



**ASSOCIATION OF *ARTEMISIA AFRA* GENETIC DIVERSITY, AND BIOACTIVE
COMPOUNDS USING SOUTH AFRICAN AND LESOTHO GENOTYPES**

By

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Thesis submitted in fulfilment of the requirements for the Degree

DOCTOR IN AGRICULTURE

in the

Department of Agriculture, Faculty of Health and Environmental Sciences

at

Central University of Technology, Free State

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JANUARY 2025

DECLARATION

I, Matumelo Alice Rafiri, student number #####, do hereby declare that this research project submitted to the Central University of Technology, Free State, for the Degree DOCTOR IN AGRICULTURE is my work. It complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State. The work has not been submitted before to any institution by me or any other person in fulfilment (or partial-fulfilment) of the requirements for the attainment of any qualification.

2025/07/10

Signature of the student

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ACKNOWLEDGEMENT

I would like to express my heartfelt appreciation to my promoters, Prof. Moosa M. Sedibe and Dr. Alina Mofokeng (may her soul rest in peace). Their continuous guidance, assistance, patience, and constructive criticisms have been invaluable to the success of my work. I am also grateful to the Central University of Technology for the financial support that made my doctoral studies possible. Additionally, I wish to thank Dr. Riel Pierneef for his analysis, along with many other scientists who have contributed to my research. My sincere thanks go to Mr. Nyakane for his assistance in sample preparation. I extend special thanks to Dr. Gabriel Vusanimuzi Nkomo for his ongoing mentorship and guidance. I am deeply grateful to my friends and all individuals who have supported me, both directly and indirectly, throughout my studies. Lastly, I would like to acknowledge the unwavering support of my husband, Palamang Sylvester Rafiri, who accompanied me during my fieldwork while collecting samples.

ABSTRACT

Artemisia afra L. (African wormwood) is a plant that grows in the northern, central, and coastal regions of South Africa, as well as in neighbouring countries such as Eswatini and Lesotho. It is mostly used for human medicine in rural areas, especially for flu, malaria, cancer, gastric/abdominal pains, steaming to prevent congestion of nose and many other health disorders. Besides being used for medicinal purposes, people use it to make cosmetics, bathing soap and tea. To maintain by-product quality as well as improvement of yield of essential oils, genetic, mineral and phytochemical analyses were performed. The samples were collected from four locations: Roma (RO), Mohale's Hoek (MH), Wepener (WE) and Hobhouse (HO). The samples were sent to Kenya for Genetic diversity analysis because the International Livestock Research Institute (ILRI) specialises in genome analysis, and mineral analysis was performed at the University of Lesotho. Analysis of variance (ANOVA), unweighted pair group method with arithmetic mean (UPGMA), and principal component analysis (PCA) were used to interpret the data obtained. Genetic diversity differences were noted within *Artemisia afra* genotypes collected from the same location. Even though many genotypes of *Artemisia afra* were similar, there were some exceptions that were different from others. The results revealed other differences on the UPGMA dendrogram, where the clades were divided into clusters and formed smaller groups. The PCA also showed a very close relationship of genotypes, with very few genotypes appearing far from the rest. The differences were also noticed in the analysis of foliar minerals and phytochemicals. Some samples had high amounts of soluble compounds. The study revealed that *Artemisia afra* samples collected from MH, RO, WE and HO had high amounts of phenolic compounds, and some differences in quantities were noted.

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ABBREVIATIONS

%	percent
°C	degrees Celsius
3,4,5-triCQA	3,4,5-tri-O-caffeoylquinic acid
3,4-diCQA	3,4-di-O-caffeoylquinic acid
3,5-diCQA	3,5-di-O-caffeoylquinic acid
3-CQA	3-caffeoylquinic acid
4,5-diCQA	4,5-di-O-caffeoylquinic acid
4CL	4-coumarate-CoA ligase
4-CQA	4-caffeoylquinic acid
5-CQA	5-O-caffeoylquinic acid
ACT	Artemisinin-based Combination Therapy
AFLP	amplified fragment length polymorphism
ANOVA	Analysis of variance
APAP	acetaminophen
ASE	<i>Artemisia sacrorum</i> Ledeb. Extract
C ₁₂ H ₇ NC ₁₂ O ₂	Dichlorophenol indophenols
C ₂ H ₆ O	Absolute alcohol
C ₃ H	p-coumaroyl shikimic acid/quinic acid 3'-hydroxylase
C ₄ H	cinnamate 4-hydroxylase
C ₆ H ₈ CO ₃	Ascorbic acid
Ca	Calcium
ChI/IAA, 24:1	chloroform-isoamyl alcohol
CQAs	caffeoylquinic acids
CTAB	cetyltrimethyl ammonium bromide
DArT	Diversity Arrays Technology
DArTseq	Diversity Arrays Technology sequence
DMADP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid

E	East
EDTA	ethylenediaminetetraacetic acid
EtOH	(ethanol/ethyl), TE / Tris, and EDTA)
g/mL	grams per millilitre
GAE	Gallic acid equivalent
h	hour
H ₂ O	Water
HCl	Hydrochloric acid
HCT/HQT	4-hydroxycinnamoyl-CoA: shikimate/quinic acid 4-hydroxycinnamoyl transferase
He	Helium
HO	Hobhouse
HO ₃ P	Metaphosphoric acid
ICS	isochlorogenic acid synthase
ILRI	International Livestock Research Institute
IPP	Independent power producer
K	Potassium
MAS	marker-assisted selection
MH	Mohale's Hoek
mL	millilitres
mM	millimetres
Mn	Manganese
N	Nitrogen
Na ₂ CO ₃	Sodium Carbonate
NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
P	Phosphorus
PAL	phenylalanine ammonia-lyase
PCA	Principal Component Analysis
PCR	Polymerase chain reaction

pH	Potential of hydrogen
Pi	phosphate
RAPD	amplified fragment length polymorphism
RFLP	restriction fragment length polymorphisms
RNAse	bovine pancreatic ribonuclease
RO	Roma
S	South
S	Sulfur
SD	Standard deviation
SNP	Single nucleotide polymorphism
SSR	microsatellites or simple sequence repeats
Std	standard
UPGMA	Unweighted pair group method with arithmetic mean
W	West
WE	Wepener

CHAPTER ONE

GENERAL INTRODUCTION

1.1 BACKGROUND

Artemisia afra L. (African wormwood) is an important medicinal shrub in large parts of Africa. It is well known, even though it is called by different names wherever it grows. For instance, it is called “Umhlonyane” in the Xhosa language, “Mhlonyane” in the Zulu language, “Lengana” in both Sesotho and Tswana, “African wormwood” in English and in Afrikaans, “Wilde als”. According to Liu *et al.* (2009), *Artemisia afra* L. is widely distributed across Southern Africa – from Kenya, Tanzania, Uganda, Ethiopia, South Africa, Namibia, Zimbabwe, Eswatini to Lesotho. That is why it is called Artemisia of Africa (*Artemisia afra* L.).

Although *Artemisia afra* thrives in temperate climates, its distribution extends to a variety of other climates as well. In temperate regions, it typically grows in specific hilly areas where the soil is gravelly and rocky. It is one of the *Artemisia* genera, and Nigam *et al.* (2019) mention that *Artemisia* comprises over 500 species, as supported by Liu *et al.* (2009), who note that they are utilised in many ways across the world to help sick people. *Artemisia afra* L. is a unique medicinal plant with its aromatic smell and bitter taste. The aromatic smell makes it a favourable fumigant to control storage pests, especially weevils, and also acts as a repellent when placed inside bags of grains. *Artemisia afra* is also used for medicinal purposes to treat infectious diseases and as a good source of essential oils (Liu *et al.*, 2009).

Mint, basil, *Asparagus aethiopicus* L. and *Citrullus colocynthis* L. are examples of other plants used for disease remedy in economically weak regions of the world, such as Africa (Elansary *et al.*, 2018). In most cases, rural communities cannot afford to buy western medicine and people desire to use what they have. That is why they use the available medicinal plants around their areas to address their own problems. This knowledge, that wild plants are used to cure people and their livestock, is passed from generation to

generation. According to Van Wyk *et al.* (2008), *Artemisia afra* L. is no exception; it is also used as a medicinal plant for various types of diseases but mostly used for treating the common cold. It is sometimes inserted into the nose to clean out and unblock the sinuses when a person is suffering from the common cold or if it is difficult to breathe. Many studies have been conducted on the morphology, taxonomy, distribution, ethnopharmacology, utilisation, chemistry, chemical variation, and biological effects of *Artemisia afra* L., and multiple uses of *Artemisia afra* L. have been mentioned in many reviews (Wake *et al.*, 2000; Thring & Weitz, 2006).

The performance of genetic mapping and diversity analysis is essential for these types of plants, as this would help to study their genetic make-up, and such information would be useful for improving the plant. According to Wenzl *et al.* (2004), the study of deoxyribonucleic acid (DNA) variation is not new, as DNA sequencing and molecular markers were developed to enable the field of genomics. However, it is wise to choose the most cost-effective, high-throughput technology that tests the most samples at once. Polymerase chain reaction (PCR)-based molecular marker methods have been noted to have a low throughput as they are time-consuming and costly for applications, especially where many samples are genotyped; thus, a large budget is needed to analyse the samples (Petroli *et al.*, 2012).

PCR-based markers and restriction fragment length polymorphisms (RFLP) markers have been around for many years, but it has been found that these methods also have their shortcomings. Research caps forced scientists to look for alternative options to address the problem. Subsequently, single nucleotide polymorphism (SNP) arrays were developed to complement these low-throughput markers. Nevertheless, limitations observed in developed SNP arrays led to the development of Diversity Arrays Technology (DArT) (Wenzyl *et al.*, 2004). According to the value of the parallel nature of the micro-array platform, instead of serial analysis marker data, DArT detects the presence versus absence of individual fragments in genomic representations (Jaccoud *et al.*, 2001). Budhlakoti *et al.* (2022) mentioned that its efficiency has been noted to be a potential tool for a quick and powerful assessment of the structure of germplasm collections and plant breeding.

The fact remains that many people rely on indigenous plants for their healthcare and use local herbs to cure different diseases because the plants are readily available at their location. It is therefore important to study all aspects of *Artemisia afra* and other essential plants. However, there have been no studies to date to assess the genetic makeup of this important, widely used herb. It is thus essential to characterise the plant to understand its genetic makeup. Levin (2002) confirms that, in plant studies, the key for systematic, evolutionary, and conservation approaches is to characterise genome organisation. Information on the *Artemisia afra* L. genome and genetic diversity of its genotypes is crucial, as they are commonly used for medicinal purposes. The relationship between genetic diversity and plant attributes, such as minerals and metabolites, are therefore considered in this thesis.

Minerals are plant constituents that play a major role in their lifespan. According to Alassane *et al.* (2013), minerals found in plants are influenced by the content of soil and the environment around them. Minerals play a major role in plant performance and its nature. Without them, some reactions in plants do not take place and such failure results in retarded growth and often death. In plants, it has been reported that different minerals play different roles. According to Rouached *et al.* (2017), there is a close correlation between genes and plant minerals. In most cases, the plant's mineral deficiency can result in mutations because plants change their behaviour for survival. Similar observations have been made in *Arabidopsis*, where specific mutations and genotypes were identified that exhibit altered sensitivities to phosphate (Pi) in regard to primary root growth (Muller *et al.*, 2015). Plants have their own mechanisms of adapting to the environment. Morphological, physiological and molecular changes trigger the formation of accession and mutant lines (Rouached *et al.*, 2017). Thus, consideration of mineral analysis in this study is essential as they have been known to influence plant genetics.

1.2 JUSTIFICATION OF RESEARCH

The first step towards efficiency in the utilisation of genetic resources is the evaluation and introduction of new genes, followed by their maintenance. Genetic resources are evaluated and characterised based on morphological and agronomic traits at the starting

point of any breeding program. Botanical gardens and natural genetic resource management departments have collected genotypes of many plants of economic importance, such as *Artemisia afra*, from different areas. Nevertheless, nothing has yet been done on genomic analysis of such plants. *Artemisia afra* is not an exception among those neglected, but there is very little information on the genomic analysis of most indigenous plants.

The use of DNA markers has had a revolutionary impact on gene mapping and, generally, on the genetics of the entire animal and plant kingdom (Dodgson *et al.*, 1997). According to Du *et al.* (2017), it is very important to understand the genetic characteristics of plant growth and development, the impact of gene expression on the growth of the plant, and the development of enzyme genes in the synthesis process of secondary metabolites. It is therefore important to develop and genotype *Artemisia afra* genotypes using molecular markers such as single nucleotide polymorphisms (SNPs).

SNPs involve the substitution of one nucleotide for another; in other words, a SNP marker is just a single base change in a DNA sequence (Beuzen *et al.*, 2000). Advantages of the SNP markers include: SNP markers are located in the coding area of DNA, therefore, they affect protein function directly; secondly, SNPs are more suitable than microsatellites for high-throughput genetic analysis; thirdly, they are more stably inherited than other DNA markers, making them more suited as long-term selection markers; SNPs can also identify the genomic diversity of species to demonstrate the speciation and evolution, and associate genomic variations with phenotypic traits (McNally *et al.*, 2009).

For a successful breeding programme to function, a complete understanding of the genetic diversity of the crop is required. Better knowledge of the genetic similarities and dissimilarities of breeding material could aid in maintaining genetic diversity and sustain long-term selection gain. Monitoring the genetic diversity among the genetic resources of elite breeding material could make crop improvement more efficient due to the directed accumulation of favoured alleles, thus reducing the amount of material to be screened.

Artemisia afra is also considered to be plant of economic importance because of its oils that are used by many people. In nature, essential oils play an important role in the

protection of the plants as antibacterials, antivirals, antifungals and insecticides, especially against weevils (Rashmi *et al.*, 2014). In human beings, essential oils are employed in aromatherapy and for the treatment of several diseases including cardiovascular disease, diabetes, Alzheimer's, and cancer (Ali *et al.*, 2015), because it contains a diverse mixture of chemicals. Bakkali *et al.* (2008) mentions that essential oils may also attract some insects to favour the dispersion of pollens and seeds or repel undesirable others. Variation in climate is noted to cause genetic variation in essential oil plants and may result in poor oil quality due to instability in chemical composition. The uniformity and quality of the produce depend on the plant make-up. According to Ehrendfeld (2013) the interaction of minerals from the soil and the plant genes was found to be essential to the plant environment. In past studies, it has been documented that there are genes involved in both nutrient uptake and root growth in *Arabidopsis* (Rouached *et al.*, 2015). Before plant utilisation and improvement, the knowledge of plant genetics, minerals, and phytochemicals plays a great role. It is always useful to mitigate against factors that lead to poor oil quality. It is therefore essential to study the genetics and phytochemistry of *Artemisia afra* as the basis of its improvement, as well as its safety to be consumed and used as a medicinal plant. The study has been thoroughly conducted by analysis of DArTseq, micro- and macro-elements, and major bioactive constituents of *Artemisia afra*.

1.3 PURPOSE OF THE STUDY

1.3.1 Aim

This study aimed to analyse the *Artemisia afra* L. genome and its related attributes, such as minerals and phytochemicals.

1.3.2 Objectives

The specific objectives of the study were:

1. To determine the genetic diversity of *Artemisia afra* L. genotypes from four agroecological zones using DArTseq.

2. To analyse the macro- and micro-elements composition of *Artemisia afra* L. genotypes from four different locations.
3. To investigate the qualitative and quantitative analysis of the major bioactive constituents of *Artemisia afra* L. genotypes.

1.3.3 Research Hypothesis

The research was conducted to prove the following:

1. There is no diversity among *Artemisia afra* genotypes based on DArTseq, regardless of the different locations.
2. There are differences in the macro- and micro-elements composition of *Artemisia afra* genotypes collected from different locations.
3. There are differences among *Artemisia afra* genotypes based on bioactive constituents.

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CHAPTER TWO

LITERATURE REVIEW

2.1 ARTEMISIA SPECIES AND ORIGIN

The *Artemisia* genus belongs to a daisy family, *Asteraceae*. According to Abad *et al.* (2013), *Artemisia* is the largest genera with more than 500 species. They are found growing in the northern temperate regions of the world to the southern region of Africa. Liu *et al.* (2022) state that members of the *Artemisia* genus grow in the temperate, frigid and subtropical regions of the northern hemisphere and a few species are distributed in the southern hemisphere. This makes it easy for *Artemisia* to survive and grow in many places, such as at high altitudes and in extremely arid areas. *Artemisia* is classified as *Artemisiinae* Less. (subtribe) and *Anthemideae* Cass. (tribe). The tribe contains aromatic plants and are valued for their pharmaceutical and pesticidal value (Oberprieler, 2022). Kane *et al.* (2019) mention that at least 80% of the population of Africa living in rural areas use traditional medicine by gathering medicinal plants from the bushes. Most of these plants grow in their habitats around the homesteads of the environment where situations are suitable for their survival.

