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The effects of elevated carbon dioxide on the secondary metabolites and biological activities in Moringa oleifera Lam. and Moringa peregrina Forssk

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The effects of elevated carbon dioxide on the secondary metabolites and biological activities in *Moringa oleifera* Lam. and *Moringa peregrina* Forssk.

by

Thato Moloi

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This dissertation is submitted in fulfilment of the requirements for the degree of

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in

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in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

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Co-supervisors**:** Dr Shalini Dukhan and Mr Phillemon Ramalepe

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Declaration

I, Thato Moloi, hereby declare that this dissertation is my original work submitted for the degree of Master of Science to the University of the Witwatersrand, Johannesburg. It has never been submitted to any other University for any other degree or examination.

Thato Moloi



(Signature of candidate)

Acknowledgement

A major research project like this one is never the work of anyone alone. The contributions of many different people, in their different ways, have made this possible. I would like to express my heartfelt thanks and gratitude to my amazing supervisors (Dr Ida Risenga, Dr Shalini Dukhan and the future Dr, Mr Philemon Ramalepe) for their timeless support, patience and love; I am really grateful. I would like to show my gratitude to my awesome friends and colleagues who have supported me and helped me push through even when I was down. I love you all and I hope your lives and careers explode with good fortunes.

Dedication

I also dedicate this work to all the people working on their dreams, skills and techniques. I dedicate this to all the people who are thinking of giving up but find a way to make things work. I dedicate this to everyone who is humble and caring. And lastly, I dedicate this to myself.

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Abstract

Climate is crucial for the distribution and survival of medicinal plants as it can influence phytochemicals and regulatory hormones that are responsible for the normal growth and development, as well as their interactions with the environment. Thus, it is important to understand how climate change will impact these crucial plant compounds and hormones that play a significant role in the plant's survival and development. With the increasing CO_2 in the atmosphere, it is expected that climate change effects will be devastating to the world and Southern Africa. The present study intended to achieve two aims, the first being to investigate the impacts of elevated CO_2 (eCO_2) on the secondary metabolites and biological activities of two important Moringa species, *Moringa oleifera* Lam. *(M. oleifera)* and *Moringa peregrina* - (Forssk.) Fiori *(M. peregrina)*. The second aim was to investigate how the use of *M. oleifera* leaf extract (MLE) based and commercial (PhytoStim®) biostimulants influence the productivity as well as the adaptability of *M. oleifera* and *M. peregrina* under elevated eCO_2. The first set of three-month-old potted plant samples were exposed to 400 ppm (control), 600 ppm and 800 ppm for three months, respectively. The second set of plants were placed in the greenhouse and sprayed (foliar application) with 200 mL of *M. oleifera* leaf extract (MLE) and 200 mL commercial biostimulant PhytoStim® every second week for three months, respectively. The control samples were unsprayed. The third set of plants were exposed 600 ppm and 800 ppm (separately) and simultaneously sprayed with 200 mL of *M. oleifera* leaf extract (MLE) and 200 mL commercial PhytoStim® (separately) every second week for three months to assess the influence of biostimulants on the adaptability of the *Moringa* species under eCO_2. The control samples under 400 ppm were unsprayed.

In this study, 80% methanolic extracts from all the above mentioned treatments of *M. oleifera* and *M. peregrina* were screened for 17 secondary metabolite groups (tannins, saponins, flavonoids, quinones, phenols, terpenoids, cardiac glycosides, coumarins, steroids, phlobatannins, anthracyanine, volatile oils, phytosterols, triterpenoids, proteins and amino acid, glycosides, carbohydrates) using qualitative methods. Quantitative analyses were performed to determine the total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC) and total proanthocyanidin content (TPAC). The antioxidant assays were performed to determine the reducing, scavenging and chelating abilities against DPPH, H_2O_2 and metal (Iron) chelating. The antimicrobial activities against gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus*, *Streptomyces albulus* were assessed by using the agar well diffusion assay. In the control samples, out of 17 screened secondary metabolites, four (phytosterols, volatile oils, anthocyanin and phlobatannins) were not detected in both species' extracts. On average, *M. peregrina* showed higher total content of tannins, phenolics, flavonoids and proanthocyanidins. *M. peregrina* showed stronger antioxidant activity against iron chelating and H_2O_2, whilst *M. oleifera* showed stronger antioxidant activity against DPPH. Both *M. oleifera* and *M. peregrina* extracts displayed an acceptable bacterial growth inhibition capability (ZOI ≥10 mm) with only *S. albulus* being resistant to the control of *M. oleifera*.

Qualitative phytochemical analysis indicated the presence of secondary metabolites such as tannins, saponins, flavonoids under 600 ppm and a slight decline under 800 ppm in both species. The quantitative analysis indicated an increase in the total content of phenols, flavonoids (flavanols), tannins, and proanthocyanidins. An increase in CO_2 resulted in an increase in the activity of antioxidants and antibacterial for both species. On average, *Moringa peregrina* showed higher accumulation of secondary metabolites, higher antioxidant and antibacterial activities in comparison to *Moringa oleifera.*

The foliar application of MLE and PhytoStim® showed an increase in some secondary metabolites and decrease in metabolites such as tannins and phenols in *M. oleifera*. The application of biostimulants (MLE and PhytoStim®) also resulted in an increase in TPC, TTC and TPAC in *M. peregrina*, with a decline in total contents of these compounds in *M. oleifera*. However, the decline did not negatively impact both species' pharmacological abilities (antioxidant and antimicrobial activities), as they exhibited stronger antioxidant and antimicrobial activities when compared to the untreated plants (control samples).

The use of the above mentioned plant based biostimulants resulted in an enhanced adaptability as indicated by the increase in the accumulation of selected screened secondary metabolites plant samples that exhibited signs of stress. The higher accumulation of secondary metabolites was observed under 600 ppm, in combination with PhytoStim® for either species. The combined CO_2 and biostimulant treatments improved the total phenolic content (TPC) of both *M. oleifera* and *M. peregrina* significantly, with *M. oleifera* showing higher TPC content when compared to *M. peregrina*. On average, both *M. oleifera* and *M. peregrina* exhibited lower total flavonoid content (TFC), total tannin content (TTC) and total proanthocyanidins (TPAC), with *M. oleifera* showing higher contents of the above-mentioned phytochemicals in comparison to *M. peregrina*. The study also highlighted a decline in biological activities for all treatments, with the controls showing higher biological activities for both species. In the three antioxidant assays conducted, the leaf extracts of the controls had significant lower IC_{50} values for DPPH and H_2O_2, when compared to the stressed *M. oleifera* and *M. peregrina*. Antimicrobial assays also showed no significant difference in the bacterial inhibition capabilities of *M. peregrina* and *M. oleifera* under 600 ppm and 800 ppm with either biostimulant application. *M. peregrina* and *M. oleifera* controls showed high ZOI for the selected

bacterium. The study has demostrated that biostimulants (MLE and PhytoStim®) enhanced the adaptability of both species under potential stress coursed by eCO_2.

The present study has demonstrated that the exposure to elevated CO_2 could alter the accumulation and biological processes (such as antioxidant activity and antimicrobial activity) in both *M. oleifera* and *M. peregrina*. *Moringa peregrina* exhibited more tolerance to elevated CO_2 when compared to *Moringa oleifera* and showed higher antioxidant and antimicrobial activity which might be attributed to the stronger presence of phytochemicals such as flavonoids, phenols and tannins. The data also suggests that both *Moringa oleifera* and *M. peregrina* can adapt to high levels of CO_2 concentrations (~600 ppm), however, as medicinal plants, it might be difficult to sustain the acclimatisation and tolerance due to membrane oxidation and DNA damage. Therefore, foliar application of the biostimulants could enhance the adaptability and productivity of both species under high levels of CO_2. This study may contribute towards better planning on conservation efforts to improve the chances of survival of the *Moringa oleifera* and *M. peregrina* and could aid with food security.

Abbreviations and symbols

APES	Animal, Plant and Environmental Sciences
TPC	Total Phenolic Content
TFC	Total Flavonoid Content
TTC	Total Tannin Content
TPAC	Total Proanthocyanidins Content
IUCN	International Union for Conservation of Nature
GC-MS	Gas Chromatography – Mass Spectrometry
GAE	Gallic Acid Equivalence
QE	Quercetin Equivalence
WHO	World Health Organisation
UV	Ultraviolet Radiation
ANOVA	Analysis of Variance
$AlCl_3$	Aluminium Chloride
$NaNO_3$	Sodium Nitrate
MeOH	Methanol

mm	Millimetres
cm	Centimetres
ml	Millilitres
L	Litres
%	Percentage
ppm	Parts per million
mg	Milligram
µm	Micrometres
rpm	Revolution per minute
DNA	Deoxyribonucleic Acid
ROS	Reactive Oxygen Species
O_2^-	Superoxide
H_2O_2	Hydrogen peroxide
HO	Hydroxyl radical
$^1O_2$	Singlet oxygen
MLE	Moringa Leaf Extract
SM	Secondary Metabolites

MVA	Mevalonate pathway
MEP	Methylerythritol 4-Phosphate pathway
UV	Ultraviolet
ANOVA	Analysis of Variance
$AlCl_3$	Aluminium Chloride
$NaNO_3$	Sodium Nitrate
MeOH	Methanol
GHG	Greenhouse Gases
UNFCCC	UN Framework Convention on Climate Change

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Contributions from this dissertation

Conference paper presentation:

T. Moloi, S. Dukhan, P. Ramalepe, I. Risenga. Effects of elevated CO_2 on the phytochemical profile, antioxidant and antimicrobial activities in *Moringa oleifera* Lam. and *Moringa peregrina* Forssk: A comparative study. 47th annual SAAB Conference North-West University, 17 -20 January 2022.

Dissertation Structure

This dissertation has five chapters. It was written in the form of scientific publication format and will be submitted for review and subsequent publication. Chapters 3, 4 and 5 are written in scientific paper format according to specific journal requirements. Therefore, please be aware that unavoidable repetition on sections such as the introduction and methodology are to be noted. In addition, there are differences in the structure of the papers according to each journal requirement, these affect the main topics, subtopics as well as the the referencing style.

Chapter 1 is the general introduction of the research which includes the background of the study.

Chapter 2 is the literature review, the rationale, research questions, aim and objectives. It is followed by three manuscripts (Chapters 3-5) which form the main body of the dissertation.

Chapter 3 provides insight on the impacts of elevated CO_2 exposure on secondary metabolites and biological activity of both *Moringa oleifera* and *Moringa peregrina.*

Chapter 4 provides insight on the influence of *Moringa* leaf extract and PhytoStim® biostimulant foliar application on the phytochemical profile and biological activities in *Moringa oleifera* and *Moringa peregrina.*

Chapter 5 is based on the the role of *Moringa* leave extract and PhytoStim® biostimulant on the adaptability of *Moringa oleifera* and *Moringa peregrina* under elevated carbon dioxide exposure.

Chapter 6 covers the general discussion, which is followed by concluding remarks and future recommendations.

I performed my research under the supervision of my supervisors; they have been included as second and third authors on papers submitted to journals

CHAPTER 1

GENERAL INTRODUCTION

This chapter introduces the background information of the study.

1. Background

Ever since the Stone Age era, humans have recognized the value of different plant groups for their healing, curative, nutritional value and their sustenance properties crucial for daily needs (Koh *et al.*, 2009; Dar *et al.*, 2017; Belo *et al.*, 2019; Gupta *et al.*, 2019). Among these plant groups are medicinal plants, which are sought after in various sectors such as cosmetic, medical, pharmaceutical, and nutritional industries for their pharmaceutical properties (Koh *et al.*, 2009; Dar *et al.*, 2017; Amponsah *et al.*, 2014; Savithramma *et al.*, 2011). According to various studies, 80% of the world population rely on medicinal plants as a source of primary healthcare due to their low cost and little to no side effects (Koh *et al.*, 2009; Dar *et al.*, 2017; Amponsah *et al.*, 2014; Savithramma *et al.*, 2011).

Plants are constantly exposed to environmental factors that may be detrimental, be it biotic or abiotic (Gupta *et al.*, 2019). Over the years, plants have evolved (morphologically and physiologically) and adapted in order to survive the ever-changing environmental conditions (Mishra *et al.*, 2011; Senthilkumar *et al.*, 2018). One way in which plants respond to these environmental changes is through the production of phytochemicals called secondary metabolites (Dar *et al.*, 2017; Koh *et al.*, 2009; Naboulsi *et al.*, 2018; Peltonen *et al.*, 2005). Secondary metabolites (SM) are compounds that are biosynthesised from primary metabolites and are not essential in sustaining cell life (Vongsak *et al.*, 2013). Secondary metabolites are produced during the non-growth phase of plants and are synthesized in response to stress exerted on plants such as herbivory or drought (Roopashree and Naik, n.d.; Yadav and Agarwala, 2011). Major groups of secondary metabolites include but are not limited to alkaloids, steroids, phenolic compounds, essential oils like oleic acid, terpenes, and tannins

(Koh *et al.,* 2009). Secondary metabolites such as phenolic compounds, for example, serve as antioxidants, UV protective screens and pigments, enzyme inhibitors, growth regulators, and as chemical signals and toxins to nematodes, fungi, microbes and herbivores (Senthilkumar *et al.,* 2018). Secondary metabolites have properties that are also beneficial to human health. Plants with these health benefits are termed medicinal plants (Dar *et al.,* 2017; Naboulsi *et al.,* 2018; Savithramma *et al.,* 2011). Medicinal plants have bioactive compounds (such as phenolic compounds) that can treat certain ailments, act as flavouring agents for food and provide therapeutic effects, thereby making them desirable in many sectors such as in pharmaceutics (Gupta *et al.,* 2019).

Moringa oleifera Lam. and *Moringa peregrina* - (Forssk.) Fiori are two of the most popular species in the *Moringa* genus that are used as medicinal plants and are widely cultivated in regions ranging from Asia, South America to Africa (Meireles *et al.,* 2020). These species are generally favoured by many communities due to their high nutritional value and medicinal purposes (Ghazali and Mohammed, 2011; Meireles *et al.,* 2020; Senthilkumar *et al.,* 2018). They are traditionally used to treat ailments such as hypertension, diarrhoea, and burns (Senthilkumar *et al.,* 2018). Like all medicinal plants, the two species could be under threat due to climate change. Climate change can be defined as unpredictable weather events that have intense weather anomalies such as cyclones, droughts, floods and heatwaves (Gupta *et al.,* 2019). Studies indicate that Greenhouse gas emissions (GHG), particularly carbon dioxide, are the main cause of climate change (Shikwambana *et al.,* 2020). Climate change can affect medicinal plants in various ways, such as altering rain patterns and frequencies, which stress the plants occurring in the affected region, leading to a decline in rate of growth and productivity, and possible extinction of a population in that region (Gupta *et al.,* 2019).

Studies have shown that, since the start of the modern industrial society, the concentration of CO_2 has steadily increased from 270 ppm to 407.4 ppm and it is projected that the CO_2 concentration will increase to 516 ppm - 970 ppm by 2100 (Collins *et al.*, 2013; Intergovernmental Panel on Climate Change, 2007). Even though elevated CO_2 concentrations may be beneficial to the plants' performance as high concentrations induce above ground biomass increases and increases in photosynthetic and by products (starch), it should be noted that elevated CO_2 exposure can have negative impacts too (Dong *et al.*, 2018; Wattal & Siddiqui, 2015). Elevated CO_2 induce stomatal closure and this limits the amount of nitrogen taken up by the plant, additionally, the increased photosynthetic products causes a reduction in leaf chlorophyll from the altered nitrogen: carbon ratio, and results in prolonged chlorosis (yellowing of leaves) and zebra-like markings on the leaves, reducing overall plant performance and stunting growth (Quick *et al.*, 1991).

Plants have the ability to adapt to changes in the environment by altering their metabolic pathways, however this affects the production of secondary metabolites, and these compounds form the basis of their medicinal activities. Therefore, CO_2 may affect medicinal plants in various ways, which is species dependant, some plants such as birch show an increase in biomass and secondary metabolite production when exposed to elevated CO_2, whereas pine trees and others demonstrates a decrease in secondary metabolite production due to nutrient starvation (Stiling and Cornelissen, 2007).

A mitigation method that is gaining attention is the use of organic fertilizers such as plant primming agents like biostimulants. Biostimulants are organically derived products that are designed to improve the plant' yields and performance by improving soil microbes, nutrient uptake, water use efficiency, soil health and consequently (du Jardin, 2015). Biostimulants are produced from an extensive variety of organic materials including microbes, humic inorganic compounds, and seaweed (see Table 1.1

for summarised information; (du Jardin, 2015). Examples of available commercial biostimulants frequently referenced in literature include Tri-Cure SP®, Rizofos Fruit®, Technical® and Premax®.

Table 1.1: Summary of different types of biostimulants and their activities and examples. Table was adapted from *(*Drobek *et al.,* 2019*).*

Source of Biostimulant	Activity	Example
Anaerobic Digestion Product	Improve nutrient uptake and promote auxin-like behavior	Animals, lignin biomass, and plants
Hydrolysis Product	Yield improvements, Increases in-leaf phosphorus and nitrogen levels. Increased macro- and micronutrients available to plants. Improve soil fertility and aids biotic and abiotic stresses tolerance	Chemical (fish waste, animal tissue, collagen, casein) and enzymatic (fruit waste or vegetable)
Beneficial Fungi	oxidative stress Plant protection, and improvements in plants yield and aids with the recruitment of microbe communities	*Heteroconium chaetospira, Glomus claroideum, Trichoderma, Glomus etunicatum, Rhizophagus intraradices, Glomus viscosum, and Rhymbocarpus aggregatus*
Marine Algae	Antioxidant capabilities, improves resistance to microorganism infections, improves post-harvest time. Improves thermal tolerance, and Improves drought tolerance	*Gelidium pectinutum, Sargassum wightii, Enteromorpha intestinalis, Ecklonia maxima, Ascophyllum nodosum*

Numerous studies have mostly focused on *M. oleifera* as it is the widely used tree in the *Moringaceae* family, but there are limited published studies on *Moringa peregrina*, where both species are compared not only for their bioactive compounds but biological activities under the influence of

various environmental factors such as elevated CO_2 (El-Awady *et al.*, 2015). To our knowledge, there are limited studies conducted on the impacts of elevated carbon dioxide on secondary metabolites and biological activities of *M. oleifera* and *M. peregrina*. Furthermore, no studies have been performed on the influence of MLE and PhytoStim® on the productivity and adaptability of the the two species under elevated CO_2. Therefore, it is essential to investigate the effects of above mentioned factors on secondary metabolites and biological activities of *M. oleifera* and *M. peregrina*.

CHAPTER 2

Literature review

This chapter contains a detailed review on the phytochemistry, biological activities of *M. oleifera* and *M. Peregrina* as well as aspects of medicinal plants and climate change. It also states the rationale, aims and objectives of the study.

2. Literature review

2.1 The *Moringa* genus

The *Moringa* genus belongs to the sole family Moringaceae (Abd Rani *et al*., 2018) and it is one of the genera along with *Hyperanthera* and *Anoma* in the family (Abd Rani *et al.,* 2018). This family is also known as the horseradish or drumstick family (Fahey, 2005) and there are 13 plant species under the *Moringa* genus, which are distributed across many regions (Table 2.1; Olson and Rosell, 2006).

Table 2.1: Species found in the *Moringa* genus and their native distribution (table adapted from Olson and Rosell, 2006).

Species	**Country**	**Common names**
M. arborea Verdcourt	Somalia, Kenya	-
M. borziana Mattei	Somalia, Kenya	-
M. concanensis Nimmo	India	-
M. drouhardii Jumelle	Southern Madagascar	-
M. hildebrandtii Engler	Southwest Madagascar	Hildebrandt's Moringa
M. longituba Engler	Somalia, Kenya, Southeast Ethiopia	*Moringa tubiflora*
M. oleifera Lam.	India	Ben-oil Drumstick, Horseradish, Kelor
M. ovalifolia Dinter ex Berger	Southwest Angola, Namibia	African Moringo, Ghost Tree, Phantom Tree

M. peregrina Forssk. Ex Fiori	Arabia, Northeast Africa, Red Sea	Al Ban, Al Yassar, Wild drumstick tree, Yusor, wispy-needled Yasar tree, Ben tree
M. pygmaea Verdcourt	North Somalia	Swanjehro
M. rivae Chiovenda	Ethiopia, Kenya	Swanjehro
M. ruspoliana Engler	Somalia, Kenya, Ethiopia	Swanjehro
M. stenopetala (Bakerf.) Cufodontis	Somalia, Kenya, Southwest Ethiopia	Haleko, Shiferaw, Shelagda, Cabbage tree

The type of tree trunks found in the different species is often used to categorize the 13 species into three distinct groups (Abd Rani *et al.,* 2018; Olson and Rosell, 2006). The first group is called the bottle trees, as they have water storing trunks and these consists of *Moringa ovalifolia*, *M. drouhardii, M. hildebrandtii* , and *M. stenopetala* (Abd Rani *et al.,* 2018). The second group has slim trunks and it includes *Moringa oleifera, M. peregrina* and *M. concanensis* (Abd Rani *et al.,* 2018). The third group, consisting of the remaining species, are tuberous shrubs (Abd Rani *et al.,* 2018). All the *Moringa* species are fast-growing plants which require little to no human care and are drought resistant (Abd Rani *et al.,* 2018; Fahey, 2005; Olson and Rosell, 2006).

The genus is traditionally used to improve human health. For example, during war times, *M. oleifera* leaves were given to Indian warriors to relieve their stress and pain and also to give them an energy boost (Abd Rani *et al.,* 2018). Other traditional uses include wound healing, anxiety relief, healing of common ailments such as sore throats, diarrhea and skin infections (Abd Rani *et al.,* 2018; Fahey, 2005; Olson and Rosell, 2006).

Moringa is known for its nutritive properties, possibly due to the presence of water-soluble vitamins. Vitamins are a group of organic compounds with low molecular weight that are crucial for facilitating crucial biological functions (both physiological and chemical) needed for the maintenance and growth of the human body (Abibu *et al.*, 2019). Currently there are 13 vitamins that can be classified into two groups, water-soluble vitamins and fat-soluble vitamins (Ball, 2005). Water-soluble vitamins include B-group vitamins and vitamin C; and under the fat-soluble group are vitamins A, D, E, and K (Abibu *et al.*, 2019; Ball, 2005). Due to the high concentrations of these vitamins and other SM in *Moringa*, there has been an increased interest from different industrial sectors (academia, pharmaceutical, food and cosmetic) in finding accurate methods to identify vitamins, as these antioxidant vitamins provide protective properties against diseases such as stroke, diabetes, cancer, etc., (Abibu *et al.*, 2019).

2.1.1. *Moringa peregrina*

2.1.1.1 Botanical information

Moringa peregrina (Forssk.) Fiori belongs to the Moringaceae family and is a fast growing deciduous tree when compared with the other 12 species (Senthilkumar *et al.*, 2018). *Moringa peregrina* is adapted to very arid conditions, has a greyish green bark and a height ranging from 3-10 m (Olson *et al.*, 2016; Robiansyah *et al.*, 2014; Senthilkumar *et al.*, 2018). The leaves are deciduous, obovate (egg shaped), alternate and 30-40 cm long (Elbatran *et al.*, 2005; Tahany *et al.*, 2010). One interesting feature of *M. peregrina* is the falling of mature leaves, leaving behind naked leaf rachis (Figure 1.1; Senthilkumar *et al.*, 2018). The plant bears pinkish-white flowers (10-15 mm long) with an axillary inflorescence (Figure 1.1; Senthilkumar *et al.*, 2018). *M. peregrina* tree produces pods ranging from 20-40 cm with one tree producing up to 1 000 pods per year (Robiansyah *et al.*, 2014). The pods carry

un-winged, ovoid, trigonous seeds with each pod containing 8-15 seeds (Figure 1.1 and 1.33; Robiansyah *et al.,* 2014; Senthilkumar *et al.,* 2018). Before the plant breaks through the ground, tubers develop to provide all the necessary nutrients and store water (Senthilkumar *et al.,* 2018).



Figure 1. 1 *Moringa peregrina* plant parts. A: seeds of the plant. B: pods. C and D: white pink flowers [images were taken from: [https://toptropicals.com/catalog/uid/Moringa_peregrina.htm; http://moringavip.com/buy-black-moringa-peregrina-seeds and Rezende *et al.* (2015)]

2.1.1.2. Distribution

Moringa peregrina is originally from Arabian Peninsula but due to its ability to grow in extreme environmental conditions such as saline, and drought stricken environments, it now has grown in wide geographical regions (Elbatran *et al.*, 2005; Olson *et al.*, 2016; Tahany *et al.*, 2010). The species is mostly abundant in the north and south of Saudi Arabia, but it can also be found in Sudan, India, Somalia, Palestine, Syria, Oman, Jordan and Africa (Figure 1.2). It mostly grows in low altitude and well-drained areas (Elbatran *et al.*, 2005; Gharibzahedi *et al.*, 2013; Olson *et al.*, 2016; Robiansyah *et al.*, 2014; Somali *et al.*, 1984; Tahany *et al.*, 2010).



Figure 1. 2 Distribution map of *Moringa peregrina*. Map outline was taken from https://www.outline-world-map.com/white-transparent-political-world-map-b5a

2.1.2 *Moringa oleifera*

2.1.2.1 Botanical information

Moringa oleifera Lam. is the most cultivated and commercialized species in the Moringaceae family (Meireles *et al.,* 2020). *Moringa oleifera* is a short growing tree, with a height ranging from 5-12 m, with an umbrella-shaped crown, which is an open canopy with wide spreading fragile branches. It has a short trunk and the wood is soft or spongy and appears white in colour (Ghazali and Mohammed, 2011; Mishra *et al.,* 2011). Depending on the growing conditions, the foliage of *M. oleifera* can be either evergreen or deciduous, with bipinnate or tripinnate compound leaves spirally arranged along the branch tips (Ghazali and Mohammed, 2011). The leaves are 20-50 cm in length and have long petiole and pinnae pairs (usually 4-6 pairs) that have elliptical opposite leaflets (Figure 1.3 and 1.33; Ghazali and Mohammed, 2011; Mishra *et al.,* 2011). *M. oleifera* bears flowers with hairy stalks in drooping panicle, that appear yellow white in colour and 10-25 cm in length (Figure 1.3; Mishra *et al.,* 2011). The flowers are bisexual and are fragrant (Mishra *et al.,* 2011). The pods (fruit) grow up to 60 cm in length, when unripe are green in colour (Figure 1.3), which then become brown and dry as they mature (Ghazali and Mohammed, 2011). The seeds (kernels) are surrounded by a brown seed coat that is winged (three papery wings) and triangular (Figure 1.3 and 1.33; Ghazali and Mohammed, 2011; Mishra *et al.,* 2011).



Figure 1. 3. *Moringa oleifera* plant parts. A: Green leaves; B: Branches (stem); C: Green pods. D: Flowers of the plant; E: Seeds with their papery wings [Image taken from Azlan *et al.* (2022)].

2.1.2.2. Distribution

Moringa oleifera is native to the Sub-Himalayan regions of West India and grows in well-drained loamy soil (Ghazali and Mohammed, 2011; Meireles *et al.*, 2020; Mishra *et al.*, 2011). The tree grows at elevations ranging from sea level to heights of 1400 m above sea level (Mishra *et al.*, 2011). Due to its adaptive capabilities, nutritional and health benefits, it is also cultivated across the world in areas such as West Asia, the Arabian Peninsula, Sri Lanka; East, West and Southern Africa, north-eastern Pakistan, Central and South America (See figure 1.4; Dania *et al.*, 2014; Ghazali and Mohammed, 2011; Mishra *et al.*, 2011).



Figure 1. 4. Distribution map of *Moringa oleifera* (Azlan *et al.*, 2022).

2.2 Uses and biological activity of *Moringa peregrina* and *M. oleifera*

M. peregrina and *M. oleifera* have various uses amongst communities, including nutritional uses, medicine, cosmetics, plant growth enhancer, livestock feed, biofuel production, and water purification (Koheil *et al.*, 2011; Mashamaite *et al.*, 2021). Leaves, pods and seeds are edible and rich in vitamins, minerals, natural antioxidants and proteins (Zhu *et al.*, 2020). They are used to treat ailments such as malaria, diabetes, arthritis and hypertension (Meireles *et al.*, 2020; Sreelatha and Padma, 2011). *M. peregrina* and *M. oleifera* seed oil is also in high demand in the cosmetic sector as it is turned into perfumes, skin and hair products (Ghazali and Mohammed, 2011). In folk medicine, different parts of the plants are used to provide relief of different ailments that are summarised below in Table 2.2 (Elbatran *et al.*, 2005; Mishra *et al.*, 2011; Senthilkumar *et al.*, 2018).

Table 2.2: Medicinal uses of *Moringa peregrina* and *Moringa oleifera.*

Species/ plant parts	Leaves	Seeds	Pods	Roots	Bark	Flowers	References
M. oleifera	Sores, anemia, skin infections, rashes, colitis, diarrhea, dysentery, malaria, arthritis, swellings, hypertension, immune booster, elicit lactation	Acute rheumatism, gout	Warts, fever	Ant paralytic, toothache and anthelmintic	Stomach pains, ulcer, poor vision, joint pains, diabetes, anemia, hypertension, aid digestion	Inflammation, tumor, hysteria	Dhongade et al., 2017; Ghazali and Mohammed, 2011; Kassaw et al., 2016; Meireles et al., 2020; Mishra et al., 2011; Sreelatha and Padma, 2011; Zhu et al., 2020; Abd Rani et al., 2018; Belo et al., 2019; Fahey, 2005; Olson and Rosell, 2006
M. peregrina	Paralysis, hypertension, stomach disorders, malaria, diabetes, fever, wound healing and skin rashes	Purify water, abdominal pain, infantile paralysis, diabetes	Convulsions	Malaria, asthma, diabetes, hypertension, expel retained placenta	Disinfectant, wound healing, muscle pains, burns, fever, constipation, labor pains, headache		Elbatran et al., 2005; Gharibzahedi et al., 2013; Koheil et al., 2011; Olson et al., 2016; Robiansyah et al., n.d.; Senthilkumar et al., 2018; Somali et al., 1984; Tahany et al., 2010; Belo et al., 2019; Olson and Rosell, 2006

M. peregrina and *M. oleifera* also have impressive pharmacological properties that have been studied to develop new drugs. *M. oleifera* and *M. peregrina* have the following biological activities: anticancer, anti-inflammatory, anti-spasmodic, anti-diabetic, lipid lowering activity, hepatotoxicity, hypertension, antimicrobial, antioxidant, and anti-neurological diseases (Gharibzahedi *et al.,* 2013; Koheil *et al.,* 2011; Meireles *et al.,* 2020; Senthilkumar *et al.,* 2018; Somali *et al.,* 1984; Tahany *et al.,* 2010). These healing capabilities in the extracts made from different plant parts, are made possible due to their phytochemical constituents (Dhongade *et al.,* 2017). These phytoconstituents include but are not limited to Niaziminin A, Niazirin and Niazirinin–nitrile glycosides, Pyrrole alkaloid (pyrrolemarumine 400-O-a-L-rhamnopyranoside); 40- hydroxyphenylethanamide (marumoside A and B); D-glucose; quercetin; kaempferitin; kaemopherol; isoquercetin and ascorbic acid; thiocarbamate; D-mannose and Niaziminin B; protein; three mustard oil glycosides: 4-(alpha-1- rhamnopyranosyloxy)-benzylglucosinolate; quercetin-3- O-glucoside and quercetin-3-O-(6’’- Malonyl- glucoside); Niazimicin; gamma-tocopherol.2; and 4-[(4’-O- acetylalpha- L-rhamnosyloxy) benzyl isothiocyanate (Dhongade *et al.,* 2017).

2.3 Antioxidants

Antioxidants can be defined as molecules with the ability to prevent oxidative stress by inhibiting the reaction of free radicals, hence preventing cell damage (Irato & Santovito, 2021). Maintaining redox homeostasis in plants is crucial as excessive oxidation of tissues is detrimental to the plant’s normal functioning. Due to their sessile nature, plants are exposed to different conditions, both biotic and abiotic that threaten their growth and development (He *et al.,* 2018; Dumanović *et al.,* 2021). In order to survive and reproduce, plants have evolved varying defence mechanisms that

enable them to constantly adapt to their ever-changing environment. These defence mechanisms are termed enzymatic and non-enzymatic antioxidant defence systems (Ahmad *et al.,* 2009; Concato *et al.,* 2022; Dumanović *et al.,* 2021; Irato & Santovito, 2021; Nimse & Pal, 2015). The enzymatic system is comprised of antioxidant enzymes such as glutathione (GSH), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), superoxide dismutase (SOD) and catalase (CAT), whilst the non-enzymatic system is made up of low molecular weight antioxidants such as glutathione, ascorbic acid (AsA), carotenoids, proline, flavonoids, phenolic acids, and high molecular weight secondary metabolites like tannins (Ahmad *et al.,* 2009; Dumanović *et al.,* 2021; Irato & Santovito, 2021; Kasote *et al.,* 2015). Both defence mechanisms are crucial for plants to be able to counteract biotic and abiotic stressors such as pathogens, high UV, herbivore etc., and neutralise excessive ROS production (Dumanović *et al.,* 2021; Irato & Santovito, 2021). Previous studies, such as (Bamagous *et al.,* 2018; Bhattacharya *et al.,* 2018; Khalofah *et al.,* 2020), conducted on species from the *moringaceae* family, specifically *Moringa peregrina* and *M. oleifera*, report impressive antioxidant capabilities exhibited by both species. Most of the climate-change related exposure stress and biostimulant studies conducted on *M. peregrina* and *M. oleifera* report an increase in the antioxidant defence systems (both enzymatic and non-enzymatic). Azeem *et al.* (2023) reported an increase in all the antioxidant enzymes and antioxidant constituents after exposing *M. oleifera* to salinity stress. Khalofah *et al.* (2020) showed that there is an increase in antioxidant enzymes such as GR, SOD, PPO, ASO and CAT; and non-enzymatic antioxidant constituents such as ASA and GSH of *Lepidium sativum* L. which aided with the mitigation of cadmium stress after the application of *M. oleifera* leaf extract (MLE).

