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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

The effects of elevated carbon dioxide concentrations on the physiological and biological characteristics of *Portulacaria afra.*

Domonique Courtney Basson

1678016

Masters Dissertation

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Supervisor: Dr. Ida Risenga

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Declaration

I, Domonique Basson, declare that this research report is my own work. It is being submitted for the degree of Master of Science in Animal, Plant and Environmental Sciences at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature:  Date: 22/03/2023

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Contributions of this dissertation

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3. **Publication** under review in South African Journal of Science "The effect of elevated carbon dioxide on the phytochemical components and biological activity of *Portulacaria afra.***"**
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Symbols and abbreviations

%	-	percentage
±	-	plus/minus
°C	-	Degrees Celsius
µ	-	micro
CO_2	-	Carbon dioxide
e.g.	-	exempli gratia (for example)
et al.	-	*et al*ia (and others)
g	-	gram
mg	-	milligram
mg/g	-	milligrams per gram
ml	-	millilitre
HCl	-	Hydrochloric acid
MeOH	-	Methanol
Na_2CO_3	-	Sodium carbonate
nm	-	nanometres
ppm	-	parts per million
P. afra	-	*Portulacaria afra*
ROS	-	Reactive Oxygen Species
SEM	-	Scanning electron microscope
TPC	-	Total Phenolic Content

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Abstract

There is a concern that rising atmospheric carbon dioxide (CO_2) concentrations may affect the medicinal or nutritional profile of medicinal plants. *Portulacaria afra (P. afra)* is a medicinal plant used by traditional healers to treat various skin conditions. The aim of this study was to determine whether elevated CO_2 concentrations will affect the physiological and medicinal properties of the leaves, stems, and roots of *P. afra*.

This was achieved by measuring the physiological, phytochemical, antioxidant, and antimicrobial activity of the various plant parts, which were exposed to ambient (420 ppm) and elevated (600 ppm) CO_2 concentrations and comparing them to samples grown in greenhouse conditions. The plant samples were placed in a Conviron climate stimulator for three months. Three harvests were completed during this time. The physiological properties measured include the weight of the various plant parts as well as the chlorophyll content within the leaves. The phytochemical profile of the plant was examined through a set of standard colour tests (qualitative analysis) and the determination of the total flavonoid content (quantitative analysis) in all three plant parts exposed to the various treatments. The antioxidant activity was determined by analysing the scavenging activity of the extracts against 1,1 diphenyl-2-picrylhydrazel (DPPH) and hydrogen peroxide (H_2O_2) with the use of a spectrophotometer. The antimicrobial activity determined the extracts' ability to inhibit the growth of six microorganisms related to skin conditions.

The results of this study revealed that there was a 65% and 39% increase in the weight of the leaves and roots, which were exposed to 600 ppm, respectively. A higher phytochemical presence was recorded in plants exposed to 600 ppm. There was a significant increase in flavonoid presence and flavonoid content in the methanolic and hot water plant samples that were exposed to elevated CO_2. The leaves, stems, and roots of *P. afra* exhibit strong scavenging activity against DPPH and even more so towards H_2O_2. The strongest antioxidant activity was

exhibited by the methanolic leaf extracts which were exposed to 600 ppm, against H_2O_2 (0.2±0.7 mg/ml). The antimicrobial activity of all three plant parts was relatively constant between the different treatments. Despite the relatively weak antimicrobial activity of *P. afra* (MIC values > 1000 (µg/ml), a notable increase in the antimicrobial activity of the leaves against *Cutibacterium. acnes* were observed in samples exposed to 600 ppm of CO_2.

Portulacaria afra is a medicinal plant that exhibits great resilience towards elevated atmospheric CO_2 concentrations. The phytochemical and biological properties in this study displayed either no change or an increase in activity, which suggests that *P. afra* may continue to provide relief against certain ailments in the future despite rising atmospheric CO_2 concentrations

Chapter 1: General Introduction

1.1 Medicinal Plants and rising atmospheric CO_2 Overview

Medicinal plants are defined as plant species that exert a positive pharmacological effect on humans or animals. Medicinal plants possess compounds within their structures that can be extracted and used for medicinal purposes (Sofowora *et al.*, 2013). These compounds are more commonly known as phytochemicals. Many chemicals produced by plants have been extracted and replicated to produce modern drugs, such as salicin (more commonly known as aspirin); which was extracted from the bark of a white willow tree (Shara and Stohs, 2015). Medicinal plants have proven to be an important resource in drug discovery, as well as, in treating and preventing human illness.