The literature has many interpretations of the origin of the *Artemisia* genus. According to Adriaenssen (2003), this genus was named after Greek goddess Artemis (Diana for the Romans), who was Zeus's daughter and Apollo's sister. However, Gledhill (2008) stated that the *Artemisia* and *Mausolea* genus look alike. The *Mausolea* genus, sister and wife of Mausolus sound similar to the *Mausolea* genus. Due to close relationship of *Artemisia* and *Mauselea*, it was also named after queen of Caria ([www.worldhistory.org/Artemisia I of Caria/](http://www.worldhistory.org/Artemisia_I_of_Caria/)). The word *Artemisia* is originally taken from the Greek goddess, Artemis (Judžentienė, 2016).

Commonly known *Artemisia* species grow worldwide, depending on the adaptation conditions of each species. Valles (2011) supports that they are found in almost all parts of the world – in Antarctica they grow in the northern hemisphere and few species are in the southern hemisphere. There are many types of *Artemisia*, such as *Artemisia*

absinthium, *Artemisia annua*, *Artemisia vulgaris* and *Artemisia afra*. Sweet-smelling *Artemisia annua* L. herb is mentioned to be widely distributed in Asia, Europe, and North America (Abad *et al.*, 2013).

The study focuses exclusively on the *Artemisia afra* species, which is widely utilized for various purposes. This plant is native to Africa and is sometimes referred to as the "*Artemisia* of Africa." According to Van Wyk *et al.* (1997), *Artemisia afra* is a perennial woody shrub that can grow up to 2 meters tall, featuring a leafy, hairy, and ridged stem. Each plant has multiple stems that are thick and woody at the base, becoming thinner and softer toward the top (van der Walt, 2004). Common names for *Artemisia afra* include Lengana (Sesotho), African wormwood (English), Umhlonyane (IsiXhosa), and Mhlonyane (IsiZulu).

2.2 ARTEMISIA BENEFITS AND USES

Most of these herbs normally grow in the homesteads and are therefore accessible to people around these areas. According to Wang *et al.* (2018), many *Artemisia* plants, such as *Artemisia annua*, *Artemisia argyi*, *Artemisia sacrorum*, *Artemisia capillaris*, and *Artemisia japonica*, are recorded in ancient Chinese pharmacopoeia as being used as traditional Chinese medicine. *Artemisia annua* has been used in Artemisinin-based Combination Therapy (ACT) for the treatment of malaria (Rombauts *et al.*, 2015). Further, *A. judaica* is used in folk medicine for a number of remedies, including colds, gastrointestinal cramps, stomach upset, abdominal disturbances, and constipation (Batanouny *et al.*, 1999). The volatile oil of *A. judaica* has antimicrobial activity and it is also used in the treatment of inflammatory-related diseases, fungal infections, diabetes, atherosclerosis, cancer, and arthritis (El-Masry *et al.*, 2002; Nofal *et al.*, 2009).

In general, *Artemisia* plants are classified as aromatic plants of economic importance due to their potential for the synthesis of essential oils. These essential oils are used in folk and modern medicine, and the cosmetics and pharmaceutical industry (Abad *et al.*, 2012; Teixeira da Silva, 2004).

Artemisia afra is most popular and commonly used as medicine in Lesotho and South Africa. It is known to treat various disorders, including coughs, colds, influenza, and malaria (du Toit & van der Kooy, 2012). *Artemisia afra* has been described as very drought resistant and hardy; commonly grown on arid soils, open to sunny situation with light, well-drained soil and needing water occasionally (Hutchings, 1996; Greenham, 2000).

Other species, such as *Artemisia scoparia*, were mentioned to be effective to treat fever, inflammation, jaundice, and infection (Ding *et al.*, 2001). Observations have revealed that the various aqueous and alcoholic extracts of *Artemisia* possess an antidiabetic effect caused by hypoglycaemic action (Dabe & Kefale, 2017). The *Artemisia* species is sometimes used for livestock feeding in dry areas where there is a lack of grazing vegetation, wind breakers in windy areas to block strong winds, as well as to combat soil erosion in open pastoral areas.

Another important genus of *Artemisia* is *A. absinthium* L., which is well known in many places for its antiparasitic effects, and it is used to treat gastrointestinal problems, anorexia, and indigestion (Nigam *et al.*, 2019). Alzweiri (2011) states that, in the Northern Badia region of Jordan, *Artemisia herba-alba* Asso is used in traditional medicine in the form of a decoction against fever, menstrual, and nervous problems. However, in Morocco, the same *Artemisia* species (*Artemisia herba-alba*) is used for a different purpose – to treat arterial hypertension and diabetes (Ziyyat *et al.*, 1997; Tahraoui *et al.*, 2007; Zeggwagh *et al.*, 2008).

2.3 IMPORTANCE OF GENETIC DIVERSITY IN PLANTS

Genetic diversity is the foundation for continued existence of plant life in nature, crop improvement and development. In most cases, genotypes are chosen because of distinct characteristics, such as high yield potential, large seed, colour, palatability, photosensitivity, pest and disease resistance. Diversity has been described as the degree of differentiation between or within species (Bhandari *et al.*, 2017) and it provides the opportunity to improve crops to meet the preferences of both farmers and breeders. Sometimes the improvement of crops is done to address the problem and demand of the

market. For instance, crops are bred for many different reasons, such as disease tolerance, yield, long shelf storage or amount and quality of essential oils. It is essential to protect genetic diversity by conserving genetic variation, especially among threatened species. Reduction in genetic diversity has led to extinction of species and many plants disappeared. According to Govindaraj *et al.* (2015), genetic diversity is conserved and maintained, and provision of tools for population monitoring and assessment can be used for conservation planning as key factors.

2.3.1 Diversity analysis methods

Biological markers can measure diversity to differentiate individuals and population from each other. The main purposes of using markers are disease identification, forensic evidence, evolution relatedness, contamination detection, assistance in breeding phenotyping and genotyping.

The availability of genetic markers is a fundamental technique within plant biology and plant breeding (Rudd *et al.*, 2005). Rudd *et al.* (2005) further explain that molecular markers are mostly associated with map-based cloning of individual genes, characterisation of quantitative multi-gene traits, the survey and the analysis of genetic diversity.

Morphological markers

Morphological markers are correlated to differences in shape, size, colour and surface of an assortment of plant parts. For instance, this includes traits such as flower colour, seed shape, growth habits, and pigmentation that can be observed by the naked eye. This does not require expensive technology. Salinity stress was found to affect germination of crops such as tomatoes, *A. thaliana*, sunflower, rice, wheat and maize (Soltabayeva *et al.*, 2021). For instance, stress causes delay in plant flowering, depending on different plants, but in some plants, it causes rapid flowering. *Kalanchoe* were characterised by their different colour features of orange, violet, and yellow (Al-Khayr *et al.*, 2022). These characteristics aid plant breeders in genotype selection and characterisation depending on phenotype, as well as in genetic improvement projects.

Cytological markers

Cytological markers detect variation in chromosome number, shape, size and banding pattern. Karyotypes and banding patterns of the chromosomes can be used as cytological markers. Karyotypes are numbers and visual appearance of the chromosomes in the cell nuclei of an organism or species. Cytological observation of the infection progression revealed that *U. virens* infection was a stepwise invasion process, which could subsequently be divided into three distinctive stages based on the morphology of the infected floral organs (Chao *et al.*, 2014).

Biochemical markers

These markers can sometimes be called biological markers as they are a measurable indicator of some biological state or condition. The markers measure the protein produced by gene expression. However, Strimbu and Tavel (2010) explain a biomarker as any substance, structure, or process that can be measured in the body or its products and influence to predict the incidence of outcome or disease. Biochemical markers are mentioned to be the first molecular tool to be used for genetic characterisation (Kumar *et al.*, 2018). These markers can be divided into two divisions, such as proteins and phytochemical markers. Protein markers can be further classified into protein isoenzymes or isozymes and alloenzymes or allozymes (Kumar *et al.*, 2018).

Protein isozymes are defined as generally selectively neutral, well distributed over the genome, and interactive in a co-dominant manner (van de Ven *et al.*, 1990). These markers have been used in various crops for classification studies, inbred line recognition, and the determination of outcrossing frequencies and cultivar identification. In analysis of *Vicia faba*, isozymes generated minor polymorphism bands and this did not contribute significantly to the understanding of its genetics (van de Ven *et al.*, 1990).

Molecular markers

Molecular markers are sometimes called DNA markers. DNA-based markers have been used for assessment of genetic diversity and identification of plant genotypes as they allow direct access to the plant genome (Gaafara *et al.*, 2017). They are specific

fragments of DNA that can be identified within the genome and found in a specific location of the genome. Molecular markers can be broadly clustered into three classes based on the method of their detection, such as hybridisation based, polymerase chain reaction (PCR) based, and DNA sequence based (Govindaraj *et al.*, 2015). According to Koopae (2014), microsatellites and SNP DNA markers are grouped into PCR based markers and the other group is called non-PCR based markers.

Restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites or simple sequence repeats (SSR) and single nucleotide polymorphism (SNP) are the commonly used molecular marker technologies. These advanced molecular markers have made crop diversity assessments more attainable and informative than before (Fu, 2015) and markers are developed to assess diversity, construct genetic maps, and perform marker assisted selection (MAS) in plants. Development of new technologies arose due to limitations of previous markers which did not give specific answers.

Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism markers make a distinction by analysis of patterns derived from the sample of DNA. RFLP markers are used for genetic mapping and have been used in analysis of many species for accurate scoring of genotypes. Analyses comprising RFLP markers are labour intensive and time consuming and it has therefore led to the development of other advanced technologies. It is based on the restricted fragment distance end to end of DNA. The restricted enzymes have the main role for their amplification and production, for example, restricted fragment length polymorphism, or RFLP markers (Avisé, 2004). However, as disadvantages, these markers have high development and running costs and require high quality and quantity of DNA (Oliveira & Azevedo, 2022).

Randomly amplified polymorphic DNA

Markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence. In this technique, DNA samples

are digested with specific endonucleases resulting in a profile of fragments of different lengths which is characteristic of each species (Oliveira & Azevedo, 2022). These DNA markers have been used to assess the genetic diversity of many crops. According to Zhang and Zhang (2014), RAPD markers were used to analyse many vegetable crops, such as cabbage, Chinese kale, mustards, cauliflower and others. However, in genetic diversity RAPD application was found to produce inconsistency in analysis of genetic markers that provide precise information than morphology and biochemical markers.

Simple sequence repeats

Simple sequence repeats (SSR), also known as microsatellites, are DNA segments with a tandem repeat motif of 1–6 nucleotides. Microsatellites are locus specific and evenly distributed along chromosome. SSR are usually used because they are found to be highly informative co-dominant markers. The markers have been successfully used for assessment of genetic diversity of *Artemisia dracunculus* from Iran (Karimi *et al.*, 2015) and *Achillea fragrantissima* in Egypt with reference to plant size and seed yield (Badr *et al.*, 2017).

Amplified fragment length polymorphism

In this technique, DNA samples are digested using two restriction enzymes after the annealing of adapters, which creates cut boundaries that act as primer binding sites for PCR amplification. Polymorphisms are recognised by the presence or absence of DNA fragments following analysis on polyacrylamide gels (Oliveira & Azevedo, 2022).

Single nucleotide polymorphism

Limitations and challenges of the RFLP, RAPD, SSR and AFLP markers in applications of genetic study resulted in the development of advanced markers to do the work that the previous markers could not do. For instance, for analysis of plants with large genomes, a certain special marker is needed to read and give sufficient information. Single nucleotide polymorphisms (SNPs) involve the substitution of one nucleotide for another, in other words, a SNP marker is just a single base change in a DNA sequence (Beuzen *et al.*, 2000). SNP with its high abundance, cost efficiency, and high-throughput scoring, has

become a powerful tool in genome mapping, association studies, diversity analysis, and tagging of important genes in plant genomics. SNPs are co-dominant markers with a high level of polymorphism and very high genomic abundance (Oliveira & Azevedo, 2022). SNPs include more than 90% of all differences between the individuals; therefore, they are the best genetic variation resource for population studies and genome mapping (Frohlich *et al.*, 2004).

Genomic selection using SNP markers is a powerful new tool for genetic selection (Seidel, 2010). Advantages of SNP markers are that they are located in the coding area of DNA; therefore, they affect protein function directly; SNPs are more suitable than microsatellites for high-throughput genetic analysis; they are more stably inherited than other DNA markers, making them more suited as long term selection markers, and they are common and provide more potential markers near the locus of interest than other types of polymorphism. The problem of SNPs is the biallelic nature of these markers, which means that there are usually only two alleles in a population. SNPs can also identify genomic diversity of species to demonstrate the speciation and evolution and associate genomic variations with phenotypic traits (McNally *et al.*, 2009).

Diversity arrays technology (DArT)

Diversity arrays technology was originally developed for crops such as rice, a diploid crop with a small genome of 430 Mbp, and it is now used in several crops (Jaccoud *et al.*, 2001). According to Akbari *et al.* (2006), DArT generates whole-genome fingerprints by scoring the presence versus absence of DNA fragments in genomic representations generated from samples of genomic DNA. DArT determines many polymorphic loci in a single assay and has been used in analysis of many crops because of its high-throughput and cost-effective nature (Jing *et al.*, 2009). It is more advantageous when compared to previously developed technologies because it lightens the limitation of gel-based technologies.

DArT involves the isolation and cloning of a random set of DNA fragments from a complexity-reduced DNA sample assembled by pooling several germplasm genotypes so that a representative collection of variable genomic sequences of one or more target

species is captured (Petroli *et al.*, 2012). The success and best results in using DArT have led to scientists trusting it in plant genomic analysis. DArT technology has been applied to the analysis of many crop species, such as rice, eucalyptus, barley, cassava, wheat, pigeon pea, banana, sugarcane, *Arabidopsis* fern and moss (Petroli *et al.*, 2012). Unlike other techniques, DArT technology does not rely on DNA sequence information but can detect allelic variation. In *Sorghum bicolor*, DArT markers have been developed, and the results have demonstrated that DArT provides high quality markers that can be used for diversity analyses and to construct medium-density genetic linkage maps (Mace *et al.*, 2008).

2.3.2 Genetic diversity measures

Allelic diversity measures

The measurement of allelic diversity is based on the number of different allelic types segregating in the population, and the number of rare alleles can be used as an indicator of the amount of gene flow between subpopulations (Caballero & García-Dorado, 2013). The diversity of alleles at single loci (allelic richness) in wild relatives is particularly vulnerable to loss due to reduction in population size (Schoen & Brown, 1993).

According to Foulley and Ollivier (2006), genetic diversity depends on allelic richness and this is regarded as very important in genetic conservation and marker-assisted methods for maximising the number of alleles conserved. Allelic richness is a good indicator of the decrease in population size, and it is the parameter used to measure genetic diversity (Foulley & Ollivier, 2006). In common beans, germplasm showed very high diversity, 91 SSR markers used revealed high diversity of 21 alleles, a very high average number of alleles/loci (7.59), and high average gene diversity ($He=0.73$) (Mir *et al.*, 2021). High diversity is a good indicator of heterozygosity of genes which is more common in landraces than on exotic germplasm.

Genetics and Genetic base

The study of genes, genetic variation and heredity in organisms is important because heredity is vital to organisms' evolution. In pattern of inheritance, genes are passed from

parents to the offspring with similar (homozygous) or different alleles (heterozygous). Application of engineering in genetics results in gene manipulation to increase yield of desired characteristics. For instance, in *Artemisia annua*, conversion of IPP and DMADP were changed into FDP to increase the accumulation of artemisinin (Kiani *et al.*, 2016).

2.4 PLANT MINERAL SALTS AND SOIL MINERALS

Plant nutrients are mineral elements found in soils, essential for plants to complete their life cycles. Nutrients are categorised into macro-elements and micro-elements – elements needed in small amounts and large amounts, respectively. However, macro-elements are further categorised as primary and secondary elements. Kabange *et al.* (2022) state that N, P, and K as primary macro-nutrients and S, Ca, and Mg as secondary macro-nutrients, play an important role in plant growth. In general, N, P, and K are important for healthy green foliage, strong roots, blooms and healthy plant growth. Limited amounts of macro-elements and micro-elements can affect plant growth. Interestingly, the accumulation of high plant minerals in plants such as *Artemisia* may be dangerous for human consumption, this supported by Stefanowicz *at el.* (2015) on analysis of plants growing on Zn-Pb ore mining.