2.3.1 Enzymatic defence system

Superoxide dismutase (SOD) is regarded as a key enzyme in the mitigation of oxidative damage caused by ROS as it plays a crucial role in scavenging of hydroxyl radicals and maintain normal physiological function (Irato & Santovito, 2021). The main redox buffers includes glutathione (GSH) which is the oxidised to GSSG (glutathione disulfide) and AsA, which are further converted to MDHA (monodehydroascorbate) and DHA (dehydroascorbate) (Dumanović *et al.,* 2021). Most of these antioxidant systems can function to compensate for each other as they partially possess overlapping functions (Dumanović *et al.,* 2021). SOD converts $^{\cdot}O^{-}$ into H_2O_2 which is then further detoxified by APX and CAT (S. Ahmed, 2020; Irato & Santovito, 2021; Kasote *et al.,* 2015). The isoforms (variant) of APX are targeted to areas where there is increased ROS production viz., mitochondria, chloroplast, cytoplasm, peroxisomes, endoplasm ER and glyoxysomes (Dumanović *et al.,* 2021). CAT primarily functions to detox H_2O_2 into O_2 and H_2O and is mainly found in the peroxisomes (Ahmad *et al.,* 2009; Dumanović *et al.,* 2021).

2.3.2 Non-enzymatic defence system (Secondary metabolites)

Plant secondary metabolites (SM), also known as specialised metabolites, are vast and diverse plant compounds that aid with the plant's interaction with its environment, to increase survival and fecundity (reproductive ability) of plants (Badyal *et al.,* 2020; Erb & Kliebenstein, 2020a; Khare *et al.,* 2020). The production of these diverse groups of compounds is dependent upon vast factors such as plant species, climate conditions (temperature, rain, wind, etc) and locality (wild or cultivated plants) (Ahmad *et al.,* 2009; Erb & Kliebenstein, 2020a). In general, SM are a product of primary metabolites with modifications to the biosynthetic pathways of primary metabolites. Thus SM are often more complex in their side chains and structural composition than primary

metabolites (Dumanović *et al.,* 2021). Many authors have suggested that SM's role is defence against both biotic and abiotic stressor such as herbivores, UV, pests, salinity, drought, high carbon and temperature (E. Ahmed *et al.,* 2017; Austen *et al.,* 2019; Erb & Kliebenstein, 2020b; Isah, 2019). Previous work has reported that SM do not play a role in the normal development and growth of the plant (Badyal *et al.,* 2020). Due to the vast beneficial biological functions of SM such as strong antioxidant agents, anticancer and anti-inflammatory, to name a few. There has been a surge of interest in how to understand their biosynthesis pathways, enzymes and both biological and ecological functions (Erb & Kliebenstein, 2020b; Khare *et al.,* 2020). Research now reveals that the SM do not only act to defend plants but also act as both primary metabolites (facilitates normal plant growth by providing the necessary resources to crucial processes such as photosynthesis) and plant hormones, giving them a "triple role" in plants, making them involved in growth and development, communication and regulatory action (Erb & Kliebenstein, 2020b).

Due to the diverse nature of SM, large-scale industrial reproduction of these compounds is proving to be difficult due to the complexity of their chemical structures and biosynthesis pathways (Kreis & Mller-Uri, 2010). As most SM share a common structural backbone, they are classified into four major classes or categories due to their structural composition (Zhao *et al.,* 2019). These four categories include phenolics (such as tannins, phenolic acid, lignans, stilbenes, lignins, coumarins, and flavonoids), terpenoids (such as sterols, plant volatile organic compounds, cardiac glycosides and carotenoids), sulphur containing compounds (such as defensins, lectins GSH, phytoalexins, thionine and GSL,) and nitrogen containing compounds (such as alkaloids, cyanogenic glucosides, and non-protein amino acids) (Falcone Ferreyra *et al.,* 2012; Hildebrandt *et al.,* 2015; Liu *et al.,* 2018; Tong *et al.,* 2022). The following SM were investigated in the present study due to their shared biosynthetic pathways that they exhibit on overlapping functions.

2.3.2.1 Phytosterols

Phytosterols (plant sterols) are found in various marine and plant material and include more than 250 different sterol compounds (Kreis & Mller-Uri, 2010). The most common sterols found in plants include campesterol (4-desmethyl), sitosterol and stigmasterol (Du *et al.,* 2022; Piironen *et al.,* 2000). Phytosterols are synthesised in the mevalonate pathway (MVA) of the isoprenoid metabolism (Schaller, 2003; Valitova *et al.,* 2016). Plant sterols are distributed randomly in cell tissues as free sterols and include cholesterol, campsterol and sitosterol (Piironen *et al.,* 2000). Plant sterols function as crucial membrane bilayer component, regulating membrane adaptability to temperature, membrane fluidity and permeability (Du *et al.,* 2022; Kreis & Mller-Uri, 2010; Piironen *et al.,* 2000; Schaller, 2003; Valitova *et al.,* 2016). They also serve as substrates to a variety of secondary metabolites (Valitova *et al.,* 2016).

2.3.2.2 Proteins and Amino acid

Proteins and amino acids are also known as osmolytes and are distributed ubiquitously throughout plant tissues and cells (Hildebrandt *et al.,* 2015). The most common types found in plants include isoleucine, leucine, phenylalanine, methionine, valine, histidine, arginine, tryptophan, lysine, alanine, cysteine, proline, glutamine, asparagine, aspartic acid, serine, glycine, tyrosine and threonine (Concato *et al.,* 2022; Hildebrandt *et al.,* 2015). Amino acids (precursors of proteins) have a plethora of functions in plants including acting as alternative substrates to respiration in times of reduced CO_2 assimilation by the stomata (Batista-Silva *et al.,* 2019). They also function in plant growth and development, production of metabolic energy during abiotic and biotic stress, intracellular pH control agents, signalling molecules, precursors for secondary metabolites and phytohormones (Hildebrandt *et al.,* 2015). During stressful conditions, proteins and amino acids

degrade to provide an influx of molecules that will be used in other plant biological processes and defense systems (Hildebrandt *et al.,* 2015).

2.3.2.3 Anthocyanins

Anthocyanins are water-soluble plant pigments and belong to the flavonoid family (Albert *et al.,* 2009; Liu *et al.,* 2018). Anthocyanins are biosynthesised in the phenylpropanoid pathway and have a wide range of colour from orange-red to blue-purple (Liu *et al.,* 2018). As plant pigments, anthocyanin are found in the leaves, flowers, fruits, and stems of the plant and have wide functions in plants (Albert *et al.,* 2009; Falcone Ferreyra *et al.,* 2012; Liu *et al.,* 2018). They play a role in pollinator attraction and seed dispersals in flowers and fruits, respectively; UV protection (prevents photoinhibition) and act as complex co-pigments (Liu *et al.,* 2018).

2.3.2.4 Carbohydrates

Carbohydrates are very crucial compounds which comprise a wide range of sugars like monosaccharides and disaccharides, polymers such as cellulose and starch; and sugar alcohols (Arnao *et al.,* 2021). Carbohydrates are crucial not only in plants and all biotic organisms as they are heavily involved in the generation of metabolic energy. Carbohydrates are produced during photosynthesis via three pathways (processes) viz., glycogenesis, gluconeogenesis and the Calvin-cycle triose-phosphate (Arnao *et al.,* 2021). Carbohydrates also have a multitude of functions in the plant, including playing a role in fruiting, phytohormonal regulation, ROS scavenging, promoting rooting, osmoregulation, seed germination, plant growth and development, photorespiration and serves as a precursor in several secondary metabolites such as simple phenols, carotenoids, flavonoids and several terpenoids (Arnao *et al.,* 2021).

2.5.2.5 Cardiac glycosides

Cardiac glycosides are biosynthesised in the mevalonic pathway and are triterpenes which have a minimum loss of three methyl groups (Kreis & Mller-Uri, 2010; Tomilova *et al.,* 2022). They are distributed in the bark, seeds, flowers, leaves and roots (Ainembabazi *et al.,* 2022; Kreis & Mller-Uri, 2010; Tomilova *et al.,* 2022). The role of cardiac glycosides in plants is still unclear but it has been shown that cardiac glycosides affect cardiac muscles, smooth muscles and the central nervous system (CNS), hence we find these compounds in most cardiotonic drugs (Ainembabazi *et al.,* 2022; Kreis & Mller-Uri, 2010; Tomilova *et al.,* 2022).

2.3.2.6 Steroids

Like cardiac glycosides, steroids are biosynthesised in the mevalonate pathway in general and plant steroids are produced from squalene-like triterpenes (Kreis & Mller-Uri, 2010). Plant steroids function as components of membranes and have biological activities such as anti-inflammatory, antitumour, antifungal and antiparasitic (Kreis & Mller-Uri, 2010; Summons *et al.,* 2006; Valitova *et al.,* 2016). The steroids exhibiting these biological activities often contain nitrogen steroidal compounds and are found in most plant tissue cells (Kreis & Mller-Uri, 2010). Plant steroids also function in normal plant development facilitating cell division and elongation, reproductive development, vascular differentiation and often causes gene expression alterations and aids in resistance against biotic and abiotic stress (Summons *et al.,* 2006).

2.3.2.7 Coumarins

The word ‘coumarin’ was derived from ‘coumarou’, which is a Tonka bean (*Dipteryx odorata* wild, Fabaceae) (Dighe *et al.,* 2010). Coumarins are a part of the Benzopyrone family and includes

simple coumarins, triscoumarins, furanocoumarins, biscoumarins, coumarinoligans and pyranocoumarins (Borges *et al.,* 2005; Dighe *et al.,* 2010; Küpeli Akkol *et al.,* 2020; Robe *et al.,* 2021). Coumarins are biosynthesised in the phenylpropanoid pathway and are found ubiquitously in vascular compartments of plant tissues (Borges *et al.,* 2005). Coumarins are crucial for environmental adaptation in plants and act as a scavenger of free radicals (ROS) (Robe *et al.,* 2021). They also function as membrane stabilizers and lipid peroxidation inhibitors; and have anti-inflammatory, anticancer and antihyperglycemic activities (Borges *et al.,* 2005; Dighe *et al.,* 2010; Küpeli Akkol *et al.,* 2020; Robe *et al.,* 2021).

2.3.2.8 Flavonoids

Flavonoids are one of the largest groups in polyphenols consisting of more than 8000 metabolites and are found in all parts of the plants (Dighe *et al.,* 2010; Falcone Ferreyra *et al.,* 2012; Tohge *et al.,* 2017). Flavonoids can be grouped into flavonols, proanthocyanidins, anthocyanidins, flavones, chalcones, isoflavones and flavanones (Falcone Ferreyra *et al.,* 2012). Flavonoids are biosynthesised in the phenylpropanoid pathway and they are also known as plant pigments (Dighe *et al.,* 2010; Falcone Ferreyra *et al.,* 2012). Flavonoids are involved in phototropic responses, pollen maturity, auxin and iron (Fe) transport, signalling during nodulation and protection against pathogens, UV, high carbon, low nitrogen, herbivores and cold stress (Falcone Ferreyra *et al.,* 2012; Tohge *et al.,* 2017).

2.3.2.9 Saponins

Saponins are highly amphipathic compounds that are steroidal of triterpenes (Mugford & Osbourn, 2012). Saponins have soap-like properties, hence the name ‘saponin’ (Kreis & Mller-Uri, 2010; Mugford & Osbourn, 2012; Thimmappa *et al.,* 2014). Saponins are randomly distributed throughout different plant tissues and function in signal transduction and protect the plant against herbivores and pathogens (Mugford & Osbourn, 2012). Saponins have anticancer, anti-inflammatory, antibacterial, anti-parasitic and antifungal activities: (Kreis & Mller-Uri, 2010; Mugford & Osbourn, 2012; Thimmappa *et al.*, 2014).

2.3.2.10 Tannins

Tannins are phenolic compounds that have a high molecular weight that ranges from 500 to 3000 Da and are found in barks, leaves, fruits, wood and roots (Tong *et al.,* 2022). Tannins are found in many plant species, both flowering and non-flowering species and have the ability to form complexes with proteins (Hassanpour *et al.,* 2011). Tannins are grouped into two main groups, condensed (contain flavonoid unit) and hydrolysed (containing phenolic groups and carbohydrate) tannins (Hassanpour *et al.,* 2011; Tong *et al.,* 2022). Tannins possess the following biological activities viz., antioxidant, anti-inflammatory, antidiarrheal, antibacterial, antiparasitic and antiviral activities (Hassanpour *et al.,* 2011; Tong *et al.,* 2022).

2.3.2.11 Terpenoids

Terpenoids are a large group of secondary metabolites with over 15 000 structures discovered (Tholl, 2015). Terpenoids are present in higher plants and found in most vegetative structures example leaves, roots and flowers (Cheng *et al.,* 2007; Tholl, 2015). Examples of terpenoids

include paclitaxes, artemisinin, and ginsenoside (Cheng *et al.,* 2007; Tholl, 2015). Terpenoids play an important role in plants such as plant-plant, plant-insect and plant-pathogen interaction, with some defense structures (thorns and trichomes) containing terpenoids (Booth *et al.,* 2017; Cheng *et al.,* 2007; Tholl, 2015). Terpenoids are biosynthesised plastidial in the methylerythritol phosphate pathway (MEP) and via the mevalonate pathway (MEV) in the cytosol (Cheng *et al.,* 2007). Terpenoids possess anti-inflammatory, antibacterial, anxiolytic and sedative effects (Booth *et al.,* 2017; Cheng *et al.,* 2007; Tholl, 2015).

2.3.2.12 Volatile organic compounds (essential oils; VOCs)

Volatile organic compounds are characterised by having low boiling point, high vapour pressure at room temperature and low molecular weight (less than 300 Da) (Dhifi *et al.,* 2016; Lange *et al.,* 2000; Santoro *et al.,* 2011). VOCs are also produced via the MEP and MEV pathways (Dhifi *et al.,* 2016). These compounds are ubiquitous in all aromatic parts of the plant, such as flowers, leaves, seeds, barks, wood and rhizomes (Dhifi *et al.,* 2016; Lange *et al.,* 2000; Santoro *et al.,* 2011). VOCs play a crucial role in communication between and within organisms, regulation of hormones (Santoro *et al.,* 2011). They also serve as antibacterial, antioxidant, anti-inflammatory, cancer chemo-protectants, cytotoxicity, repellent and insecticidal; and allelopathic agents (Dhifi *et al.,* 2016; Lange *et al.,* 2000; Santoro *et al.,* 2011).

2.4 Microorganisms

Microorganisms, also known as microbes, are microscopic living organisms that are found in almost any environment or condition and cannot be seen with a naked eye (Masterton, 2008). Microbes are found in almost every environment thanks to their ability to live and thrive in hostile environments such as animal intestines, deep-sea volcanoes, and ice caps. They include organisms

such as bacteria, protists, viruses, archaea, fungi, and prions; and are Earth's most diverse group of organisms. Over the years, there has been a growing need to study bacterium due to the increasing cases of drug resistance bacteria and pathogen prevalence (Gerba, 2009; Peterson, 1996). Drug resistant bacteria (medication does not aid with bacterial growth inhibition) are a major health concern for humans as they jeopardize modern health care advances. Even though bacteria can be harmful to humans (such as *Staphylococcus aureus, Streptomyces albulus, Escherichia coli, Listeria and Salmonella),* there are much more bacteria that are beneficial to humans (such as Bifidobacterium and Lactobacillus) (Schuchat *et al*., 1997). Different *in vitro* methods have been developed to test if plant extracts have antimicrobial activities, including agar or broth dilution, antimicrobial gradient method (Etest) and disk-diffusion method (Balouiri *et al.*, 2016; Inouye *et al.*, 2001). Various authors have used the above mentioned methods to test antimicrobial activities of *M. oleifera* and. *M. peregrina*. For example, Bukar *et al*. (2010), showed that *M. oleifera* leaf ethanol extract exhibited activity against *Enterobacter spp, S. aureus, P. aeruginosa and E. coli.* Mansour *et al.,* (2019) also showed that *M. peregri*na has antimicrobial activity against *S. aureus, S. mutans, E. coli, P. aeruginosa* and *K. pneumonia*. However, very little is known about how the impact of elevated atmospheric CO_2 may alter the antimicrobial activities of *M. oleifera* and *M. peregrina*. Bacteria are separated by their cell envelopes (protective sophisticated layered structure, that is selectively permeable to allow diffusion of nutrients in and waste out of the organism) into two categories: gram-negative and gram-positive bacteria (Peterson, 1996).

Gram-negative (GM-) bacteria have a thin peptidoglycan cell wall, that is surrounded by an outer lipopolysaccharide membrane, and they do not dye crystal violet with the gram staining method (Oliveira & Reygaert, 2023). Gram-negative bacteria include *Chlamydia trachomatis*, *Escherichia*

coli and *Pseudomonas aeruginosa.* Gram-positive (GM+) bacteria contain a thick peptidoglycan cell wall and no outer membrane, causing them to have surface receptors and they retain the violet stain (Sizar *et al.*, 2023). Gram positive bacteria include *Staphylococcus aureus, Streptomyces albulus, Hay bacillus* and *Listeria monocytogenes.* There are various bacterial strains (a slightly different form of the species) discovered over the past century and they are very important in the advancement of drug development. Bacterial strains are collected and cultured (used as a standard for comparison) and are often associated with abbreviated letters followed by numbers, for example ATCC (American Type Culture Collection) or NCTC (National Collection of Type Cultures) (Nakano, 2007). Even thought the strains are from one species, they often exhibit varying degrees of illness causing capabilities, allowing varying antimicrobial tests to be rapidly conducted, saving time and resources (Berns *et al.*, 1996; Gerba, 2009; Nakano, 2007).

2.4.1 Escherichia coli (E. coli)

E. coli a large group of bacteria (often harmless) that lives in the intestines of humans and animals and play a crucial role in the healthy functioning of the gut. Even though most *E. coli* bacteria are harmless to humans, some *E. coli* strains can cause severe sicknesses such as urinary tract infections, diarrhoea, and respiratory illness (Masterton, 2008; Nakano, 2007; Oliveira & Reygaert, 2023).

2.4.2 Streptomyces albulus (S. albulus)

S. albulus is a Gram-positive soil bacterium which has a slow growth rate and often grows at a pH of 6.8-7.0 and temperatures between 25-30°C. *S. albulus* has become important due to scientist

discovering it can produce important secondary metabolites. *S. albulus can also induce infections such as* pulmonary infections (Sizar *et al.*, 2023).

2.4.3 Staphylococcus aureus (S. aureus)

S. aureus is Gram-positive bacteria that can grow at temperatures between 18 °C and 40° C aerobically or anaerobically (Sizar *et al.*, 2023; Balouiri *et al.*, 2016). *S. aureus* are often found on mucous membranes and the skin, and cause multiple human infections, including skin and soft tissue infections (cellulitis, carbuncles), infective endocarditis, urinary tract infections, meningitis, and gastroenteritis toxic shock syndrome (Taylor & Unakal, 2023).

Studies have shown that *M. oleifera* and *M. peregrina* leaves possess high levels of nutrients and phytochemicals which enable the two species to have potent anti-inflammatory, anticancer, procoagulant, antifungal, and antibacterial properties (Al-Asmari *et al.*, 2015; Chuang *et al.*, 2007; Doughari *et al.*, 2007; Ezeamuzie *et al.*, 1996; Mansour *et al.*, 2019; Raubilu *et al.*, 2020). Mansour *et al.* (2019) reported that *M. oleifera* and *M. peregrina* leave extracts have impressive antibacterial properties against Gram-negative and Gram-positive bacteria. The study also highlighted that *M. oleifera* has high affinity for inhibiting the growth of Gram-positive like *S. aureus* whilst *M. peregrina* has a high affinity for inhibiting the growth of Gram-negative bacteria like *E. coli*. Raubilu *et al. (2019)* also reported similar results as Mansour *et al.* (2019), where *M. oleifera* showed to possess antimicrobial properties against *Staphylococcus aureus, Escherichia coli, aeruginosa, Enterobacter aerogenes, Shigella spp, S. typhimurium and Salmonella typhi*. The strong antimicrobial properties of *M. oleifera* and *M. peregrina* can be attributed to some bioactive phytochemicals such as retinol (used in the treatment of cancer), long chain fatty acids (such as

linoleic acid, palmitic acid and myristic acid) and phenolic compounds (such as ascorbic acid and thymol) (Mansour *et al.,* 2019)

2.5 Biostimulants

Recently, there has been increased interest and use of biostimulants due to the shift to more sustainable and technological innovations in agriculture and horticulture. Biostimulants are any organic material that can improve the productivity of plants by improving soil microbiomes, nutrient use, water use and plants' responses to stress (Vasconcelos & Chaves, 2019). Biostimulants can be either a group of compounds or a single chemical compound that has an established origin such as plant extract (Ugena *et al.,* 2018). In this regard, biostimulants can be found in varying form and mixtures but they are generally grouped into seven major groups (du Jardin, 2015). These groups include chitin and chitosan derivatives, nitrogen containing compounds and protein hydrolysates, beneficial fungi, Inorganic compounds, seaweed extracts, humic substances and beneficial bacteria (du Jardin, 2015). Even with the complexity of biostimulants from mixing different organic matter, it is known that biostimulants possess amino acids, phytohormones, polyamines and osmolytes (D'Amato & Del Buono, 2021; Phiri, 2010). With such potent compounds that are crucial in normal functioning of plants, the exogenous application of biostimulants is reported to aid in the amelioration of stress induced effects (Phiri, 2010).

2.6 Climate change

Due to the exponential increase in the human population, there has been an exponential demand for energy, food, and transportation (Marchal *et al.,* 2012). Climate change is the result of the

increased emissions of greenhouse gases (GHG) produced either naturally or by anthropogenic sources such as burning of fossil fuels and cattle farming (Marchal *et al.,* 2012; Meehl *et al.,* 2007). With these increases, there has been an apparent surge in the emission and accumulation of greenhouse gas CO_2 in the atmosphere since pre-industrial times (1850-1900). For example, in 2010 there were already 30.6 gigatonnes (Gt) of GHG emitted, with predictions stating that GHG will increase by 50% in 2050, with CO_2 contributing 70% to that figure (Marchal *et al.,* 2012; Meehl *et al.,* 2007). This increase stems from the estimated 80% increase in demand for energy, meaning more fossil fuels are utilized, as it is much easier and cheaper for countries to burn fossil fuels at large scales than to invest in green energy such as solar and wind energy power (Marchal *et al.,* 2012). Transportation, which already accounts for about 28% GHG emissions today in the United States (US) alone, is also estimated to double as cars and aviation become in demand in developing countries (Agrawala, 2011). The Organisation for Economic Co-operation and Development (OECD) report states that without any ambitious interventions, the ambient CO_2 concentrations in our atmosphere will reach 685 ppm by 2050 and more than 800 ppm by the end of the 21st century (Marchal *et al.,* 2012). This drastic CO_2 increase is above the goal set in 2010 at the UNFCCC (UN Framework Convention on Climate Change) conference held in Cancún. It was decided to limit CO_2 concentrations from going above 450 ppm to have a 50% chance of managing global mean temperatures at an increase of 2°C (Bond and Sun, 2005; Marchal *et al.,* 2012; Meehl *et al.,* 2007). Under these climate models and predictions (from the IPCC and OECD), the global mean temperatures will surpass the goal set at the UNFCCC, with temperatures as high as 3°C to 6°C more than those of pre-industrial levels observed at the end of the 21st century (Agrawala, 2011; Bond and Sun, 2005). These extreme temperature increases might deem catastrophic for all life on Earth as this high temperature will continue to intensify extreme weather

events like hurricanes, droughts, alter precipitation levels and cause increases in sea level due to the melting of glaciers (Bauer *et al.,* 2011). This extreme temperature might also push some of the earth's systems to their tipping point, causing them to irreversibly change how they function which will affect both nature and societies in a negative way (Gupta *et al.*, 2019).

South Africa (SA), due to its reliance on fossil fuels, specifically coal, is the world's 14th largest emitter of GHG (Mcsweeney and Timperley, 2018). In 2019, the CO_2 concentrations of SA were just under 500 million tons of carbon (MtC) due to an increase in energy demands and an increase of coal stocks despite the shift away from coal carried out by most countries to renewable energy (Karombo, 2020), (Figure 1.5). This increase is predicted to continue until the end of the decade if new measures are not taken (Karombo, 2020; Lüthi *et al.*, 2008; Mcsweeney and Timperley, 2018; Shikwambana *et al.*, 2020). For example, in 2019 SA planned to reduce its CO_2 emissions but resulted in a 1.5% increase instead (Climate Transparency Report, 2020; Department of Environmental Affairs, 2019; Karombo, 2020). These GHG increments are predicted to bring a temperature increase of about 4 to 6^{o}C in SA which might be detrimental to medicinal plants, as prolonged exposure to abiotic stresses such as temperature denatures enzymes crucial in physiological processes of plant's life (Lüthi *et al.*, 2008). However, it is not all bad, for example in 2020 reports have shown that due to the pandemic and reduced mining activities in the country, there was a 7% decline of GHG emissions (Le Quéré *et al.*, 2020).

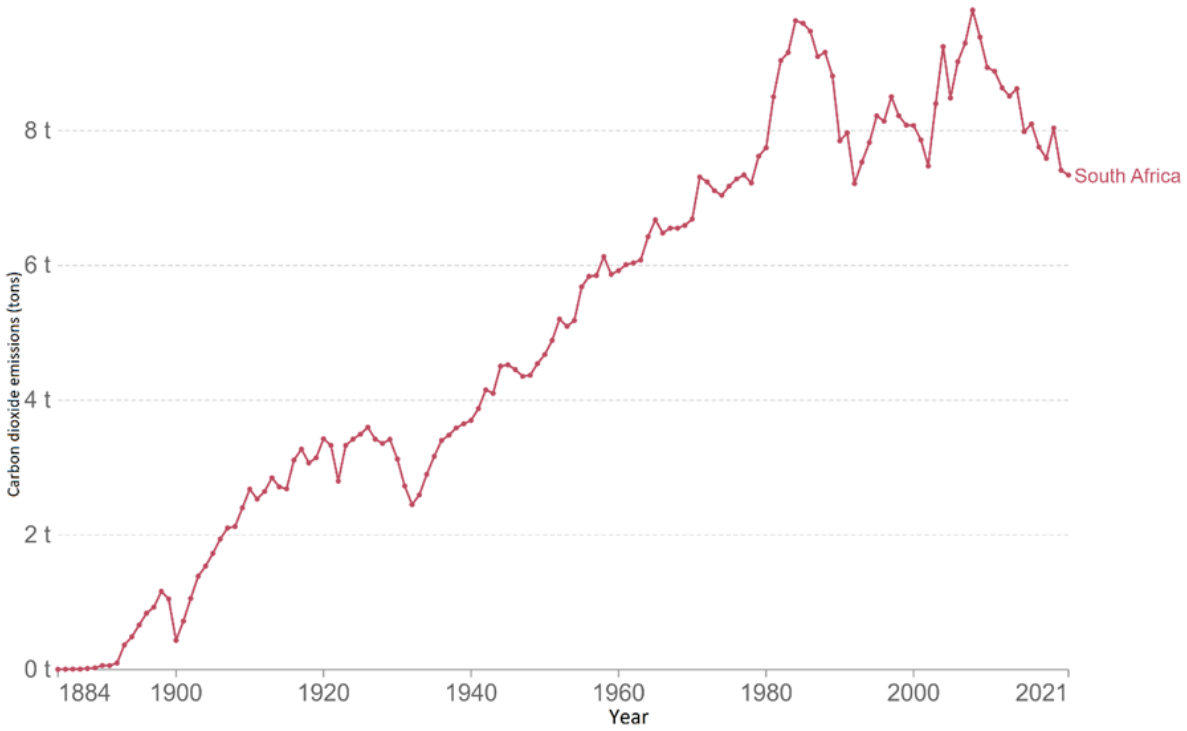


Figure 1. 5. South Africa's CO2 emissions from 1884 to 2021 [image taken from https://ourworldindata.org/co2/country/south-africa]

2.6.1 Effects of elevated CO_2 (eCO_2) on secondary metabolites of medicinal plants

Human activities have increased CO_2 emission levels since 1750 (Marchal *et al.*, 2012). Medicinal plants have elastic metabolic pathways, stemming from secondary metabolites which allow them to adapt to the changing environment (Gupta *et al.*, 2019; Idso *et al.*, 2000; Lavola and Julkunen-Tiitto, 1994; Matros *et al.*, 2006). Drastic changes in the environment affect the plants secondary metabolite production and their activity (Gupta *et al.*, 2019). Various studies have reported that there is an increase in the production of secondary metabolites when the plants are exposed to elevated CO_2 (Castells *et al.*, 2002; Goufo *et al.*, 2014; Matros *et al.*, 2006). Another crucial factor

to CO_2 concentration, is the duration in which plants are exposed to elevated CO_2, as this will determine the amount and quality of secondary metabolite produced in a plant (Goufo *et al.*, 2014; Gupta *et al.*, 2019; Idso *et al.*, 2000). For example, Idso *et al.* (2000) observed an initial (first year) increase of three alkaloids produced by *Hymenocallis littoralis* and observed a significant decline over the following years (Gupta *et al.*, 2019).

Although plants have been reported to grow under elevated CO_2 and benefit from it in terms of increased productivity (increased photosynthesis), an increase in metabolites in some species and an increase in biomass accumulation (faster growth), there are some drawbacks associated with elevated CO_2 on plants (Taub, 2010). Stomatal conductance in plants is regulated by CO_2 concentrations in the atmosphere, meaning that under ambient CO_2 the stomata are open to capture CO_2 and in doing so, transpiration also takes place. Under elevated CO_2, there is a decrease in transpiration taking place due to the decrease in stomata size because the stomata can capture the same amount of CO_2 as it did under ambient CO_2 even with it slightly opened (Ainsworth and Rogers, 2007; Taub, 2010). This means that plants compromise stomatal conductance of water for maintaining increased photosynthesis (Ainsworth and Rogers, 2007). This will in turn increase leaf temperature, affecting the integrity of the cells; and it will decrease water usage by the plant, affecting the plant's water cycle (Ainsworth and Rogers, 2007). This decreased water use means that water is no longer drawn from the ground, leaving the soil saturated which might lead to surface runoffs, affecting the whole ecosystem (Leakey *et al.*, 2009). Plants grown under elevated CO_2 exhibit low leaf nitrogen levels and this might be explained by three factors, i.e. increased photosynthates (sugar and starch) might dilute leaf nitrogen levels; there is a decrease in the amount of minerals drawn from the soil due to the affected water cycle, and decreased nitrate assimilation (Ainsworth and Long, 2005; Bloom *et al.*, 2010; Taub, 2010; Taub and Wang, 2008;

Zvereva and Kozlov, 2006). Because protein content in plants is tied to the level of nitrogen in plants, protein concentrations is seen to decline in plants grown under elevated CO_2 (Ainsworth and Long, 2005; Bloom *et al.*, 2010; Taub, 2010; Taub and Wang, 2008; Zvereva and Kozlov, 2006). Photorespiration of plants grown under elevated CO_2 also decline which is associated with stress level in plants, meaning plants are producing less antioxidants (such as Vitamin C and E) and proteins; meaning the increased growth is at an expense of poor nutritional quality (Stiling and Cornelissen, 2007). This in turns leads to increased herbivory or consumptions as herbivores are trying to compensate for the decreased food quality (Stiling and Cornelissen, 2007).

2.6.2 Reactive oxygen species

Reactive oxygen species (ROS) is a term used to describe free radicals and reactive molecules that originates from molecular oxygen (an oxygen atom with one unpaired electron) (Das & Roychoudhury, 2014). ROS have dual function, causing them to be constructive (physiological) at low concentrations and destructive (pathological) at elevated levels. In plants, ROS are mainly produced during photorespiration, with chloroplast, mitochondria and peroxisomes being their main site of production (Tripathy & Oelmüller, 2012). The photosystem I (PSI) and photosystem II (PSII) in the chloroplast generates most ROS in plants (Concato *et al.*, 2022; Das & Roychoudhury, 2014). At low concentrations, ROS play a crucial role in maintaining homeostasis, aging and act as signalling molecules (Tripathy & Oelmüller, 2012). At elevated levels, primarily induced by abiotic stressors such as salinity, high temperature and light etc., electrons from the electron transport chain (ETC) escape and react with oxygen to produce ROS (Hasanuzzaman *et al.*, 2019, 2019; Juan *et al.*, 2021). In the event of elevated CO2, the increase in photosynthetic rate causes an influx of ROS which increases oxidative damage to cells, causing damage to crucial structures. ROS damage includes DNA and RNA damage, lipid peroxidation of fatty acids within

cell membranes and protein oxidation (Roldán-Arjona & Ariza, 2009). In short, ROS take electrons from the DNA, proteins and cell membranes, altering their physiological and chemical properties, affecting how they function.

