichrysum aureonitens* demonstrated excellent antifungal activity as observed from this study and as presented in chapter three. Of all the five fungal species tested (*Aspergillus flavus, Aspergillus nomius, Cladosporium cladosporioides, Fusarium oxysporum* and *Penicillum halotolerans*), all but one showed good antifungal activities with MIC values ranging from 0.39-1.56 µg/mL. Similar result was reported by Mathekga, (2004) where twenty-seven out of twenty-eight *Helichrysum* species including *H. aureonitens* showed antifungal activity. Many other studies have reported the antifungal activities of *Helichrysum* species (Tomás-Barberán et al., 1990; Angioni et al., 2003; Tchoumbougnang et al., 2010; Kutluk et al., 2018). Further investigation is needed for possible antifungal product development based on the reported antifungal activities of *Helichrysum* genus.
6. The study further supports earlier reports on the antiviral potentials of *H. aureonitens* as contained in chapter four. Seventeen out of twenty-six extracts (mostly from wet sites and wet season) demonstrated good activity against HSV-1 has been observed in the study. Earlier studies have reported the presence of phenolic compounds such as galangin and chlorogenic acids (which are well known for their antiviral activities) from *H. aureonitens* (Meyer et al., 1996, 1997a; Khan et al., 2005; Ikeda et al., 2011; Prinsloo and Vervoort, 2018). Based on the antiviral activity of *H. aureonitens* as observed in this study and as corroborated by reports from earlier studies, it is recommended that

the result of this study be used as a springboard for more elaborate studies in the development of antiviral products from *Helichrysum* species.

7. Another important finding from this study which has not been addressed yet is the toxic properties of the extracts irrespective of region and season as determined by the selectivity index of less than 1 for a greater percentage of the extracts. This is quite concerning, as use of these extracts at therapeutic level are basically all toxic. This certainly warrants further research.

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