2.5 PLANT PHYTOCHEMICALS

In general, medicinal plants play a great role in the health of people and communities. Their medicinal value lies in some of the chemical substances that they produce. Plants produce phytochemicals, such as alkaloids, tannins, flavonoids, and phenolic compounds (Amin *et al.*, 2013). The use of traditional medicine has been supported by the World Health Organization, provided they are proven to be efficacious and safe (WHO, 1985). However, due to insufficient knowledge and understanding, communities use plants without them having been tested and analysed for their safety. The main cause is that rural people are often far from health clinics and they use indigenous medicinal plants as an alternative to treat diseases.

Differences in qualitative and quantitative composition of bioactive compounds might be correlated with environmental conditions, species variation, geographic, climatic, and

genetic conditions, plant age, soil and phase of vegetation, anatomical parts of the plant, harvesting season, and method of harvesting. Metabolism and gene regulation are two fundamental biological processes that are essential to all living organisms. Metabolism is the total of all chemical reactions in cells and organisms that maintain life and it can be divided into two classes: catabolic processes (the breakdown of molecules that usually results in the release of energy) and anabolic processes (the synthesis of components such as proteins, lipids, and nucleic acids, which costs energy).

Plants are considered factories of secondary metabolites (Li *et al.*, 2020). Formation of metabolites occurs during growth and development processes. These intermediate products are catalysed by enzymes within plant cells during metabolism. These metabolites are the sources of many commercial pharmaceutical drugs and herbal remedies derived from medicinal plants. Ahn (2017) mentions that Tamiflu, Aspirin, Taxol, Ginkgo Biloba extract, and plantain extract are the more commonly used and sold to generate income. Botanical drugs made from medicinal plants, however, need to be analysed and proven for their safety and clinical efficacy. According to Talib *et al.* (2022), phytochemicals can be classified into different groups, such as flavonoids, alkaloids, phytosterols, terpenoids, sulfides, polyphenols, and other natural chemicals.

The production of metabolites is believed to be influenced by genes. In sweet potato plants, phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C₄H), 4-coumarate-CoA ligase (4CL), 4-hydroxycinnamoyl-CoA: shikimate/quinic acid hydroxycinnamoyl transferase (HCT/HQT), p-coumaroyl shikimic acid/quinic acid 3'-hydroxylase (C₃H) and isochlogogenate synthase (ICS) were identified to be involved in production of secondary metabolites (Vasyutkina *et al.*, 2022). Vasyutkina *et al.* (2022) further mention that, with the aid of these primary genes, the following plant chemicals were then produced: caffeoylquinic acids (CQAs), namely 5-O-caffeoylquinic acid (5-CQA), 3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA), 3,4-di-O-caffeoylquinic acid (3,4-diCQA), 3,5-di-O-caffeoylquinic acid (3,5-diCQA), 4,5-di-O-caffeoylquinic acid (4,5-diCQA), and 3,4,5-tri-O-caffeoylquinic acid (3,4,5-triCQA).

Phytochemicals in Artemisia species

Artemisia gmelinii (Gmelin's wormwood) leaves and stems contain flavonoids and sesquiterpenes used to treat hepatitis, hyperlipaemia, and infected cholecystitis (Mamatova *et al.*, 2019). Juan (2009) has also revealed that *Artemisia sacrorum* Ledeb. extract (ASE) was more effective against acetaminophen (APAP)-induced hepatotoxicity in mice and, again, if ASE is administered before APAP, significant results were obtained. Yuan *et al.* (2016) found ten flavonoids with similar structures from *Artemisia sacrorum* Ledeb. extract; however, the results revealed that certain flavonoids revealed different cytotoxic activity against the two human liver cancer SK-HEP-1 and human cervical cancer HeLa cells under study.

The most popular and well-known phytochemical in the *Artemisia* species is artemisinin. The presence of artemisinin has been reported by many authors (Hsu *et al.*, 2006; Aryanti *et al.*, 2001; Arab *et al.*, 2006; Zia *et al.*, 2007; Mannan *et al.*, 2008) in many *Artemisia* species such as *Artemisia apiacea*, *Artemisia lancea*, *Artemisia cina*, *Artemisia sieberi*, *Artemisia absinthium*, *Artemisia dubia* and *Artemisia indica*. In a study that was done in Pakistan, Mannan *et al.* (2010) found the highest concentration of artemisinin from *A. annua* flowers and leaves compared to other *Artemisia* species. *Artemisia* species are dominated by terpenoids (in particular, sesquiterpene lactones), flavonoids, coumarins and other shikimate metabolites (Brown, 2010).

According to Brown (2010), *A. annua* is the only Asteraceae species having high concentrations of sesquiterpenoids (Terpenoids) and flavonoids, coumarins and other shikimate metabolites. Like other *Artemisia* species, *Artemisia afra* phytochemicals were identified as sesquiterpenes, glaucolides, guaianolides, flavonoids, glycosides, coumarins and others (Liu *et al.*, 2009). Liu *et al.* (2009) further mention that chemical variation is influenced by major factors such as geographical variation, different plant parts used, drying methods and variation within the natural population. All these factors may result in qualitative and quantitative differences in phytochemicals. It is therefore important to find out the effect of the metabolites' change on the plant genome. It is thus essential to consider phytochemicals and minerals when studying genetic diversity, and

variation as they are somehow directly interrelated, and genes are hereditary materials influencing chemical reactions inside the cells.

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CHAPTER THREE

DIVERSITY ANALYSIS OF *ARTEMISIA AFRA* USING A SINGLE NUCLEOTIDE POLYMORPHISM DERIVED FROM DIVERSITY ARRAYS

3.1 ABSTRACT

Artemisia afra is an aromatic shrub used as a herbal remedy in southern Africa for various disorders, including coughs, colds, influenza and, most notably, for the prophylaxis and treatment of *Plasmodium falciparum* infections. The infrageneric classification of such a large genus was challenging until recently. Modern high-throughput genotyping by sequencing, which combines next-generation sequencing and diversity array technology (DArTseq) complexity reduction methods, allows for the generation of high-quality whole-genome profiles without any previous organismal genetic information that may hamper the diversity analyses. This study investigated the feasibility of incorporating DArTseq-derived single-nucleotide polymorphisms (SNPs) and bioactive compounds into a geographic information system to predict the spatial distribution of *A. afra* gene diversity in selected southern African environs. DNA extraction, sequencing and SNP calling mineral analyses (using R Studio) were performed on a total of 80 genotypes collected around Roma and Mohale's Hoek, Lesotho, and Wepener and Hobhouse, South Africa. Unweighted pair group method with arithmetic mean (UPGMA) and neighbour-joining dendrograms revealed three clusters of variation among genotypes; however, geographic distribution did not influence *A. afra* genetic diversity. Mean heterozygosity among genotypes was 7.7%. Genotypes collected in Roma and Mohale's Hoek exhibited high levels of genetic diversity. Local *A. afra* genetic diversity was relatively low, indicating uniformity of subspecies per locality. The diversity among genotypes indicated that *A. afra* SNPs may occur despite the location. Genomic heterozygosity influences the survival of an organism because genotypes contain two alleles of different types. The diversity makes them adapt better in changing environments. Given that genetic diversity enhances crop performance, our findings can be useful in germplasm management, industries producing byproducts of *A. afra*, and plant breeding programs. For the long-

term conservation of medicinal plants in South Africa, significant sampling and regional data are required.

Keywords: aromatic shrub, DNA fingerprinting, ethnomedicine, germplasm, next-generation sequencing, UPGMA dendrogram

3.2 INTRODUCTION

Artemisia afra Jacq. ex Willd (African wormwood) is part of the Magnoliophyta division, Magnoliopsida class, Asteroideae sub-class, Asterales order, and Asteraceae family. Its morphology, taxonomy, distribution, ethnopharmacology, utilisation, chemistry, and biological effects were reported by Thring and Weitz (2006). *A. afra* is an important medicinal shrub found in Africa and is widely distributed in South Africa, Kenya, Tanzania, Uganda, Ethiopia, Namibia, Zimbabwe, Eswatini, and Lesotho (Liu *et al.*, 2009). *A. afra* occurs in Gauteng, Limpopo, and the eastern regions of South Africa, as well as in Eswatini and Lesotho, extending all the way to the Western Cape. The plant also occurs widely in the KwaZulu-Natal province, ranging from the coastline to the Drakensberg Mountain range (Van Wyk *et al.*, 1997).

A. afra is used as a fumigant against pests of stored products (such as weevils) and as a moth repellent (Nigam *et al.*, 2019). Liu *et al.* (2009) highlight its importance as a medicinal herb used by rural communities to cure infectious diseases primarily the common cold (Van Wyk, 2008). A mixture of *A. afra* and *Eucalyptus globulus* is utilised for treating flu, while a blend of *Lippia asperifolia* and *A. afra* leaves and stems is applied as a remedy for fever, flu, measles, and a preventative measure against lung infection (Liu *et al.*, 2009).

Genetic diversity is important for the adaptability and survival of plant species (Kujane *et al.*, 2019). While there have been several genomic studies of the *Artemisia* genus (Chen *et al.*, 2022; Shen *et al.*, 2018), reports of the *A. afra* genome and its sequencing remain scarce (Hu & Zhen, 2020). The work has been published in accredited journals.

This study aimed to investigate the feasibility of diversity arrays technology sequencing (DArTseq)-derived single-nucleotide polymorphisms (SNPs).

3.3 MATERIALS AND METHODS

3.3.1 Plant material collection

The modified Whittaker nested vegetation sampling method was used to collect 80 leaf samples from four different locations in Lesotho, including Mohale's Hoek (located at 30.1426° S, 27.4674° E) and Roma (located at 29.5212° S, 27.7755° E). In South Africa, leaf samples were collected in Wepener (located at 29.7294° S, 27.0206° E) and Hobhouse (located at 29.5258° S, 27.1452° E). This was carried out systematically by placing ten 0.5 × 2 m (1 m²) subplots along the inside border, two 2 m × 5 m subplots in alternative corners, and a 5 m × 20 m subplot at the center of the plot. The subplots were used to collect plants and surrounding soil from a depth of 60 cm (Ghorbani *et al.*, 2011).

Fresh leaves were frozen in liquid nitrogen and powdered with a mortar and pestle. Each sample was replicated four times, and a total of eighty samples were obtained. DArTseq was used to evaluate the genetic diversity of the genotypes. The DArTseqTM protocol (Diversity Arrays Technology, Canberra, Australia) was used for the extraction and sequencing of *A. afra* DNA. Genomic DNA was extracted from 1 mg of leaf tissue from each accession. Isolation was then carried out using a modified cetyltrimethyl ammonium bromide (CTAB)/chloroform/isoamyl alcohol method (Doyle, 1987). The ground leaf tissue was mixed with 2% CTAB isolation buffer (1.4 M NaCl, 100 mM Tris [pH 8.0], and 20 mM ethylenediaminetetraacetic acid [EDTA]) that had been pre-warmed (60°C; Sigma-Aldrich, St. Louis, MO, USA). The mixture was then transferred to a 2 mL microcentrifuge tube and incubated for 1 hour at 60°C. DNA was extracted once with chloroform-isoamyl alcohol (ChI/IAA, 24:1) and precipitated with 2 volumes of isopropanol. The pellet was washed thoroughly with 70% EtOH (ethanol/ethyl), dried, and dissolved in 100 mL TE buffer (consisting of Tris and EDTA) containing 50 g/mL RNase A (bovine pancreatic ribonuclease; Sigma-Aldrich, South Africa). For DArTseq and SNP genotyping, the extracted DNA was quantified using 0.8% agarose gel electrophoresis adjusted to 50 mg/L.

3.3.3 Diversity arrays technology sequencing

To process the DNA in digestion/ligation reactions, a single restriction enzyme (PstI) compatible adaptor was replaced with two adaptors corresponding to two different restriction enzymes, PstI and SphI-compatible adaptors (Kilian *et al.*, 2012). An Illumina flow cell attachment sequence was combined with staggered sequences of varying length barcode regions to create the PstI-compatible adaptor (Elshire *et al.*, 2011). In 30 rounds of polymerase chain reaction (PCR), only mixed fragments (PstI-SphI) were amplified using the following reaction conditions: 94°C for 1 min., 30 cycles of 94°C for 20 s, 58°C for 30 s, 72°C for 45 s, and 72°C for 7 min. Following PCR, equimolar amounts of amplification products from each of 96-well microtiter plate samples were bulked and applied to a c-Bot (Illumina) bridge PCR, followed by sequencing on an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA). The single-read sequencing was repeated 77 times.

Each lane's sequences were processed using proprietary DArT analytical pipelines (PLs). In the primary PL analysis, poor-quality sequence fragments with a reproducibility of <90% and a read depth <3.5 for SNPs, or 5 for presence-absence markers, were removed. The barcode region was subjected to more stringent selection criteria than the other sequences. Sequences were identified and used in marker callings for each barcode/sample. For reference and SNP alleles, we recorded the average browse depth across loci per individual per locus. Fastqcoll files were groomed using DArT PL's proprietary algorithm, which converts a low-quality base from a singleton tag into a correct base using collapsed tags with multiple members as a template. DArTsoft14 was used in the secondary PL to call DArT PL's proprietary SNP and SilicoDArT (presence/absence of restriction fragments in representation; PA markers) algorithms (Diversity Arrays Technology).

SNP calling was performed at a brink distance of 3 for all tags from all libraries contained within the DArTsoft14 analysis and clustered using DArT PL's C++ algorithm. Clusters were divided into separate SNP loci using the balance of read counts for allelic pairs. To

facilitate the selection of technical parameters to effectively distinguish true allelic variants from paralogous sequences, alleles were tested for deviations using the Hardy-Weinberg equilibrium. Multiple samples were processed as technical replicates from DNA to allelic calls, and scoring consistency was used as the primary selection criteria for high-quality/low-error-rate markers. The high average browse depth per locus (over 30 reads/locus across all markers) ensured for high calling quality. For genotyping by sequencing (GBS) analyses, DNA was diluted to 50 mg/L.

3.3.4 Data analysis

SNP analyses were conducted using DArTseq. For population genetic analyses in R Studio, large SNP sequencing sets were obtained by GBS. GBS was also used in high-throughput sequencing (HTS) experiments. The genome was reduced in GBS using restriction enzymes and sequencing with HTS (Pojskić, 2018). The analysis was performed for 80 *A. afra* genotypes. *A. annua* L., the closest relative to *A. afra*, was used as a reference in the DNA analysis.

Individual and population genetic distance matrices were created. To assess branch support, a distance tree was built using the unweighted pair group method with arithmetic mean (UPGMA) algorithm with 100 bootstrap replicates. Minimum spanning networks were used to visualise the population structure and determine the number of allelic differences that existed between two samples. Principle component analysis (PCA) was used to convert the observed SNP data into principal components, which visualises variation between samples. A discriminant analysis of principle components, a multivariate statistical approach that maximises variance among populations in samples, was used to investigate population assignments.

3.4 RESULTS AND DISCUSSION

It remains challenging to initially assemble large genomes that have a high degree of heterozygosity (Schatz *et al.*, 2012; Kajitani *et al.*, 2014; Shen *et al.*, 2018). GBS DArTseq represents a useful approach to comparing the composition of genomic representations of different genotypes. DArTseq can be used for profiling of whole plant genomes in broader taxonomic spectra, rather than just focusing on cultivated plants, to reveal variable hybridisation signal intensities for different individuals. Despite the species being used as a multipurpose ethnomedicinal plant in many African countries, the current study serves as a benchmark for *A. afra* spp. genomic profiling. We used *A. annua* as the reference species in the genomic analyses, which is one of only a few species sequenced from the family Asteraceae (Shen *et al.*, 2018).

The UPGMA method (Fig. 3.1) revealed three major clusters and subclusters (Kalinowski, 2009). The dendrogram assembled 80 genotypes into three clusters, grouping similar genotypes together, regardless of the sampling locality. The hierarchical cluster dendrogram represents the relationships between genotypes in terms of approximate distances and is based on genetic relatedness. Cluster 1 comprised nine genotypes, while cluster 2 comprised six. Clusters 1 and 2 contained only those genotypes collected from WE. However, most (n=63) genotypes appeared in Cluster 3, the largest cluster in the dendrogram. Cluster 1 included 20 genotypes from MH, RO, and HO. Five genotypes collected from WE also appeared in Cluster 3. The UPGMA-constructed tree indicated variation in genetic distance (the height), ranging from 53.1 to 100. Variations in distance were noted in both clusters and clades (Fig. 3.1), and the observed differences between the genotypes may be ascribed to genetic variation.