2.6.3 Mitigation of climate change

There are few studies that investigate the mitigating impact of elevated CO_2 on plants, instead, many available reports have conducted studies on the exposed plant adaptive response to elevated CO_2. It is shown that plants might mitigate the impacts of elevated CO_2 by inducing and modifying (gene expression) the alternative oxidase (AOX) pathway (Dahal and Vanlerberghe, 2018; Florez-Sarasa *et al.,* 2020; Selinski *et al.,* 2018; Vanlerberghe, 2013). Elevated CO_2 induces ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH^-$) and the superoxide anion (O_2^-) (Cassia *et al.,* 2018) due to the resulting imbalances between redox reaction (ROS production and antioxidant defenses). Many of these ROS originate from sources such as chloroplast, mitochondria, the NADPH oxidase in the plasma membrane and the peroxisome (Florez-Sarasa *et al.,* 2020). AOX pathway lowers the level of ROS from the above-mentioned sources and provides metabolic flexibility, thus improving plant performance (Florez-Sarasa *et al.,* 2020; Selinski *et al.,* 2018; Yoshida and Noguchi, 2009).

Recently, research on the use of *Moringa* leaf extract (MLE) biostimulant as a mitigator agent for climate change and abiotic stressors has gained traction. Due to the origin of MLE, it is reported that MLE biostimulant constitute of sugars, proline, high antioxidants and mineral content, amino acids, cytokines, gibberellins, and auxins (Khalofah *et al.,* 2020; Mashamaite *et al.,* 2022; Nobossé *et al.,* 2018). The constituents of MLE may explain how it acts as a mitigator of climate change stress as the high content of antioxidants and enzymes it possesses, scavenge the elevated ROS

generated by abiotic stressful conditions (Yasmeen *et al.,* 2013). The high minerals and osmolytes provides an increased influx of nutrients to the stressed plants (Hafeez *et al.,* 2022). The influx of nutrients provided to the stressed plants (in this case, *Moringa oleifera* and *M. peregrina*), are incorporated into important systems such as photosynthesis and antioxidant biosynthesis pathways, influencing the production of protective antioxidants that further aid in the amelioration of the stressed plants (Mazrou, 2019).

2.7 Rationale

Carbon dioxide is undeniably one of the major greenhouse gases that affects medicinal plants, however little research has been conducted to investigate the impacts of elevated carbon dioxide on medicinal plants. As the levels of CO_2 continue to increase, it predicted that climate change will have adverse effects in southern Africa, therefore this aspect of research is relevant because a sizable proportion of the population in developing countries such as South Africa relies heavily on medicinal plants to treat various ailments. Although there are extensive studies on *M. oleifera* and *M. peregrina*, many of these studies focused on individual species by assessing their secondary metabolites, antioxidant potential and how they are utilised either traditionally or commercially. Limited research exists on how abiotic factors such as eCO_2 affect the phytochemical profile and antioxidant activity of *M. oleifera* and *M. peregrina*. Therefore, it is necessary to assess how eCO_2 will affect these economically important species.

There has been a growing interest in the use of plant based biostimulants in order to improve crop quality and productivity. They improve nutrient availability in the soil, enhance plant use efficiency of nutrients, and promote degradation and humification of organic matter in the soil. Medicinal plants can also be incorporated in this aspect of research to enhance the production of

phytochemical groups with medicinal value. However, there are no available reports on how the use of plant biostimulants affect productivity of medicinal plants as such *M. oleifera* and *M. peregrina*, and as well as their potential use to mitigate and /or reverse the effects of abiotic stress such as elevated CO_2.

2.8 Aims

The aims of this study were to examine the effects of elevated carbon dioxide on the secondary metabolites and biological activities of *M. oleifera* and *M. peregrina*. Additionally, the study aimed to assess the impact of PhytoStim® and MLE on the productivity (metabolites and biological activities) of *M. oleifera* and *M. peregrina*. Lastly, the study aimed to investigate the potential of MLE and PhytoStim® on the adaptability of *M. oleifera* and *M. peregrina* under elevated carbon dioxide.

To achieve these aims, the following objectives were tested:

2.9 Objectives

- To screen the presence of various phytochemicals and quantify selected phytochemical groups in leaf extracts of *M. oleifera* and *M. peregrina* under elevated carbon dioxide (600 and 800 ppm).
- To determine the antioxidant and antibacterial activities of leaf extracts of *M. oleifera* and *M. peregrina* under elevated CO_2 (600 and 800 ppm).
- To screen the presence of various phytochemicals and quantify selected phytochemical groups in leaf extracts of *M. oleifera* and *M. peregrina* under different biostimulant foliar treatments (MLE and PhytoStim®).

- To determine the antioxidant and antibacterial activities of leaf extracts of *M. oleifera* and *M. peregrina* under different biostimulant foliar treatments (MLE and PhytoStim®).
- To assess the role of MLE and PhytoStim® on the phytochemical profile (qualitative and quantitative) of *M. oleifera* and *M. peregrina* under elevated CO_2 (600 and 800 ppm).
- To evaluate the role of MLE and PhytoStim® on the antioxidant and antibacterial activities in *M. oleifera* and *M. peregrina* under elevated CO_2 (600 and 800 ppm).

2.10 Research questions

- How will elevated carbon dioxide impact the phytochemical profile and biological activities of *M. oleifera* and *M. peregrina*?
- Will *Moringa* leaf extract and PhytoStim® biostimulants influence the productivity of *M. oleifera* and *M. peregrina*?
- What is the role *Moringa* leaf extract and PhytoStim® biostimulants on the adaptability of *M. oleifera* and *M. peregrina* under possible toxic effects of elevated carbon dioxide?

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CHAPTER 3

The chapter provides insight on the impacts of elevated CO_2 exposure on secondary metabolites and biological activity of both *Moringa oleifera* and *Moringa peregrina*.

Phytochemical analysis and biological activities of *Moringa oleifera* and *Moringa peregrina* under short-term elevated carbon dioxide exposure

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Abstract

Background: *Moringa oleifera* and *Moringa peregrina* are known as miracle trees as they have both great nutritional and medicinal benefits. The aim of this study was to investigate the effects of elevated CO_2 on the secondary metabolites and biological activities in *Moringa oleifera* and *Moringa peregrina.*

Results: Qualitative phytochemical analysis indicated the presence of numerous secondary metabolites such as tannins, saponins, flavonoids under 600 ppm and a slight decline under 800 ppm. The quantitative analysis indicates an increase in the total content of phenols, flavonoids (flavanols), tannins, and proanthocyanidins. An increase in CO_2 resulted in an increase in the activity of antioxidants and antibacterial for both species. On average, *Moringa peregrina* showed higher accumulation of secondary metabolites, higher antioxidant and antibacterial activities in comparison to *Moringa oleifera* under elevated CO_2 concentrations.

Conclusion: The data suggests that short-term exposure to elevated CO_2 could potentially alter the accumulation and biological processes (such as antioxidant activity and antimicrobial activity) of both *Moringa oleifera* and *Moringa peregrina*. *Moringa peregrina* is more tolerant to elevated CO_2 when compared to *Moringa oleifera* and hence it has higher antioxidant and antimicrobial activity which might be attributed to the presence of phytochemicals such as flavonoids, phenols and tannins.

Keywords: Secondary metabolite, Moringa, CO_2, Antioxidant, Antibacterial, Phenylalanine ammonia lyase (PAL), Climate Change, Medicinal Plants

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3.1 **Background**

Moringa (*M.*) is one of the most traditionally utilized, scientifically studied and publicized genus (Abd Rani *et al.,* 2018; Senthilkumar *et al.,* 2018). It is widely known for its superior medicinal and nutritional properties. The genus consists of 13 species with medicinal and nutritional value which are distributed globally (Abd Rani *et al.,* 2018). Within the genus, *Moringa stenopetala, Moringa oleifera, Moringa peregrina*, and *Moringa concanensis* are the four most studied species (Abd Rani *et al.,* 2018). Both *M. oleifera* and *M. peregrina* are fast growing trees that thrive in loamy soil that dry well (Pakade *et al.,* 2013; Abd Rani *et al.,* 2018; Senthilkumar *et al.,* 2018). All plant parts (leaves, roots, stems, seeds and flowers) of the two species are used in medicinal healing practices. The two species contain potent antioxidant, antimicrobial, anti-inflammatory, anticancer and antiviral properties, which causes them to have vast traditional and commercial utilization (Abd Rani *et al.,* 2018; Alaklabi, 2015; Mehta *et al.,* 2003; Nobossé *et al.,* 2018, 2018; Pakade *et al.,* 2013). This is due to the broad range of phytochemicals they possess including but not limited to phenols, flavonoids, terpenes, glucosides, and niazirini (Pakade *et al.,* 2013).

Medicinal plants are sensitive to changes in the environment and are climate dependent, i.e. the plants have adapted to the type of climate of that region, and are distributed where they thrive well (Gupta *et al.,* 2019). Climate change is the prolonged alterations in the weather patterns that is caused by the increase in greenhouse gas (GHG) emissions, which causes global annual temperature increases (global warming) and climate change as a result (Intergovernmental Panel on Climate Change, 2007; OECD,2012). Climate change poses a threat to medicinal plants as it brings about unpredictable climate with severe weather anomalies that can affect medicinal plant distribution, reproduction, growth and their overall quality. Climate change brings about new stressful environments, which facilitates the production of phytochemicals in exposed plants and this might have devastating effect on their pharmacological properties (Gupta *et al.,* 2019). This is a serious threat not only to medicinal plants but also crops and has particular relevance for food security, especially in developing countries such as South Africa (SA). South Africa is a region with limited clean drinking water, inequality and high unemployment rates (Bauer & Scholz, 2010). It is predicted that SA will experience severe drought spells (worse than the 2015/16 drought season) and see a six-degree Celsius temperature increase which will exacerbate the impact of climate change (Lüthi *et al.,* 2008).

Stressful environments may induce an increase and accumulation of reactive oxygen species (ROS) within plants (AbdElgawad *et al.,* 2021). Plants produce ROS in normal amounts and these ROS molecules carry out various biological processes within the plant but when they accumulate in large quantities, they have detrimental effects on plant organelles and thus have to be reduced and/or scavenged by various antioxidant agents (Gulcin *et al.,* 2003; Marslin *et al.,* 2017; Robinson *et al.,* 1980).

Studies have been conducted to investigate the impact of CO_2 on the phytochemicals of *M. oleifera* and *M. peregrina.* They report varying results, for example Castells *et al.* (2002), Matros *et al.* (2006) and Goufo *et al.* (2014) report an increase in the phytochemical profile, while Gupta *et al.* (2019) reported a decline in the plants' phytochemicals. But, there is no known study on the two Moringa species that is similar to the current study as most of the studies conducted look at how CO_2 will increase or decrease the phytochemicals. Thus, the aim of the study was to investigate the effects of elevated CO_2 on the medicinal value of *M. oleifera* and *M. peregrina*.

3.2 Methods

Experimental design

Moringa oleifera seeds were purchased in a town called Hammanskraal (25.4132^o S, 28.2578^o E), Gauteng, South Africa. The seeds of *M. peregrina* were obtained from the University of Tabuk, Saudi Arabia (28, 38444° N, 36, 48111° E). The seeds were grown at the University of the Witwatersrand (WITS) greenhouse and the seedlings were transferred to the growth chambers after 2 weeks of germinating. The growth chambers were running three different CO_2 concentrations, whilst relative humidity (60%), light intensity (160 nits) and temperature (27 ^{o}C day/ 25^{o}C at night) remained constant. The CO_2 concentrations settings were 400 ppm (control), 600 ppm (moderate concentration) and 800 ppm (high concentration). These experiments were conducted on short term CO_2 exposure (3 months) basis. The plant samples were watered after every two days.

Plant material preparation

The leaves of *M. oleifera* and *M. peregrina* were collected after three months of exposure, weighed, washed with distilled water and air dried. The dried leaves were weighed and ground to

fine powder using an electric blender (Bennet Read kitchen boss), labelled, sealed in plastic bags and stored in a dark place for later use.

Methanol extract preparation

80% methanol extracts (%v/v) were prepared using the powdered samples. In preparation of 80% methanol solvent, 80 ml of 99.9% (analytical grade) methanol, manufactured by Merck® was diluted with distilled water (H_2O_d) to 100 ml. 3g of the powdered samples were weighed and transferred onto 26 mL of 80% methanol and allowed to mix on a shaker for 72 hours. After 72 hours, the extracts were filtered using BOECO grade 1 filter paper and the filtrate was re-extracted while the filtered extract was decanted into dark bottles and stored until the second extraction was completed. After the completion of the second extraction, it was filtered and the two filtered extracts were combined to make up the total extract, and mixed by rigorous shaking. The extracts were then centrifuged at 6000 rpm for 15 minutes. The supernatant was collected into dark bottles and stored for use for various assays.

Qualitative phytochemical colour assay

Qualitative assays only indicate the presence of a targeted molecule or compound by an indication of a colour change (the colour change is also dependent on the concentration of the compound, for instance a darker shade of a colour will be observed if there is an increased concentration of the molecule). For this assay, a total of 17 phytochemicals were investigated *viz.*, tannins, saponins, flavonoids, quinones, phenols, terpenoids, cardiac glycosides, coumarins, steroids, volatile oils, phlobatannins, anthracynine, carbohydrates, glycosides, proteins and amino acids, phytosterols and triperpinoides. Methods from Roghini and Vijayalakshmi (2018) and Shaikh and Patil (2020) were slightly modified for the suitability of this current study.

Total phenolic content (TPC) assay

Sodium carbonate (7.5%) ($NaCO_3$) was mixed with 0.3 mL of the plant extracts. A volume of 750 µL of Folin–Ciocalteu′s phenol reagent was added to the mixture and the total mixture was diluted with distilled water (H_2O_d) to 7 mL, then the mixture was incubated in a dark place at room temperature for 2 hours. Absorbance measurements were recorded at 765 nm against the blank using the Genesys 10s UV-Vis spectrophotometer (Thermo-Fisher Scientific®) . A standard gallic acid curve was produced and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg/g GAE). The determination of TPC was repeated three times for both *M. oleifera* and *M. peregrina*. The following formula obtained from the gallic acid standard curve was used to calculate the TPC (Pakade *et al.,* 2013):

$Y= 0.0495x – 0.0259, R^2= 0.9994$

Total flavonoid content (TFC) assay

The TFC of the extracts was determined using the aluminium chloride colorimetric assay method. 0.3 mL of the plant extracts were added to a test tube, this was followed by an addition of 4 mL of 5% (w/v) sodium nitrate ($NaNO_3$) that was prepared by dissolving 5g of $NaNO_3$ into 100 mL of distilled water. The mixture was allowed to stand for 5 minutes, then 3 mL 10% (w/v) aluminium chloride ($AlCl_3$), prepared by dissolving 10 g of $AlCl_3$ into 100 mL of distilled water, was added to the mixture and allowed to stand for 6 minutes. After that period, 2 mL of 1M sodium hydroxide (NaOH) was added and the solution was topped-up with 10 mL distilled water and incubated at room temperature in a dark place for one hour. The absorbance was measured at 510 nm against

the blank using the Genesys 10s UV-Vis spectrophotometer (Thermo-Fisher Scientific®). This was performed in triplicates. Quercetin was used as a standard curve and the results of the assay were reported as quercetin equivalents (mg/g QE) by the use of the linear equation obtained from the calibration curve (quercetin standard). The following formula obtained from the quercetin standard curve was used to calculate the TFC (Phuyal *et al.,* 2020):

$Y= 0.2388x – 0.0019; R^2= 0.9997$

Total tannin content (TTC) assay

The TTC of the extracts were determined using the Folin–Ciocalteu's phenol reagent with gallic acid used as a standard. The method was adapted from Lahare *et al.* (2021) with slight modifications. 0.1 mL of the plant extracts was diluted with 7.5 mL of distilled water and then 0.5 mL Folin–Ciocalteu′s phenol reagent was added to the mixture. Then 1 mL of 35% (w/v) of Na_2CO_3 was added to the solution and the mixture was topped up to 10 mL with distilled water and shaken rigorously. It was then incubated at room temperature in a dark place for 30 minutes. Absorbance measurements were recorded at 725 nm using Thermo-Fisher Scientific® spectrophotometer (Genesys 10s UV-Vis). The TTC results were reported in GAE (same as TPC) (Lahare *et al.*, 2021). The following formula obtained from the gallic acid standard curve was used to calculate the TTC:

$Y= 0.046x – 0.0264; R^2= 0.9833$

Total proanthocyanidin content (TPAC) assay

The TPAC of the plant extracts was determined following Oyedemi *et al.* (2010) methods, with slight modifications. 0.5 mL of the plants' extracts were added to 3 mL of 4% vanillin-methanol

(w/v) and then 1.5 mL of hydrochloric acid (HCL). This was then vortexed and thereafter incubated in a dark place at room temperature for 15 minutes. The absorbance against the blank was measured at 500 nm with the Genesys 10s UV-Vis (Thermo-Fisher Scientific®). The results were reported in catechin equivalent (CE). The following formula obtained from the catechin standard curve was used to calculate the TPAC (Oyedemi *et al.*, 2010):

$Y = 0.9554x - 0.0003$; $R^2 = 0.9927$

DPPH scavenging assay

The DPPH scavenging activities of the plant extracts were determined following methods reported by Gochev *et al.* (2012) with slight modifications. To prepare the DPPH solution, 50 mg of DPPH was added to 100 mL of 80% methanol and rigorously mixed. It was then further diluted in a 1:5 (v/v) ratio with 80% methanol solvent. The prepared DPPH was added (700 µL) to the plants' extract aliquots (10, 20, 30, 40 and 50 µL). A blank was also prepared. This method was perfomed in triplicates for both *M. oleifera* and *M. peregrina*. Both the blank and the sample DPPH solutions were incubated in a dark place at room temperature for 45 minutes. Following incubation, absorbance readings were measured at 517 nm with the Genesys 10s UV-Vis (Thermo-Fisher Scientific®). The following equation was used to calculate the DPPH scavenging percentage of the extract:

$$\text{DPPH \%} = [(\hat{A}_{blank} - \hat{A}_{sample} / \hat{A}_{blank})] \times 100$$

Where $\hat{A}_{blank}$ represents the absorbance of the control (has all reagents except DPPH). $\hat{A}_{sample}$ is the test compound (with plant extract) absorbance (Gochev *et al.*, 2012).

Metal chelating assay

The metal (iron) reducing power of the plant extracts was determined following methods described by Gulcin *et al.* (2003) with slight modifications to suit this current study. Plant extract aliquots (10, 20, 30, 40 and 50 µL) were added to 0.05 mL of 2 mM Iron chloride $FeCl_2$, followed by 0.2 mL of 5 mM ferrozine to initiate the reaction. These solutions were mixed and allowed to stand for 10 minutes at room temperature in a dark cardboard. Absorbance of the solutions was measured at a wavelength of 562 nm using the Genesys 10s UV-Vis (Thermo-Fisher Scientific®) and compared against the blank. These steps were repeated in triplicates. The chelating effect (in percentage) was calculated using the following equation:

Chelating %= $[(A_o-A_s/A_o)] \times 100$

Where A_o is the absorbance of the control, i.e. it has all reagents except ferrozine and $FeCl_2$, and A_s is the absorbance of the solution with plant extract (Gulcin *et al.,* 2003).

Hydrogen peroxide (H_2O_2) assay

The H_2O_2 reducing power of the extracts was determined following methods reported by Gulcin *et al.* (2003) with slight modifications. Both the H_2O_2 solution and the phosphate buffer (pH 7) were prepared and both solutions were mixed (4.53 mL H_2O_2 and 995.47 mL phosphate buffer) to produce 40 mM H_2O_2 solution. 40 nM H_2O_2 solution (600 µL) was added to aliquots of plant extracts (10, 20, 30, 40 and 50 µL) and allowed to stand for 10 minutes. Absorbance against the blank was measured at 230 nm. The H_2O_2 reducing percentage was determined using the following formula:

H_2O_2 %= $[(A_o-A_s/A_o)] \times 100$

Where A_o is the absorbance of the control (has all reagents except 40 mM) and A_s is the absorbance of the solution with plant extract (Gulcin *et al.,* 2003).

Anti-bacterial assay

The antimicrobial activity of the two species was determined using the disk well method described by Gonelimali *et al.* (2018) with slight modifications. The bacterial activity of the extracts was tested using 3 bacterial strains, two of which are gram-positive bacterial strains [*Staphylococcus aureus* (ATCC 25923), *Streptomyces albulus* (ATCC 12757)] and one is a gram-negative bacteria strain [*Escherichia coli* (ATCC 25922)]. The bacterial strains were obtained from Thermo-Fisher's laboratory specialists in Johannesburg LTD. (PTY), South Africa, while the Mueller-Hinton (MH) agar was purchased from Sigma Aldrich LTD., South Africa. The bacterial strains (*S. aureus, S. albulus,* and *E. coli*) were cultured in the MH agar (Gonelimali *et al.,* 2018).

To conduct the antimicrobial screening, cooled MH agar petri dish plates were inoculated with their respective bacterial strains and incubated at 37°C for 24 hours to achieve a "bacteria lawn". Sterile pipette tips with a diameter of six millimetres were used to punch holes (wells) in the Agar for each treatment. Plant extracts (100 µL) were then transferred to the wells and incubated at 37°C for 48 hours. 80% methanol solvent was also added as a negative control. After incubation, the antimicrobial activity of the extracts was determined by calculating the zones of inhibition (ZOI).

Data analysis

Statistical analysis was conducted with IBM SPSS statistics version 23 (SPSS Inc., Chicago, IL, USA). A Shapiro-Wilks test was conducted to check for normality in our data. The results (bar

graphs) were represented as mean ± SE and a two-way analysis of variance (ANOVA) was conducted to test for the difference between treatments and species. This was followed by a tukey's HSD post hoc test, to see where the differences lie. The significant difference was set to $P<0.05$.

3.3 Results

Qualitative phytochemical screening

Qualitative screening of phytochemicals allows the detection (presence or absence) of bioactive compounds in the plants extract. The qualitative phytochemical analysis of 17 phytochemicals under different CO_2 concentration levels show that there is an increase in the detection of secondary metabolites of the leaf extract of *M. oleifera*, represented by three positive signs, that result from increasing the CO_2 concentration from 400 ppm (control) to 600 ppm (see table 3). Under 800 ppm exposure, a slight decline was observed in the detection of phytochemicals for *M. oleifera* species. *Moringa peregrina* exhibited the same trends as reported above for *M. oleifera*, with the difference in the darker shades of blue for *M. peregrina*, indicating on average, a high detection of phytochemicals in the species plant extract (see table 3.1). Table 3.1 shows the results of the assays and shows that phytochemicals were present under the three different treatments.

Table 3.1: Qualitative phytochemical analysis of the leaf extracts of *M. oleifera* and *M. peregrina* under different CO_2 concentrations.

Key:

Not detected

Low

Moderate

High

Compounds	*Moringa oleifera*			*Moringa peregrina*		
	400 ppm Control	**600 ppm**	**800 ppm**	**400 ppm Control**	**600 ppm**	**800 ppm**
Tannins						
Saponins						
Flavonoids						
Quinones						
Phenols						
Terpenoids						
Cardiac Glycosides						
Coumarins						
Steroids						
Phlobatannins						
Anthocyanin						
Volatile oils						
Phytosterols						
Triterpenoids						
Proteins and amino acid						
Glycosides						
Carbohydrates						

Quantitative assays

Total Phenolic Content (TPC)

The total phenolic content of the leaf extracts from both *M. oleifera* and *M. peregrina* fluctuated as the concentration increased. When the CO_2 concentration increased from the control levels (400 ppm) to 600 ppm, there was a decline in the content of phenols and flavanols for *M. oleifera*, whereas the content of phenols and flavanols slightly increases when the CO_2 concentration increased to 800 ppm (Figure 1.6). In *M. peregrina*, the phenolic content initially increased and then slightly declined as the CO_2 concentration increased from 600 ppm to 800 ppm (Figure 1.6).

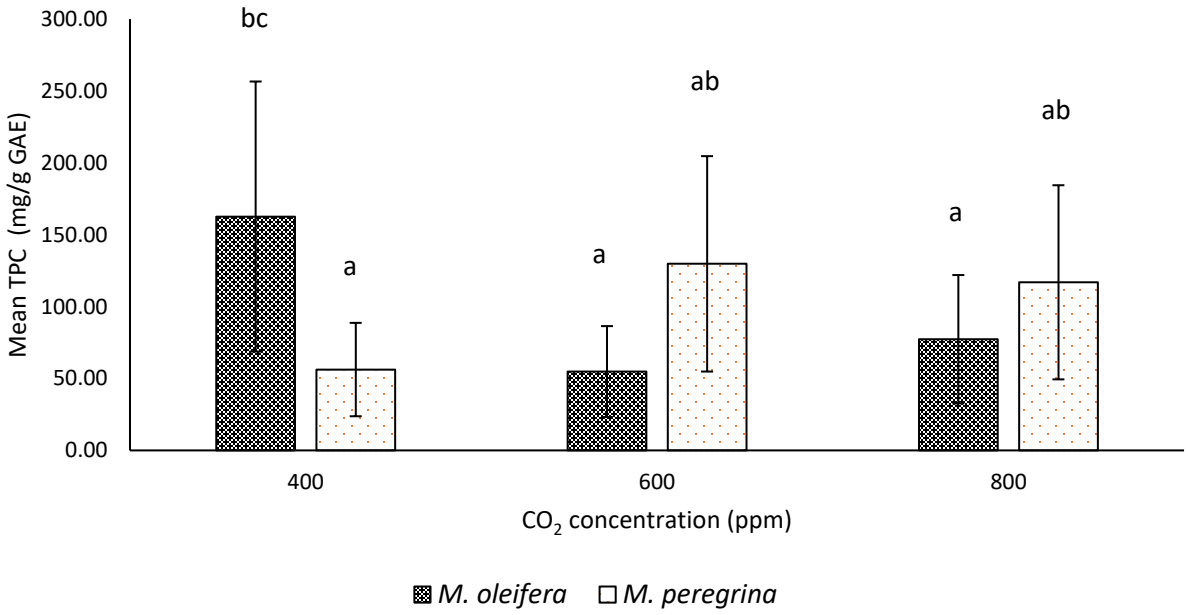


Figure 1. 6 The effects of CO_2 on the total content of phenolics for both *M. oleifera* and *M. peregrina*. The bar graphs follow the mean ± SE (*n=3*) standard with the different alphabets representing a significant difference *P<0.05*. The error bars represent the standard error.

Total flavonoid content (TFC)

Similar to figure 1.6, figure 1.7 shows that *M. peregrina* TFC increased with increasing CO_2 concentration, with the highest TFC (243 QE/g) observed under 800 ppm in comparison to *M. oleifera* where the highest TFC is observed under the control (400 ppm) experiment (figure 1.7).

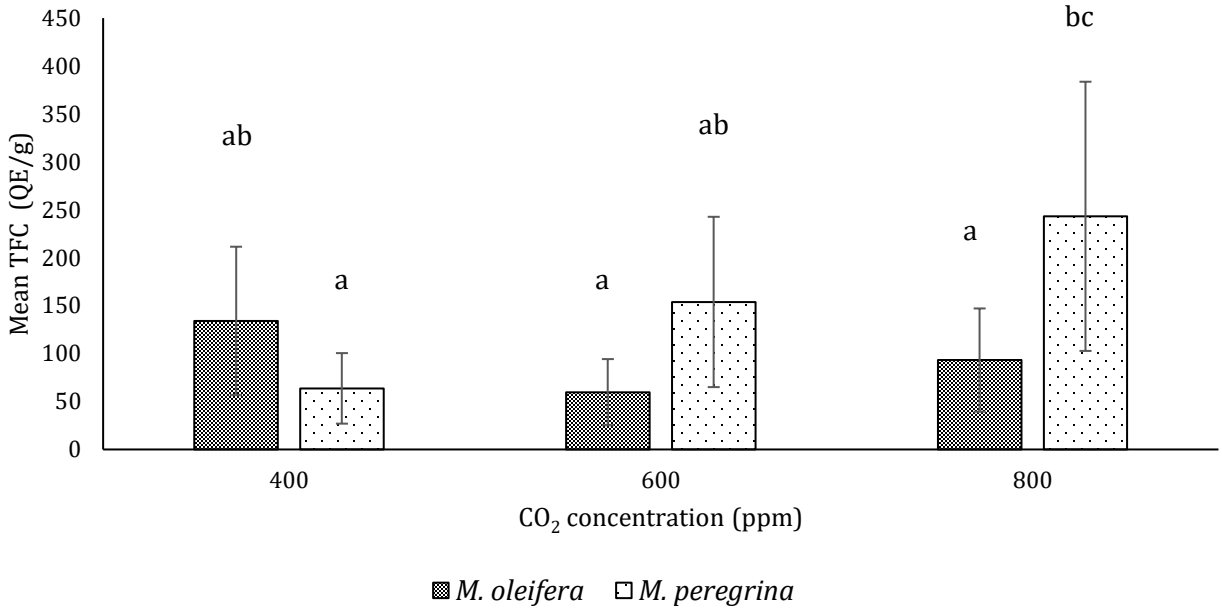


Figure 1. 7 The effects of CO_2 on the total content of flavonoid for both *M. oleifera* and *M. peregrina*. The bar graphs follow the mean ± SE (*n=3*) standard with the different alphabets representing a significant difference (*P<0.05*). The error bars represent the standard error.

Total Tannin Content (TTC)

The TTC of *M. oleifera* showed a steady increase as the CO_2 concentration levels increase with 800 ppm showing the highest TTC for *M. oleifera* (figure 1.8). *Moringa peregrina's* TTC also increased with increasing CO_2 concentration with a slight decrease in TTC under 600 ppm and the highest TTC can be found under 800 ppm followed by the control for *M. peregrina* (figure 1.8).

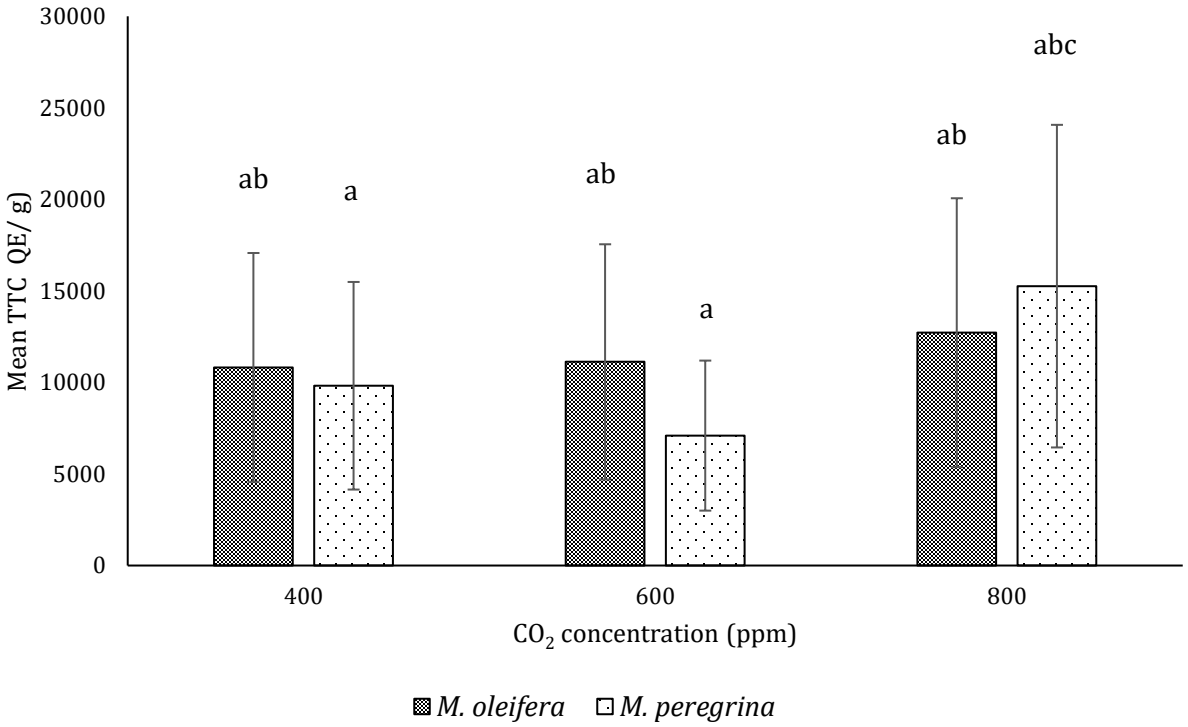


Figure 1. 8 The effects of CO_2 on the total content of tannins for both *M. oleifera* and *M. peregrina*. The bar graphs follow the mean ± SE (*n=3*) standard with the different alphabets representing a significant difference (*P<0.05*). The error bars represent the standard error.

Total proanthocyanidins (TPAC)

The TPCA of *M. oleifera* shows a slight increase from control levels (400 ppm) and plateaus as the CO_2 concentrations were increased further to 600 and 800 ppm (figure 1.9). *Moringa peregrina* TPAC decreased when the CO_2 concentration was increased to 600 ppm and the TPAC values increase to its highest when the CO_2 levels are at 800 ppm (Figure 1.9).