Genetic diversity in wild plant species is related to the life form, mating/breeding system, seed dispersal, geographic range, and population size (Li *et al.*, 2020). Ferreira *et al.* (1995) report that *A. annua* is cross-pollinated by both wind and insects, which may have influenced the genetic diversity among Asteraceae species. However, there is no reported information on the mode of pollination of *A. afra*.

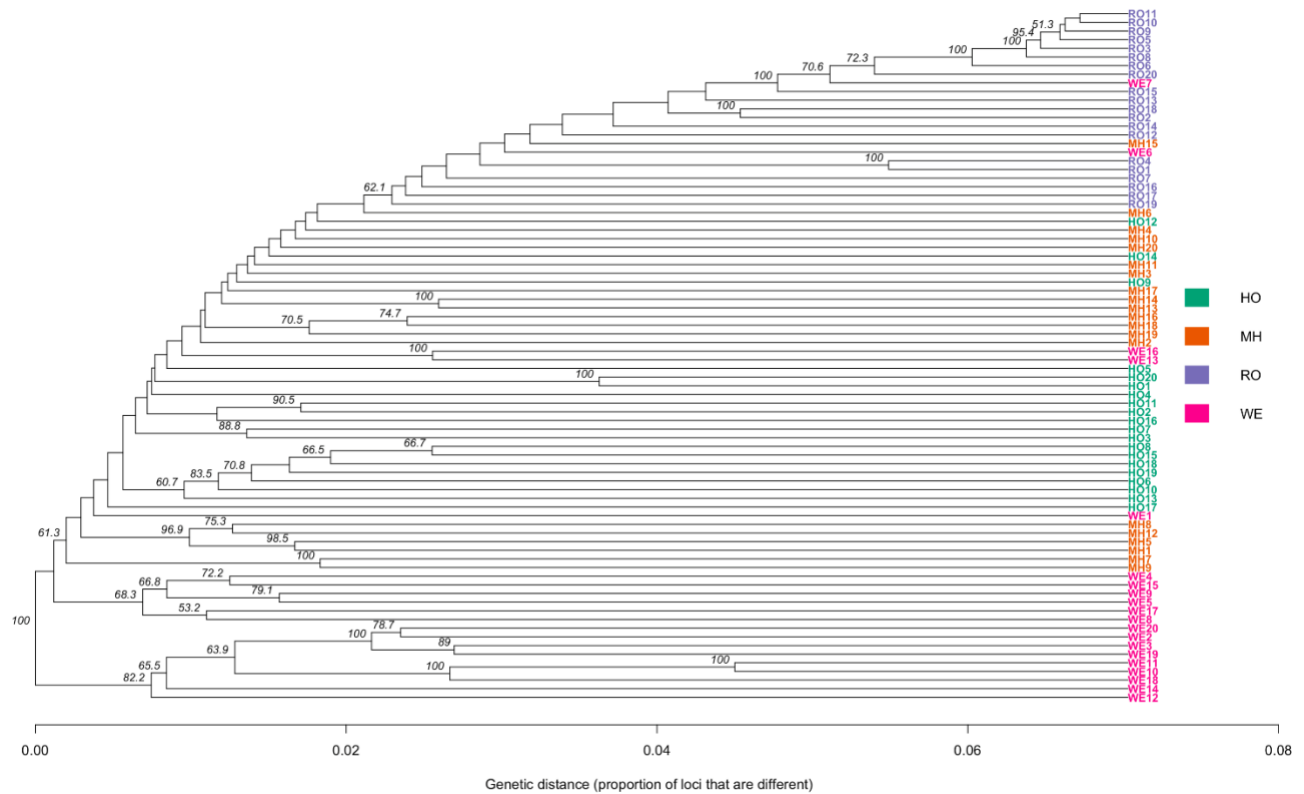


Figure 3.1: Dendrogram of *Artemisia afra* collected from Lesotho (Mohale’s Hoek [MH]: 30.1426° S, 27.4674° E, and Roma [RO]: 29.5212° S, 27.7755° E) and South Africa (Wepener [WE]: 29.7294° S, 27.0206° E, and Hobhouse [HO]: 29.5258° S, 27.1452° E).

Approximately fifteen *A. afra* genotypes collected from WE were closely related to six genotypes from MH. Cluster 2 contained 17 genotypes from HO. Five genotypes from WE were not closely related with the other genotypes collected at the same location, which were grouped in Cluster 3. However, they showed similarities with the genotypes from RO, MH, and HO.

In Cluster 1, the genetic distance in the first clade was 81.9 while that of the second clade in Clusters 2 and 3 was 59.8, further confirming the variation between these genotypes (Fig. 3.1). Clades within Cluster 1 displayed a single-leafed branch that was distinct from the other genotypes. In Cluster 2, the genetic distance between the clades was 63.4, but the genetic distance increased from 63.4 to 78.8. Cluster 3 included many genotypes, but few appeared to be different from each other. High variation in genetic distances was

observed, ranging from 59.8 to 100, though some genotypes showed a shorter distance of 53.1.

Figure 3.2 depicts the minimum spanning network of *A. afra* population clusters based on genetic distances between genotypes. Most *A. afra* genotypes were closely clustered, with a genetic distance ranging from 0.006 to 0.037 bp. Only a few genotypes from HO, RO, and WE were far apart from the remaining genotypes. There were three groups, one central group containing most genotypes, a second cluster in the upper left, and a third cluster in the lower left of the minimum spanning network.

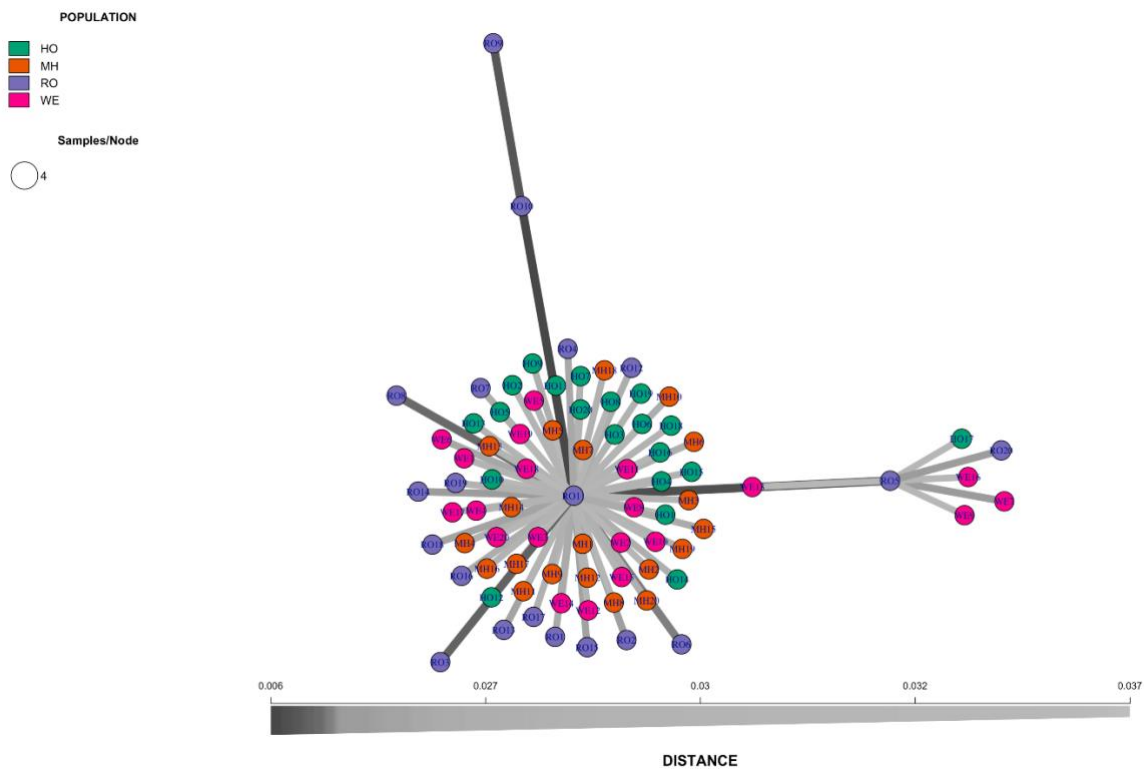


Figure 3.2: Genetic distance relationships of *A. afra* populations obtained from four locations.

The PCA analysis showed genetic variability between the *A. afra* genotypes (Fig. 3.3). The PCA of the entire population was 11.07 with a sum of 22.15. However, the highest and lowest recorded PCA values were 21.15 and 0.79, respectively. The PCA showed that genotypes from the same location were similar. MH genotypes occurred in one

quadrant, showing a positive relationship. However, genotypes from WE and HO were divergent and distant from genotypes collected at the same locations. RO genotypes occurred in the second quadrant, while most genotypes occurred in the WE quadrant. The eigenvalues revealed three clusters, consistent with the dendrogram. Similar results were obtained from a genetic diversity analysis in different populations of *A. annua* native to northern Iran (Reza *et al.*, 2016).

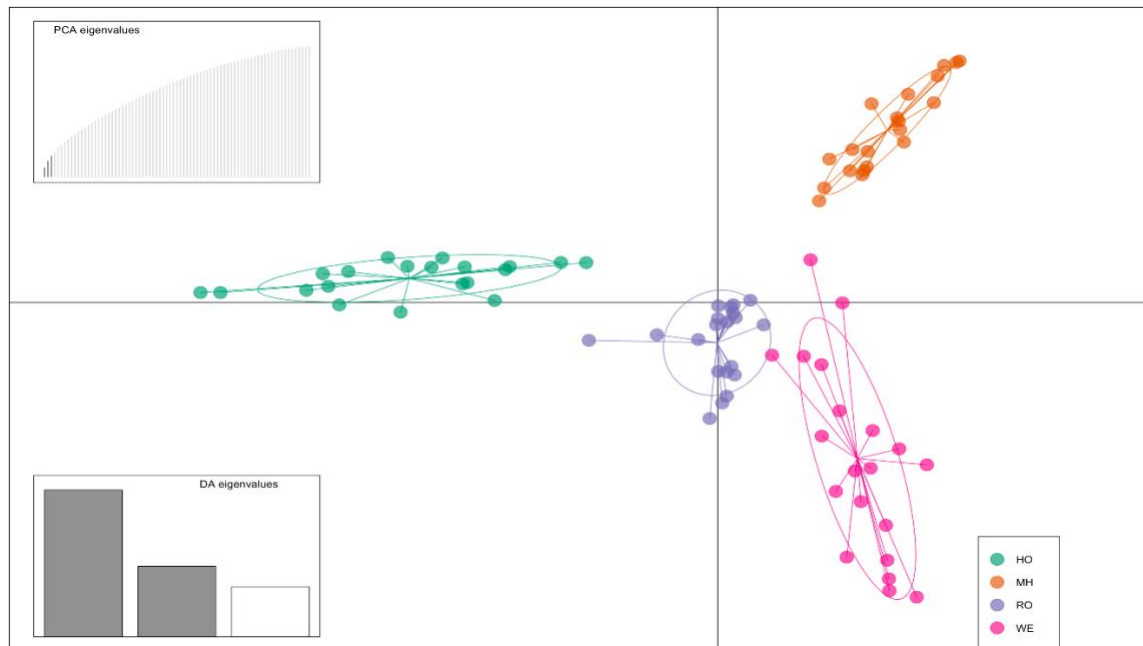


Figure 3.3: Genetic variability assessed via principal component analysis (PCA) of 80 *A. afra* genotypes from four locations.

Population structure and membership probability analyses grouped 80 *A. afra* genotypes into four populations (Fig. 3.4). Each bar in Fig. 3.4 signifies a distinct genotype. Admixed genotypes are indicated by multiple colours. Population 1 (HO) contained 18 pure genotypes and two admixtures. Population 2 (MH) contained 20 pure genotypes. Population 3 (WE) had 16 pure genotypes with four admixtures. Population 4 (WE) had 14 pure genotypes and six admixtures. Three groups were confirmed by the PCA ordination, where HO and WE genotypes were distinct while RO and MH had admixed with some similarities (Fig. 3.5).

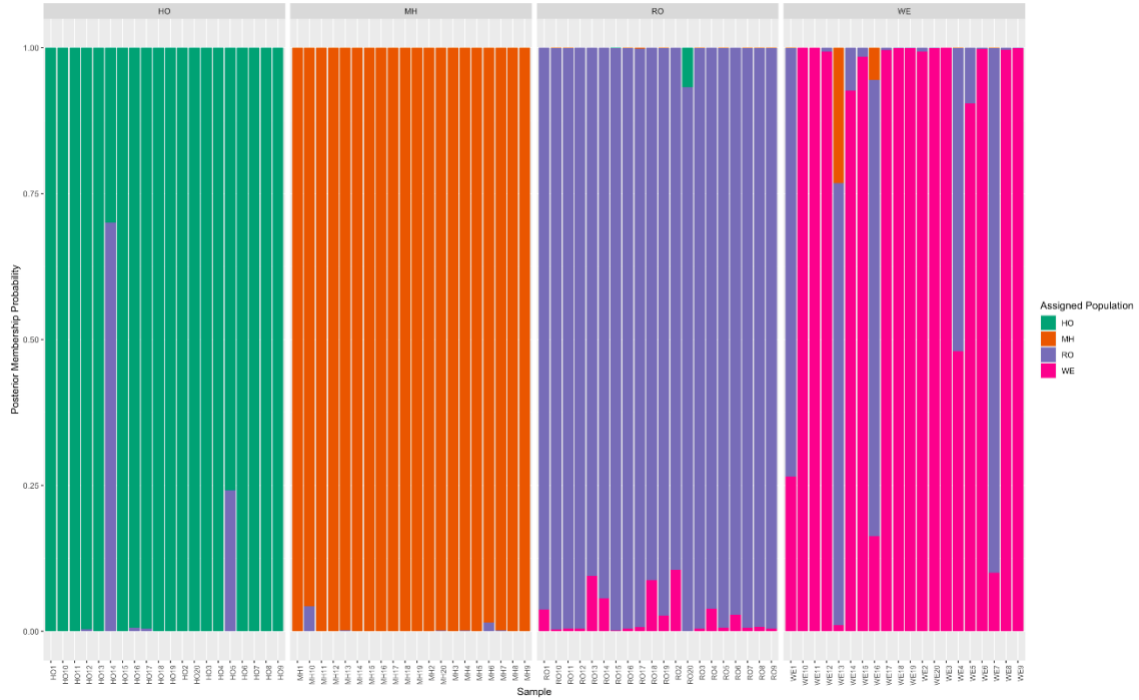


Figure 3.4: Population structure and membership probability of 80 *A. afro* genotypes based on diversity arrays technology. The x-axis corresponds to the genotypes and the colour coding corresponds to the sampling location. Lesotho (Mohale's Hoek [MH], Roma [RO]) and South Africa (Wepener [WE], Hobhouse [HO]).

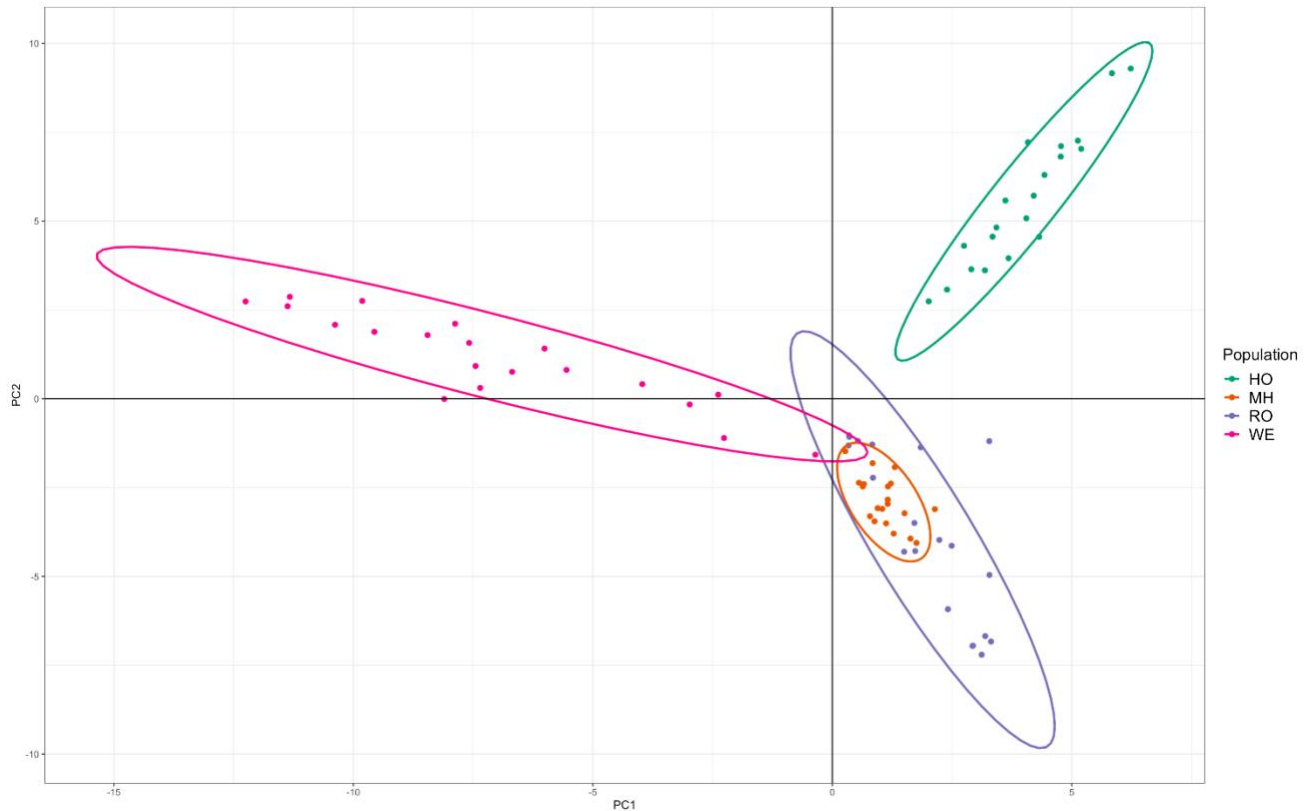


Figure 3.5: PCA ordination of 80 *A. afra* genotypes from four locations.