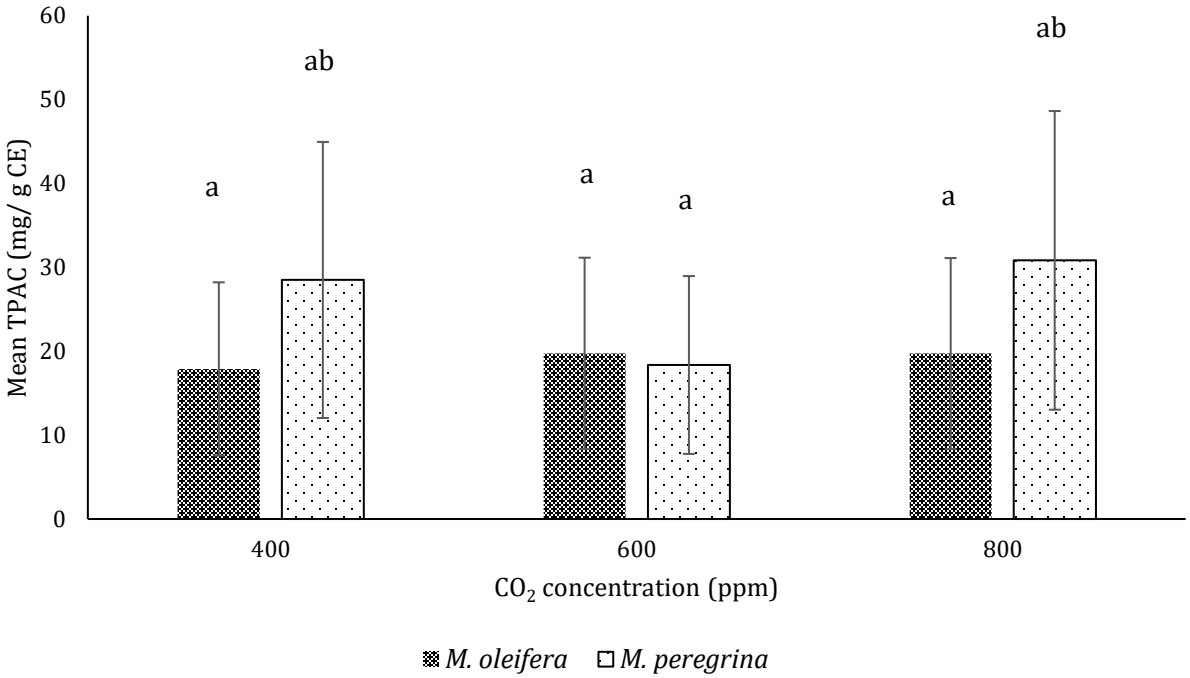


Figure 1. 9 The effects of CO_2 on the total content of proanthocyanidins for both *M. oleifera* and *M. peregrina*. The bar graphs follow the mean ± SE (*n=3*) standard with the different alphabets representing a significant difference ($P<0.05$). The error bars represent the standard error.

Antioxidant assays

2,2-diphenyl-1-picrylhydrazyl (DPPH)

Antioxidant assays showed that the extracts from *M. peregrina* exhibited better antioxidant activity in comparison to *M. oleifera* for all three treatments. *Moringa oleifera* appears to show a reduction

in its antioxidant activity as the CO_2 concentration. *Moringa peregrina* showed a decline in its antioxidant activity under 600 ppm for all three assays however when the CO_2 concentration was increased to 800 ppm, an increase in the antioxidant activity was observed (figure 1.10). There was a significant difference between the antioxidant activity of the plant extracts for all treatments (p<0.05) (see figure 1.10).

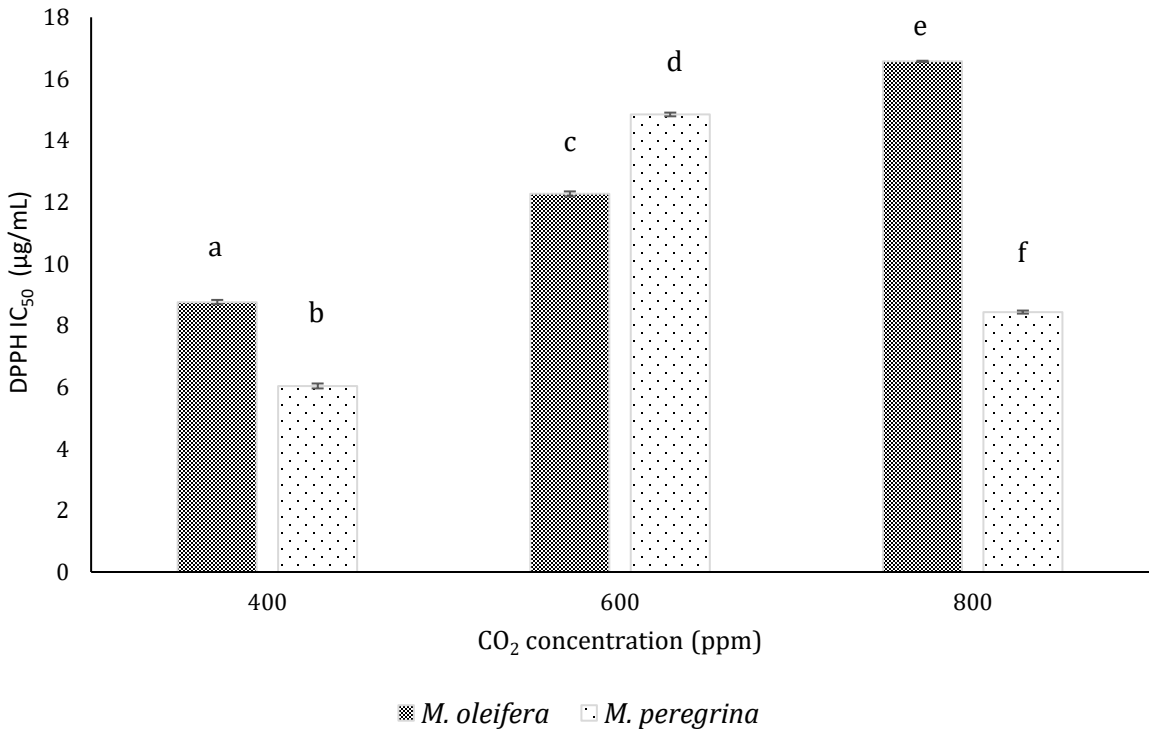


Figure 1. 10 Comparison of the IC50 values for *M. oleifera* and *M. peregrina* under different CO_2 concentrations. Figure 1.10 shows the scavenging activity against DPPH. The bar graph follows the mean ± SE ($n=3$) standard with the different alphabets representing a significant difference ($P<0.05$). The error bars represent the standard error.

Hydrogen peroxide (H_2O_2)

The H_2O_2 reduction of *M. peregrina* increased as the CO_2 concentrations increased with an exception for the 600-ppm treatment where there was a decline in its reducing abilities (figure

1.11). *Moringa oleifera* had the highest reducing capabilities under the control treatment conditions with a rapid decline observed as CO_2 levels increase (Figure 1.11).

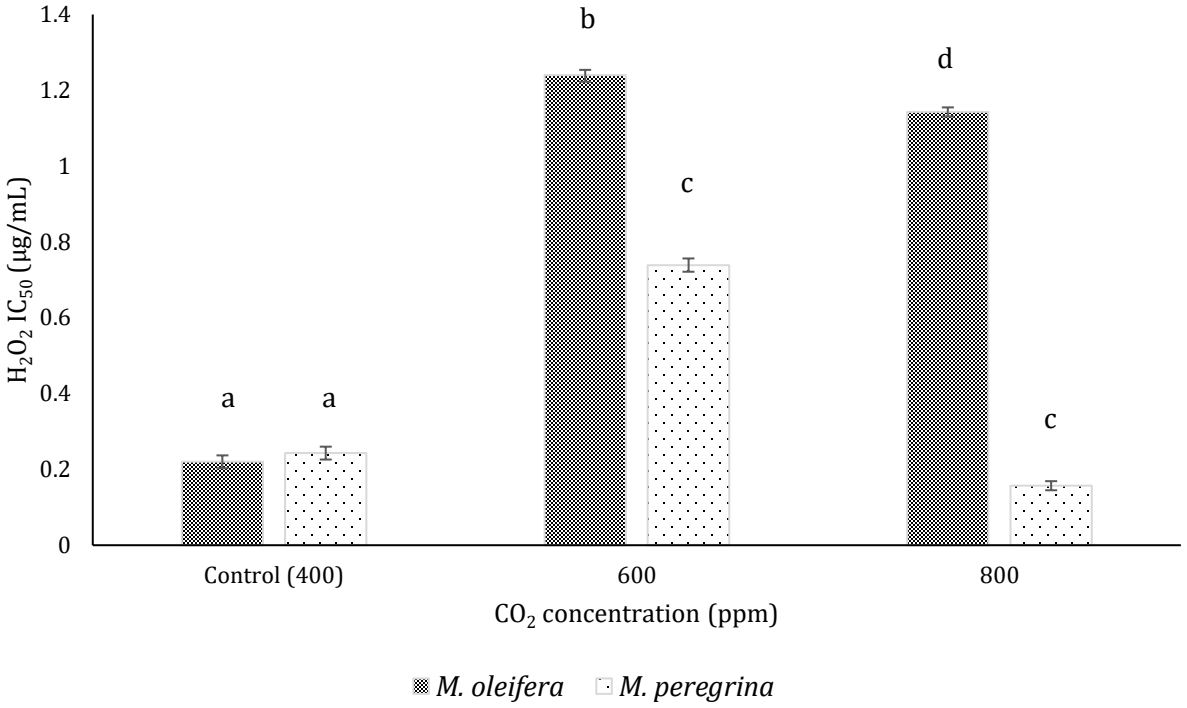


Figure 1. 11 Comparison of the IC_{50} values for *M. oleifera* and *M. peregrina* leaf extracts under different CO_2 concentrations against H_2O_2. The bar graph follows the mean ± SE (*n=3)* standard with the different alphabets representing a significant difference ($P<0.05$). The error bars represent the standard error.

Metal (iron) chelating

The chelating ability against ferrozine of both species increased with an increase in CO_2 concentrations, with both species showing a decline in their chelating activity under the 600 ppm treatment (figure 1.12).

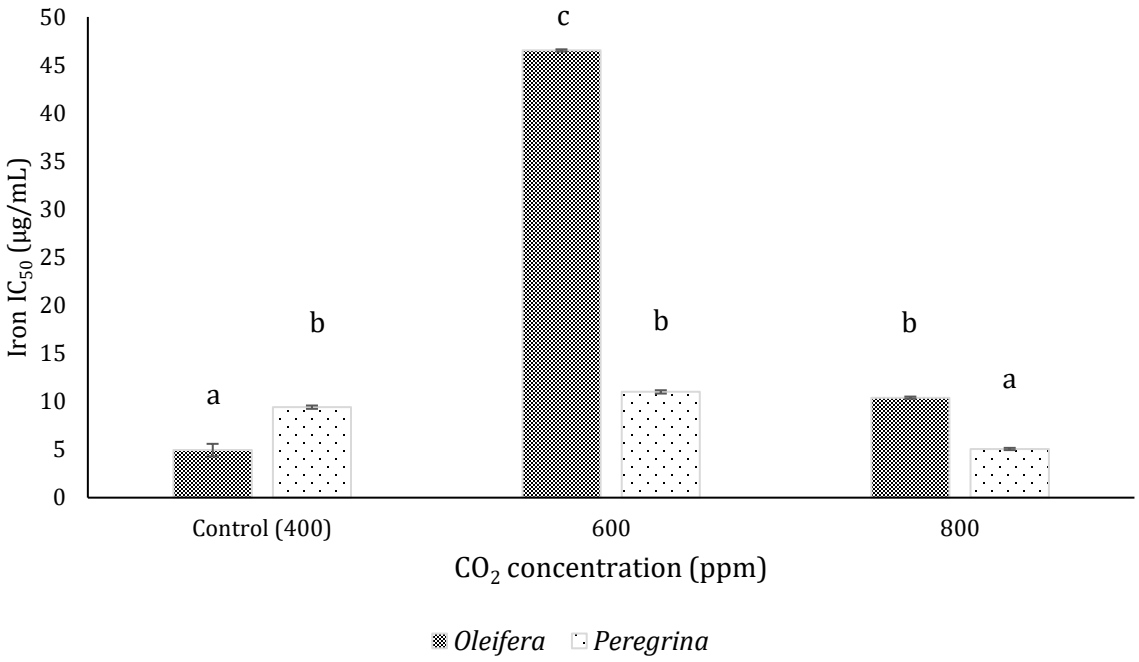


Figure 1. 12 Comparison of the IC_{50} values for *M. oleifera* and *M. peregrina* leaf extracts under different CO_2 concentrations against Iron. The bar graph follows the mean ± SE (*n=3*) standard with the different alphabets representing a significant difference (*P<0.05*). The error bars represent the standard error.

Anti-microbial assay

Similar to the antioxidant activity results, *M. peregrina* on average appeared to have slightly better antimicrobial activity under elevated CO_2 (Table 3.2). There was no significant difference in the ZOI (antioxidant activity) of the extracts under the different CO_2 concentrations, with *M. peregrina* showing a greater antioxidant activity against *S. aureus* and *E. coli* under 800 ppm.

Table 3.2: Antibacterial zone of inhibition (ZOI) of *Moringa oleifera* and *Moringa peregrina* against three bacterial strains at three different CO_2 concentrations. The asterisk (*) show significant difference (*P<0.05*).

Extracts	**Bacterial strain**	**Zone of Inhibition (mm)**			
		Negative control 80% methanol	**400 ppm**	**600 ppm**	**800 ppm**
M. oleifera	***E. coli***	0±0.0	17±0.47	18±0.12	16±0.05
M. peregrina		0±0.0	19±0.02	20±0.21	21±0.03*
M. oleifera	***S. aureus***	0±0.0	17±0.01	19±0.12*	17±0.05
M. peregrina		0±0.0	19±0.36	18±0.02	23±0.01*
M. oleifera	***S. albulus***	0±0.0	11±0.02	15±0.13	16±0.07
M. peregrina		0±0.0	15±0.05	18±0.36	17±0.05

3.4 Discussion

Secondary metabolites are necessary for plant survival as plants are constantly exposed to both biotic (pathogens and herbivory) and abiotic threats (droughts, high temperatures, floods and salinity) (Gasparini *et al.*, 2019; Ghosh *et al.*, 2022; Gochev *et al.*, 2012). These secondary metabolites (source of exogenous vitamins, minerals and essential oils) are also crucial for human survival as changes in their chemical composition might be detrimental to humans if they are not well understood (Cavazos *et al.*, 2021). Thus, the aim of this study was to assess how elevated CO_2 will affect the widely utilized and propagated medicinal plants, *M. oleifera* and *M. peregrina*.

There was an observed increase in the detection of secondary metabolites under elevated CO_2 conditions. This is in correlation with what other authors observed in their studies (AbdElgawad

et al., 2021; Bloom *et al.,* 2010; Dong *et al.*, 2018; Matros *et al.,* 2006; Mehta *et al.,* 2003; Quick *et al.,* 1991). Although an increase in some secondary metabolites was observed, no saponins were detected under 800 ppm for *M. oleifera*. Anthocyanins, phlobatannins, volatile oils and phytosterols were also not detected. The mechanisms by which these secondary metabolites increase due to an increase in CO_2 are highly complex and still not fully elucidated. The increased exposure to elevated CO_2 upregulates Ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) (Chollet & Ogren, 1975; Wattal & Siddiqui, 2015; Xu *et al.*, 2015). Rubisco is an important enzyme in photosynthesis which fixes the carbon molecules from the atmospheric CO_2 (Keys, 1986; Quick *et al.,* 1991; Wattal & Siddiqui, 2015). Under ambient CO_2 concentrations, CO_2 is in competition with O_2 for Rubisco. Studies show that under ambient conditions, it is energetically costly for plants (Rubisco) to assimilate carbon and thus Rubisco binds to O_2 and favours photorespiration but under elevated CO_2, the O_2 and CO_2 ratio shifts and Rubisco binds with CO_2 and the rate of carbon assimilation then increases as a result (Chollet & Ogren, 1975; Bravdo & Canvin, 1979; Keys, 1986).

The impressive antioxidant and antimicrobial activity observed from *Moringa oleifera* and *Moringa peregrina* is attributed to vast phytochemicals that possess antioxidant properties such as phenols, flavonoids, tannins and proanthocyanidins, etc (Phuyal *et al.,* 2020). Phenolic compounds possess an aromatic ring, with an ability to have one or more substituents. Phenols are crucial as they are responsible for a range of functions such as defense, odour production and colour production and they are found in many plant species (Ahmed, 2020). Flavonoids are a naturally occurring group of substances that have varying phenolic structures and can be found in roots, tea, fruits, bark, flowers, wine, vegetables and stem. Just like phenols, flavonoids possess the following medicinal properties viz., anti-mutagenic, anti-oxidative agents, anti-inflammatory

and anti-carcinogenic properties with the ability to modulate important cellular enzyme functions (Roghini & Vijayalakshmi, 2018). Tannins are water soluble polyphenols that are found in many foods with the following medicinal properties viz., anticarcinogenic and antimutagenic (Chung *et al.,* 1998). Proanthocyanidins are a subgroup of polyphenols which branched from flavonoids and are also found in a vast number of foods and structures of plants. Due to their high antioxidant agents, they have been linked to treat cardiovascular-related ailments (Rauf *et al.,* 2019).

Even though *M. oleifera* showed a decline in its antioxidant and antimicrobial activities in comparison to *M. peregrina*, it should be noted that its IC_{50} values all fall under 50 µg/mL, signifying potent antioxidant capabilities. The observed results are similar to AbdElgawad (2021) where they reported that CO_2 enhanced the antioxidant activity of the caraway plants (Carum carvi L.). The increase in the biological processes of *M. peregrina* can be explained by the increase in secondary metabolites, such as phenols, proanthocyanidins, flavanols, tannins, terpenoids, coumarins, glycosides and saponins which all give the plants antioxidant and antibacterial capabilities. The observed increased activities might be caused by the increase in photosynthates caused by the increase in CO_2 concentration (Shahidul Islam *et al.,* 1996). With the possible increased rate of photosynthesis, there is increased accumulation of sugar granules (Araya *et al.,* 2006) and with prolonged exposure to CO_2, these sugar granules grow and rupture the mesophyll cells in the leaves. Thus, resulting in the release of H_2O_2 in the leaves as a response (Sousa *et al.,* 2019). This released H_2O_2 will signal for upregulation of abscisic acid (ABA) which will further upregulate the following enzymes: catalase (CAT), superoxide dismutase (SOD), peroxidase (POX), dehydroascorbate reductase (DHAR), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), ascorbate peroxidase (APX), and glutathione peroxidase (GPX) (Finkelstein, 2013; Ghosh *et al.,* 2022; Shu *et al.,* 2016; Sousa *et al.,* 2019; Staneloni *et al.,* 2008;

Younus, 2018). Stressful conditions disrupt homeostasis and ion distribution of plants, causing an increase in osmotic stress which results in an increase in the accumulation and production of reactive oxygen species (ROS) (Shu *et al.,* 2016). Increased accumulation of ROS in plants can cause damage to the DNA and membranes as ROS contain free radicals such as hydroxyl radicals (OH), superoxide radicals (O_2^-) and perhydroxyl radicals (HO_2^-) (Rajput *et al.,* 2021a). Rajput *et al.* (2021) reported that under stress, there is an increase of the above-mentioned enzymes which scavenge the ROS, produced under these stressful conditions. The study reported that CAT binds with H_2O_2 and remain in this inactive state until an increase in H_2O_2 is observed where the compound will then react to the H_2O_2 ROS to produce H_2O and O_2 (Govender *et al.,* 2017, 2017; Hasanuzzaman *et al.,* 2019; Rajput *et al.,* 2021a; Sousa *et al.,* 2019). SOD is reported to react with the superoxide anion to produce H_2O and O_2 (Hasanuzzaman *et al.,* 2019; Rajput *et al.,* 2021b). POX is responsible for the catabolism of H_2O_2 in the extracellular spaces (Hasanuzzaman *et al.,* 2019). The remaining enzymes are actively involved in the Ascorbate–Glutathione pathway (antioxidant defense pathway). APX is involved in sensing the redox levels in the plant and it also reacts with H_2O_2 to form H_2O and O_2 (Hasanuzzaman *et al.,* 2019). MDRA is responsible for the recycling of ascorbate acid (AsA) by reducing MDHA (Hasanuzzaman *et al.,* 2019). DHAR improves the reduction of DHA to form AsA and oxidised glutathione (Hasanuzzaman *et al.,* 2019; Rajput *et al.,* 2021b). GR speeds up the reduction of oxidised glutathione to glutathione (a strong antioxidant) (Hasanuzzaman *et al.,* 2019). This might explain the observed increase in secondary metabolites as the above-mentioned enzymes are often observed to upregulate the production of secondary metabolites, which further aids in scavenging ROS.

The increased CO_2 stress might also upregulate the expression of Phenylalanine ammonia lyase (PAL). PAL is the first important enzyme for the phenylpropanoid pathway (Govender *et al.,*

2017). Studies show that the increase in CO_2 shift the C:N ratio, causing an increase in nitrogen assimilation and a reduction is nitrogen uptake, triggering PAL to be upregulated as the enzyme is limited by nitrogen (Assis *et al.,* 2001; Govender *et al.,* 2017; Jaafar *et al.,* 2012). PAL is crucial under elevated CO_2 for plants as it diverts aromatic acids which were meant for primary metabolism to the phenylpropanoid pathway (El Houari *et al.,* 2021). For the plant to be able to cope with multiple abiotic stresses as one stress is bound to be followed by another abiotic stress, for example high temperatures are often related to drought stresses that follow (He *et al.,* 2018), non-enzymatic antioxidants are crucial in the regulation of normal plant functioning under abiotic stress conditions (Ahmad *et al.,* 2009). Non-enzymatic antioxidants include ascorbate (vitamin c), vitamin E, phenols, flavonoid etc (Irato & Santovito, 2021). This can explain *M. oleifera* and *M. peregrina's* ability to cope with the stressful conditions they were growing under, as the results highlighted accumulation of some of these antioxidant agents in both species' leaves.

3.5 Conclusion

The current study highlights that short term exposure to elevated CO_2 has an impact on the content and accumulation of secondary metabolites in *M. oleifera* and *M. peregrina*. It also highlights that there was an increase in the antioxidant and antibacterial activities of the two species. It is proposed that PAL together with regulatory enzymes, is upregulated to shunt resources into the phenylpropanoid pathway, which then produced more secondary metabolites that assist in the scavenging of ROS. The study is beneficial to conservation efforts as it provides a proxy of how Moringa species might react and respond to the increasing CO_2 in the atmosphere. These increases are still not fully understood and thus further studies are required to fully understand how CO_2 alter the secondary metabolites concentrations (toxicology studies), biosynthesis pathways and

how these secondary metabolites structures change with exposure to CO_2, which might help in the treatments of ailments not previously described to be treatable using *M. oleifera* and *M. peregrina*.

Abbreviations

CO_2: Carbon dioxide; SA: South Africa; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; GAE: Gallic acid equivalent; CE: Catechin equivalent; TAE: Tannic acid equivalent; TPC: Total phenolic content; TFC: Total flavonoid content; TTC: Total tannin content; TPAC: Total proanthocyanidins; ROS: Reactive oxygen species; PAL: Phenylalanine ammonia lyase; SEM: Standard error of the mean; SD: Standard deviation

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Authors' contributions

All the authors contributed ideas and designs of the experiments. Thato Moloi collected and prepared the samples and performed all the experiments. Thato Moloi analyzed the data and wrote the manuscript. Dr Ida Risenga, Dr Shalini Dukhan and Mr Philemon Ramalepe reviewed the manuscript. Dr Risenga conceptualised and funded the research concept. All the involved authors read and approved the manuscript.

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Availability of data and materials

All data or results obtained in this study are included in this manuscript. Upon a reasonable request, data from the current study is available from the corresponding author.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

There are no competing interests.

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CHAPTER 4

The chapter provides insight on the influence of *Moringa* leaf extract (MLE) and PhytoStim® biostimulant foliar application on the phytochemical profile and biological activities in *Moringa oleifera* and *Moringa peregrina*.

The influence of *Moringa* leaf extract and PhytoStim® biostimulant foliar application on the phytochemical profile and biological activities in *Moringa oleifera* and *Moringa peregrina*

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Abstract

The investigation on the use of moringa leaf extract (MLE) and commercial PhytoStim®, to improve the productivity of medicinal plants (*M. oleifera* and *M. peregrina*) is highlighted in the present study. The analysis used in the present study included the qualitative screening of 17 secondary metabolites; three antioxidant assays [2,2-diphenylpicrylhydrazyl (DPPH) assay, hydrogen peroxide (H_2O_2) assay, iron chelating assay]; quantitative assays for TPC, TFC, TTC and TPAC; and antioxidant assay using four bacterial strains. The study showed that the application of biostimulants caused an increase in some secondary metabolites, and decreased some metabolites, such as tannins and phenols in *M. oleifera*. The application of biostimulants also resulted in an increase in the TPC, TTC and TPAC in *M. peregrina*, with a decline in total contents of these compounds in *M. oleifera*. However, this decline did not impact both species' pharmacological abilities (antioxidant and antimicrobial activities), as they had comparable antioxidant and antimicrobial activities when compared to the untreated plants (controls). The present study demonstrated that the foliar application (MLE) and commercial PhytoStim® enhanced the productivity of *M. oleifera* and *M. peregrina*.

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4.1 Introduction

Over the years, the use of chemical fertilizer has been the norm for supplying the required minerals to crops. But these chemical fertilizers are not very effective in their deliverance of these required nutrients as they are not readily available to crops due to the additional steps required by the microbes to physically, chemically, or even biologically transform these nutrients to a way accessible to plants (Del Buono, 2021). Consequently, a higher dose of these chemical fertilisers (more than the plant needs) are often applied to compensate for this shortfall, causing excess chemical fertiliser to wash off the field in a process called runoff, polluting the environment (destroys the microbial communities in the soil), water bodies and the atmosphere (Bhupenchandra, *et al.,* 2022). In addition to polluting the biosphere, the manufacturing procedure of these chemical fertilizer is energy intensive, further contributing to the emission of greenhouse gases due to the large quantities of carbon dioxide (CO_2) produced (Ugena *et al.,* 2018). Unlike chemical fertilisers, organic fertilisers are more stable when foliar or soil applied, making them less likely to runoff and pollute other systems and they are not energy-intensive to produce, making their carbon footprint small (Xu & Geelen, 2018). Similarly to chemical fertilizers, some organic fertilizer have nutrients that are not water-soluble and readily absorbed by plants, however unlike chemical fertilizers, organic fertilizers stimulate, promote and recruit soil microbial communities that can break down these nutrients and make them readily available to plants at an exceptional way (Vasconcelos & Chaves, 2019).

An example of organic fertilizers that have received attention lately in the science community due to their ability to do the above-mentioned plant growth promoting processes are biostimulants. Biostimulants are products that improve natural processes in and on the plant, and also around the plant's root system to promote nutrient efficiency, nutrient uptake, increased tolerance to abiotic stresses and improve plant (crop) vigour, quality and yield (du Jardin, 2015). The term 'plants' productivity or performance describes improvements observed after application be it crop production, quality, and yield (Nephali *et al.,* 2020b; Povero *et al.,* 2016; Ricci *et al.,* 2019). There are different types of biostimulants, viz.: humic (and fulvic acids), protein hydrolysates, seaweed extracts, biopolymers (Chitosan), inorganic compounds, beneficial fungi, beneficial bacteria, and plants extract (Bello *et al.,* 2021; Stewart *et al.,* 2020a). These biostimulants are different in their mode of action and their constituents, and they are often named from the type of organic material from which they are manufactured (Bhupenchandra *et al.,* 2022; du Jardin, 2015; Lephatsi *et al.,* 2022; Nephali *et al.,* 2020; Povero *et al.,* 2016; Yakhin *et al.,* 2016a). Moringa leaf extract (MLE) is reported to contain plant growth promoting constituents,

To date, there are limited studies conducted on how biostimulants influence the productivity of *M. oleifera* and *M. peregrina*, which are important due to their vast uses by local communities and many commercial industries. Consequently, our study aimed to investigate the influence MLE and PhytoStim® foliar application on the productivity *M. oleifera* and *M. peregrina*.

4.2 Methods

Experimental design

In this study, *Moringa oleifera* seeds were purchased in Hammanskraal (25.4132º S, 28.2578º E), Gauteng, South Africa, while *M. peregrina* seeds were obtained from the University of Tabuk,

Saudi Arabia (28,38444° N, 36,48111° E). All seeds were germinated in a mixture of potting soil, clay soil and vermiculite (40%: 50%: 10%) at the University of the Witwatersrand, in Hofmeyer greenhouse with ambient light conditions and temperatures ranging between 10°C (night) and 30 °C (day). Seeds were watered twice a week, with 200 mL of water per plant. After two weeks, the potted seedlings were grouped by the type of treatment viz., control/untreated plants, MLE treatment and PhytoStim® treatment. Each experimental treatment had 16 pots in total, with both species having 8 5L pots for each treatment. Each pot had 3 seedlings, equally spaced apart (*n = 72* plants per species). The two biostimulant foliar application treatments were conducted twice a week for up to three months and the leaf material harvesting was performed after three months.

Plant material preparation

M. oleifera and *M. peregrina* leaves were collected, weighed, washed with distilled water and air dried for two weeks. The dried leaves were weighed and ground to fine powder using an electric blender (Bennet Read kitchen boss), labelled, sealed in plastic bags, and stored in a dark place for later use.

Biostimulant preparation

PhytoStim® (Commercial biostimulant)

The commercial biostimulant (PhytoStim®) was purchased from a local distributor [Moringa Nutrition (PTY) LTD (2018/392318), P.O Box 62, Manezhe, 0967, South Africa] and its made of the following contents: Potassium, Calcium, Magnesium, Sulphur, Valine, Isoleucine, Leucine, Phenylalanine, Aspartic acid, glutamic acid, Serine, Glycine, Threonine, Alanine, Proline (see table 4.1).

Table 4.1: Chemical composition of PhytoStim®

22 Amino acids totalling 4.198 g/L	
Isoleucine	246.6 mg/L
Valine	323.8 mg/L
Glutamic acid	507.6 mg/L
Serine	269.2 mg/L
Proline	222.1 mg/L
Alanine	365.2 mg/L
Threonine	249.9 mg/L
Aspartic acid	315.0 mg/L
Glycine	244.6 mg/L
Phenylalanine	259.1 mg/L
Leucine	437.4 mg/L
Fertiliser group III REG. NO: M121	

Preparation of Moringa leaf extract (MLE) biostimulant

The following method was adopted from Mazrou (2019) with slight modifications to suit the present study. The *M. oleifera* leaves were obtained from the Moringa farm situated in Hammanskral (25.4132° S, 28.2578° E), Gauteng province, South Africa.

The MLE biostimulant was made in a laboratory by using 80% ethanol and finely ground *M. oleifera* leaves. The harvested leaves were washed and allowed to air dry in a dark place for two weeks. After drying, 60g of the dry leaves was weighed and ground to fine powder and stored in an air sealed labelled plastic container for later use. The ground leaves were transferred to an

electric blender (Bennet Read kitchen boss blender) and 1L of 80% ethanol solvent was decanted to the blender and the mixed for 10 minutes. The blended contents (leave extract) were transferred to a 1L Schott glass bottle. The 1L bottle was then placed on a shaker for 48 hours at 120 rpm. After the 48 hour period had lapsed, the extract was filtered using the BOECO grade 1 filter paper and the filtrate was centrifuged at 15000 rpm for 15 minutes. The resulting supernatant was re-filtered and transferred to the rotary evaporator to evaporate the alcohol (80% ethanol). The dry concentrated extract was re-suspended with 1L distilled water and the solution was stored in a 1L bottle and covered with foil to avoid light degradation and stored in the fridge at 5^{o} C for later use.

Application of Biostimulant

50 ml of MLE was decanted in 1L of tap water and 30 ml of PhytoStim® was decanted in 1 L of tap water. The different volumes are due to the biostimulant concentrations (they had equivalent concentrations at those volumes, $60gL^{-1}$ (dw/v)).

The resulting mixture was sprayed on the leaves twice a week, during the mornings using the 4L Kaufmann pressure sprayer. The plants were housed in an out-door glass greenhouse opposite "Hofmeyr's House" at Wits. The average humidity was 60% with day temperatures ranging from 10-26 °C. The plants were watered everyday by adding 200 mL H_2O in each pot plant. Leaf harvesting was performed after three months, and the leaves were air dried and ground to fine powder and stored for later use in a sealed plastic container.

Methanol extract preparation

80% methanol extracts (%v/v) were prepared using the powdered samples. 3g of the powdered samples were weighed and transferred onto 26 mL of 80% methanol and were allowed to mix on

a shaker for 72 hours. After 72 hours, the extracts were filtered using BOECO grade 1 filter paper and the filtrate was re-extracted while the filtered extract was decanted (transferred) into dark bottles and stored until second extraction was completed. After the completion of the second extraction, it was filtered and the two filtered extracts were combined to make up the total extract and mixed by rigorous shaking. The extracts were then centrifuged at 6000 rpm for 15 minutes. The supernatant was collected into dark bottles and stored for various assays (laboratory tests).

Qualitative phytochemical colour assay

Qualitative assays only indicate the presence of a targeted molecule or compound by an indication of a colour change (the colour change is also dependent on the concentration of the compound, for instance a darker shade of a colour was be observed if there is an increased presence of the compound). A total of 17 phytochemicals were investigated viz., tannins, saponins, flavonoids, quinones, phenols, terpenoids, cardiac glycosides, coumarins, steroids, volatile oils, phlobatannins, anthocyanins, carbohydrates, glycosides, proteins and amino acids, phytosterols and triterpenoids. Methods from Roghini and Vijayalakshmi (2018) and Shaikh and Patil (2020) were slightly modified for the suitability of this study.

Total phenolic content (TPC) assay

7.5% sodium carbonate ($NaCO_3$) was mixed with 0.3 mL of the plants' extracts. A volume of 750 µL of Folin–Ciocalteu′s phenol reagent was added to the mixture and the total mixture was diluted with distilled water (H_2O_d) to 7 mL, then the mixture was incubated in a dark place at room temperature for 2 hours. Absorbance measurements were taken at 765 nm against the blank using the Genesys 10s UV-Vis spectrophotometer (Thermo-Fisher Scientific®). A standard gallic acid curve was produced and the results expressed as milligrams of gallic acid equivalents (GAE) per

gram of dry weight (mg/g GAE). The determination of TPC was repeated three times for both *M. oleifera* and *M. peregrina*. The following formula obtained from the gallic acid standard curve was used to calculate the TPC (Pakade *et al.,* 2013):

$Y= 0.0495x – 0.0259$, $R^2= 0.9994$

Total flavonoid content (TFC) assay

The TFC of the extracts was determined by using the aluminium chloride colorimetric assay method. 0.3 mL of the plants' extract were added to a test tube, this was followed by an addition of 4 mL of 5% (w/v) sodium nitrate ($NaNO_3$) that was prepared by dissolving 5g of $NaNO_3$ into 100mL of distilled water. The mixture was allowed to stand for 5 minutes, then 3 mL 10% (w/v) aluminium chloride ($AlCl_3$), prepared by dissolving 10 g of $AlCl_3$ into 100 mL of distilled water, was added to the mixture and allowed to stand for 6 minutes. Thereafter, 2 mL of 1M sodium hydroxide (NaOH) was added and the solution was topped to 10 mL with distilled water and incubated at room temperature in a dark place for one hour. The absorbance was measured at 510 nm against the blank using the Genesys 10s UV-Vis spectrophotometer (Thermo-Fisher Scientific®). This was perfomed in triplicates. Quercetin was used as a standard curve and the results of the assay were reported as quercetin equivalents (mg/g QE) by the use of the linear equation taken from the calibration curve (quercetin standard). The following formula obtained from the quercetin standard curve was used to calculate the TFC (Phuyal *et al.,* 2020):

$Y= 0.2388x – 0.0019$; $R^2= 0.9997$

Total Tannin content (TTC) assay

The TTC of the extracts were determined using the Folin–Ciocalteu's phenol reagent with gallic acid used as a standard. The method was adapted from Lahare *et al.* (2021) with slight modifications. 0.1 mL of the plant extracts was diluted with 7.5 mL of distilled water and then 0.5 mL Folin–Ciocalteu′s phenol reagent was added to the mixture. Then 1 mL of 35% (w/v) of Na_2CO_3 was added to the solution and the mixture was topped up to 10 mL with distilled water and shaken rigorously. It was then incubated at room temperature in a dark place for 30 minutes. Absorbance measurements were recorded at 725 nm using Thermo-Fisher Scientific® spectrophotometer (Genesys 10s UV-Vis). The TTC results were reported in GAE (same as TPC) (Lahare *et al.*, 2021). The following formula obtained from the gallic acid standard curve was used to calculate the TTC:

$Y= 0.046x – 0.0264$; $R^2= 0.9833$

Total proanthocyanidin content (TPAC) assay

The TPAC of the plant extracts was determined following Oyedemi *et al.* (2010) methods, with slight modifications. 0.5 mL of the plant extracts were added to 3 mL of 4% vanillin-methanol (w/v) and then 1.5 mL of hydrochloric acid (HCL). This was then vortexed and thereafter incubated in a dark place at room temperature for 15 minutes. Absorbance against the blank was measured at 500 nm with the Genesys 10s UV-Vis (Thermo-Fisher Scientific®). The results were reported in catechin equivalent (CE). The following formula obtained from the catechin standard curve was used to calculate the TPAC (Oyedemi *et al.*, 2010.):

$Y= 0.9554x – 0.0003$; $R^2= 0.9927$

DPPH scavenging assay

The DPPH scavenging activities of the plant extracts were determined following methods reported by Gochev *et al.* (2012) with slight modifications. To prepare the DPPH solution, 50 mg of DPPH was added to 100 mL of 80% methanol and rigorously mixed. It was then further diluted in a 1:5 (v/v) ratio with 80% methanol solvent. This prepared DPPH was added (700 µL) to the plants' extract aliquots (10, 20, 30, 40 and 50 µL). A blank was also prepared. This method was perfomed in triplicates for both *M. oleifera* and *M. peregrina*. Both a blank and the sample DPPH solutions were incubated in a dark place at room temperature for 45 minutes. Following incubation, absorbance readings were measured at 517 nm with the Genesys 10s UV-Vis (Thermo-Fisher Scientific®). The following equation was used to calculate the DPPH scavenging percentage of the extract:

$$\text{DPPH \%} = [(\hat{A}_{blank} - \hat{A}_{sample} / \hat{A}_{blank})] \times 100$$

Where $\hat{A}_{blank}$ represents the absorbance of the control (has all reagents except DPPH). $\hat{A}_{sample}$ is the test compound (with plant extract) absorbance (Gochev *et al.*, 2012).

Metal chelating assay

The metal (iron) reducing power of the plant extracts was determined following methods described by Gulcin *et al.* (2003) with slight modifications to suit our study. Plant extract aliquots (10, 20, 30, 40 and 50 µL) were added to 0.05 mL of 2 mM iron chloride $FeCl_2$, followed by 0.2 mL of 5mM ferrozine to initiate the reaction. The mixture was mixed and allowed to rest for 10 minutes at room temperature in a dark cardboard. Absorbance of the solutions was measured at a wavelength of 562 nm using the Genesys 10s UV-Vis (Thermo-Fisher Scientific®) and compared against the blank. These steps were repeated in triplicates. The chelating effect (in percentage) was calculated using the following equation:

Chelating %= $[(A_o-A_s/A_o)] \times 100$

Where A_o is the absorbance of the control (has all reagents except ferrozine and $FeCl_2$) and A_s is the absorbance of the solution with plant extract (Gulcin *et al.,* 2003).

Hydrogen peroxide (H_2O_2) assay

The H_2O_2 reducing power of the extracts was determined following methods reported by Gulcin *et al.* (2003) with slight modifications. Both the H_2O_2 solution and the phosphate buffer (pH 7) were prepared and both solutions were mixed (4.53 mL H_2O_2 and 995.47 mL phosphate buffer) to produce 40 mM H_2O_2 solution. 40 nM H_2O_2 solution (600 µL) was added to aliquots of plant extracts (10, 20, 30, 40 and 50 µL) and allowed to rest for 10 minutes. Absorbance against the blank was measured at 230 nm. The H_2O_2 reducing percentage was determined using the following formula:

H_2O_2 %= $[(A_o-A_s/A_o)] \times 100$

Where A_o is the absorbance of the control (has all reagents except 40 mM) and A_s is the absorbance of the solution with plant extract (Gulcin *et al.,* 2003).

Anti-bacterial assay

The antimicrobial activity of the two species was determined using the agar well diffusion method described by Gonelimali *et al.* (2018) with slight modifications. The bacterial activity of the extracts was tested using four bacterial strains, two of which are gram-positive bacterial strains [*Staphylococcus aureus* (ATCC 25923), *Streptomyces albulus* (ATCC 12757)] and two are gram-

negative bacterial strains [*Escherichia coli* (*ATTC87396*) and *Escherichia coli* (*ATTC11775*)]. The bacterial strains were obtained from Thermo-Fisher's laboratory specialists in Johannesburg LTD. (PTY), South Africa, while the Mueller-Hinton (MH) agar was purchased from Sigma Aldrich LTD., South Africa. The bacterial strains *(S. aureus*, *S. albulus* and *E. coli*) were cultured in the MH agar (Gonelimali *et al.*, 2018).

To conduct the antimicrobial screening, cooled Mueller Hinton agar petri dish plates were inoculated with their respective bacterial strains and incubated at 37°C for 24 hours to achieve a "bacteria lawn". Sterile pipette tips with a diameter of six millimetres were used to punch holes (wells) in the Agar for each treatment. Plant extracts (100 µL) were then transferred to the wells and incubated at 37°C for 48 hours. 80% methanol solvent was also added as a negative control. After incubation, the antimicrobial activity of the extracts was determined by calculating the zones of inhibition (ZOI).

Data analysis

Statistical analysis was conducted with IBM SPSS statistics version 23 (SPSS Inc., Chicago, IL, USA). Normality of the data was investigated with the Shapiro-Wilk normality test. The results were represented as *mean* ± *SE* and the analysis of variance (ANOVA) was conducted to test the difference between treatments and species. This was followed by a Turkey post hoc test, to see where the differences lie. The significant difference was set to $P<0.05$.

4.3 Results

Qualitative phytochemical screening

Qualitative screening of phytochemicals detects (presence or absence) of secondary metabolites in the plant's extract. The screening 17 phytochemicals under two different biostimulant treatments is presented in Table 4.2. There was an increase in the presence of secondary metabolites in the leaf extract of *M. oleifera* and *M. peregrina,* sprayed with PhytoStim® in comparison to the untreated plants and plants sprayed with MLE. There was no significant difference in the secondary metabolites accumulation after the application of either MLE or PhytoStim® biostimulants (*P>0.05*; see table 4.2). The accumulation of tannins and phenols was not detected in *M. oleifera* after the application of both biostimulant when compared to untreated plants (see table 4.2). On average, *M. peregrina* showed more accumulation of the secondary metabolites in comparison *M. oleifera.* The following secondary metabolites: Phlobatannins, anthocyanins, volatile oils and triterpenoids were not detected in all plant extracts. Table 4.2 shows the results of the assays and shows phytochemicals that were present under the MLE and PhytoStim® foliar application.

Table 4.2: Qualitative phytochemical analysis of *Moringa oleifera* and *Moringa Peregrina* that was under foliar treatment of MLE and PhytoStim® biostimulants. The control represents untreated plants.

Key:

Not detected

Low

Moderate

High

Test	Control *M. oleifera*	Control *M. peregrina*	MLE *M. peregrina*	PhytoStim® *M. peregrina*	MLE *M. oleifera*	PhytoStim® *M. oleifera*
Tannins						
Saponins						
Flavonoids						
Quinones						
Phenols						
Terpenoids						
Cardiac Glycosides						
Coumarins						
Steroids						
Phlobatannins						
Anthracyanine						
Volatile oils						
Phytosterols						
Triterpenoids						
Proteins and amino acid						
Glycosides						
Carbohydrates						

Quantitative assays

Total Phenolic Content (TPC)

There were significant changes in TPC of the leaf extracts from both *M. oleifera* and *M. peregrina* under the two different biostimulant treatments. There was a decline in the content of phenols in both species under MLE application as compared with TPC in the untreated plants. The application of PhytoStim® resulted in a decrease in TPC in *M. oleifera* and an increase in TPC in *M. peregrina* when compared to untreated plants (Figure 1.13; $P<0.05$). On average, *M. peregrina* exhibited the highest TPC when compared to *M. oleifera* under all treatments ($P<0.05$; see figure 1.13).

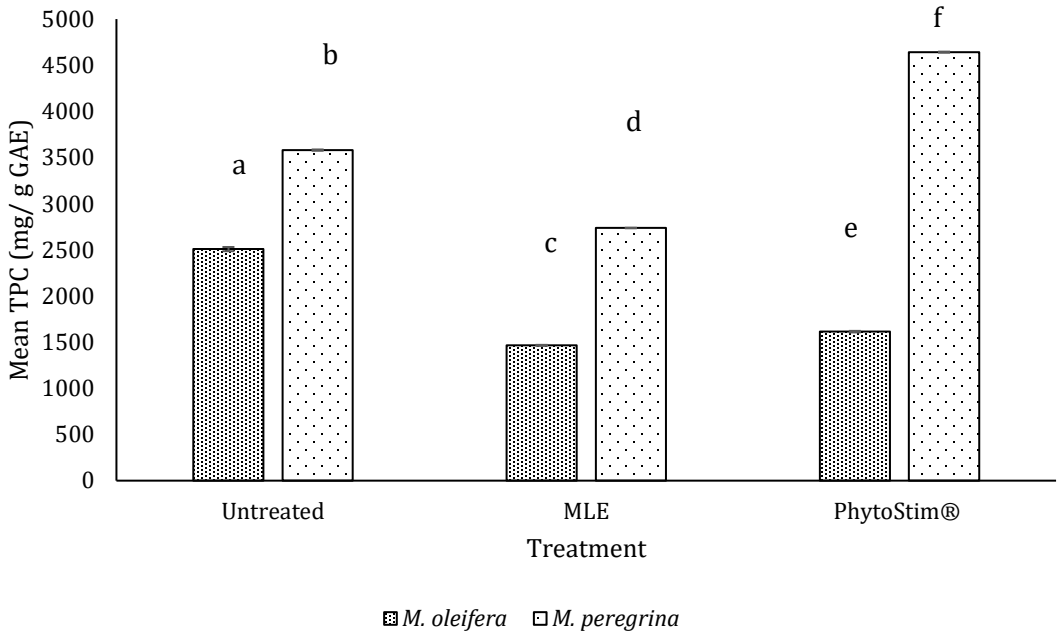


Figure 1. 13 The effects of MLE and PhytoStim® on the total content of phenolics in *M. oleifera* and *M. peregrina*. The bar graphs follow the mean ± SE ($n=3$) standard with the letters representing where significant differences between each treatment ($P<0.05$). The untreated samples represent the controls.

Total flavonoid content (TFC)

Similar, to figure 1.13, application of MLE slightly lowered the TFC of *M. oleifera* when compare to *M. peregrina* (figure 1.14; $P<0.05$). The untreated *M. oleifera* had the highest TFC when compared to the plants treated with MLE and PhytoStim® ($P<0.05$). TFC of *M. peregrina* increased significantly after the application of MLE and PhytoStim® when compared to untreated plants. No significant difference was observed in the TFC in *M. peregrina* under both biostimulant treatments ($P>0.05$; See figure 1.14).

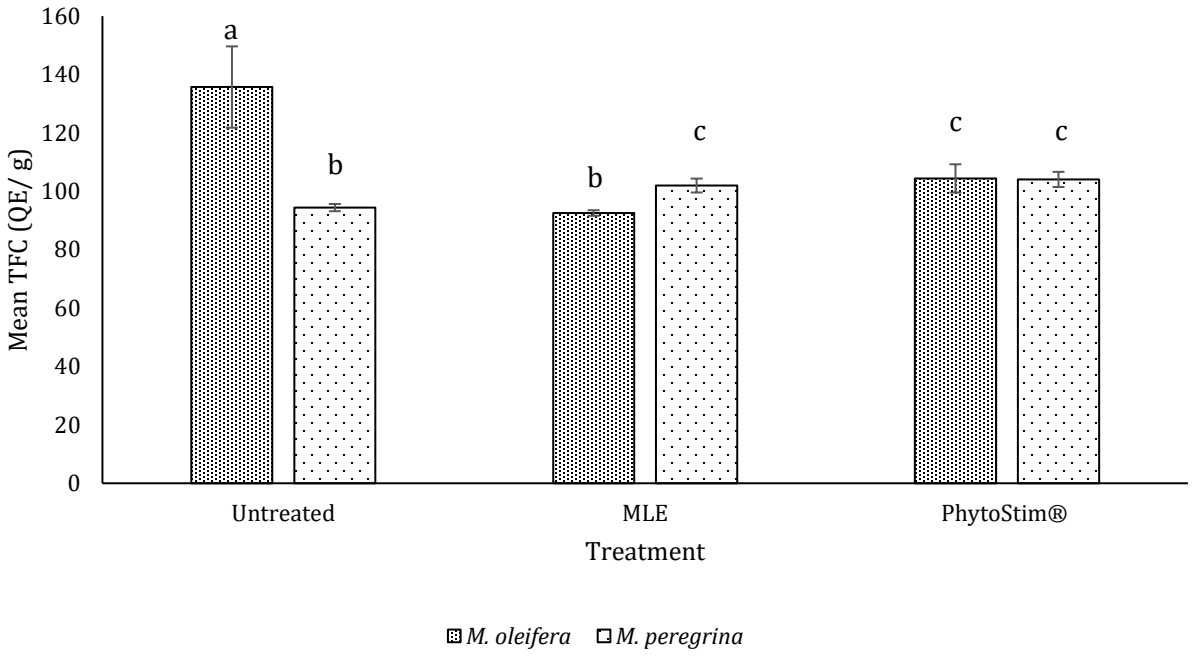


Figure 1. 14 The effects of MLE and PhytoStim® on the total content of flavonoids for both *M. oleifera* and *M. peregrina*. The bar graphs follow the mean ± SE ($n=3$) standard with the letters representing a significant difference between each treatment ($P<0.05$). The untreated samples represent the control.

Total tannin content (TTC)

The TTC in *M. peregrina* had significantly increased with the application of either biostimulant treatment in comparison to the untreated *M. peregrina*, with the highest TTC observed under PhytoStim® treatment ($P<0.05$; figure 1.15). The application of either biostimulant on *M. oleifera* resulted in a decreased TTC when compared to untreated plants, with the lowest TTC observed under MLE application (figure 1.15). On the contrary, *M. peregrina* showed an increase under both biostimulant treatment (see figure 1.15; *P<0.05*).

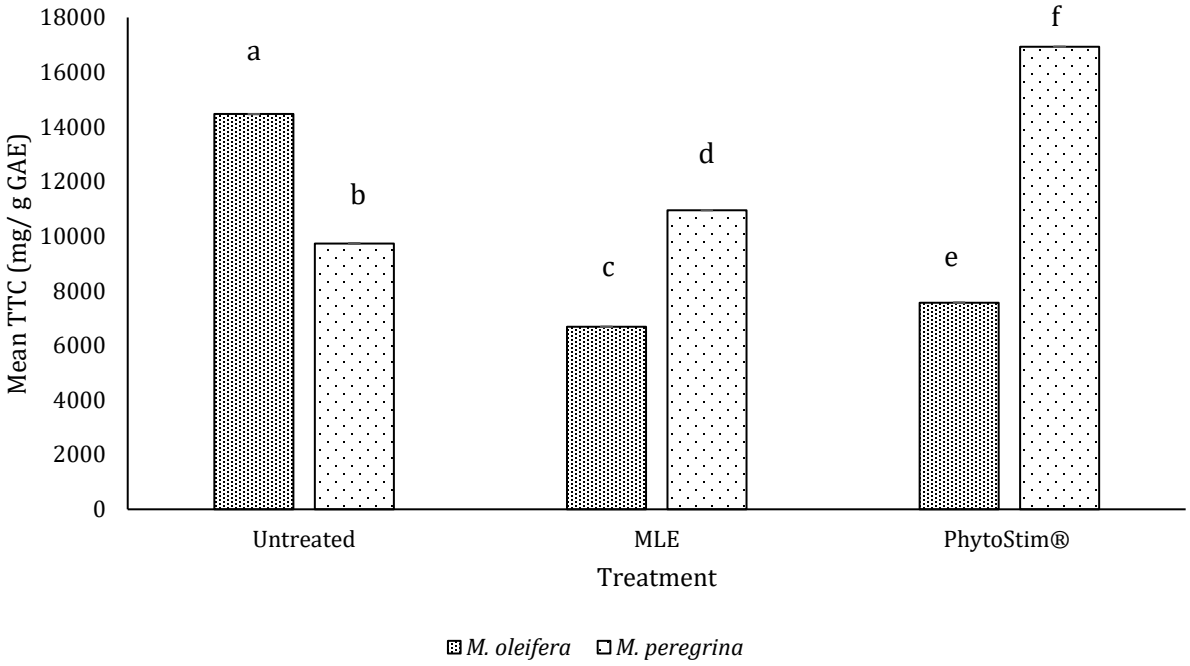


Figure 1. 15 The effects of different biostimulant treatments on the total content of tannins for both *M. oleifera* and *M. peregrina*. The bar graphs follow the mean ± SE (*n=3*) standard with the letters representing a significant difference between each treatment (*P<0.05*). The untreated samples represent the control.

Total proanthocyanidins (TPAC)

The application of MLE slightly increased and decreased the TPAC of *M. oleifera* and *M. peregrina*, respectively, when compared to the untreated plants. *M. peregrina* accumulated the highest TPAC when compared to *M. oleifera* (*P<0.05*). The application of PhytoStim® resulted in a slight decrease in TPAC in *M. oleifera* (*P>0.05*, see figure 1.16).

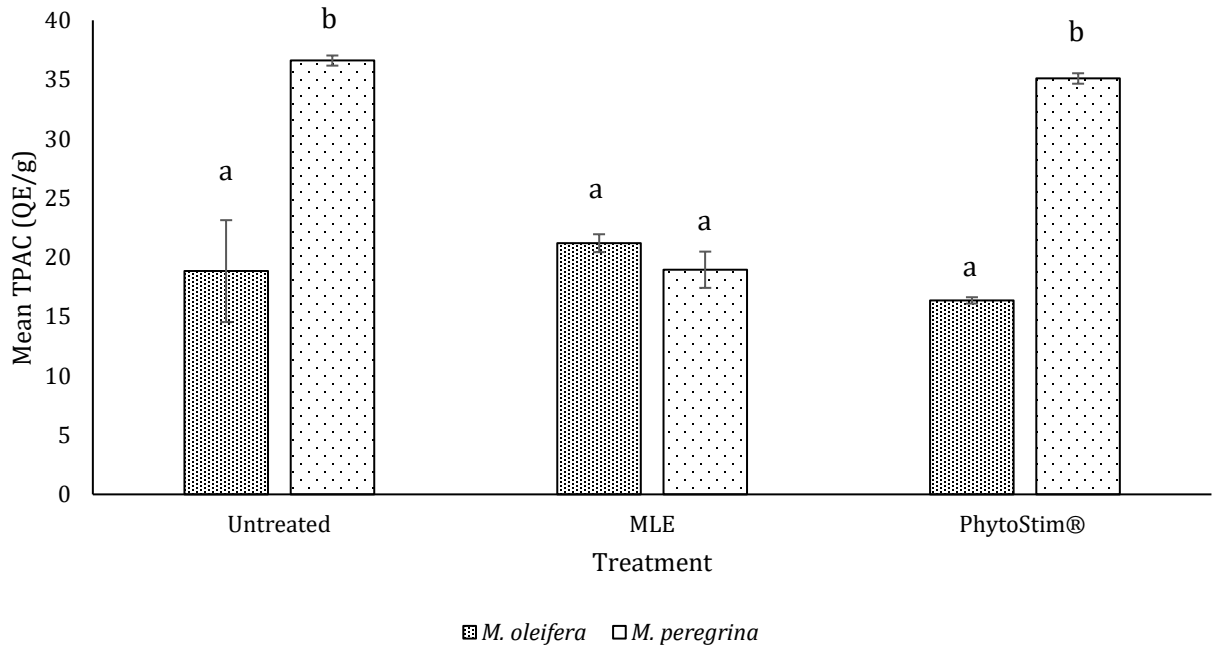


Figure 1. 16 The effects of MLE and PhytoStim® on the total content of proanthocyanidins for both *M. oleifera* and *M. peregrina*. The bar graphs follow the mean ± SE (*n=3*) standard with the letters representing a significant difference between each treatment (*P<0.05*). The untreated samples represent the control.

Antioxidant assays

2,2-diphenyl-1-picrylhydrazyl (DPPH)

The untreated plants showed better DPPH scavenging properties when compared to that of the biostimulant treated samples, with the exception of *M. peregrina* under the PhytoStim® treatment (*P<0.05*). The DPPH scavenging capabilities of both species declined under the MLE treatment application when compared to untreated plants, with a significant decline observed for *M. peregrina (P<0.05)*. On the contrary, the PhytoStim® treatment resulted in a stronger scavenging ability against DPPH in *M. peregrina* (*P<0.05*, see figure 1.17).

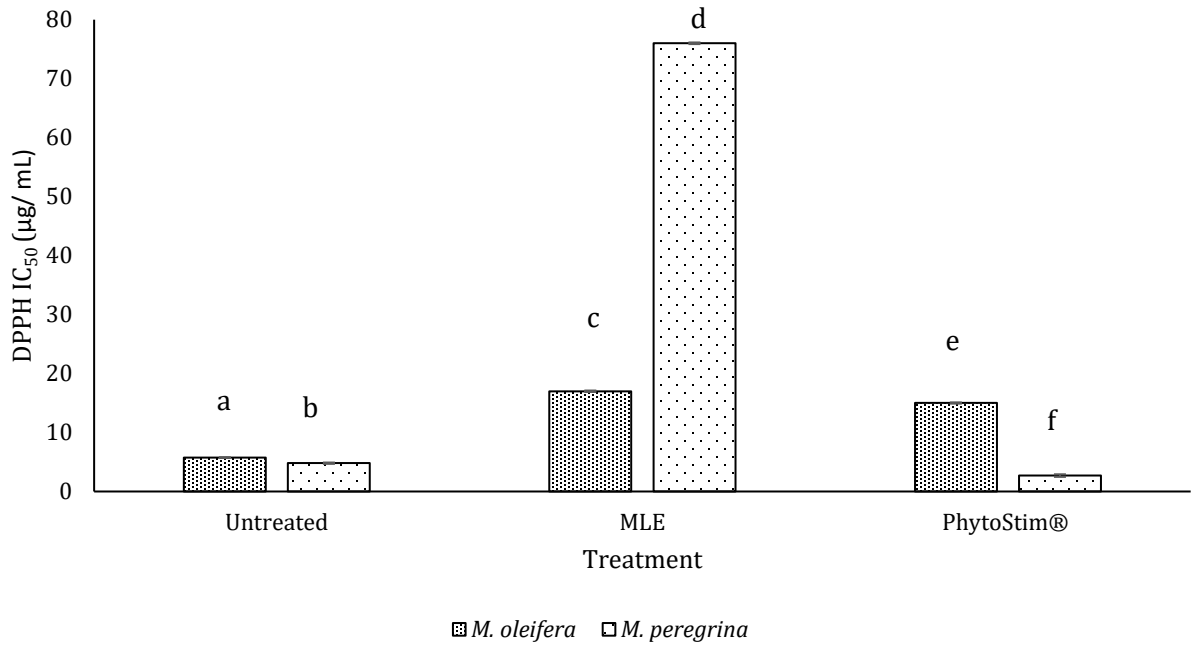


Figure 1. 17 Comparison of the DPPH IC_{50} values for *M. oleifera* and *M. peregrina* leaf extracts under MLE and PhytoStim®. The untreated samples represent the control. The bar graphs follow the mean ± SE (*n=3*) standard with the letters representing a significant difference (*P<0.05*).

Hydrogen Peroxide (H_2O_2)

The H_2O_2 reducing capability of *M. oleifera* was stronger as compared to *M. peregrina* under all treatments (*P<0.05*), with no significant difference observed fin *M. peregrina* under both biostimulant treatments (see figure 1.18). The biostimulant treated *M. oleifera* and *M. peregrina* showed slightly stronger H_2O_2 reducing capability when compared to the untreated samples, with the highest H_2O_2 reducing capability observed in *M. oleifera* plant extract under MLE treatment (*P<0.05*; Figure 1.18).

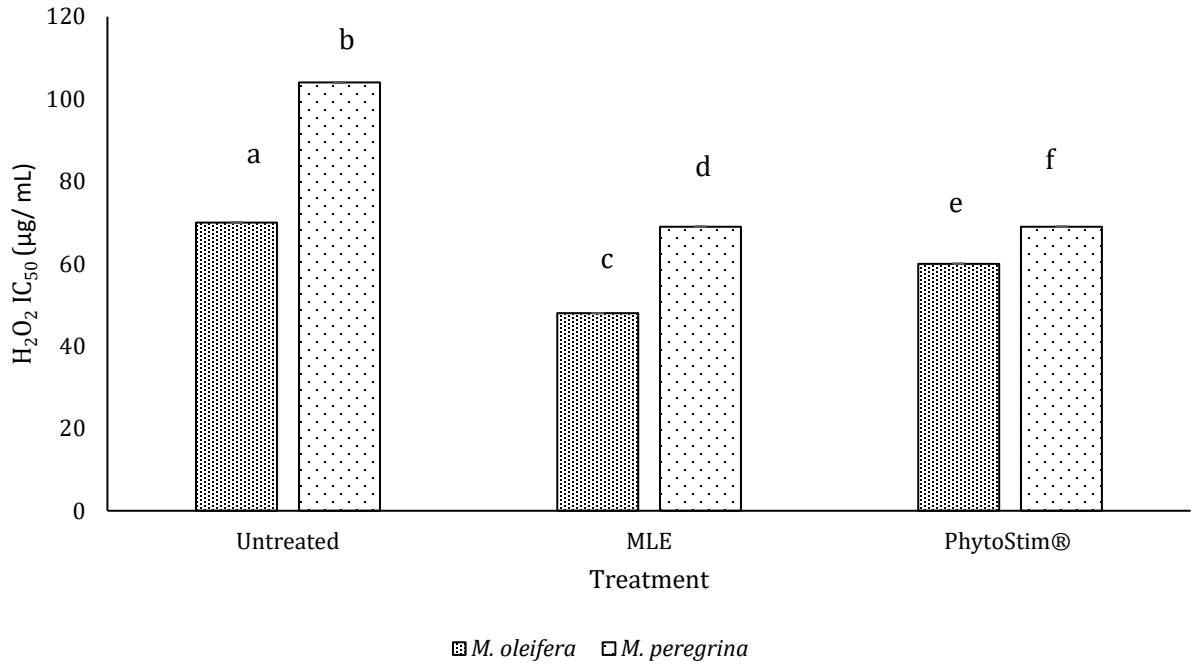


Figure 1. 18 Comparison of the H_2O_2 IC_{50} values for *M. oleifera* and *M. peregrina* leaf extracts under the MLE and PhytoStim® foliar treatments. The untreated samples represent the control. The

bar graphs follow the mean ± SE ($n=3$) standard with the letters representing a significant difference ($P<0.05$).

Metal (iron) chelating

The chelating ability against ferrozine of both species, was at its strongest under PhytoStim® treatment, with *M. oleifera* showing the strongest iron chelating capabilities when compared with *M. peregrina* ($P<0.05$). On average the application of either biostimulant treatment resulted in stronger reducing capabilities of both species when compared to the untreated *M. oleifera* and *M. peregrina*, with the exception of *M. oleifera* under MLE treatment application. There was a significant decline in the chelating capabilities of *M. oleifera* under MLE treatment ($P<0.05$; see figure 1.19).

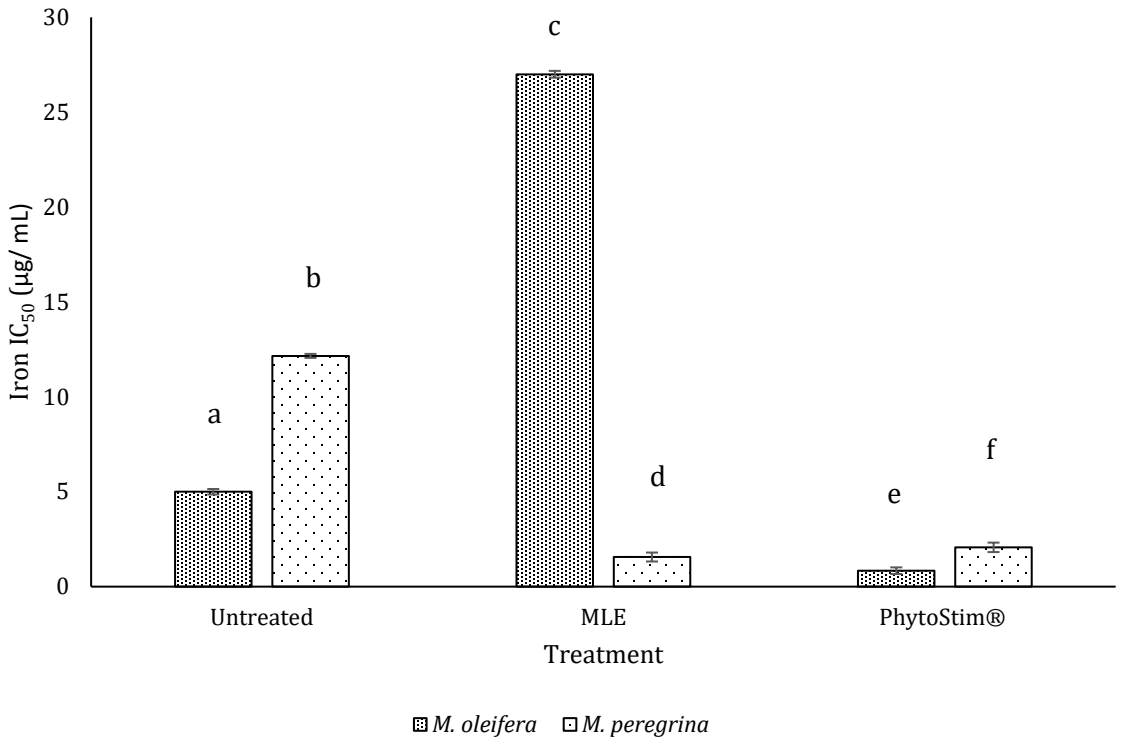


Figure 1. 19 Comparison of iron chelating IC_{50} values for *M. oleifera* and *M. peregrina* leaf extracts under MLE and PhytoStim® biostimulant foliar treatments. The untreated samples represent the control. The bar graphs follow the mean ± SE (*n=3*) standard with the letters representing a significant difference (*P<0.05).*

Anti-Microbial assays

The antibacterial assay results of methanol leaf extracts of *M. oleifera* and *M. peregrina* applied with two different biostimulant treatments (MLE and PhytoStim®) are displayed in table 4.3. There was no significant difference in the antimicrobial activity in most extracts under both biostimulant treatments (*P*>0.05) against *Staphylococcus aureus* (ATCC 25923), *Streptomyces albulus* (ATCC 12757), *Escherichia coli* (*ATTC87396*) and *Escherichia coli* (*ATTC11775*), with the exception of

M. peregrina under PhytoStim® and untreated samples (see table 4.3). *S. albulus* was resistant (R) to the untreated *M. oleifera* leaf extract. On average, *S. albulus* had less ZOI rate in comparison to other bacterial strains such as *S. aureus* (see table 4.3).