A total of 22,948 SNPs were generated from the 80 *A. afra* genotypes (Tab. 3.1). Polymorphic locus per chromosome varied from 1 to 7. Some of the chromosomes shared the same locus, with chromosomes 4, 9, 10, and 12 sharing locus 2. A similar occurrence was observed on chromosomes 6 and 8, which shared locus 3. Chromosomes 1, 3 and 11 occurred on locus 5, 6, and 7, respectively. Heterozygosity within populations ranged from 0.0078 to 0.1319 per chromosome. The highest heterozygosity within populations was found on chromosome 10 and the lowest on chromosome 12. Chromosome 10 had a higher within-population genetic diversity (H_s : 0.2085), overall gene diversity (H_t : 0.2565), sample-to-sample gene diversity (D_{st} : 0.0479), high-throughput phenotyping (H_{tp} : 0.2725), corrected D_{st} (D_{stp} : 0.0639), fixation index (F_{st} : 0.1869), corrected fixation index (F_{stp} : 0.2346), inbreeding coefficient per overall locus (F_{is} : 0.3673), and population differentiation value (D_{est} : 0.0808). Very low values were observed for D_{st} , H_{tp} , D_{stp} , F_{st} , F_{stp} , F_{is} , and D_{est} on chromosomes 6 and 12. Chromosome 5 did not show any heterozygosity.

Table 3.1: Genetic diversity within and among 80 *Artemisia afra* genotypes based on 22,948 single nucleotide polymorphism markers from Lesotho (Mohale's Hoek, 30.1426° S, 27.4674° E; Roma, 29.5212° S, 27.7755° E) and South Africa (Wepener, 29.7294° S, 27.0206° E; Hobhouse, 29.5258° S, 27.1452° E).

Chromosome No.	Locus No.	Ho	Hs	Ht	Dst	Htp	Dstp	Fst	Fstp	Fis	Dest
1	5	0.011	0.039	0.041	0.002	0.042	0.003	0.053	0.069	0.716	0.003
3	7	0.041	0.067	0.068	0.001	0.068	0.002	0.019	0.025	0.381	0.002
4	2	0.098	0.107	0.116	0.009	0.119	0.013	0.081	0.106	0.081	0.014
6	3	0.093	0.083	0.083	0.000	0.083	0.000	-0.002	-0.003	-0.117	0.000
8	3	0.057	0.062	0.066	0.003	0.067	0.005	0.051	0.067	0.078	0.005
9	2	0.010	0.028	0.028	0.000	0.028	0.000	0.004	0.005	0.630	0.000
10	2	0.132	0.209	0.257	0.048	0.273	0.064	0.187	0.235	0.367	0.081
11	6	0.058	0.082	0.082	0.001	0.082	0.001	0.007	0.010	0.295	0.001
12	2	0.008	0.023	0.023	0.000	0.023	0.000	-0.002	-0.003	0.654	0.000
Mean		0.057	0.078	0.085	0.007	0.087	0.010	0.044	0.057	0.343	0.012
Standard deviation		0.044	0.056	0.071	0.016	0.076	0.021	0.061	0.077	0.290	0.026
Standard error		0.0147	0.0188	0.0236	0.0052	0.0252	0.0069	0.0204	0.0256	0.0967	0.0088

* Chr: chromosomes; Ho: heterozygosity within populations; Hs: genetic diversity within populations; Ht: overall gene diversity; Dst: gene diversity among samples; Htp: high-throughput phenotyping; Dstp: corrected Dst; Fst: fixation index; Fstp: corrected fixation index; Fis: inbreeding coefficient per overall loci; Dest: measure of population differentiation.

Population genetic analysis is important for detecting gene diversity within a population and identifying genetic drift. Due to their reproducibility, reliability, and independence from environmental conditions, DNA markers are used for precise genetic characterisation, as well as in determining genotype origin and dispersal routes (Sütyemez *et al.*, 2021). DArTSeq-derived SNPs are especially useful in the profiling of whole-plant genomes. In this study, a total of 22,948 SNPs were generated for *A. afra*, with a mean heterozygosity of 7.7% within populations. In studies of *A. annua*, extremely low within-population heterozygosity was observed, with variation among individual chromosomes. Gene gain or loss may be important factors influencing genetic diversity and reducing the numbers of similar genes in the host. In *Kalmia latifolia* L., the mean heterozygosity within populations was 19% (Li *et al.*, 2020), which was higher than what we estimated here. Likewise, *A. annua*, a popular species of Asteraceae from China, showed heterozygosity of 21% (Czechowski *et al.*, 2016).

3.5 CONCLUSIONS

This study outlines the importance of DArTseq-derived SNPs in characterising the genetic diversity of *A. afra* genotypes found in different environs of southern Africa. A PCA, minimum spanning network, UPGMA-cluster dendrogram, population structure, membership probability test, and PCA ordination test confirmed the genetic relativity among genotypes from a single location. However, geographic distribution was not correlated with the genetic diversity among *A. afra* genotypes collected in the four locations. The diversity among genotypes clearly indicated that *A. afra* SNPs may occur despite the location. Given that genetic diversity enhances crop performance, our findings can be useful in germplasm management, industries producing byproducts of *A. afra*, and plant breeding programs. In order to maintain byproduct standard of *A. afra*, collection must be done in consideration of the location. For the long-term conservation of medicinal plants in South Africa and Lesotho, significant sampling and regional data are required.

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CHAPTER FOUR

COMPARATIVE STUDY OF MINERAL PROPERTIES OF *ARTEMISIA AFRA* L. FROM DIFFERENT LOCATIONS

ABSTRACT

Artemisia afra L. is used as a medicinal plant because of its components. It contains micronutrients and macronutrients depending on the environment and soil they are growing. Large parts of Africa's population make concoctions by mixing *Artemisia afra* with other plants. The collection of medicinal plants from diverse areas is assumed to result in different ingredients. This is triggered by abiotic stress, which negatively influences respiration, transpiration, and photosynthesis. During growth and development, nutrient uptake, the plant system plays a significant role in coordinating several physiological and developmental processes. The study aims to analyse the macro- and micro-element composition of *Artemisia afra* L. genotypes. The samples were collected for four locations (Mohale's Hoek, Roma, Wepener, and Hobhouse). The soil pH, soil cation exchange capacity, and elements were determined in the laboratory. The results revealed differences in the composition of elements; in some plants accumulation of elements was very high. However, in certain locations, some *Artemisia afra* had very low levels. Cation exchange capacity and pH were found to affect the number of elements absorbed by plants. It is therefore very important to consider plant analysis to find out the plant components.

Keywords: *Artemisia afra*, minerals analysis, soil analysis, UPGMA dendrogram, and PCA.

4.1 INTRODUCTION

Medicinal plants are used to prepare herbal concoctions among rural people in South Africa and Lesotho to treat human and livestock infectious diseases. In some cases, some medicinal plants are used solely or sometimes mixed with herbs such as mint, peppermint, Eucalyptus, and others. Many of these plants have promising characteristics. *Artemisia afra* is widely used in southern African countries as traditional medicine for the treatment of many ailments and diseases. In Lesotho, *Artemisia afra* is a very important plant used for treating the flu, as well as to control and eliminate grain weevils in storage. These medicinal plants are therefore classified as complementary and alternative medicines (CAMs) in traditional African societies. CAMs continue to play an important role in the treatment of many diseases and ailments (Nweze & Eze, 2009).

For plant growth and development, 17 essential elements are needed, and among them are 14 mineral elements (Ma & Tsay, 2021). These elements are absorbed by the roots from soil solution, and they are translocated to different parts of the plants. In plants, a very low concentration of minerals results in active transportation. The plant leaves use these essential elements, water and sunlight to manufacture its own food. Minerals alone cannot be used by plants without air and sunlight. All these processes happen at cellular and subcellular level, where mineral elements are further transported to different organelles, such as vacuoles, chloroplasts, mitochondria and Golgi apparatus for use in their physiological functions. According to Wang *et al.* (2021), a shortage of plant nutrients such as N and P in soils limits plant growth and development. The yield of the plant depends directly on macro- and micro-nutrients; however, maintenance and improvement of soil fertility is important in obtaining the most profitable yields (van Maarschalkerweerd & Husted, 2015).

Literature indicates that some heavy metals, such as Fe, Co, Cu, Mn, Zn, Mo and others, can act as micro-nutrients and play a significant role in plant development; however, when they exceed threshold concentrations, they become poisonous (Angulo-Bejarano *et al.*, 2021). Heavy metals occur naturally from the earth's crust. Their consumption is poisonous to human health and some of them affect yield in plants (Trentin *et al.*, 2022).

Plant growth requires soluble nutrients and plants easily utilise dissolved ions. Primary minerals release several nutrient elements into the soil solution by means of physical, chemical and biological weathering. The roots then facilitate the mineral ions' movement through the xylem and phloem by means of active transport, and it happens against the concentration transport. Velasquez *et al.* (2018) state that the mineral nutrients play a potential role in supporting plant wellness and work together with light, humidity and temperature. According to literature, nitrogen, phosphorus and potassium are utilised by plants at different environmental statuses; uptake of Nitrogen is influenced by high temperature (Lukac *et al.*, 2011), Potassium directly depends on soil moisture (Wang *et al.*, 2013), and light intensity contributes towards the intake of phosphorus (Wen *et al.*, 2017). In plants, minerals decrease pathogen load as well as imparting resistance to the plants against the invading pathogens (Tripathi *et al.*, 2022).

Essential minerals from the soil are accumulated in different parts of the plant. For instance, *Hibiscus cannabinus* leaves are mentioned to be highly nutritious and are a considerable source of substances such as beta-carotene, vitamin C, protein, iron, and potassium (Michael, 2010). The knowledge of essential minerals is very important in determining the quality of medicinal plants as well as the safety of their consumption by humans. The study is aimed to analyse the macro- and micro-element composition of *Artemisia afra* L. genotypes from four different locations.

4.2 MATERIALS AND METHODS

4.2.1 Soil analysis

The soil samples were taken from the depth of 30 cm at the same places where *Artemisia afra* samples were collected (Roma, Mohale's Hoek, Wepener and Hobhouse). The analysis took place in the Soil Science Laboratory of the National University of Lesotho.

4.2.1.1 Soil pH Determination

Soil sample of 20 g (air-dried <2 mm) was weighed and transferred to a 250 ml Erlenmeyer flask. Then, 50 ml of water was added, and the solution was shaken in a reciprocal shaker at the rate of 150 rpm for 15 minutes. The suspension was then allowed to stand until the soil particles had settled before measuring the pH of the suspension with a calibrated pH-meter. Next, 3.73g KCl was added to the soil-water suspension and shaken once again for 15 minutes. The suspension was again allowed to settle, and pH was measured.

4.2.1.2 Soil Cation Exchange Capacity (CEC)

The soil sample of 5 g of air-dried soil was weighed and sieved through a 2 mm sieve, into the beaker. A little NH_4OAc was added; it was swirled to stir and then allowed to stand for approximately 10 minutes. A 250ml Erlenmeyer flask was mounted together with a funnel and Whatman No. 1 filter paper. The soil was washed onto the filter paper in the funnel with NH_4OAc and 100 ml of the filtrate was collected; NH_4OAc was added to make to required volume and stored (Filtrate 1). The soil residue was washed again with 100 ml of NH_4OAc solution to saturate all the exchange sites with Na^+ and the filtrate was discarded. Excess Na^+ was washed with approximately 100 ml (~4 full funnels) of pure alcohol and filtrate was discarded. The adsorbed Na^+ was replaced by washing the soil with approximately 100 ml of NH_4OAc and stored (Filtrate 2). The cations (Ca^{++} , Mg^{++} , K^+ and Na^+) were read in filtrate 1 and CEC as Na^+ in filtrate 2 on the Atomic Absorption Spectrometer.

Iron, manganese, copper and zinc in DTPA extractant

Chelating agent, Diethylenetriaminepentaacetic acid (DTPA) is increasingly used for the determination of Fe, Mn, Cu and Zn in the soil (Lindsay Norvell method).

The soil sample of 20 g was air-dried in a polythene bottle of 100 ml. One standard sample and two blanks were included with each series. Next, 40 ml of DTPA extractant was also added. The reciprocal shaker was shaken lengthwise in horizontal position at the speed of 150 rpm at 20°C for 2 hours. They were then filtered through a fine filter.

4.2.2 Plant Sample preparation

Plant materials used for mineral analysis were collected from four locations where they had been collected before. Foliar parts of the plants were oven-dried at 60°C for 2 to 3 days until the required weight was obtained (Singh & Praharaj, 2017). Chemical analysis was conducted at the National University of Lesotho's Soil Science Laboratory.

4.2.3 Plant Mineral Analysis

4.2.3.1 Sample preparation

The 0.5000g of air-dried plant material <1 mm (to nearest 0.1 mg) was weighed. The material was transferred to a digestion tube using a glass tunnel with a long stem. This included two samples and two blanks. Five carborundum boiling stones were added.

Next, 2.5 ml of the digestion mixture was added in such a way as to wash down any soil adhering to the tube wall, it was mixed and heated at 100°C for 2 hours. Then, 1 ml of H₂O₂ was added three times and immediately mixed carefully after each reaction. The second and the third portion were added after the reaction of the previous addition had ceased; 2 ml of H₂O₂ was added and mixed well. The digestion tubes were placed in a block digester and heated at 300°C just below the point of the digestion mixture for 1 hour.

Then, the digestion tubes were removed from the block digester, and they were allowed to cool for 5 minutes. After cooling, it was mixed well. The digestion tubes in the block digester were replaced and heated again at 300°C (digest was repeated when digestion organic matter still present). After digestion, the tubes were removed from the block digester and were allowed to cool. Then, 50 ml of water was added and mixed well. After cooling to room temperature, it was diluted to the mark with water and the mixture was filtered through a fine filter paper into a 50 ml polypropylene Erlenmeyer flask.

Measurements of Potassium, Calcium, Phosphorus, Magnesium, Iron, Zinc, Manganese, Boron, Copper and Nitrogen were determined using atomic emission spectrometry with Inductive Coupled Plasma Optical Emission Spectrometric (ICP-OES) (Optima 4300 DV ICP-OES, PerkinElmer Inc. Wellesley, USA) (Maathuis, 2013). However, Duma's combustion

method was used to determine N using a Leco FP-528 combustion nitrogen analyser (LecoCorp, St. Joseph, MI, USA) (Maathuis, 2013).

4.2.4 Data Analysis

ANOVA was performed using a Randomised Complete Block Design (RCBD) for analysis of plant mineral samples (Sodium, Potassium, Calcium, Magnesium, Phosphorus, Sodium, Iron, Copper, Zinc and Manganese). Principal component analysis was performed to draw the relationship among minerals of same samples and Unweighted Pair Group Method (UPGMA) was used to generate the dendrogram.

4.3 RESULTS AND DISCUSSION

Plants contain mineral elements in different forms and these elements are essential for plants to perform various functions (Qin *et al.*, 2017). They get these elements from the soil as soils are a potential sink for minerals, and supply plants with these essential minerals. According to Qin *et al.* (2017), minerals act as go-between to facilitate vital biochemical reactions and act as a co-factor or catalyst for many enzymes. Lack of minerals may trigger failure of certain processes, and this may cause the by-pass of some reactions. Differences in mineral content might lead to heterozygosity as they are observed in Table 4.1. The deficiency of plant minerals in the soil results in stress, which can lead to complementary substance production (Rouached *et al.*, 2017). In most cases, distribution of plants is influenced by the availability of minerals. That is why plants do not grow everywhere but are found in special habitat. The same occurred for *Artemisia afra* plants as they were collected from rocky soils.