Table 4.3: Antibacterial zone of inhibition (ZOI) of *Moringa oleifera* and *Moringa peregrina* against four bacterial strains. R means resistant. The asterisk (*) show significant difference ($P<0.05$).

Sample	**Zone of Inhibition (mm)**			
	***S. aureus* (ATCC 25923)**	***S. albulus* (ATCC 12757)**	***E. coli* (ATTC87396)**	***E. coli* (ATTC11775)**
Negative control (80% methanol)	0±0.0	0±0.0	0±0.0	0±0.0
Control : *M. oleifera*	12±0.05	R (0±0.0)	13±0.56	11±0.03
Control: *M. peregrina*	16±0.05*	12±0.03	11±0.01	10±0.13
MLE: *M. oleifera*	15±0.23*	11±0.45	12±0.03	12±0.01
MLE: *M. peregrina*	16±0.09*	12±0.3	14±0.87	13±0.3
PhytoStim®: *M. oleifera*	12±0.45	10±0.35	13±0.73	13±0.35
PhytoStim®: *M. peregrina*	11±0.01	10±0.37	12±0.57	16±0.02*

4.4 Discussion and conclusion

The discovery of organic fertilizers in the scientific community has caused a rippling effect, as now potent chemical fertilizers can be naturally substituted with a more environmentally friendly and sustainable fertilizer that does not destroy the biosphere when used in moderate or recommended amounts. In this study, Moringa leaf extract (MLE) and commercial PhytoStim® were used as biostimulants in order to evaluate their influence on the productivity of *M. oleifera* and *M. peregrina*.

The application of the biostimulants treatments on both species, showed a slight increase (on average) in some accumulated secondary metabolites. Even though most of the secondary metabolites increased in *M. peregrina*, it was noted that not all secondary metabolites increased in equal amounts in *M. oleifera*, as observed in some bioactive compounds from the secondary metabolite screening assays. This is in contradiction to what most studies have found, as they report an increase in the total accumulation of secondary metabolites after application of some biostimulants (Ben Mrid *et al.,* 2021; Idowu *et al.,* 2022; Singh *et al.,* 2013; Toscano *et al.,* 2021). This observable difference between the response of *M. oleifera* and *M. peregrina* can be attributed to biostimulants being species specific (meaning not all plants will produce the same response to the same type of biostimulant) (Bhupenchandra, *et al.,* 2022); and the type of biostimulant used is different from what has been used in other studies, as biostimulant contain different types of constituents that induce a specific response in the plant. Another possible explanation is that the biostimulant can be specific or induce different responses according to the plant part (Mazrou, 2019; Phiri, 2010; Povero *et al.,* 2016; Stewart *et al.,* 2020b; Yakhin *et al.,* 2016b). The growth stage of the plant may also affect the overall secondary metabolites detected, also the conditions under which the plant was grown in, may have contributed towards the phytochemicals that the plant produced depending on the energy costs and the plant's need for those bioactive compounds (Del Buono, 2021).

MLE and PhytoStim® application slightly decreased the total content of phenolics, flavonoids, tannins and proanthocyanidins of *M. oleifera*, with the opposite being observed for *M. peregrina,* with slight improvements in the TPC, TFC and TTC. MLE application seemed to decrease the secondary metabolites more than PhytoStim®. The untreated plants had the highest TPC, TFC, TTC and TPAC in the present study, which is contradictory to what other authors have found when

they applied biostimulants to their study plants (du Jardin, 2015; Kauffman *et al.*, 2007; Merwad, 2018; Povero *et al.*, 2016; Vasconcelos & Chaves, 2019). To fully understand the responses of the above-mentioned secondary metabolites, the mode of action of each biostimulant needs to be explored and comprehended first in order to establish the exact biosynthesis pathways (Erb & Kliebenstein, 2020). Mazrou (2019) attributed the increase in the TPC observed in coriander to the high antioxidants agents such as phenolics, flavonoids and phytohormones detected in the MLE. Other bioactive constituents that might explain these increases in TPC observed in many studies might be due to the influx of vitamins and minerals MLE has which cause an "overflow" of readily available constituents that are shafted to the biosynthesis of the secondary metabolites needed in the antioxidant pathway, hence stimulating the accumulation of secondary metabolites (Bello *et al.*, 2021; Erb & Kliebenstein, 2020; Mazrou, 2019; Phiri, 2010; Stewart *et al.*, 2020a; Toscano *et al.*, 2021). Another reason that might explain the lower accumulation of these secondary metabolites is the trade-off of investing resources in either growth, maintenance and/or defences. As both *M. peregrina* and *M. Oleifera* were both relatively young (3 months), the application of biostimulant treatments on both species, might have favoured both species to invest resources into growth.

Even though the application of PhytoStim® biostimulant slightly lowered the TPC, TFC, TTC and TPAC in *M. oleifera*, there was a significant improvement in the scavenging and reducing capabilities of both species against DPPH, H_2O_2 and iron chelating. Application of MLE also improved the antioxidant capacities of both species against H_2O_2. Furthermore, MLE improved the scavenging and reducing capabilities against DPPH and iron chelating, but this is observed to be species specific, with *M. peregrina* noting improvements against iron chelating and *M. oleifera* against DPPH. This observation further supports the specificity of biostimulants to certain species

(Bhupenchandra, *et al.,* 2022; Erb & Kliebenstein, 2020). Even though the application of both biostimulant improved the antioxidant capabilities for both species, the untreated plants had the highest DPPH scavenging capabilities in comparison to the treatments. This could possibly indicate that either the biostimulant treatment had no effect in the biochemical processes that are responsible for the antioxidant activities in *M. oleifera* and *M. peregrina* in this case. Another interesting observation was that, even though the decline was noted in the common bioactive compounds (TPC and TFC) responsible for the antioxidant properties, there was no significant loss of antioxidant activities of the two species, which could be attributed to the upregulation and downregulation of some genes, biosynthesis pathways and/or biological processes (Erb & Kliebenstein, 2020). Li (2018) reported that during herbivory attack, secondary metabolites also act as defensive switches (upregulating and downregulating some defensive compounds) depending on the stressor type even though it is known that hormones are responsible for initiating or facilitating defensive compounds ("cross-talking").

The antimicrobial activity against four bacterial strains [*S. albulus, S. aureus, E. coli* (ATTC11775) and *E. coli* (ATTC87396)] of the treatments showed limited significant improvements in comparison to the untreated plants. Most of the bacterial strains showed susceptibility to the plant extracts except for the untreated *M. oleifera* extract where *S. albulus* showed resistance (R). The four bacterial extracts were mostly sensitive to extracts from *M. peregrina*. This antimicrobial activity of *M. peregrina* might be explained by the increased accumulation of secondary metabolites observed in the screening secondary metabolites after the application of the biostimulant treatments. Other studies reported that MLE provides fatty acids such as linoleic acid, this is often known for its role in maintaining the heart and lowering LDP cholesterol and have been found to have antimicrobial activities (Donati *et al.*, 2022).

In conclusion, the study concurs with what other studies observed in terms of noting the above ground mass improvements such as larger leaves, stem, and improved chlorophyll (deeper shades of green in this study) after the application of biostimulants to respective plant species. It is noteworthy that biostimulants are made from different organic matter that have complex phythochemicals and phytohormones, which react and form different pathways that produce secondary metabolites that incite a different response in biostimulants from what is known about them and their dedicated roles. Hence, in the application of MLE and PhytoStim®, it was noted that lower accumulation of secondary metabolites commonly used for antioxidant activities (TPC, TFC, and TTC); pharmacological processes such as the ROS scavenging and reducing properties of both species applied with either biostimulant was not reduced in comparison to untreated plants. This response could be attributed to the secondary metabolites that influence plant systems to react in a positive or negative pathway (positive and negative feedbacks). Various studies reported improvements in tolerance against both biotic and abiotic stress, therefore biostimulants may be used in the mitigation of climate change impacts on medicinal plants.

The application of either biostimulant on both species slightly improved the antimicrobial activities against *S. albulus, S. aureus* and *E. coli*, with *M. peregrina* extracts showing better ZOI in comparison to *M. oleifera,* however there was no significant difference between the two species. This response may also be attributed to the downregulation (phytochemicals not well detected during screening) of secondary metabolites involved in biological processes. Nonetheless, there was no significant loss on the antimicrobial properties of both *M. peregrina* and *M. oleifera*.

Future research could explore the changes to biological processes and biosynthesis pathways after biostimulant application in order to provide targeted improvements without compromising the productivity and health of the plant.

Due to the steady increase of CO_2 observed each year across the globe due to human activities such as the burning of fossil fuels, there is an increase in greenhouse gases (GHG) emitted into the atmosphere and the possibility of elevated GHG stress (specifically eCO_2 stress). Therefore, the application of biostimulants could be considered for future studies for mitigation strategies.

Authors' contributions

The all the authors contributed ideas and designs of the experiments. Thato Moloi collected and prepared the samples and performed all the experiments. Thato Moloi analyzed the data and wrote the manuscript. Dr Ida Risenga, Dr Shalini Dukhan and Mr Philemon Ramalepe reviewed the manuscript. All the involved authors read and approved the manuscript.

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Availability of data and materials

All data or results obtained in this study are included in this manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

There are no competing interests.

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CHAPTER 5:

This provides insight on the influence of *Moringa* leaf extract and PhytoStim® biostimulant foliar application on the phytochemical profile and biological activities in *Moringa oleifera* and *Moringa peregrina.*

The role of *Moringa* leave extract and PhytoStim® biostimulant on the adaptability of *Moringa oleifera* and *Moringa peregrina* under elevated carbon dioxide exposure

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Abstract

Since the industrial revolution, there has been an increase in the emission of greenhouse gases such as CO_2 which trap heat and contribute to global warming. As a consequence, there are alteration in the ordinary, predictable climatic conditions, making them unpredictable and more intense in their frequency and impact severity. Studies show that Africa will be severely impacted by climate change due to limitations in resources needed to cope with the effects of climate change. Medicinal plants such as *Moringa oleifera* and *M. peregrina* are also at risk as they are sensitive to changes in their environment. Over the years, the use of organic fertilizers such as plant based biostimulants have gained attention not only for plant enhancers but as climate change mitigator agents. In the present study, the aim was to investigate the role of *Moringa* leaf extract (MLE) and PhytoStim®) on the adaptability of *M. oleifera* and *M. peregrina* under elevated CO_2 (eCO_2) . The analysis used in the present study include qualitative screening of 17 secondary metabolites; three antioxidant assays [2,2-diphenylpicrylhydrazyl assay, hydrogen peroxide assay, iron chelating assay]; quantitative assays for total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC) and total proanthocyanidin content (TPAC); and antioxidant assay using four bacterial strains. The application of the biostimulant treatment showed that MLE and PhytoStim® mitigated the potential plant stress that was caused by concurrent exposure to eCO_2 in both *Moringa* species. The results showed a drastic increase in the presence of compounds such as phytosterols, terpenoids, cardiac glycosides and coumarins, as well as weaker presence of tannins, phenols and triterpenoids. The pharmacological activities (antioxidant and antimicrobial) of both species were however not significantly elevated, suggesting the stress mitigation effects of both MLE and PhytoStim®. In conclusion, the MLE and PhytoStim® biostimulants mitigated the effects of eCO_2 and enhanced biological plant processes. Thus, the foliar application improved the

adaptability of *M. oleifera* and *M. peregrina* under eCO_2. Therefore, plant based biostimulants can be recommended to mitigate stress in regions heavily affected by climate change.

Keywords: Antioxidant, biostimulant, climate change, mitigation, *Moringa*

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5.1 Introduction

Abiotic stress resulting from climate change impairs the growth and health of many plant species (Gupta *et al.,* 2019). Plants are constantly exposed to harsh conditions with a large variety of both physically and chemically hostile factors such as ultraviolet (UV) radiation, varying extreme temperatures, excess salt, and elevated CO_2 (eCO_2) among others (Gupta 2019). These factors alter plant response and ultimately survival, causing mass decline in the plant's diversity communities (Gupta *et al.,* 2019). Climate change is the long-term alterations of weather patterns brought about by the release of greenhouse gases (GHG) emitted from man-made activities such as the burning of fossils fuels like coal, which releases harmful chemical compounds into the atmosphere that aid in heat trapping, contributing to global warming (Davis-Reddy & Vincent, 2017; Scholes *et al.,* 2020). This is of extreme concern in developing world countries especially Southern African

countries such as South Africa, as many of the people have no means of coping with the impacts of climate change, with most climate change studies focused on Africa suggesting the continent will observe less rainfall and experience a 4 to 6 °C temperature increase over the subtropics during the 21st century with a further 3 to 5 °C temperature increase in the tropics by the end of the century, double the global projected 1.5°C temperature increase (Engelbrecht *et al.,* 2015; Davis-Reddy & Vincent, 2017; Scholes *et al.,* 2020).

Medicinal plants, in particular, are highly sensitive to their environment and are rich in bioactive phytocompounds (such as phenols, flavonoids, iridoids and essential oils) that have the ability to treat numerous ailments (Mathabe *et al.,* 2006; Mentreddy, 2007; Gupta *et al.,* 2019). Medicinal plants are highly utilised by poor, indigenous communities, and tribes of each continent as they cannot afford western medicine thus medicinal plants and herbs become the only choice as most medicinal plants are readily accessible, inexpensive, and thus culturally accepted (Mathabe *et al.,* 2006; Mentreddy, 2007). Medicinal plants have formed an important component of traditional medicine over the past decades in South Africa and around the world (Mathabe *et al.,* 2006; Pakade *et al.,* 2013). The increase in frequency of intense extreme weather events such as drought, floods brought about by climate change alters phyotchemicals, thus affecting medicinal plant effectiveness (Backer *et al.,* 2018; Nephali *et al.,* 2020a; Zulfiqar *et al.,* 2020; Ben Mrid *et al.,* 2021; Zulfiqar, 2021; Bhupenchandra *et al.,* 2022; Lau *et al.,* 2022).

Moringa oleifera Lam (syn. *M. ptreygosperma* Gaertn.) and *M. peregrina* (Forssk.) Fiori are two of 13 species belonging to the family Moringaceae, also known as the horseradish or drumstick family (Abd Rani *et al.,* 2018). They are both widely distributed and studied as they possess excellent nutrients and secondary metabolites which gives them amazing nutritional value and potent medicinal properties (Al-Owaisi *et al.,* 2014; Mashamaite *et al.,* 2021). *Moringa Oleifera*

and *M. peregrina* are natively from India and Saudi Arabia, respectively (Al-Owaisi *et al.,* 2014; Mashamaite *et al.,* 2021). The two species are heat tolerant and grow well in a hot to arid climate with well-draining soils, thus they are highly recommended by many governments from Africa, Madagascar, North and South America (Abd Rani *et al.,* 2018). Due to the vast phytochemicals (bioactive plant chemical compounds) such as tannins, phenols, flavonoids etc., amino acids and macro and micro nutrients, they are used in folk medicine to treat numerous ailments such as diabetes, malaria, arthritis, hypertension and skin diseases (Abd Rani *et al.,* 2018).

Over the years there has been a growing interest to finding sustainable ways to mitigate the effects of climate change on plants by priming plant defences with the use of natural products agents such as biostimulants. Plant priming is a process that aids in altering the physiological state of plants to prepare them to rapidly respond after exposure to both biotic and abiotic stresses (Ugena *et al.,* 2018; Zulfiqar, 2021; Lephatsi *et al.,* 2022; Sujeeth *et al.,* 2022). The term "biostimulant(s)" is used to describe any naturally derived product that has the ability to improve microbe diversity, soil health and subsequently plant health and yields (du Jardin, 2015a). Biostimulants achieves this by stimulating the plant's natural biological processes that enhances the plant's ability to take up nutrients, and increase its tolerate to stress, regardless of the plant's nutrient content. Biostimulants are produced from a wide variety of materials including but not limited to inorganic compounds, humic, seaweed and microbes (du Jardin, 2015a; Povero *et al.,* 2016; Vasconcelos & Chaves, 2019). Among those MLE based biostimulants are prepared from *M. oleifera* leaves and have shown potential as an alternative source that can naturally improve the productivity of such stressed plants (Singh *et al.,* 2013; Zulfiqar *et al.,* 2020; Yap *et al.,* 2021; Yuniati *et al.,* 2022).

MLE achieves this due to the contents it possesses *viz*: flavonoid, amino acids, zeatin, macro and micro-nutrients, ascorbic acid, carotenoids, phenolics, vitamins A and C; making it the most

natural environmentally friendly alternative to fertilizers (Singh *et al.,* 2013). Foliar application of MLE has been shown to improve the productivity of plants both grown under stress and non-stressful conditions (Zulfiqar *et al.,* 2020; Yap *et al.,* 2021; Yuniati *et al.,* 2022). Ndubuaku *et al.* (2015) also reported that application of MLE on Cassava plants considerably increased the productivity of the plant. This is due to the upregulation of genes responsible for crucial biological processes such as photosynthesis (Erb & Kliebenstein, 2020). Aslam *et al.,* (2016) reports that MLE treatment on spinach caused an increase in protein and total chlorophyl. PhytoStim®® is a local commercial biostimulant that is made from *M. oleifera* leaves. It is a locally manufactured and reported to enhance plant growth, stimulate bigger fruits, show improved water use and improved resistance to pest and abiotic stress.

There are no studies that have investigated how the application of MLE biostimulant to medicinal plants can aid plants mitigate the effects of climate change, specifically *M. oleifera* and *M. peregrina,* which look to mitigate the impact of eCO_2 exposure. Thus, with the unlimited potential of MLE, the aim of the study was to investigate whether two different types of biostimulant treatments (MLE and PhystoStim) can alleviate the potential effects of eCO_2 (600 ppm and 800 ppm) stress on phytochemicals (such as tannins, phenols etc.) and biological activities in *M. oleifera* and *M. peregrina*.

5.2 Methods

Experimental design

Moringa oleifera seeds were purchased locally at a Moringa farm in Hammanskraal (25.4132º S, 28.2578º E), Gauteng, South Africa whereas *M. peregrina* seeds were obtained from the University of Tabuk, Saudi Arabia (28.38444° N, 36.48111° E). The seeds were germinated in the greenhouse

at the University of the Witwatersrand, Oppenheimer Life Sciences Building. Seeds from each species were grown in a mixture of clay soil, potting soil and vermiculite (50%: 40%: 10%) in 100 1.5L pots (50 pots for *M. oleifera* and 50 for *M. peregrina*). The study was replicated three times to validate the results *(n=100*). The seeds were incubated at 37°C in a growth chamber until germination occurred. Germinated seedlings were then transferred to the Oppenheimer Life Sciences (OLS) rooftop greenhouse at WITS university and watered every two days (200 mL), acclimatized for one month. The plants were then transferred to the walk-in convirons (Conviron, Winnipeg, Canada; model No.: PGW40) where the short-term CO_2 exposure experiments were performed. The convirons were running three different CO_2 concentrations separately: 400ppm (control), 600 ppm (moderate) and 800 ppm (high). The relative humidity (60%), light intensity (160 nits) and temperature (27 °C Day/ 25 °C night) were kept constant for all three growth chambers. The experiment was conducted over a period of three months. The plant samples were watered (200 mL per plant) every two days. The foliar application of MLE and PhytoStim® was performed twice a week.

Biostimulant treatments for foliar application

Commercial biostimulant

The commercial biostimulant (PhytoStim®) was purchased from a local distributor [Moringa Nutrition (PTY) LTD (2018/392318), P.O Box 62, Manezhe, 0967] and it constitutes of the following contents: potassium, calcium, magnesium, sulphur, valine, isoleucine, leucine, phenylalanine, aspartic acid, glutamic acid, serine, glycine, threonine, alanine, proline (see table 5.1).

Table 5.1: PhytoStim® chemical composition.

22 Amino acids totalling 4.198 g/L	
Isoleucine	246.6 mg/L
Valine	323.8 mg/L
Glutamic acid	507.6 mg/L
Serine	269.2 mg/L
Proline	222.1 mg/L
Alanine	365.2 mg/L
Threonine	249.9 mg/L
Aspartic acid	315.0 mg/L
Glycine	244.6 mg/L
Phenylalanine	259.1 mg/L
Leucine	437.4 mg/L
Fertiliser group III REG. NO: M121	

Preparations of Moringa oleifera extract biostimulant

The following method was adopted from Mazrou (2019) with slight modifications (such as using different solvents, and study species) to suit the present study. The MLE was made by using 80% ethanol and finely ground up *M. oleifera* leaves obtained from mature trees at Moringa farm in Hammanskraal. In preparation of 80% methanol solvent, 80 ml of 99.9% (analytical grade) methanol, manufactured by Merck® was diluted with distilled water (H_2O_d) to 100 mL. The harvested leaves were washed and allowed to air dry in a dark place for two weeks. After drying, 60 g of the dry leaves were weighed, ground to fine powder and stored in an air sealed labelled plastic container for later use. The ground leaves were transferred to an electric blender (Bennet Read kitchen boss and 1L of 80% ethanol solvent was decanted to the blender and the mixture was blended for 10 minutes, and the blender contents (i.e. leave extract) was transferred to a 1L Schott

glass bottle. The 1L bottle was then placed on a shaker for 48hrs at 120 rpm. After the 48 hours, the extract was filtered using the BOECO grade 1 filter paper and the filtrate was centrifuged at 15000 rpm for 15 minutes. The resulting supernatant was re-filtered and transferred to the rotary evaporator to evaporate the alcohol (80% ethanol). The dry concentrated extract was resuspended in 1L distilled water and the solution was stored in a 1L bottle and covered with foil to avoid light degradation and stored in the fridge (7°C) for later use.

Application of biostimulant treatments

About 50 mL of the prepared MLE was decanted in 1L of tap water and 30 mL of PhytoStim® was decanted in 1L of tap water. Tap water was used instead of distilled water for practicality since this is what most agricultural farms and local farmers mix with their fertilizers. The different volumes are due to the biostimulant concentrations (they had equivalent concentrations at those volumes, $60gL^{-1}$ (dw/v)).

A 4L Kaufmann pressure spray was used to spray the MLE and PhytoStim® plants' foliage twice a week for up to three months. The MLE and PhytoStim® foliar application was performed on samples that were exposed 600 ppm and 800 ppm in that same period. The control samples (400ppm) were left unsprayed. The plants were watered everyday by hand in the walk-in growth chambers (Conviron, Winnipeg, Canada, model No. PGW40). The harvesting of sprayed leaves occurred once a after three months and the leaves were air dried and ground to fine powder, and stored for later use in a sealed plastic container.

Plant material preparation

Young leaves of *M. oleifera* and *M. peregrina* were harvested once after three months. The harvested leaf material was weighed, washed with distilled water and air dried for two weeks. The air-dried leaves were weighed and ground to fine powder using an electric blender (Bennet Read kitchen boss), labelled, sealed in plastic bags, and stored in a dark place for later use.

Methanol extract preparation for quantitative and qualitative analysis

80% methanol extracts (% v/v) were prepared using the powdered samples as mentioned above. An amount of 3g of the powdered samples were weighed and transferred into 26 mL of 80% methanol and allowed to mix in a shaker for 72 hours. After 72 hours, the extracts were filtered using BOECO grade 1 filter paper and the filtrate was re-extracted. The filtered extract was decanted into dark bottles and stored until second extraction was completed. After the completion of the second extraction, it was filtered, and the two filtered extracts were combined to make up the total extract (50 mL) and mixed by shaking rigorously. The extracts were then centrifuged at 6000 revolutions per minute (rpm) for 15 minutes. The supernatant was collected into dark bottles and stored for various assays (Pakade *et al.*, 2013).

Qualitative phytochemical colour assay

Qualitative assays only indicate the presence of a targeted compound by an indication of a colour change. The colour change is also dependent on the concentration of the compound, where darker shades are indicative of an increase in compound concentration. For this assay, a total of 18 phytochemicals were investigated viz., tannins, saponins, flavonoids, quinones, phenols, terpenoids, cardiac glycosides, coumarins, steroids, volatile oils, phlobatannins, anthracynine, carbohydrates, glycosides, proteins and amino acids, phytosterols and triterpenoids. Methods from

Roghini and Vijayalakshmi (2018) and Shaikh and Patil (2020) were slightly modified for the suitability of this study.

Total phenolic content assay

Diluted sodium carbonate (7.5%; $NaCO_3$) was mixed with 0.3 mL of the plants' extracts. 750 µL of Folin–Ciocalteu′s phenol reagent was added to the mixture and the total mixture was diluted with distilled water (dH_2O) to 7 mL, then the mixture was incubated in a dark place at room temperature for 2 hours. Absorbance measurements were recorded at 765 nm against the blank using the Genesys 10s UV-Vis spectrophotometer (Thermo-Fisher Scientific®). A standard gallic acid curve was produced, and the results expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg/g GAE). The determination of TPC was repeated three times for both *M. oleifera* and *M. peregrina*. The following formula obtained from the gallic acid standard curve was used to calculate the TPC. The following method was adopted from Pakade *et al.*, (2013).

$$Y = 0.0495x - 0.0259,\ r^2 = 0.9994$$

Total flavonoid content assay

The total flavonoid content (TFC) of the extracts was determined using the aluminium chloride colorimetric assay method. 0.3 mL of the plant extract was added to a test tube, along with 4 mL of 5% (w/v) sodium nitrate ($NaNO_3$), 3 mL of 10% (w/v) aluminium chloride ($AlCl_3$) and 2 mL of 1M sodium hydroxide (NaOH) was added and the solution was topped to 10 mL with distilled water and incubated at room temperature in a dark place for one hour. The absorbance was measured at 510 nm against the blank using Thermo-Fisher Scientific® Genesys 10s UV-Vis

spectrophotometer. This was performed in triplicates. Quercetin was used as a standard curve and the results of the assay were reported as quercetin equivalents (mg/g QE) using the linear equation taken from the calibration curve (quercetin standard). The following formula obtained from the quercetin standard curve was used to calculate the TFC (Phuyal *et al.,* 2020).

$$Y= 0.2388x – 0.0019; r^2= 0.9997$$

Total tannin content (TTC) assay

The total tannin content (TTC) of the extracts were determined using the Folin–Ciocalteu's phenol reagent with gallic acid used as a standard (*modified* Lahare *et al.,* 2021). 0.1 mL of the plant extracts were diluted with 7.5 mL of distilled water and then 0.5 mL Folin–Ciocalteu′s phenol reagent was added to the mixture. 1 mL of 35% (w/v) of Na2CO3 was added to the solution and the mixture was topped up to 10 mL with distilled water and shaken rigorously. Then incubated at room temperature in a dark place for 30 minutes. Absorbance measurements were recorded at 725 nm using Thermo-Fisher Scientific® spectrophotometer (Genesys 10s UV-Vis). The TTC results were reported in GAE (Lahare *et al.,* 2021). The following formula obtained from the gallic acid standard curve was used to calculate the TTC.

$$Y= 0.046x – 0.0264; r^2= 0.9833$$

Total proanthocyanidins content (TPAC) assay

The TPAC of the plants' extracts was determined following Oyedemi *et al.* (2010) methods, with slight modifications. 0.5 mL of the plants' extracts were added to 3 mL of 4% vanillin-methanol (w/v) and then 1.5 mL of hydrochloric acid (HCL). This was then vortexed and thereafter incubated in a dark place at room temperature for 15 minutes. Absorbance against the blank was

measured at 500 nm with the Genesys 10s UV-vis (Thermo-Fisher Scientific®). The results were reported in catechin equivalent (CE). The following formula obtained from the catechin standard curve was used to calculate the TPAC (Oyedemi *et al.,* 2010.).

$$Y= 0.9554x – 0.0003; r^2= 0.9927$$

DPPH scavenging assay

The DPPH scavenging activities of the plant extracts were determined following methods reported by Gochev *et al.* (2012) with slight modifications. To prepare the DPPH solution, 50 mg of DPPH was added to 100 mL of 80% methanol and rigorously mixed. It was then further diluted in a 1:5 (v/v) ratio with 80% methanol solvent. The prepared DPPH was added (700 µL) to the plants' extract aliquots (10, 20, 30, 40 and 50 µL). A blank was also prepared. This method was performed in triplicates for both *M. oleifera* and *M. peregrina*. Both the blank and the sample DPPH solutions were incubated in a dark place at room temperature for 45 minutes. Following the incubation, absorbance readings were measured at 517 nm with the Genesys 10s UV-Vis (Thermo-Fisher Scientific®). The following equation was used to calculate the DPPH scavenging percentage of the extract:

$$\text{DPPH \%} = [(Â_{blank} – Â_{sample} / Â_{blank})] \times 100$$

Where $Â_{blank}$ represents the absorbance of the control (has all reagents except DPPH). $Â_{sample}$ is the test compound (with plant extract) absorbance (Gochev *et al.*, 2012).

Metal chelating assay

The metal (iron) reducing power of the plant extracts was determined following methods described by Gulcin *et al.* (2003) with slight modifications to suit our study. Plants' extract aliquots (10, 20, 30, 40 and 50 µL) were added to 0.05 mL of 2 mM Iron chloride $FeCl_2$, followed by 0.2 mL of 5mM ferrozine to initiate the reaction. The mixture was mixed and allowed to stand for 10 minutes at room temperature in a dark cardboard. Absorbance of the solutions was measured at a wavelength of 562 nm using the Genesys 10s UV-Vis (Thermo-Fisher Scientific®) and compared against the blank. These steps were repeated in triplicates. The chelating effect (in percentage) was calculated using the following equation:

Chelating %= $[(A_o-A_s/A_o)] \times 100$

Where A_o is the absorbance of the control (has all reagents except ferrozine and $FeCl_2$) and A_s is the absorbance of the solution with plant extract (Gulcin *et al.,* 2003).

Hydrogen peroxide (H_2O_2) assay

The H_2O_2 reducing power of the extracts was determined following methods reported by Gulcin *et al.* (2003) with slight modifications. Both the H_2O_2 solution and the phosphate buffer (pH 7) were prepared and both solutions were mixed (4.53 mL H_2O_2 and 995.47 mL phosphate buffer) to produce 40 mM H_2O_2 solution. 40 nM H_2O_2 solution (600 µL) was added to aliquots of plant extracts (10, 20, 30, 40 and 50 µL) and allowed to stand for 10 minutes. Absorbance against the blank was measured at 230 nm. The H_2O_2 reducing percentage was determined using the following formula:

H_2O_2 %= $[(A_o-A_s/A_o)] \times 100$

Where A_o is the absorbance of the control (has all reagents except 40 mM) and A_s is the absorbance of the solution with plant extract (Gulcin *et al.,* 2003).

Anti-bacterial assay

The antimicrobial activity of the two species was determined using the agar disk well diffusion assay described by Gonelimali *et al.* (2018) and Mathabe *et al.* (2006) with slight modifications. The bacterial activity of the extracts was tested using four bacterial strains, two of which are gram-positive bacterial strains [*Staphylococcus aureus* (ATCC 25923), *Streptomyces albulus* (ATCC 12757)] and two are gram-negative bacterial strains [*Escherichia coli* (*ATTC87396*) and *Escherichia coli* (*ATTC11775*)]. The bacterial strains were obtained from Thermo-Fisher's laboratory specialists in Johannesburg LTD. (PTY), South Africa, and the Mueller-Hinton (MH) agar was purchased from Sigma Aldrich LTD., South Africa. The bacterial strains *(S. aureus, S. albulus and E. coli*) were cultured in the MH agar (Gonelimali *et al.,* 2018).

To conduct the antimicrobial screening, cooled MH agar petri dish plates were inoculated with bacterial strains using sterile swab sticks and incubated at 37°C for 24 hours to achieve a "bacteria lawn". Sterile pipette tips with a diameter of six millimetres were used to punch holes (wells) in the Agar for each treatment. Plant extracts (100 µL) were then transferred to the wells and incubated at 37^{o}C for 48 hours. 80% methanol solvent was also added as a negative control. After incubation, the antimicrobial activity of the extracts was determined by calculating the zones of inhibition (ZOI) with a 30cm ruler by reading the diameter (ZOI).