Soils from Wepener had a higher content of nitrogen (7.49 mg kg^{-1}) and phosphorus (3.43 mg kg^{-1}) than the other three sites. High potassium content of 354 mg kg^{-1} was found in Hobhouse, and Wepener was the least (100 mg kg^{-1}) of all four locations. Mohale's Hoek soils had much higher calcium content (8665 mg kg^{-1}) than Roma (6410 mg kg^{-1}), Wepener (4597 mg kg^{-1}) and Hobhouse (895 mg kg^{-1}). It was also observed that soils in Wepener had a considerable amount of Magnesium compared to other locations where

collection was done. Roma soils had a high Zinc nutrient (8.12 mg kg^{-1}) and very little Zinc was found in soils from Mohale's Hoek (0.99 mg kg^{-1}).

Regarding soils' heavy metals, Copper and Iron were also present in soils collected from Roma, Mohale's Hoek, Wepener and Hobhouse. High amounts of 1.64 mg kg^{-1} of Copper were obtained from Mohale's Hoek soils with lower content of Iron of 13.84 mg kg^{-1} . The results in pH of Roma, Mohale's Hoek and Wepener were all acidic. Only the Hobhouse soil was slightly basic with an organic carbon percentage of 10.74, higher than the organic carbon percentages of Roma, Mohale's Hoek and Wepener. Even though there are differences in soils collected from different locations, the cation exchange capacity of four samples seemed to be slightly different. Higher CEC was obtained from Mohale's Hoek soils, followed by Wepener, then Hobhouse and, lastly, Roma.

Soils with a high organic carbon percentage were obtained from Hobhouse soils (10.74%), followed by 8.95% organic carbon from Wepener soils. Roma (1.59%) and Mohale's Hoek (4.77%) soils were found to contain very low organic carbon percentages. However, the soil pH of samples taken from Hobhouse was 7.06 and the rest of the soil sampled from Roma (6.86), Wepener (5.37) and Mohale's Hoek (5.45) were acidic. The capacity of the soils to hold exchangeable cations was also different. CEC of Mohale's Hoek soil was 35.56, followed by 34.12 of Wepener, 32.75 of Hobhouse and then 31.83 of Roma. These results indicate that *Artemisia afra* grows well in different environments.

Table 4.1: Soil chemical analysis of Lesotho region (Mohale's Hoek, 30.1426° S, 27.4674° E and Roma, 29.5212° S, 27.7755° E) and South Africa's (Wepener, 29.7294° S, 27.0206° E and Hobhouse, 29.5258° S, 27.1452° E) soils.

Location	P	K	Ca	Na	Mg	Cu	Zn	Fe	Mn	Available N	pH (H ₂ O)	*OC %	**CEC
mg kg ⁻¹													
Wepener	3.43	100.60	4597.00	50.04	1130.0	1.37	3.75	36.52	7.49	112.00	5.37	8.95	34.12
Roma	2.75	332.00	6410.00	45.46	318.84	1.28	8.12	34.46	5.13	112.00	6.86	1.59	31.83
M/Hoek	2.16	212.50	8665.00	54.28	663.00	1.64	0.99	13.84	6.52	84.00	5.45	4.77	35.56
Hobhouse	2.29	354.00	895.00	49.30	582.50	1.25	5.89	18.75	4.52	140.00	7.08	10.74	32.75

*OC=Organic carbon, **CEC=cation exchange capacity

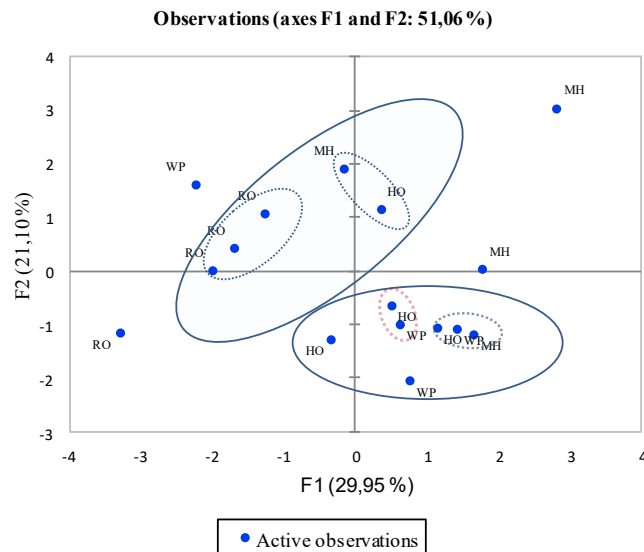
Nitrogen, Phosphorus and Potassium are major essential nutrients in plant growth. The results of Nitrogen from four locations were significantly different. High Nitrogen percentage of 56% was observed in Mohale's Hoek *Artemisia afra*. However, Roma (43%) and Hobhouse (39%) plants' Nitrogen content were slightly lower. Wepener was the least of all locations by 30%. A difference was also found in Phosphorus analysis. In this case, Wepener (2.27mg/Kg^{-1}) had higher amounts of Phosphorus than other places. This was followed by Mohale's Hoek and Hobhouse. Roma's *Artemisia afra* Phosphorus content was the smallest. Statistically, there was no significant difference between the four locations. The foliar results of Potassium, Sodium, Iron, Copper and Zinc were not significantly different in the four locations. *Artemisia afra* samples from Mohale's Hoek (26.93) and Wepener (24.15) had the highest Manganese content compared to Hobhouse (15.25) and Roma (21.75).

Table 4.2: *Artemisia afra* (wormwood) foliar minerals collected from four different locations in Lesotho (Mohale's Hoek, 30.1426° S, 27.4674° E, and Roma, 29.5212° S, 27.7755° E) and South Africa (Wepener, 29.7294° S, 27.0206° E, and Hobhouse, 29.5258° S, 27.14520 E).

Foliar minerals (mg kg ⁻¹)					
Location	N%	P	Ca	Mg	K
Hobhouse	0.39±0.07 ^{bc}	1.92±0.16 ^{bc}	17.27±0.37 ^a	6.52± 1.36 ^b	1289.00±102.47 ^a
M/Hoek	0.56±0.12 ^a	2.09±0.24 ^{ab}	16.26±2.46 ^a	4.66± 0.51 ^b	1346.93±145.60 ^a
Roma	0.43±0.08 ^{ab}	1.72±0.06 ^c	10.20±1.30 ^b	10.37±1.98 ^a	1166.87±165.04 ^a
Wepener	0.30±0.02 ^c	2.27±0.26 ^a	14.67±2.84 ^a	10.81±1.64 ^a	1278.25±55.49 ^a
LSD _{T0.05}	0.12	0.31	2.96	2.48	223.3
p-value	**	*	***	***	ns
Location	Na	Fe	Cu	Zn	Mn
Hobhouse	109.14±6.05 ^a	102.60±114.3 ^a	3.68±2.31 ^a	35.43±6.97 ^a	15.25±1.78 ^b
M/Hoek	103.68±7.02 ^a	79.75±60.66 ^a	9.02±4.15 ^a	36.05±5.18 ^a	26.93±7.35 ^a
Roma	105.69±3.49 ^a	178.78±38.69 ^a	5.33±1.16 ^a	32.60±4.97 ^a	21.75±3.23 ^{ab}
Wepener	104.21±10.02 ^a	110.38±121.49 ^a	5.77±3.19 ^a	32.25±4.28 ^a	24.15±2.96 ^a
LSD _{T0.05}	11.44	154.73	3.89	8.82	6.65
p-value	ns	ns	ns	ns	*

Degrees of freedom = 8. Statistical significance was at the 0.05 p-value. *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$, ns = not significant at $p > 0.05$, \pm = standard deviation, LSD_T 0.05 = least significant difference at the 5% level of significance. Means with the same letter as superscripts within a column are not significantly different ($p < 0.05$).

The principal component analysis on mineral data loading is shown in Figure 4.5. Phosphorus, calcium, potassium, iron and zinc were positively loaded to the first PCA, while Cu was loaded to the second PCA with variability of 29.95% and 21.12%, respectively. Furthermore, the foliar mineral relationships among populations of *A. afra* were further shown in Figure 4.6 using UPGMA, and the NJ method. Diverse grouping was found on species obtained in RO, WE and another diverse group occurred in MH. However, most of the RO species were relatively close to each other, located in the positive quadrants. Only one RO species occurred on the negative quadrant. Similar results were observed on three MH species even though they were scattered on two upper quadrants. One MH species was found on the far lower quadrant. Some *Artemisia afra* species from HO and WE appeared to be closely related on the lower side fourth quadrant.



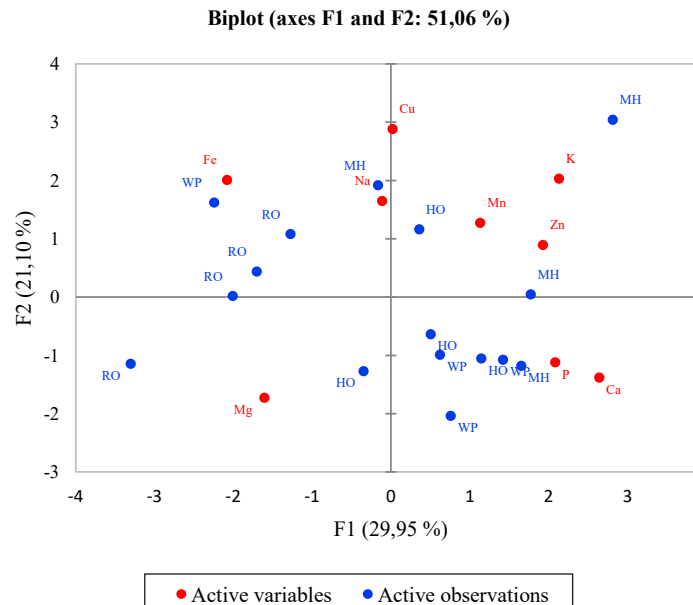


Figure 4.1: Foliar mineral content variability principal component analysis of *Artemisia afra* L. genotypes from four locations (HO – Hobhouse; MH – Mohale’s Hoek; RO – Roma; WE – Wepener).

Unweighted Pair Group Method (UPGMA) was used to generate the dendrogram of foliar minerals for *Artemisia afra* species collected from Roma, Wepener, Mohale’s Hoek and Hobhouse. The dendrogram showed two distinct clades originating from the same point. One clade had only two leaves of Roma species, their wavelength was estimated to be 300 and both were 75. The second clade was separated into two clades, a simplicifolius and bifolius. A single clade observed on one of the MH species showed the differences. MH species separated from the second clade at about 375 wavelengths. Bifolius clades contained many different species which seemed to be different by dividing into two clades where the species were further classified at different wavelengths of 125 and 175.

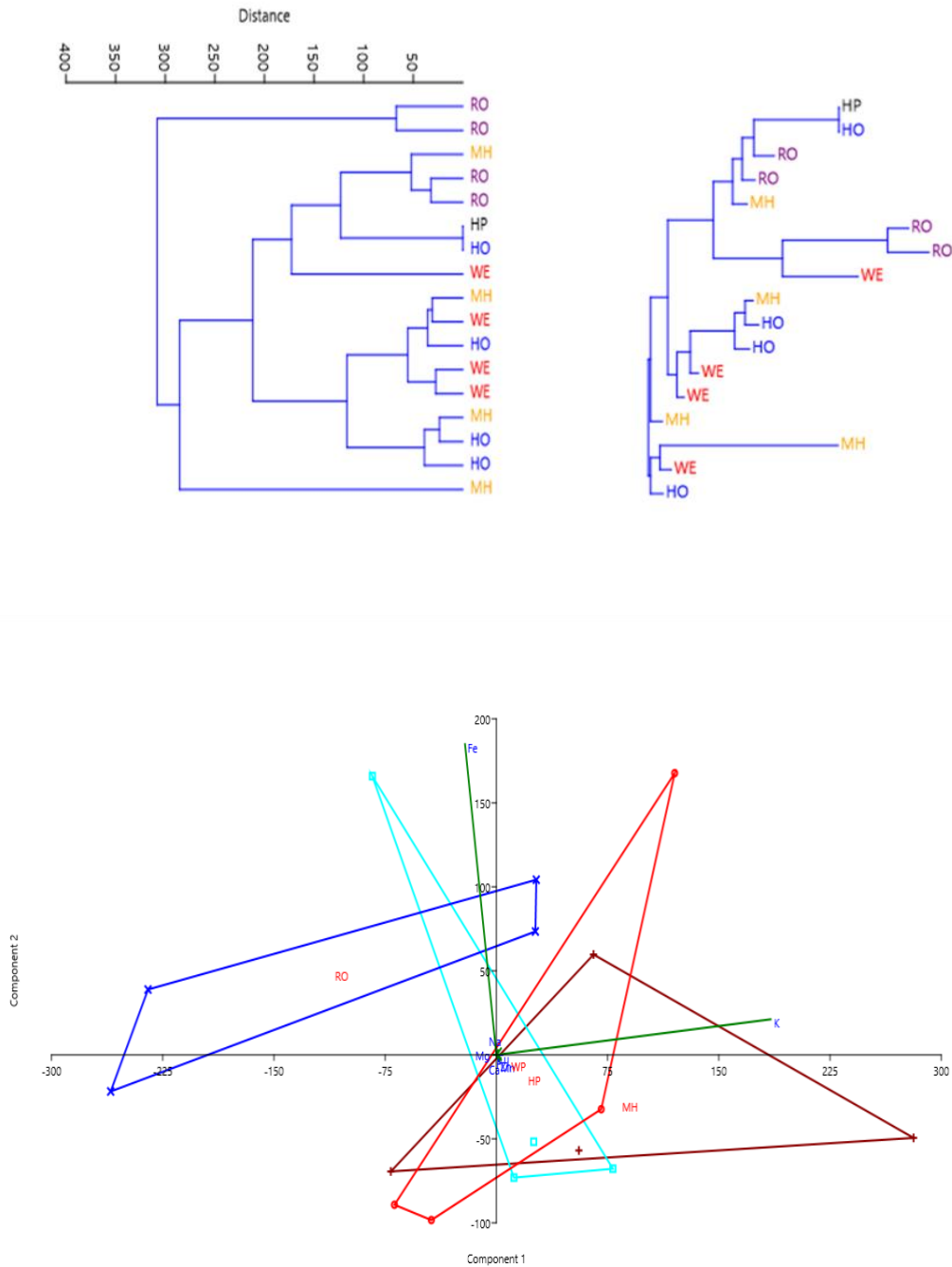


Figure 4.2: Foliar mineral relationship among populations of *Artemisia afra* L. using unweighted pair group method with arithmetic (UPGMA) and the neighbour joining (NJ) methods (HO – Hobhouse; MH – Mohale’s Hoek; RO – Roma; WE – Wepener).

All living organisms solely depend on mineral elements for growth as well as their immune systems. Shortages of these elements normally result in diseased plants and sometimes stunted or poor growth. In plants, mineral elements are naturally present in different forms and some of these elements are essential for the body to perform different functions (Qin *et al.*, 2017). Soils are a potential sink for minerals, and they supply plants with these essential minerals. Soil chemical analysis from Table 4.1 showed different amounts of NPK and other minerals. According to Qin *et al.* (2017), minerals mediate vital biochemical reactions by acting as a co-factor or catalyst for many enzymes. Differences in minerals content might lead to heterozygosity, as it was observed in Table 4.2. The deficiency of plant minerals in the soil results in stress, which can lead to complementary substance production (Rouached *et al.*, 2017). In most cases, distribution of plants is influenced by the availability of minerals.

According to Lula *et al.* (2022), the Copper element is essential to both respiration and photosynthesis processes as it plays a signalling role in protein transcription and Fe mobilisation. Excessive levels of mineral elements may cause damage in plants, for instance, a high concentration of copper may influence plant morphology, nutritional disorders, physiological, morphological and biochemical alterations (Adrees *et al.*, 2015). In Mohale's Hoek, a high concentration of Cu (9.02 mg kg^{-1}) might affect the safety of *Artemisia afra* growing in the area when they are consumed. Copper is classified as a heavy metal even though is one of the essential nutrients. It is needed in minute amounts so that it does not become harmful to plant and people. However, Adrees *et al.* (2015) state that very small amounts of it may hold back the growth rate of plants. At Hobhouse, where the amount of Cu is 3.68 mg kg^{-1} , is a good example of where Cu is very low. Situations where fertilisers, fungicides, herbicides and pesticides containing Cu^+ are intensively used, contribute to higher results of copper in the soil.