Data analysis

Statistical analysis was conducted with IBM SPSS statistics version 23 (SPSS Inc., Chicago, IL, USA). The data was tested for normality using the Shapiro-Wilk normality test. The results (bar graphs) were represented as *mean* ± *SE* and a two-way analysis of variance (ANOVA) was conducted to test the for difference between treatments and species. This was followed by a Turkey post hoc test, to see where the differences lie. The significant difference was set to $P<0.05$

5.3 Results

Qualitative phytochemical screening

Qualitative screening of phytochemicals detects presence or absence of secondary metabolites in the plants extract. The qualitative phytochemical analysis of 17 phytochemicals under two different CO_2 concentration levels and different Biostimulant treatments showed that there was an increase in the detection of secondary metabolites of the leaf extract of *M. oleifera and M. peregrina*, sprayed with PhytoStim® under 600 ppm. The accumulation of flavonoids, terpenoids, steroids, proteins, amino acids and glycosides was more prevalent in *M. oleifera* under 600 ppm when sprayed with biostimulants. Under 800 ppm, a slight decline in the presence of triterpenoids, phenols and tannins was observed in both species, with either biostimulant application resulting in almost similar results for specific metabolites. Interestingly, phytosterols were detected consistently in all treated samples under 600 ppm and 800 ppm except for control samples. Table 5.2 shows the assay results and phytochemicals presence under varying CO2 and biostimulant treatments.

Table 5.2: Qualitative phytochemical analysis of *Moringa oleifera* and *Moringa peregrina* leaves with 80% methanol solvent under elevated CO_2 concentrations and biostimulant treatments.

Key:

Not detected

Low

Moderate

High

Test	400 ppm *M. oleifera*	400 ppm *M. peregrina*	600 ppm MLE *M. oleifera*	600 ppm PhytoStim® *M. oleifera*	600 ppm PhytoStim®), *M. peregrina*	600 ppm MLE *M. peregrina*	800 ppm MLE *M. oleifera*	800 ppm MLE *M. peregrina*	800 ppm PhytoStim® *M. oleifera*	800 ppm PhytoStim® *M. peregrina*
Tannins										
Saponins										
Flavonoids										
Quinones										
Phenols										
Terpenoids										
Cardiac Glycosides										
Coumarins										
Steroids										
Phlobatannins										
Anthocyanins										
Volatile oils										
Phytosterols										
Triterpenoids										
Proteins and amino acid										
Glycosides										

Carbohydrates

Quantitative assays

Total phenolic content

There were significant changes observed in total phenolic content (TPC) of the leaf extracts from both *M. oleifera* and *M. peregrina* under different CO_2 concentrations and biostimulant treatments. There was a decline in the content of phenols for *M. peregrina* under the two biostimulant treatments for both CO_2 concentrations when compared to *M. oleifera*, with PhytoStim® showing lower TPC when compared to MLE under the two CO_2 concentrations ($P < 0.05$). *M. oleifera* showed the highest TPC when compared to *M. peregrina* for both CO_2 concentration and biostimulant treatments. Similarly, in *M. oleifera*, PhytoStim® treatment showed lower TPC in *M. oleifera* when compared to MLE ($P < 0.05$) under the two CO_2 concentrations (see figure 1.20).

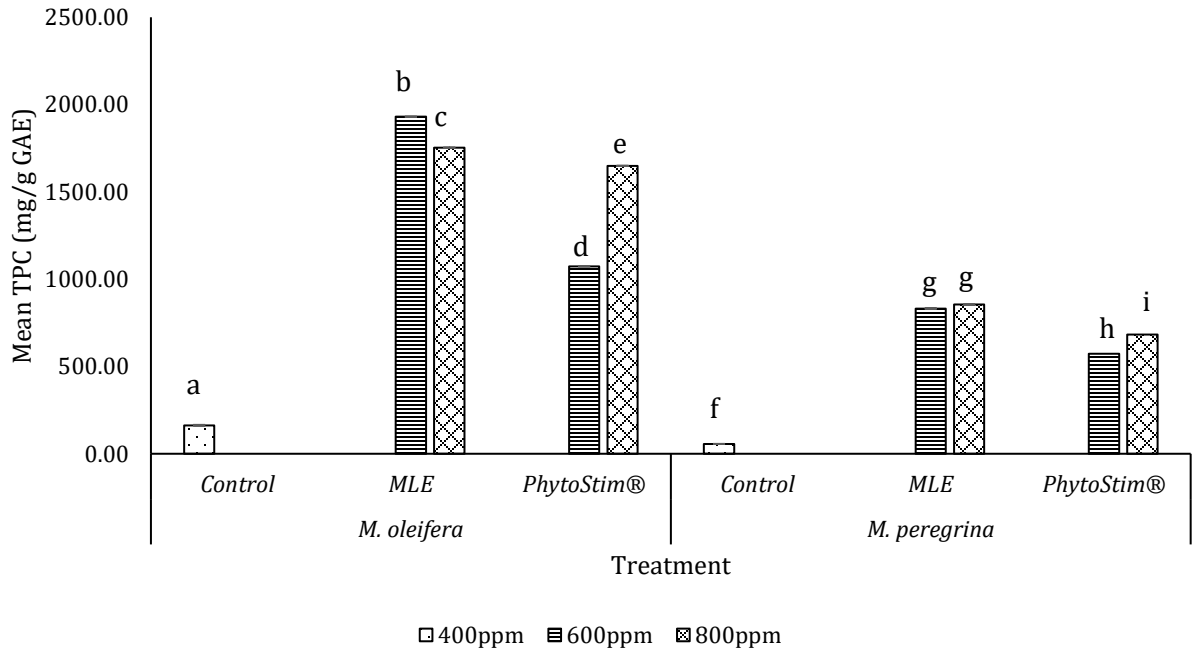


Figure 1. 20 The effects of different CO_2 concentrations and different biostimulant treatments on the total content of phenolics for both *M. oleifera* and *M. peregrina*. The bar graphs follow the

mean ± *SE* (*n = 3*) standard with the letters representing a significant difference between the treatments ($P < 0.05$).

Total flavonoid content

M. oleifera showed the highest total flavonoid content (TFC) when compared to *M. peregrina* in both biostimulant treatments under both CO_2 concentrations (600 ppm and 800 ppm; $P < 0.05$). There was a slight difference observed in *M. oleifera* under both CO_2 concentrations when MLE was applied, whereas PhytoStim® application resulted in drastically reduced TFC under 600 ppm in *M. oleifera* (see figure 1.21; $P < 0.05$). On average, *M. peregrina* showed the lowest TFC, with slight difference observed when PhytoStim® was applied under both CO_2 concentrations ($P > 0.05$), whereas MLE application showed a significant difference under 600 ppm, exhibiting the lowest TFC (See figure 1.21; $P < 0.05$).

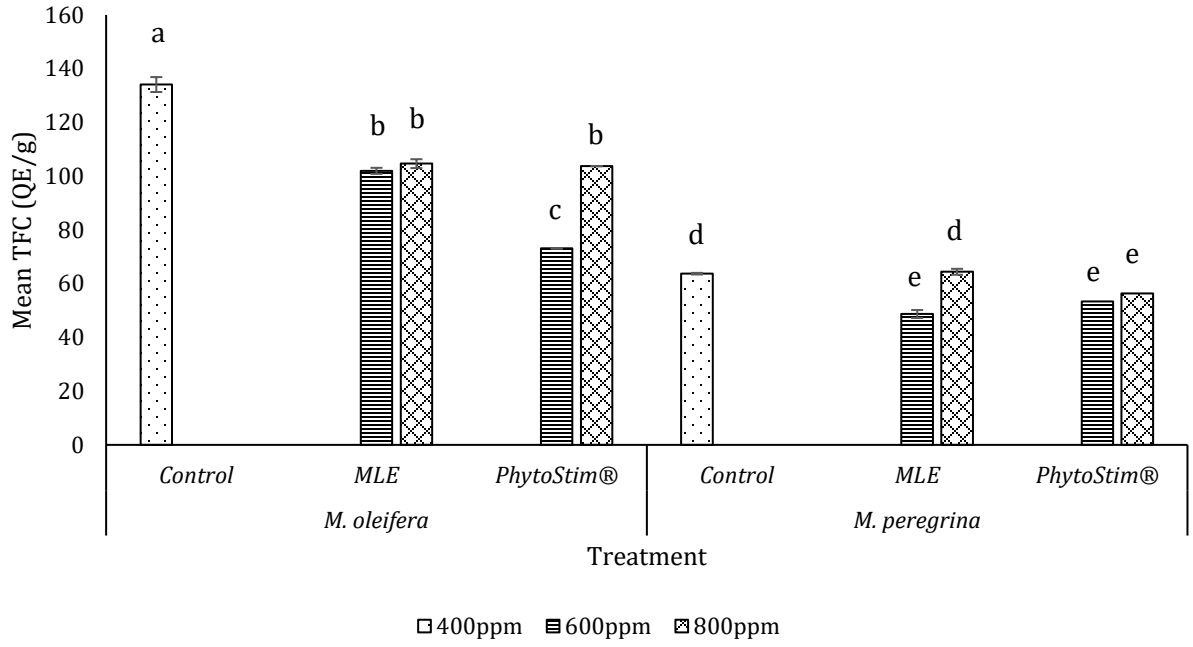


Figure 1. 21 The effects of different CO_2 concentrations and different biostimulant treatments on the total content of flavonoids in both *M. oleifera* and *M. peregrina*. The bar graphs follow the *mean* ± *SE* (*n = 3*) standard with the letters representing a significant difference between the treatments *(P < 0.05*).

Total tannin content

The total tannin content (TTC) of *M. oleifera* was significantly higher than that in *M. peregrina* under both CO_2 concentrations and biostimulant treatments ($P < 0.01$), with TTC being the highest under 800 ppm for both species under both biostimulant treatments (figure 1.22; $P < 0.05$). TTC was at its lowest under 600 ppm in both species under MLE and PhytoStim® treatments, with PhytoStim® treatment showing the lowest TTC when compared to MLE for both species ($P < 0.05$), see figure 1.22.

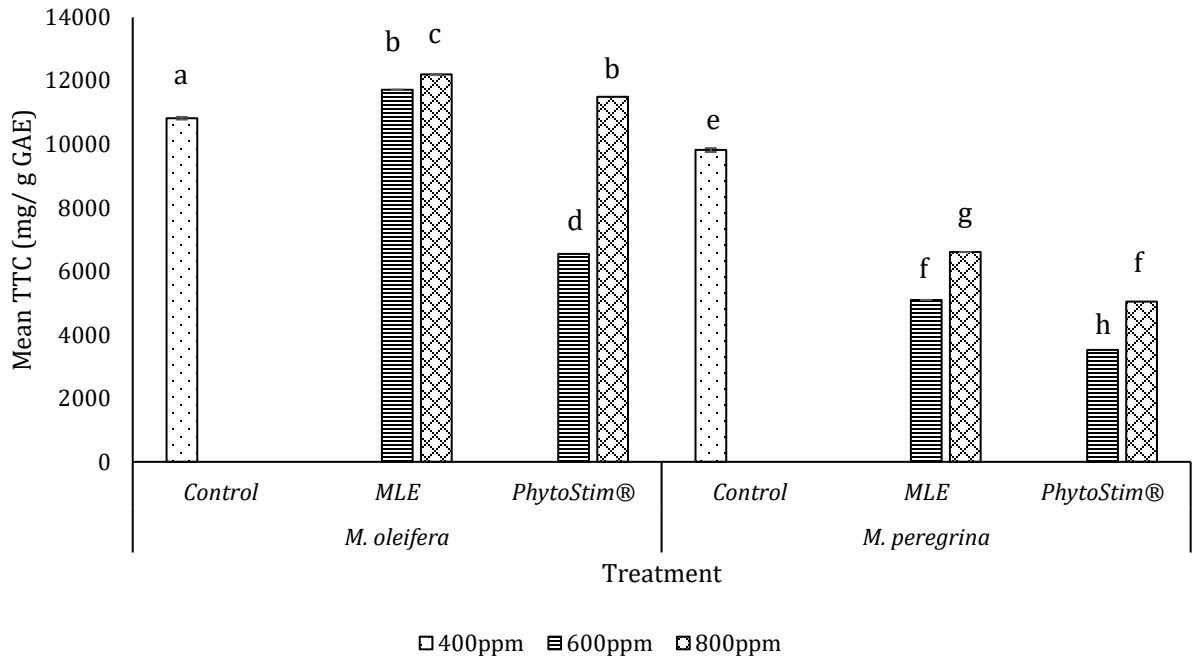


Figure 1. 22 The effects of different CO_2 concentrations and different biostimulant treatments on the total content of tannins for both *M. oleifera* and *M. peregrina*. The bar graphs follow the *mean ± SE* (*n= 3*) standard with the letters representing a significant difference between the treatments ($P < 0.05$).

Total proanthocyanidins

M. oleifera exhibited the highest total proanthocyanidins (TPAC) when compared with *M. peregrina* under the MLE and PhytoStim® application in both CO_2 concentrations ($P < 0.05$). There was a slight difference observed when MLE was applied under both CO_2 concentrations in *M. oleifera*, with 600 ppm showing a slight decline of TPAC under MLE treatment. There was a significant difference in PhytoStim® application in *M. oleifera* under both CO_2 concentrations with 600 ppm showing lower TPAC ($P < 0.01$). Another significant difference was observed between the two biostimulant application when applied to *M. peregrina* under the two CO_2 concentrations,

with both biostimulant treatments slightly reducing TPAC under 800 ppm ($P < 0.05$), see figure 1.23.

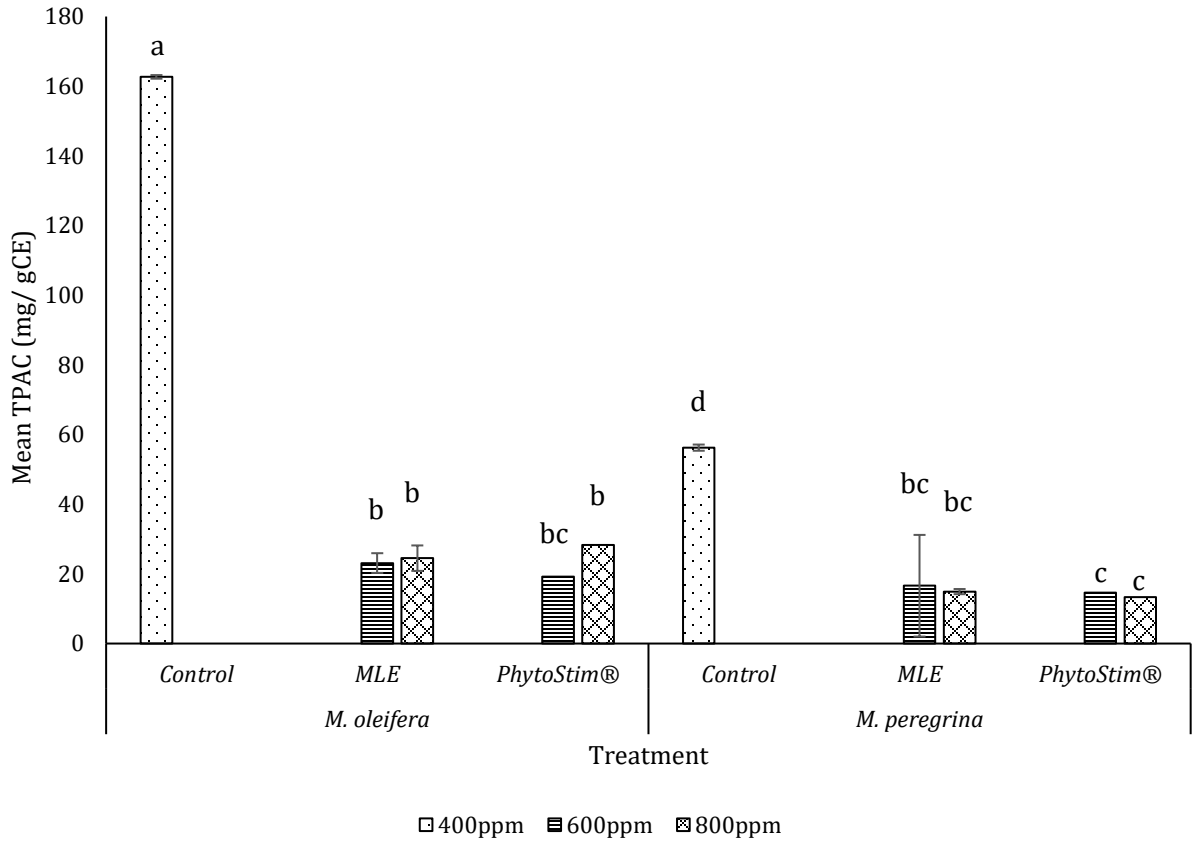


Figure 1. 23 The effects of different CO_2 concentrations and different biostimulant treatments on the total content of proanthocyanidins for both *M. oleifera* and *M. peregrina*. The bar graphs follow the *mean* ± *SE* (*n = 3*) standard with the letters representing a significant difference between the treatments (*P < 0.05*).

Antioxidant assays

2,2-diphenyl-1-picrylhydrazyl

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capabilities of both species exhibited a drastic decline in scavenging activity under 800 ppm in both biostimulant treatment when compared to 600 ppm ($P < 0.01$). However, both biostimulant treatments enhanced the scavenging abilities against DPPH of both species under 600 ppm, with PhytoStim® application exhibiting a high affinity for *M. oleifera,* and MLE having a high affinity to *M. peregrina* ($P < 0.05$). On average, DPPH scavenging abilities of *M. peregrina* was slightly better than that of *M. oleifera* ($P < 0.05$) (figure 1.24).

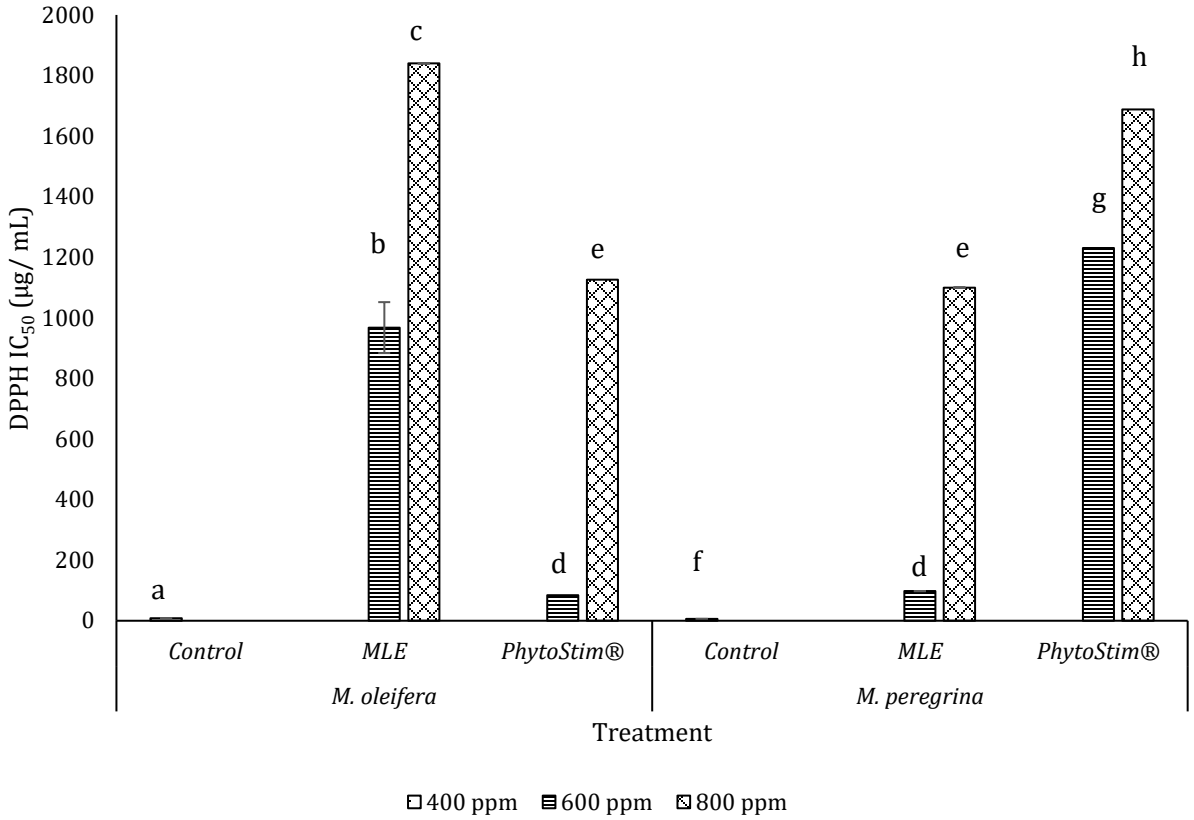


Figure 1. 24 Comparison of the IC_{50} values for *M. oleifera* and *M. peregrina* leaf extracts under different CO_2 concentrations and different biostimulant treatments against DPPH. The letters representing a significant difference between the treatments ($P < 0.05$).

Hydrogen peroxide

On average, the hydrogen peroxide (H_2O_2) reducing capabilities of *M. peregrina* was better than that of *M. oleifera* under both CO_2 concentrations and both biostimulant treatments, except for 800 ppm exposure, where *M. oleifera* showed a stronger activity as compared to *M. peregrina* when PhytoStim® was applied, further suggesting the species specificity of the biostimulant treatments ($P < 0.05$; Figure 1.25).

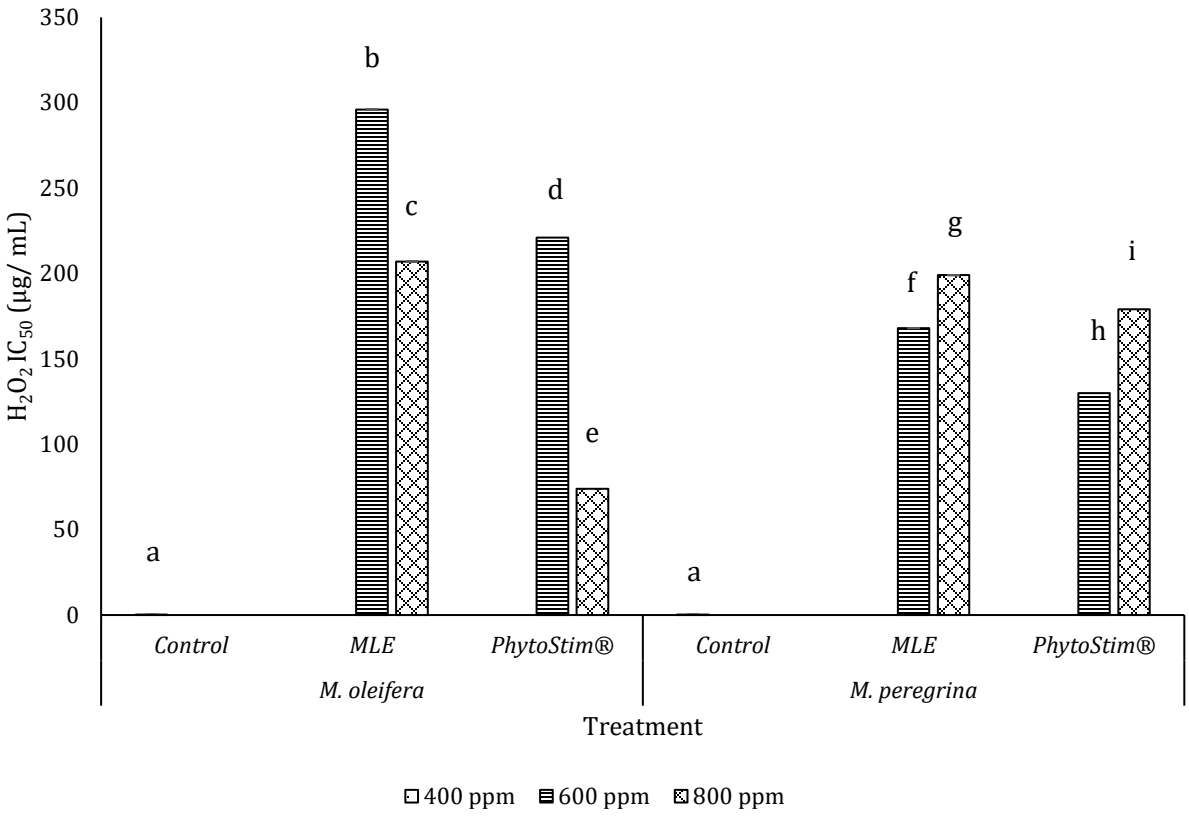


Figure 1. 25 Comparison of the IC_{50} values for *M. oleifera* and *M. peregrina* leaf extracts under different CO_2 concentrations and different biostimulant treatments against H_2O_2. The letters representing a significant difference between the treatments ($P < 0.05$).

Metal (iron) chelating

The chelating ability of both species against ferrozine, was at its strongest under 600 ppm, with PhytoStim® showing the highest reducing capabilities when compared with MLE ($P < 0.01$). On average *M. peregrina* exhibited the highest chelating capabilities when compared to *M. oleifera* under both CO_2 concentrations under MLE application, whereas PhytoStim® application enhanced the chelating capabilities of *M. oleifera* under both CO_2 concentration ($P < 0.05$). There was a

significant decline in the chelating capabilities of *M. peregrina* under 800 ppm and PhytoStim® application (see figure 1.26).

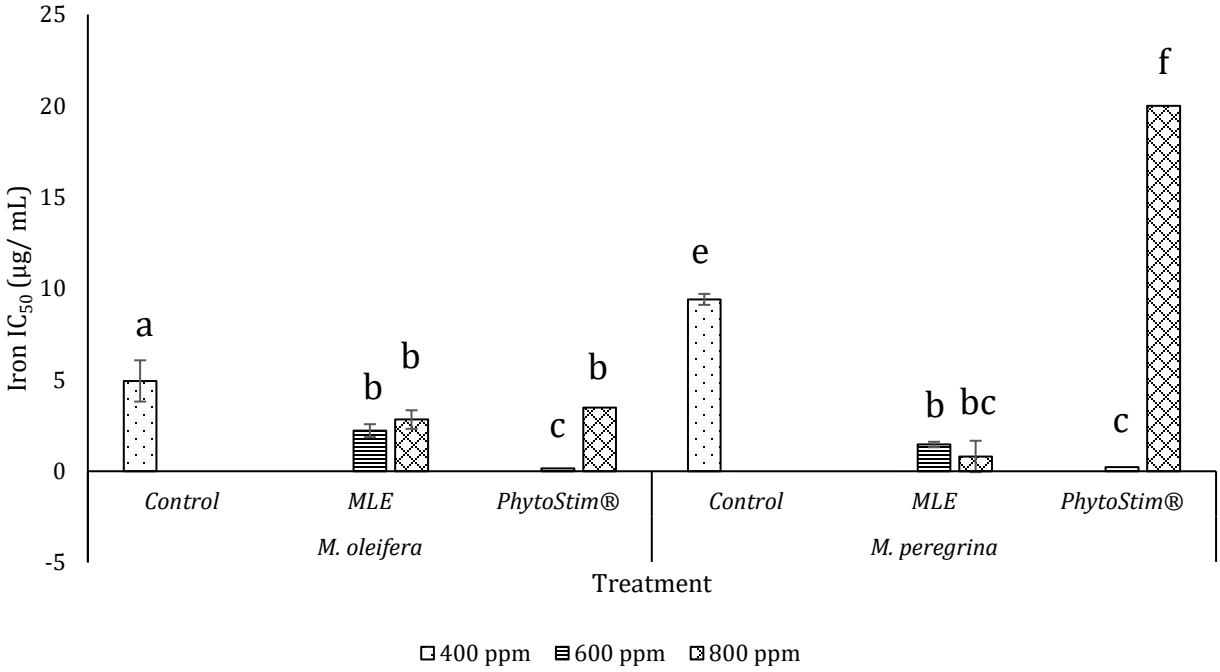


Figure 1. 26 Comparison of the IC_{50} values for *M. oleifera* and *M. peregrina* leaf extracts under different CO_2 concentrations and different biostimulant treatments against iron chelating. The letters representing a significant difference between the treatments ($P < 0.05$)

Anti-microbial assays

The agar well diffusion assay results showed no significant differences in inhibiting the growth of *S. aureus*, *S. albulus*, *E. coli*, *E. coli*. The zone of inhibition (ZOI) of the extracts under the different CO_2 concentrations and both biostimulant treatments remained under 20mm, showing an

intermediate response. However, extracts under 600 ppm showed slightly larger zone of inhibition (ZOI) when compared to plants under 800 ppm, see table 5.3.

Table 5.3: Bacterial zone of inhibition (ZOI) of *Moringa oleifera* and *Moringa peregrina* against four bacterial strains. The asterisk (*) show the largest ZOI in mm. The asterisk (*) show significant difference ($P<0.05$).

Sample	**Zone of Inhibition (mm)**			
	***S. aureus* (ATCC 25923)**	***S. albulus* (ATCC 12757)**	***E. coli* (ATTC87396)**	***E. coli* (ATTC11775)**
Negative control; 80% Methanol	0±0.0	0±0.0	0±0.0	0±0.0
400 ppm & MLE; *M. oleifera*	15±*0.23	11±0.45	12±0.03	12±0.01
400 ppm& MLE; *M. peregrina*	16±*0.09	12±0.3	14±0.87	13±0.3
400 ppm & PhytoStim®; *M. oleifera*	12±0.45	10±0.35	13±0.73	13±0.35
400 ppm & PhytoStim®; *M. peregrina*	11±0.01	10±0.37	12±0.57	16±0.02*
600 ppm & MLE; *M. oleifera*	13±0.47	10±0.08	12±0.01	10±0.06
600 ppm & PhytoStim®; *M. oleifera*	15±0.01*	10±0.32	14±0.21	12±0.09
600 ppm & PhytoStim®; *M. peregrina*	15±0.01*	11±0.03	10±0.02	14±0.21
600 ppm & MLE; *M. peregrina*	14±0.07	13±0.1	13±0.01	15±0.04*
800 ppm & MLE; *M. oleifera*	12±0.05	11±0.12	11±0.02	14±0.08
800 ppm & MLE; *M. peregrina*	14±0.01	10±0.02	12±0.06	15±0.03*
800 ppm & PhytoStim®; *M. oleifera*	12±0.7	10±0.36	10±0.45	11±0.65
800 ppm & PhytoStim®; *M. peregrina*	10±0.35	11±0.69	11±0.47	13±0.61

5.4 Discussion and conclusion

The present study aimed to investigate the role of MLE and PhytoStim® on the adaptability of *Moringa oleifera* and *M. peregrina* under elevated CO_2 (eCO_2) exposure. Amelioration of

medicinal plants under environmental factors that may cause stress is crucial not only in horticulture but agriculture as well, as unusual environmental factors may alter the plants' quality and phytochemical composition. The use of natural plant biostimulants have received attention lately as a substitute to commercial fertilizer due to their ability to not leach harmful chemicals that can degrade the soils by destroying microbial communities in the soils (du Jardin, 2015b; Povero *et al.,* 2016; Razzaq *et al.,* 2019; Vasconcelos & Chaves, 2019). Plant biostimulants can also be used as possible climate mitigators as they have many beneficial properties due to high antioxidant agents and plant regulating constituents such as osmoprotectants (Yakhin *et al.,* 2016; Ugena *et al.,* 2018; Vasconcelos & Chaves, 2019 Nephali *et al.,* 2020b). In this study, *M. oleifera* leaf extract (MLE) and PhytoStim® biostimulants were used in order to determine their role on the adaptability of *M. oleifera* and *M. peregrina* under elevated carbon dioxide.

The foliar application of both biostimulants influenced an increase of secondary metabolites in both species under 600 ppm exposure and a slight decline in the detected secondary metabolites under 800 ppm. PhytoStim® increased the secondary metabolites detected for *M. Oleifera* while *M. Peregrina* showed to be more responsive to the application of MLE. This suggests that the biostimulant may be species specific (Bhupenchandra, *et al.,* 2022; Erb & Kliebenstein, 2020). These findings correlate with previous work that suggested that the application of biostimulants on plants may not always bring about a desired response in plant growth and performance (Bhupenchandra, *et al.,* 2022; Erb & Kliebenstein, 2020). Laboratory observations showed that plants under 600 ppm were responding significantly well under the application of the biostimulant treatments as they maintained high biomass (with reduced abscission) and growth at the end of the experiment. Even though both *M. oleifera* and *M. peregrina* under 800 ppm maintained high biomass with the application of both biostimulants, they exhibited slowed growth rate in

comparison with *M. oleifera* and *M. peregrina* grown under 600 ppm. This response indicates the effects of eCO_2 on the physiology and biological processes, such as photosynthesis, as leaf abscission was induced quicker than those under 600 ppm (Austen *et al.,* 2019; Campobenedetto *et al.,* 2021; Donati *et al.,* 2022; Erb & Kliebenstein, 2020; Nephali *et al.,* 2020a; Zulfiqar *et al.,* 2021). The high vigour that was observed in plant samples under 600 ppm may be correlated with the stronger presence of secondary metabolites detected under the application of the biostimulants (Donati *et al.,* 2022). Various studies have shown that secondary metabolites are not only responsible for defence against biotic or abiotic stress but they also play a role in normal growth and development of plants (Austen *et al.,* 2019; El Houari *et al.,* 2021; Erb & Kliebenstein, 2020; Li *et al.,* 2017; Marslin *et al.,* 2017; Matros *et al.,* 2006; Shah & Smith, 2020).