Furthermore, Mn, Cu, Fe and Zn are known to play a major role in plant defence (Fones & Preston, 2013). These micronutrients are noted to play a major role in regulating many cellular metabolic activities in promoting proper plant growth and development (Arif *et al.*, 2021). In plants, growth and development are solely dependent on photosynthesis, respiration, chlorophyll synthesis, transcription, translocation, cell division and cell

elongation. All these processes are activated by micronutrients and macro-nutrients, as well as the environment around them. Arif *et al.* (2021) state that their deficiencies and abundance in plants may hinder their growth and development. Plants regulate the movement and amount of nutrients taken in and out, as well as amounts stored by plants to avoid toxicity, shortage and abundance at a cellular level. In the case of this study, samples collected from all four locations were not significantly different on Cu, Fe and Zn. This shows that *Artemisia afra* from different locations had similar amounts of these micro-elements. According to Arrivault *et al.* (2006), very high concentrations of Zinc influence shortage of Iron in plants. However, adequate amounts of each element balance the required amount in plants. They are required in small amounts because they become toxic to plants when present above their threshold (Angulo-Bejarano *et al.*, 2021).

Cation exchange capacity (CEC), soil pH and soil organic matter are useful in soil fertility as they have a positive influence on plant growth. It has been noted that there are higher CEC values in calcareous soils when pH is neutral (Saidi *et al.*, 2008). CEC has been described as the measure of the ability of the soil to hold and exchange cations (Saidi, 2012). According to Saidi (2012), soil CEC is the measure of surface electric charge of soil components. Mohale's Hoek and Wepener soils had higher CEC than Roma and Hobhouse soils. This means the soils have higher nutrient holding capacity of positively charged elements than where the CEC is low. The soils can store and release nutrients so that plants are able to use the nutrients. Mattila and Rajala (2021) categorised nutrients into cationic nutrients (Calcium, Magnesium, Potassium, Iron and Ammonium) and non-nutrients (Aluminium and Hydrogen). Mattila and Rajala (2021) further confirm that soil cation exchange capacity is the gauge of the ability of the soil to hold and release nutrients. Therefore, the soil structure influence CEC of the soil and *Artemisia afra* plants growing in different habitats might be different in chemical.

4.4 CONCLUSION

Soils are plant mineral element reservoirs as they supply plants with nutrients in the form of solution. Absorption of nutrients takes place by three processes, namely diffusion, mass transport, and root interception. However, some factors may deter smooth movement of nutrients from the soil. In this study, mineral element data designated different amounts from *Artemisia afra* samples collected from four areas. *Artemisia afra* foliar minerals seemed to be related to availability of mineral elements in the soil where they were growing. The results confirmed that mineral element uptake in plants depends on soil pH and CEC, as well as organic carbon. It has been found that, in highly acidic soils, Manganese becomes more available to plants, as evident in Wepener, Mohale's Hoek, Roma and Hobhouse. This affected the intake of other essential elements, such as Calcium, Phosphorus and Magnesium because very low amounts were noted during analysis. The restriction of mineral element intake is controlled by soil pH and cation exchange capacity. Therefore, the high CEC of the soil from Mohale's Hoek showed that N% samples outperformed samples from Wepener, Roma and Hobhouse.

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CHAPTER FIVE

QUANTITATIVE ANALYSIS OF *ARTEMISIA AFRA* L. (AFRICAN WORMWOOD) BIOACTIVE COMPOUNDS

ABSTRACT

Medicinal plants contain secondary metabolites which lead them to possess diverse useful properties over other plants. *Artemisia* plants are mostly known in Africa for their pharmacologically active compounds used to cure health problems such as colds, blood pressure, flu and many other diseases. *Artemisia afra* is one of these important plants containing phytochemicals such as flavonoids, phenolic acids, coumarins and many others. The objective of the study was to investigate the qualitative and quantitative analysis of the major bioactive components of *Artemisia afra* L. genotypes. The samples were collected from South Africa (Wepener and Hobhouse) in the Free State and Lesotho (Roma and Mohale's Hoek). Testing of qualitative and quantitative compounds was done and four secondary metabolites, namely ascorbic acid, flavonoids, tannins and phenolics, were disclosed from the samples. Much higher amounts of phenolics were noted from *Artemisia afra* samples than other chemicals. Extremely low amounts of ascorbic acids were also noted during analysis of samples. It is therefore concluded that *Artemisia afra*'s total phenolic amounts outperformed all other metabolites evaluated.

Keywords: *Artemisia afra*, phytochemicals, qualitative and quantitative analysis.

5.1 INTRODUCTION

Plants produce chemical substances known as phytochemicals for their defence as they are rich in antioxidants. Even though there are many phytochemicals, few have been isolated and identified from plants (Cao *et al.*, 2017anthraqui). According to Xiao and Bai (2019), phytochemicals can be grouped into carotenoids, flavonoids, coumarins, indoles, isoflavones, lignans, organosulfures, catechins, phenolic acids, stilbenoids, isothiocyanates, saponins, procyanidins, phenylpropanoids, nones, ginsenosides and many other groups. Phytochemicals play a major defensive role in plants, such as protecting them from diseases and adverse environmental conditions. Plants are therefore major sources of natural chemicals which are used to make medical concoction to be used to treat humans.

Medicinal plants' functions play an important role in food and pharmaceutical industries for disease prevention and treatment (Yu *et al.*, 2021). Vegetables and fruits are however also used for food and medicinal purposes as they also constitute a higher content of phytochemicals. Since ancient times, medicinal plants have been used to treat and diagnose diseases and infections (Agidew, 2022). Naseem (2014) notes that phytochemical components determine the power of the medicinal plant which causes definite pharmacological action on the human body. Phytochemicals are derived from different parts of the plants, such as leaves, stems, roots and fruits. Banu and Cathrine (2015) further explain that medicinal plants that are used as medicine contain phytochemicals that are used to cure diseases without any harm. This confirms that some naturally occurring chemicals are safe to be used because they do not leave behind residuals. Residuals cause free radicals which contribute towards the increased incidence of diseases such as cancer.

Medicinal plants such as *Artemisia afra* and others are used for their phytochemicals. A lot of work has been done to explore the *Artemisia afra* phytochemicals. In a study by Kane *et al.* (2019), the presence of the following substances was noted: terpenoids, alkaloids, tannins, saponins and cardiac glycosides; and in Burundi, results showed the highest total content of phenols, flavonoids and the highest level of antimalarial activity

compared to the other plant extracts studied. Due to *Artemisia* and their related species' importance, many people across the world are advocating for using them to manufacture conventional medicine which will address the problems of numerous diseases, such as diabetes, high blood pressure, and so forth (Bisht *et al.*, 2021).

Furthermore, a lot of work has been done on *Artemisia annua*, the economic plant for antimalaria, because of its potential to produce artemisinin. That is why it has been declared as a traditional medicine in Africa and Asia for a long time (Septembre-Malaterre *et al.*, 2020). The best-known *Artemisia* species in Europe, North Africa, the Middle East and Asia are *A. absinthium* L., *A. annua* L., *A. austriaca* Jacq., *A. pontica* L. and *A. vulgaris* L. *A. absinthium* L. (Trifan *et al.*, 2022). When antioxidant activity analysis was conducted on *A. annua*, *A. absinthium*, *A. pontica*, *A. austriaca* and *A. vulgaris*, the results revealed better antioxidant activity from aerial part extracts than from root extracts (Trifan *et al.*, 2022). Basically, most *Artemisia* species are mentioned to be aromatic as they are rich in aromatic and volatile compounds, but at the same time, non-aromatic and non-volatile compounds are present (Umam *et al.*, 2023). The possession of the heavy scent that originates from essential oils makes their extracts and ingredients appealing to be used in cosmetics, food, drinks, and herbal medicines. *Artemisia* contains compounds such as terpenoids, flavonoids, coumarins, and many other compounds. The results showed that the major compounds in the *A. annua* essential oils were monoterpenoids, including 30.7% *Artemisia* ketone, 15.8% camphor, and 18.2% sesquiterpenes (Umam *et al.*, 2023).

However, *Artemisia* species differ in qualitative and quantitative composition of bioactive compounds. Environmental conditions, species variation, geographic, climatic, and genetic conditions, plant age, soil, phase of vegetation, anatomical part of the plant, harvesting season, and method of harvesting are mentioned to be some of the most influential factors (Nigram *et al.*, 2019). All these factors make different *Artemisia* species unique, even if they sometimes look similar. According to Abad *et al.* (2012), in *A. nilagirica*, the oil extracted was different from lower (caryophyllene oxide, 28.6%), middle (borneol 35.8%) and higher altitudes of camphor (46.9%). *A. annua* constitutes various content, such as camphor (22.8–42.6%), 1,8-cineole (3.7–8.4%), linalool (0.1–11.9%), β -

caryophyllene (2–9.2%), (*E*)- β -farnesene (1.3–8.5%), germacrene D (0.5–7.3%) and 1-*epi*-cubenol (0.7–5.2%) (Abad *et al.*, 2012).

The study is intended to analyse *Artemisia afra* species to determine the main components. Compounds in medicinal plants influence the byproducts. *Artemisia* species are used to produce medicine. Analysis of phytochemicals would therefore be useful to producers to use more precise amounts. *Artemisia afra* is again used for its essential oil and the study would be a reference for the optimal altitude at which the extractors can get the highest quality oil.

The aim of this study is to investigate the qualitative and quantitative analysis of the major bioactive components of *Artemisia afra* L. genotypes.

5.2 MATERIALS AND METHODS

5.2.1 Sampling and collection of plant samples

Artemisia afra leaves were collected from four places – South Africa (Wepener and Hobhouse) and Lesotho (Mohale's Hoek and Roma). The collection was done to perform laboratory phytochemical analysis.

5.2.2 Preparation of plant extract

Leaves of *Artemisia afra* collected from the wild were thoroughly washed under running tap water to remove the soil and undesirable dirt as well as debris. The leaves were shade dried for 3 days. Dried leaves were crushed to a fine powder with an electric kitchen grinder. Then, the powder was stored in Eppendorf tubes/plastics for analysis. Next, 100 g of each powdered sample was soaked in 150 ml methanol in a conical flask. The mixture was shaken well to mix, soak and left for 72 hours at room temperature. The aim of soaking the sample was intended to break down the plant wall cells to release the cell contents.

5.2.3 Analysis of Total phenols

The analysis was adopted from Mohammed *et al.* (2015). The extract of 200 μL , 800 μL of deionized water, and 100 μL of Folin-Ciocalteu reagent were mixed and incubated for 3 min. at room temperature. Sodium carbonate (Na_2CO_3) (20 % w /v) 300 μL was added to the extract and incubated for 2 hours at room temperature whilst kept under dark conditions. The Genesys 10S UV-V spectrophotometer was used to determine the absorbance at 765 nm. A blank was prepared with distilled water instead of aliquot extract. Gallic acid standard curve was first prepared from 0–200 mg/L and total phenolic content was expressed in mg gallic acid (GA) equivalent / g dry matter. The analyses of both samples and standard were made in triplicates and the mean value with \pm standard deviation (SD) was presented. Then the expressed total phenolics in mg gallic acid equivalent (GAE)/g dry matter, and the prepared standard curve with 0 to 100 mg/gallic acid was used for calculations.

5.2.2 Qualitative Analysis of Phytochemicals

The analysis methods were done according to Sharma *et al.* (2020). Ten plants were used to prepare the samples to test the phytoconstituents of *Artemisia afra*. Several tests were conducted with different chemical reagents, depending on the phytochemical test. The following procedures were followed to test *Artemisia afra* phytochemicals:

Tannin/polyphenol test: 3-4 drops of 10% FeCl_3 were added to the diluted extract, in presence of gallic tannins and catechol tannin blue colour turned the solution green.

Flavonoid Test/Shinoda test: 4 ml of extract solution, 1.5 ml of 50% methanol solution and a small magnesium chunk were warmed. 5-6 drops of concentrated HCl were added, red colour was observed for flavonoids.

Ascorbic acid test:

Chemicals and reagents used

Metaphosphoric acid (HO_3P), 2, 6 dichlorophenol indophenols ($\text{C}_{12}\text{H}_7\text{NCl}_2\text{O}_2$), Sodium bicarbonate (NaHCO_3), Ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$), Absolute alcohol ($\text{C}_2\text{H}_6\text{O}$), Xylene

(C₈H₁₀), 2, 2'-dipyridyl (C₁₀H₈N₂), Ferric chloride (FeCl₃), α- Tocopherol (C₂₉H₅₀O₂), Sulphuric acid (H₂SO₄).

Sample preparation

Leaves were cleaned with running water and, finally, in distilled water. They were freeze-dried with liquid nitrogen and then ground into a fine powder with pestle and mortar. These powdered materials were used for ascorbic acid analysis.

Estimation of ascorbic acid content

The ascorbic acid present was estimated by direct colorimetric determination adopted from Devi *et al.* (2016). The sample of 0.5 g was homogenized with 6% metaphosphoric acid to produce an aliquot of the macerate to 50 ml in test tubes. The test tubes were pipetted out in the requisite volumes of standard ascorbic acid solution-1, 2, 2.5, 3, 4 up to 5 ml and until maintaining the solution to be 2% meta-phosphoric acid. Then, 10 ml of dye was added with a rapid delivering pipette, then shaken and the reading taken within 15 to 20 sec. The instrument was set to 100% transmission using a blank consisting of 5 ml of 2% meta-phosphoric acid solution and 10 ml of water and measured the red colour at 518 nm and plot absorbance against concentration. Then, we placed 5 ml of the extract in a dry test tube and added 10 ml of dye and measured as in standard.

Formula to calculate Ascorbic acid:

Ascorbic acid per 100g of sample =

$$\frac{\text{Ascorbic acid content x volume made up} \times 100}{\text{Solution taken for estimation} \times 1000 \times \text{weight of the sample (ml)}}$$

(Devi *et al.*, 2016).

5.2.3 Data Analysis

Data were subjected to analysis and means were computed to find out the variance between treatments. ANOVA (Analysis of Variance) was done to determine significant differences of the means.

5.3 RESULTS AND DISCUSSION

The phytochemical screening of *Artemisia afra* samples revealed the presence of four secondary metabolites, namely ascorbic acid, flavonoids, tannins and phenolics (Tab. 5.3). The minimum of 0.140 ascorbic acid and the maximum of 0.370 ascorbic acid were obtained from *Artemisia afra* leaf samples. Higher amounts of flavonoids were found, that is a minimum of 438.170 and maximum of 951.62. However, the tannins were lower (191.010) than flavonoids (691.096), but higher than ascorbic acid (0.239). Very high amounts of total phenolic content were analysed from *Artemisia afra* samples, from as low as 1255.03 and higher amounts of 4977.93. Therefore, these results indicated that phenolics dominated over other phytochemicals.

Table 5.3: Total phytochemicals of *Artemisia afra*

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
Ascorbic Acid	40	0	40	0.140	0.370	0.239	0.062
Flavonoids	40	0	40	438.170	951.620	691.096	144.000
Tannins	40	0	40	82.320	322.980	191.010	65.669
Phenolics	40	0	40	1255.030	4877.90	2880.75	953.079

The points in the graph are rising, moving from left to right (Fig. 5.4). Very high proportions of cumulative variability show the importance of the component. The curve levels off after the first component. The scree plot shows that the eigenvalues start to form a straight line after the first principal component. In this scree plot, the first eigenvalue is between 2.5 and 3 at

cumulative variability of 80%. The second, third and fourth points are between 3 and 3.5. Cumulative variability in this data set varies from 80% to 100%.