The decline in the content of antioxidant bioactive compounds such as TPC, TFC, TPAC and TTC are associated with the upregulation when plants are exposed to potentially stressful conditions (Marslin *et al.,* 2017; Robinson *et al.,* 1980; Shu *et al.,* 2016). This suggests a diversion of resources away from the phenylpropanoid pathway to the non-mevalonate pathway (MEP) since the application of the biostimulant treatments and excess CO_2 skeleton (from eCO_2) provides essential antioxidants, nutrients and micro and macro nutrients the plant requires to reallocate the resources to maintaining the organelles and overall crucial plant processes (Austen *et al.,* 2019). This is due to the presence of strong antioxidant agents and hormones in *M. oleifera* leaves such as proline, linoleic acid and carotenoids which aid in preventing peroxidation of lipids in membranes (Donati *et al.,* 2022). Lipid peroxidation is a process whereby reactive oxygen species (ROS) take electrons from lipids found in membranes, resulting in cell damage and ultimately cell death (Ayala *et al.,* 2014). This may cause cell membrane leakage which results from a lack of

essential fatty acids, cholesterol, proteins, and phospholipids needed to construct and support the cell membrane and all of its critical functions (Ayala *et al.,* 2014).

Despite the increment in the observed accumulated secondary metabolites under 600 ppm and slight increase of the same bioactive phytochemicals of *M. oleifera* and *M. peregrina* under the 800 ppm, the pharmacological activities (antioxidant and antimicrobial activities) of the two Moringa species was not significantly high (except for iron chelating capabilities of both species). This is due to the presence of regulatory hormones and ability of secondary metabolites that can act as modulators of genes responsible for the biosynthesis of important phytochemicals (Austen *et al.,* 2019; Erb & Kliebenstein, 2020). Therefore, these results suggest that the application of the biostimulant treatments could have influenced the upregulation of biosynthesis of secondary pathways such as non-mevalonate pathway (MEP) and down regulated the pharmacological processes that facilitate the scavenging of ROS and antimicrobial properties of both species with the exception of the ability to reduce iron metals (Austen *et al.,* 2019).

Even though there was no significant difference in the ability of both species to inhibit the growth of *S. albulus, S. aureus* and both *E. coli* bacterial strains, the ZOI of both species are greater than 10mm, suggesting that the species could maintain the moderate antimicrobial properties and can inhibit the growth of most commonly infection causing bacterium (Mathabe *et al.,* 2006). *M. peregrina* generally showed a stronger antibacterial activity against the growth of the four bacterial strains when compared to *M. oleifera*. *S. albulus* showed some resistance towards the plant extracts while *S. aureus*, and both *E. coli* strains were more sensitive towards the plant extracts.

In conclusion, the use of MLE and PhytoStim® on *M. oleifera* and *M. Peregrina* played a mitigatory role under the elevated carbon dioxide exposure and thus enhanced the species'

adaptability. The study highlighted crucial information and knowledge gaps about the application of fertilizer plant based biostimulants to mitigate the impacts of climate change on medicinal plants. The application of biostimulants on *M. oleifera* and *M. peregrina* that resulted in an enhanced adaptability was indicated by the observed reduction in TPC, TTC, TFC and moderate pharmacological activity assays such as the antimicrobial assay and antioxidant assays under 800 ppm. This may be interpreted as the occurrence of low levels. The study noted physiological effects that were reported by most biostimulant studies such as greener, bigger leaves, and increased tolerance to abiotic stress, in this study, the exposure to eCO_2 concentrations viz., 600 ppm and 800 ppm. The study highlighted that biostimulants are species specific, meaning that the application of biostimulants might upregulate or downregulate the secondary metabolites accumulating at the point of administration (I.e., leaves) due to secondary metabolites having more influence on both primary metabolites and phytohormones (regulatory plant hormones like auxins and ABA). Thus more studies needs to be done not only to understand the effects of biotic and abiotic stress mitigation biostimulants have on medicinal plants but to identify all phytochemicals present in each biostimulant and also quantify the phytochemicals. The produced *Moringa oleifera* leaf extra based biostimulant (MLE) seems to show increased affinity for *M. peregrina*, whilst PhytoStim® has an increased affinity for *M. oleifera* in the current study. This may be due to the different production methods employed in the making of the two biostimulants, which impact their permeability and rate of absorptions by both species. Given the results obtained in this study, we have confidence that biostimulants have climate change stress mitigation effects when applied to the site known the stressor will act on (i.e. the leaves in this study). In hindsight, application of either biostimulant treatment (MLE or PhytoStim®) mitigated the possible effects of eCO_2 exposure on both *M. peregrina* and *M. oleifera*, and with these types of studies, we can quantify

the effects of biostimulant on mitigating the impacts of eCO_2 on medicinal plants, which are wildly distributed and used across the globe.

Authors' contributions

The all the authors contributed ideas and designs of the experiments. Thato Moloi collected and prepared the samples and performed all the experiments. Thato Moloi analysed the data and wrote the manuscript. Dr Ida Risenga, Dr Shalini Dukhan and Mr Philemon Ramalepe reviewed the manuscript. All the involved authors read and approved the manuscript

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Availability of data and materials

All data or results obtained in this study are included in this manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

There are no competing interests.

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CHAPTER 6:

General discussion and Conclusion

This Chapter highlights the general discussion on the objectives, conclusion, and future remarks.

6.1 General discussion

It is known that higher plants experience stress from a wide biotic and abiotic factor such as insects, herbivory, fungi, drought, high temperatures, salinity and greenhouse gases (He *et al.,* 2018; Jiménez-Arias *et al.,* 2022; Suzuki *et al.,* 2014). As such, they have developed mechanisms such as bioactive phytochemicals (also referred as secondary metabolites in this dissertation) to be able to cope, tolerate, resist and in some cases mitigate the effects of these stresses (Ahmad *et al.,* 2009; Concato *et al.,* 2022; Irato & Santovito, 2021). The current study had three aims, the first one aimed to investigate the impact of elevated CO_2 (eCO_2) on *M. oleifera* and *M. peregrina*'s secondary metabolites and their pharmacological activities (antioxidant and antimicrobial activities). The study also aimed at investigating the effects of plant based biostimulant on productivity of *M. oleifera* and *M. peregrina*. Lastly, the study aimed to investigate the role of plant based biostimulant on the adaptability *M. oleifera* and *M. peregrina* under eCO_2.

Exposure to eCO_2 increased the secondary metabolites for both *M. oleifera* and *M. peregrina* under 600 ppm, with both species grown under 800 ppm yielding little accumulation of secondary metabolites when compared to 600 ppm. The increase of detected secondary metabolites under 600 ppm can be explained by the possible increase in photosynthesis activity which increase carbohydrates, which are one of the important components that are involved in the biosynthesis of some secondary metabolites of plants under stress (Becker & Kläring, 2016; Shahidul Islam *et al.,* 1996; Wattal & Siddiqui, 2015). Previous work have shown that eCO_2 causes a shift in the nitrogen ratio of the plant, which decreases the longevity of the leaves and increases the period of senescence (Bloom *et al.,* 2010). This correlates to our study's observations and might explain the

lower accumulation of secondary metabolites within the species leaves. The low accumulation could be due to many processes undergoing senescence, reducing the availability of resources that are needed to biosynthesize secondary metabolites.

The use of biostimulants to improve species plant productivity and improve species tolerance to both abiotic and biotic stresses has gained traction in the scientific community over the past years. With *M. oleifera* being widely studied and favoured tree species due to its impressive nutritional and medicinal properties, the use of its leaf extract as a biostimulant also called MLE has gained attention from various research bodies (Mazrou, 2019; Merwad, 2018; Toscano *et al.,* 2021; Yap *et al.,* 2021; Yuniati *et al.,* 2022). In this study, two plant based biostimulant treatments were applied to *M. oleifera* and *M. peregrina*. The two biostimulant treatments included the commercial PhytoStim® and MLE made from 80% ethanol solvent at the laboratory. Following application of both biostimulant treatments on *M. oleifera* and *M. peregrina*, there was an increased accumulation of secondary metabolites for both species with PhytoStim® showing a higher secondary metabolites recruitment in *M. peregrina* when compared to the controls. Even though PhytoStim® showed greater accumulation of secondary metabolites, there was no huge significant difference in secondary metabolites detected between the two biostimulant treatments. This might be due to the origin of the biostimulants (*M. oleifera* leaves), meaning both plant species will receive bioactive compounds that are relatively the same with differences stemming from the growth environment of the *M. oleifera* that was used for the biostimulant treatments (Ricci *et al.,* 2019; Toscano *et al.,* 2021). Other studies attributed the increased accumulation of secondary metabolites to the extra influx of vitamins, minerals and antioxidant molecules present in biostimulants (Mazrou, 2019). This can also be observed on the pharmacological activities of both species showing improvements against reactive oxygen species (ROS).

In addition to biostimulants being utilised as a plant productivity improvement treatment, it can also be used as a climate change stress mitigator (Goñi *et al.,* 2018; Jiménez-Arias *et al.,* 2022; Zulfiqar *et al.,* 2020). The eCO_2 exposed plants (see chapter 5, fig. 8) were foliar sprayed with either biostimulant to assess the ability of biostimulants (MLE and PhytoStim) to enhance the adaptability of the species to eCO_2. As *Moringa oleifera* and *M. peregrina* are both highly nutritional and medicinal trees, which are widely cultivated, studied and used by many communities and industries, they were selected as the study species. The spray application of the biostimulant treatments initiated species productivity processes such as leaf production and photosynthesis. The secondary metabolites of *M. oleifera* and *M. peregrina* foliar applied with either biostimulant treatment whilst under eCO_2 showed stronger presence of essential bioactive compounds, with *M. oleifera* and *M. peregrina* grown under 600 ppm showing stronger presence than those exposed to 800 ppm conditions (Bhupenchandra *et al.,* 2022). Even though there is a significant difference in the accumulated secondary metabolites of both *M. oleifera* and *M. peregrina*, they both exhibited excellent antioxidant activities and antimicrobial activities against three antioxidant assays and four bacterial strains, respectively. This is due to the potent antioxidants in the leaves of both species and the extra bioactive compounds from the biostimulants that aid in the protection and biosynthesis of protective phytochemicals (Del Buono, 2021; Nephali *et al.,* 2020; Zulfiqar *et al.,* 2020).

The study results also showed the presence and quantification of the following secondary metabolites: TPC, TFC, TTC and TPAC. These secondary metabolites are biosynthesised to counteract the over production of ROS by the peroxisomes, chloroplast and mitochondria in *M. oleifera* and *M. peregrina* (Irato & Santovito, 2021). This over production of ROS occurs from matrix and membrane of peroxisomes; the electron transport chain (ETC) of the mitochondria

(Complex I, Ubiquinone and Complex III); and both the photosystems (PSI and PSII) of the chloroplast (Ahmad *et al.,* 2009; Concato *et al.,* 2022; Irato & Santovito, 2021). Together the enzymatic and non-enzymatic antioxidant defence mechanisms aided in protecting the plant from excessive stress, ameliorated the tolerance to stress and mitigated the effects of eCO_2 on *M. oleifera* and *M. peregrina* (Irato & Santovito, 2021). The strong antioxidant activities observed *in vitro*, in all experimental studies (chapter 3, 4 and 5) for both *M. oleifera* and *M. peregrina*, can be attributed to the presence of numerous non-enzymatic antioxidants which include ascorbate (vitamin c), carotenoids, vitamin e, phenolic, flavonoids and many more. Non-enzymatic antioxidants are crucial to the management and regulation of ROS that are produced during abiotic stress exposure (Ahmad *et al.,* 2009). The presence of compounds such as phenols, tannins, flavonoids, terpenoids were higher when both species were under eCO_2 might explain the antioxidant and antimicrobial activities observed under the eCO_2 study. The lower accumulation observed on plants foliar sprayed in combination to being exposed to eCO_2, can explain the ability of both MLE and PhytoStim® to mitigate the effects caused by exposure to abiotic stress and hence lower accumulation of secondary metabolites in the leaves (Rao *et al.,* 1995). With the foliar spray application of PhytoStim® and MLE, the external input of enzymes, vitamins and minerals from the biostimulants enhanced the species' adaptability by eradicating the ROS from exposure to eCO_2, ameliorate membranes, protein processes and crucial systems like the vascular system and photorespiration.

In future, there should be toxicology studies done on the safety consumption *M. oleifera* and *M. peregrina* particularly after biostimulant applications. Climate change studies are important in order to understand how the earth biodiversity will respond to the changing world, and hence more

attention and skillset will be advantageous to not only the scientific community but to everyone as it affects life on earth.

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Supplementary data

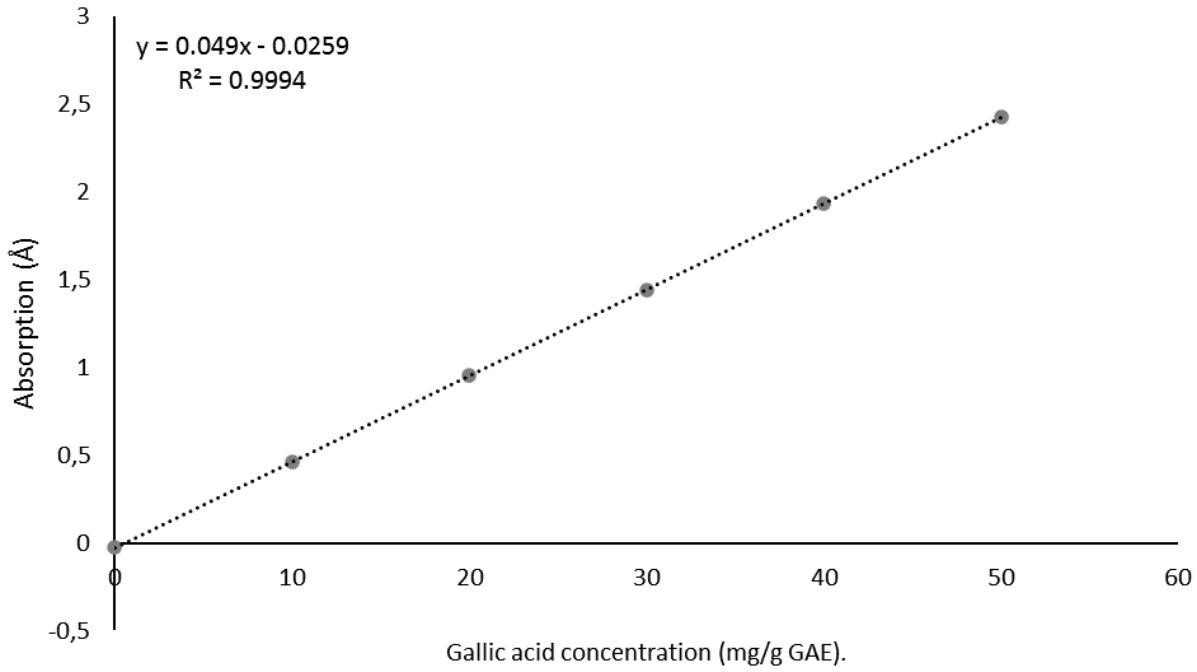


Figure 1. 27 Gallic acid standard curve for the quantification of TPC.

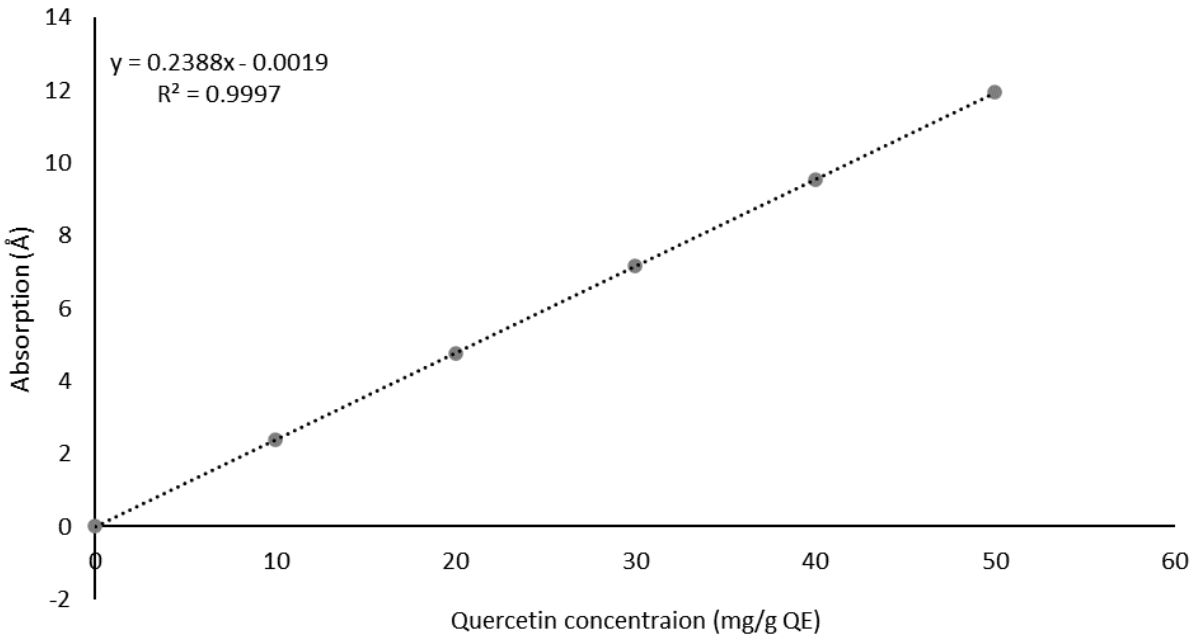


Figure 1. 28 Quercetin standard curve for the quantification of TFC.

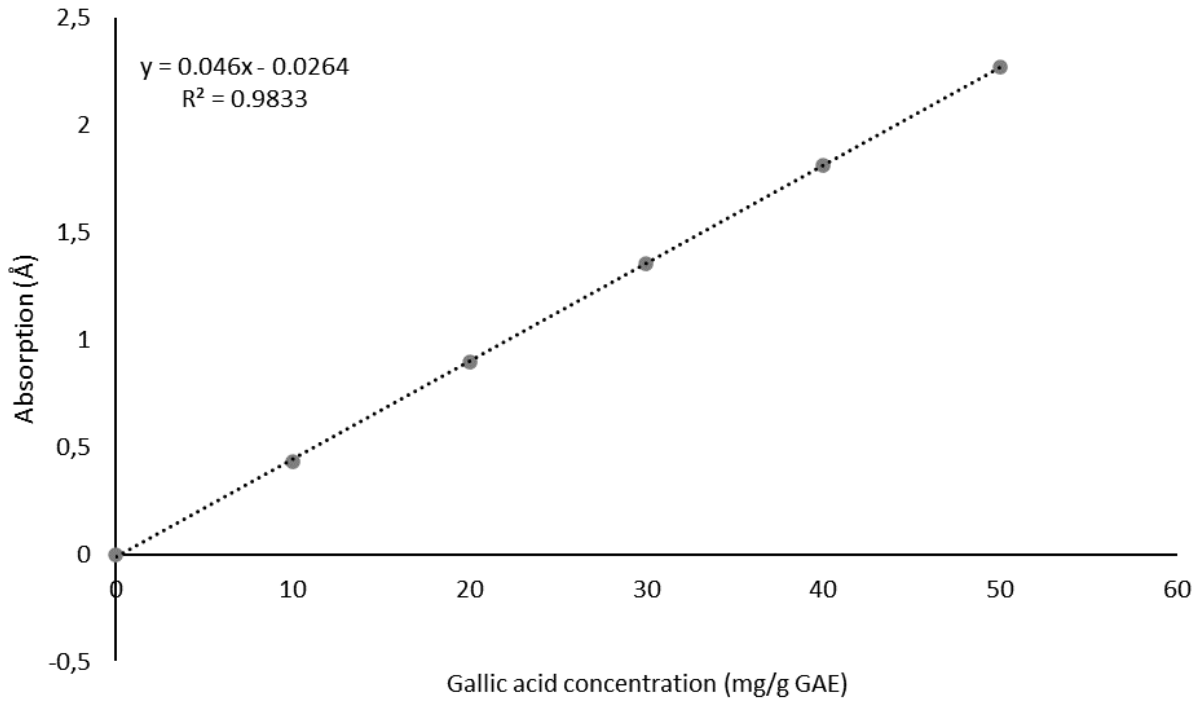


Figure 1. 29 Gallic acid standard curve for the quantification of TTC.

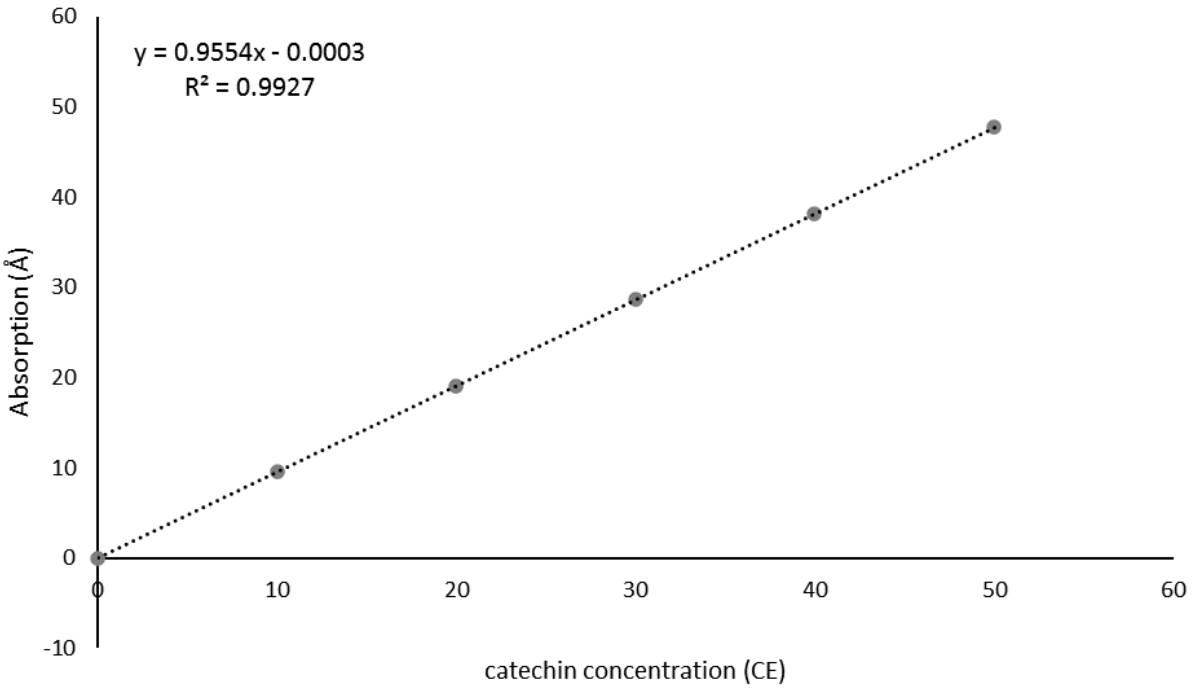


Figure 1. 30 Catechin standard curve for the quantification of TPAC.

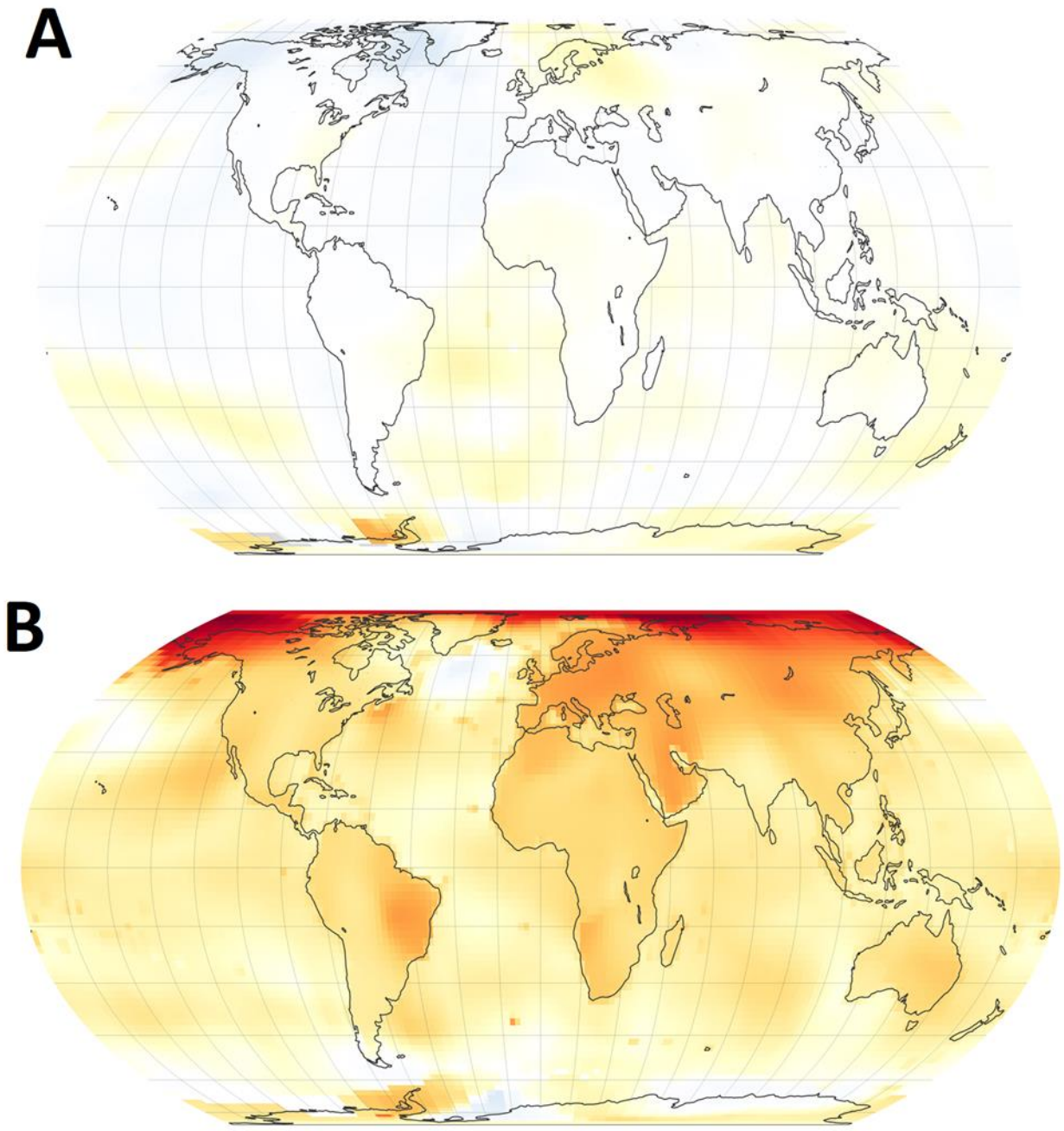


Figure 1. 31 Global mean temperature changes in correlation to increases in global atmospheric CO_2. A: the mean global temperatures between 1970-1974; B: the temperature from 2015-2019. Images taken from statistica.

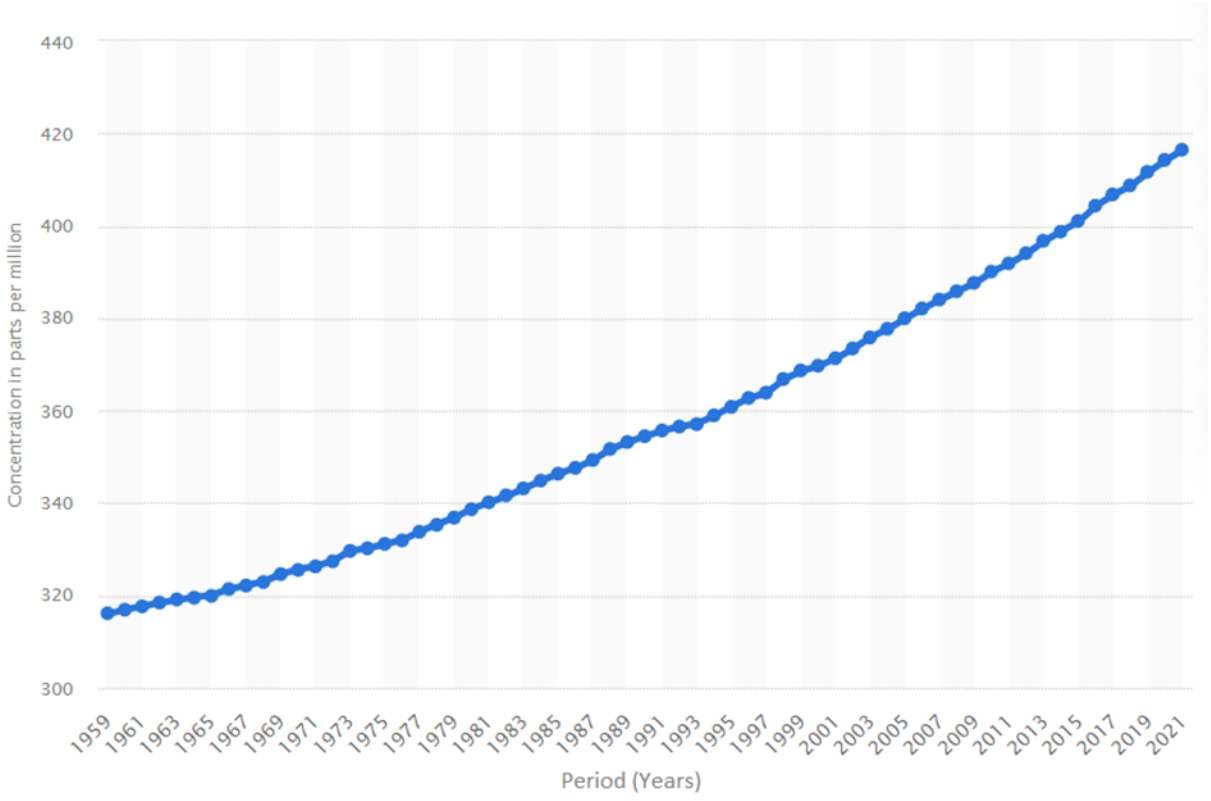


Figure 1. 32 Global mean carbon dioxide concentrations with corresponding years.

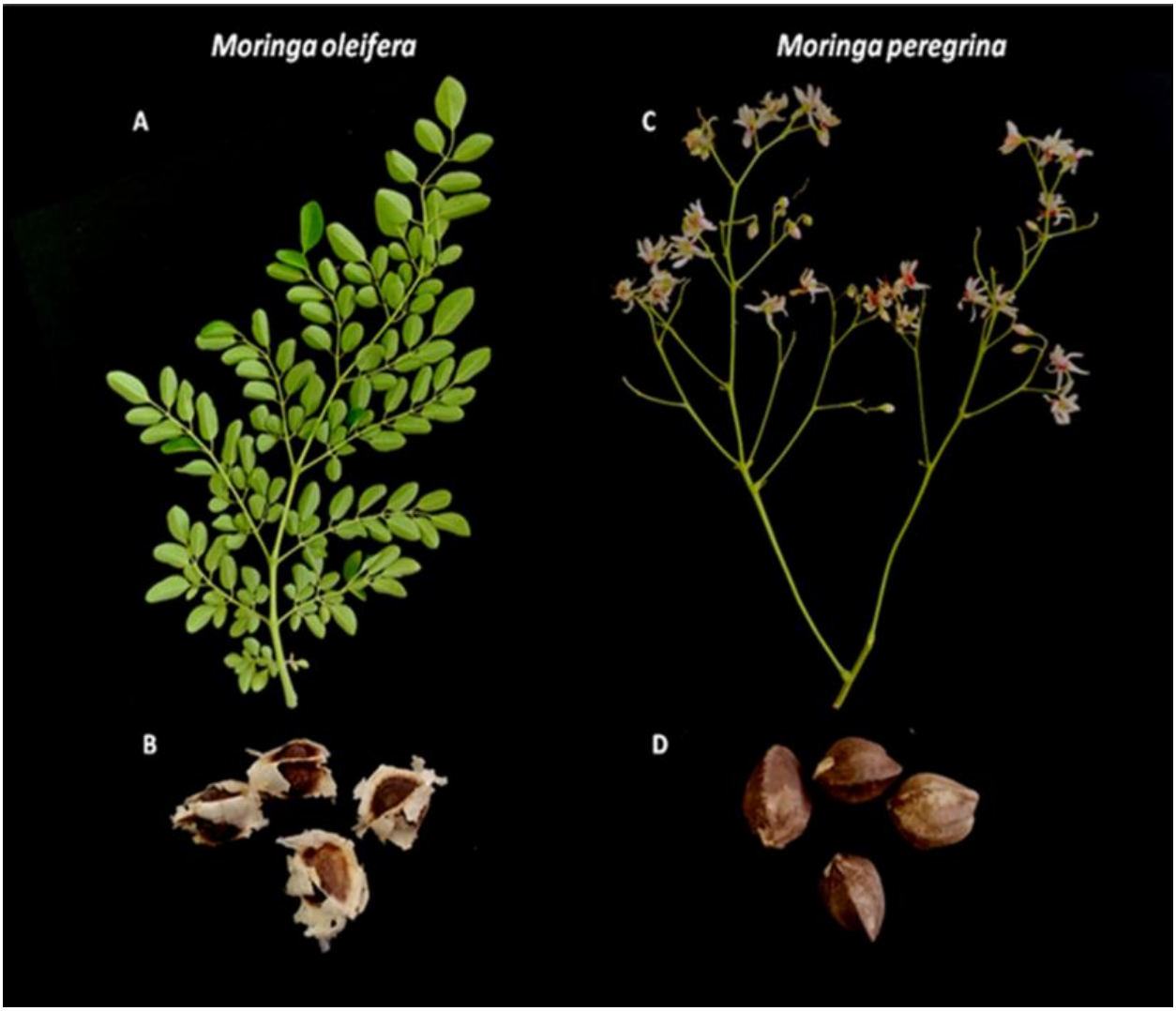


Figure 1. 33 Difference between *M. oliefera's* leaves (A) and Seeds (B); and *M. peregrina's* leaves (C) and seeds (D).