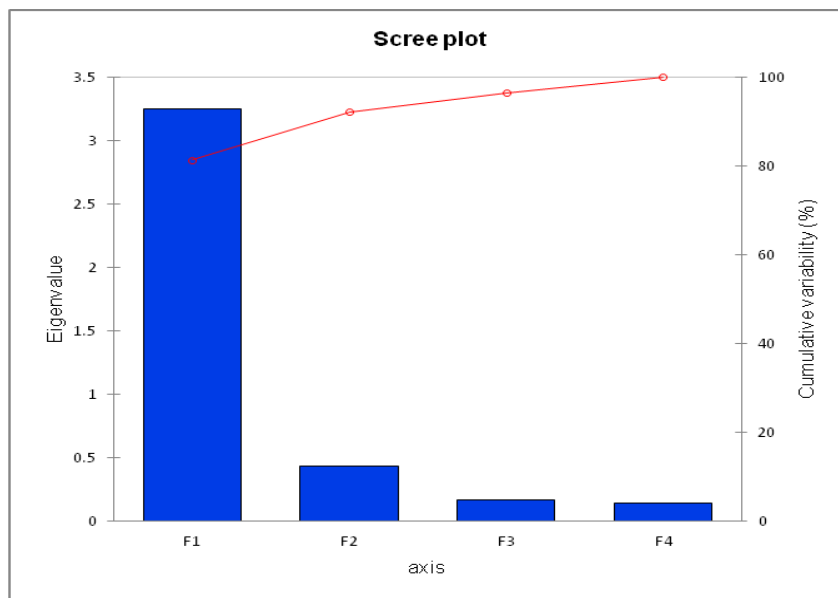


Figure 5.1: PCA scree plot of *Artemisia afra* samples

All the four phytochemicals – ascorbic acid, phenolics, flavonoids and tannins – appeared in the positive quadrants. Flavonoids and tannins were relatively close to each other but far from phenolics and ascorbic acid. Ascorbic acid is located at around F1 (75 %) on the x-axis and F2 (50%) on the y-axis. The loading plot showed the very close correlation between tannins and flavonoids. Therefore, similar loadings have been revealed, and positive correlation was observed. Different loadings are negatively correlated from both tannins and flavonoids.

Figure 5.2: Principal component loading plot of *Artemisia afra* phytochemicals

Figure 5.6 shows the observations of phytochemicals extracted from *Artemisia afra*. The points close to each other represent observations with similar values. Most of the observations are closely packed even though they appear in different upper two quadrants. Roma samples are only scattered in upper quadrants. However, Mohale's Hoek data appears on one upper and lower quadrant. Some of the Mohale's Hoek observations are extremely far from each other, showing that the values are not similar. A similar situation was observed on the Hobhouse data, and some of the observations are not close.

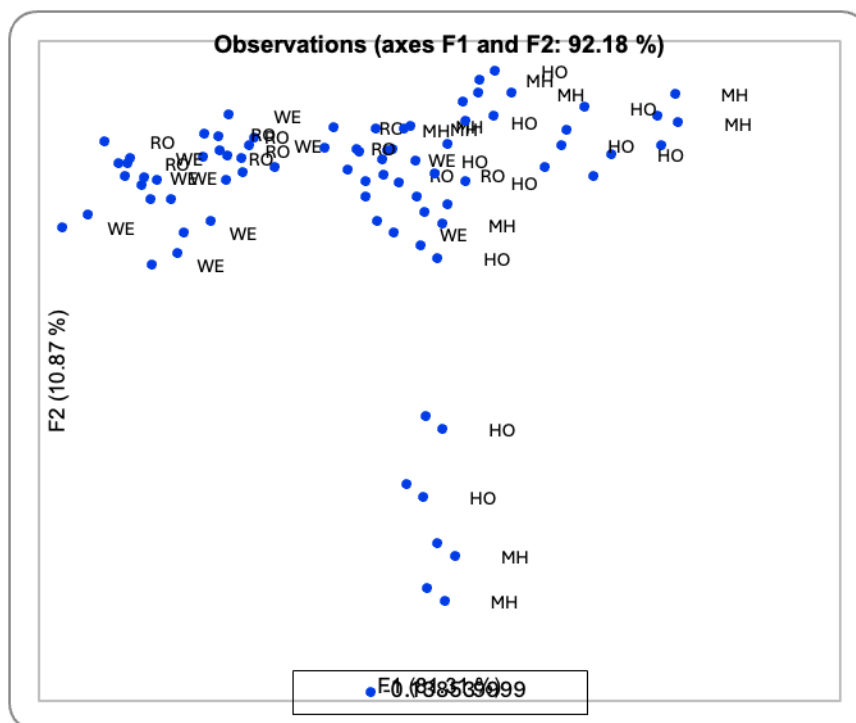


Figure 5.3: Observations of phytochemicals extracted from *Artemisia afra* obtained from four locations

The biplot in Figure 5.7 explains PCA in the scatterplot and data variance using the loading vectors and PC scores. In the below biplot, each point represents the sample of phytochemical. Principal component scores are shown on axes and variables are displayed as vectors. All the vectors (ascorbic acid, phenolics, flavonoids and tannins) originate from zero. Quadrant 1 contains more MH and HO points than other quadrants.

There was a close correlation of the samples. RO and WE populated quadrant 2, and again, here the points were closely packed together. In quadrants 3 and 4, there were very few samples. Only WE samples are displayed in the third quadrant and WE, MH and HO appeared in the fourth quadrant. The arm of the ascorbic acid vector is positive and long. Phenolics' arm is very short and rises up towards positive. However, flavonoids and tannins went downwards.

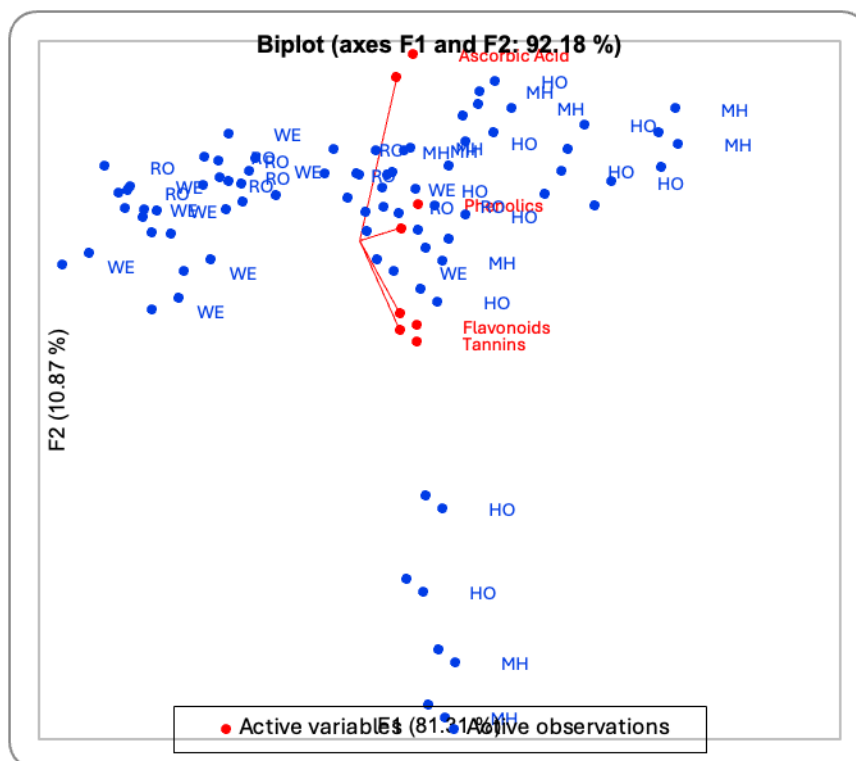


Figure 5.4: Biplot showing scatter points of *Artemisia afra* samples

Plants are sources of natural antioxidants or free radical scavengers, phenolic compounds like phenolic acids, flavonoids, tannins, stilbenes and anthocyanin. *Artemisia afra* is not the exception as it is one of the medicinal plants rich in phytochemicals and essential oils. It possesses medicinal properties that qualify it to be used in pharmaceutical industries for disease prevention and treatment. According to Liu *et al.* (2023), flavonoids, phenolic acids, lipids, amino acids and their derivatives, organic acids, alkaloids and terpenes, are noted to be the main metabolites. However, four compounds – flavonoids, phenolic acids, artemisinin and coumarin compounds – are important pharmacologically active compounds (Liu *et al.*, 2023).

The results obtained have shown that *Artemisia afra* had ascorbic acid, flavonoids, tannins and phenolics. Phenolic amounts were dominating the number of other phytochemicals with very high amounts. According to Ferriera *et al.* (2010), phenolics are naturally manufactured during normal development of plant growth, and sometimes these compounds are triggered by stress imposed by pests, UV and wounding. That is why plants are a good source of phenolics and are used to cure or as treatment of diseases such as coronary heart disease, stroke, and certain types of cancers in humans (George *et al.*, 2009). In the analysis of *Artemisia annua*, flavonoids, coumarins and other compounds were found, as well as artemisinin which is known to be used against parasitic diseases such as malaria (Ferreira *et al.*, 2010). Different plants produce different types of phytochemicals, regardless of being in the same family.

In general, very small amounts (0.140) and a maximum (0.37) of ascorbic acid obtained from samples is a good indication that *Artemisia afra* contains little ascorbic acid. Vitamin C in plants is essential for controlling cell elongation, division, differentiation of cells and programming cell death (Paciolla *et al.*, 2019). Smirnoff's (2000) results revealed that ascorbic acid synthesis is normally performed in leaves and controlled by the amount of light and photosynthesis, and photoprotection processes are responsible. The light intensity is very important in production of ascorbic acid; as very poor light intensity affects the amount of ascorbic acid in plants.

The samples contained a high number of flavonoids with a maximum of 915.62. Flavonoids were noted in abundance in the study of Liu *et al.* (2023) where a total of 227 flavonoids were obtained. Flavonoids are mostly found in various medicinal plants, like the *Artemisia* species, and flavonoids control the UV and diseases in plants. According to Kale *et al.* (2008), flavonoids play a major role in human health, such as inhibition of activation of pro-carcinogens, proliferation of cancer cells, apoptosis, and angiogenesis, activation of immune response triggered by cancer cells and modulation of inflammatory responses.

Tannins are also antioxidants found in many medicinal plants and possess antimicrobial abilities. Antimicrobial indications in plants have an influence on a very high content of

tannins (Siqueira *et al.*, 2011). The content of tannins from samples of *Artemisia afra* was considered reasonable, from as low as 82.32 and as high as 322.98. According to Van der Kooy and Sullivan (2013), extensive study done on *Artemisia annua* showed that several secondary metabolites have been discovered and, among them, Tannins were identified. Other studies mention that tannins have many biological properties such as anti-carcinogenic, antiviral, anticancer, antibacterial, anti-inflammatory and antioxidant, leading to it being used in medicinal products (Das *et al.*, 2020).

Plants synthesise phenolics and they work as antioxidants, classified in different forms, such as polyphenols (tannins and flavonoids), simple phenols (phenolic acids), and a miscellaneous group (Ferreira *et al.*, 2010). The result of the study showed very high amounts of phenolics obtained from *Artemisia afra*, which means that it has a high chance of antioxidant activity. *Artemisia annua* is one member of the *Artemisia* genus which has been stated to have very high antioxidant activity due to very high amounts of phenolics. That is why this group is mostly used for pharmaceutical purposes, for making medicine to treat many diseases, disorders and alleviate pests.

5.4 CONCLUSIONS

The analysis of phytochemicals in this study discovered the presence of secondary metabolic compounds from *Artemisia afra*. The study investigated ascorbic acid, flavonoids, tannins and phenolics from *Artemisia afra* leaves. The amount of phenolics was extremely high when compared with other phytochemicals. Again, high flavonoid quantities were noted from the analysis, followed by tannins. However, ascorbic acid was the least of all the phytochemicals measured. It is therefore concluded that the samples of *Artemisia afra* are higher in phenolics than other phytochemicals.

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CHAPTER SIX

GENERAL DISCUSSION AND RECOMMENDATION

Artemisia plants are mostly used to make medicine because of their beneficial properties. In maintenance of byproduct quality and safety, *Artemisia afra* genotypes collected from different locations was anticipated to have similar phytochemicals as they are the same species. However, very close plant inspection revealed that even within the location some plants differ in colour of leaves and stems. Then laboratory analyses were done to reveal the secret hidden behind. Furthermore, Diversity Analysis Technology sequencing (DArTseq) is therefore used to detect the DNA polymorphism of organisms. The study analysis targeted the diversity of *Artemisia afra* from different locations of Southern Africa. However, the study considered plants metabolites and minerals to be factors which can influence diversity in *Artemisia afra* genotypes. Environment plays a great role on species' genetic diversity as the DNA is environmentally sensitive. That is why mutation of cells is possible in certain situations where mutagens delete or add some genes, resulting in DNA sequence alterations. In most cases, plant constituents are responsible for chemical reactions inside the cell where DNA carries the message to different parts of the cell.

Diversity analysis results presented on the dendrogram showed the diversity of genotypes of *Artemisia afra*. The same genotypes of *Artemisia afra* from the same location showed differences by appearing in different clades of the dendrogram. The same situation was noticed on pea (*Pisum sativum* L.) phenotyping and genotyping characterisation analysis study (Uhlarik *et al.*, 2022). The decrease in similarity between genotypes was indicated by the principal component analysis (PCA). Heterozygosity of genotypes was noted to be unrelated to location, because even the samples from the same location were not similar. The clustering showed closely related genotypes, although some of the genotypes were collected in different locations. In *Artemisia annua*, when using PCR-based “retrotransposon-microsatellite amplified polymorphisms”, genetic variation was discovered from samples obtained from the same geographical regions (Holasou *et al.*, 2023). The results of this study revealed that SNPs occur despite the locations where *A. afra* genotypes grow. However, PCA, minimum spanning network, UPGMA-cluster

dendrogram, population structure, membership probability test and PCA ordination test confirmed relativity of genotypes. Similarly, in Walnut (*Juglans regia* L.) genotyping array, genotypes from different locations showed relationships and genetic variability was observed among cultivars of different origins: Europe, California and Asia (Marrano *et al.*, 2019). Organisms' adaptation is thus the great source of variability, and sometimes mutation and knockout of genes occur to allow for survival in a challenging environment.

Soil plays another major role in supplying nutrients for plant growth, and it is the main source of essential elements for processes of photosynthesis and respiration. Therefore, the soil is useful to plants as their main food source, because plants depend on essential elements like Nitrogen, Phosphorus and Potassium and some other elements which are required in minute amounts. Wepener (WO) soils were rich in Nitrogen, Phosphorus and Potassium. However, *Artemisia afra* foliar analysis results showed lower amounts of nitrogen. The absorption of elements is facilitated by pH of the soil and the cation exchange capacity. Plants possess a robust network of interaction of cells, tissues and organs which is influenced by harsh environmental factors. According to Lei (2023), three essential components of the soil are C, N, and P of soil nutrients, and they are noted to be necessary elements for plant growth. Their major role is to regulate nutrient mechanisms between plants and soil.

It is common to have fertile soil, but plants sometimes fail to access the nutrients from the soil due to many factors that affect the efficiency of nutrient acquisition. Chemistry and composition, availability, soil properties (water content and pH) and compaction of the soil are stated to contribute to poor absorption of nutrients (Morgan & Connolly, 2013). The results of this study revealed that, in some locations, *Artemisia afra* plants were growing on rich soils but plant analysis showed low nutrients. The mobility of nutrients seemed not to be effective from soil to plant.

Artemisia species contain monoterpenes, sesquiterpenes and a variety of secondary metabolites resulting in a characteristic scent or taste that makes them smell different from other plants (Gouveia & Castilho, 2013). Gouveia and Castilho (2013) further state that flavonoids and hydroxycinnamates are the main phenolic compounds when *Artemisa*

annua is analysed. In the *Artemisia* genus, high amounts of phenolic compounds influence the antioxidative effects (Bordean *et al.*, 2023). In our study, the results revealed that flavonoids were negative from the samples obtained in Wepener, Mohale's Hoek and Roma, but present in slight amounts in Hobhouse samples when using NaOH test. However, FeO₂ results indicated the presence of flavonoids in all samples. Total phenolics were mostly highly present because they were found in very high amounts, even though there were differences from different locations. Many other compounds were found in *Artemisia afra*, such as tannins, terpenoids, steroids, saponins, glycosides and alkaloids. However, Anibogwu *et al.* (2021) mention phenolic compounds, terpenoids, and alkaloids, coumarins, acetylenes, sterols, and caffeoylquinic acids as the primary classes of secondary metabolites detected for *Artemisia* species. Due to different chemical constituents obtained from *Artemisia* species, each species is used for different purposes. Some species are good remedies for cancers, flu, inflammation, digestion and other diseases. Nigam *et al.* (2019) also discovered that *Artemisia* possesses several active ingredients or secondary metabolites from different species, which work through various modes of action. That is why different species address different problems if they are used for medicinal purposes. The information would be utilised by people who use *Artemisia afra* for medicinal purposes, students and scientists.

In general, the results revealed a correlation between genetic diversity, foliar minerals, and phytochemicals. The differences in chemical composition indicate that some *Artemisia afra* genotypes within the same location are genetically and phytochemically different. It is therefore specified that they can withstand harsh climatic conditions and can be easily improved. This study did not cover the biological activities of essential oils of *Artemisia afra* as they play major roles in plant protection, transpiration rate, and many other processes. It is therefore recommended to continue with the extraction and analysis of essential oils

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APPENDICES



ARTEMISIA AFRA (ref <https://pza.sanbi.org/artemisia-afra>)