The World Health Organisation (WHO) has reported that approximately 80% of the world's population relies on traditional medicine as its primary source of health care (Mahomoodally, 2013). Medicinal plants form a large portion of traditional medicine practices. There are several reasons for the large and growing global demand for traditional medicine (Hamilton, 2004). Medicinal plants are more affordable and elicit fewer effects than synthetic modern medicine (Zahra *et al.*, 2020). In South Africa, specifically, the extensive use of traditional medicine can also be linked to the strong cultural and traditional beliefs being passed down through generations, as well as the limited access to modern medicine (Smith-Hall *et al.*, 2012) The growing demand for medicinal plants means that a greater effort must be placed on documenting and validating new medicinal plant species and conserving those which have already been identified. Factors such as urbanisation, overexploitation, and increasing atmospheric CO_2 concentrations threaten the biodiversity and efficacy of these natural resources (Wyk and Prinsloo, 2018).

The atmospheric CO_2 concentration has increased significantly over the last century and is expected to continue rising. Botanists have long been concerned about the effect that rising CO_2 concentrations may have on plants (Thompson *et al.*, 2017). The research conducted to determine the effects that rising CO_2 may have on the efficacy of medicinal plants is limited and results vary across the different species (Rajashekar, 2018). It is important to study the effect that rising CO_2 concentrations may have on medicinal plants, in order to predict the stability of this resource in the future.

1.2 Aim of study

This study aimed to assess the effects of elevated carbon dioxide concentration on the physiological properties, phytochemical profile, and biological activity of the leaves, stems, and roots of *P. afra*.

1.3 Objectives of study

The objectives of this study are:

- To determine the effects of elevated CO_2 concentrations on the weight of leaves, stems, and roots of *P. afra* plants exposed to the greenhouse (control), 420 ppm (Conviron control), and 600 ppm (experiment). As well as the effects on the chlorophyll content in the leaves of *P. afra.*
- To assess the effects of elevated CO_2 concentration (600 ppm) on the qualitative and quantitative phytochemical profile of the leaves, stems, and roots of *P. afra*, and compare it to the control (greenhouse and 420 ppm) leaves, stems and roots.
- To assess the change in antioxidant activity in the leaves, stem and roots of *P. afra* in response to elevated atmospheric CO_2 concentrations and compare it to the control (greenhouse and 420 ppm) leaves, stems and roots.
- To determine the effects of elevated carbon dioxide concentration on the antibacterial

properties of *P. afra* against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 27853), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella aerogenes* (ATCC 13048) and *Cutibacterium acnes (*ATCC 10231).

- To determine the effects of elevated carbon dioxide concentration on the antifungal

1.4 Research questions

- Does elevated CO_2 modify the physiological and phytochemical characteristics of the leaves, stems and roots of *P. afra*?
- What are the effects of elevated CO_2 concentrations on the biological activities of the leaves, stems and roots of *P. afra*?

Chapter 2: Literature Review

2.1 Background of *Portulacaria afra*

2.1.1 Botanical Overview of *Portulacaria afra*

Portulacaria afra is a plant species more commonly referred to by locals as "spekboom" (Afrikaans) or "igwanitsha" (Xhosa) (Oakes, 1973). The plant is also commonly known as "elephant bush". This name is a reference to the fondness of elephants for the plant. *Portulacaria afra* forms a part of the *Didiereaceae* family along with 21 other succulent angiosperms (Van Jaarsveld and Le Roux, 2021). *Portulacaria afra* is classified as a large shrub or small tree species and can grow up to a height of five meters (Van Jaarsveld and Le Roux, 2021). *Portulacaria afra* is characterised by its succulent leaves and a red stem. The small, flat leaves grow on opposite sides of the branching peduncles. In favourable conditions, the plant produces small, rose-coloured flowers (Applewuist and Wallace, 2003).

A  B 

Figure 1: An image of the leaves, stem (A) and root (B) of *Portulacaria afra.*

The much-branched plant species is evergreen and has been described as "incredibly resilient" due to its ability to withstand many different climatic conditions, including droughts. Despite the plant's resilience, it grows more readily in frost-free areas (Oakes, 1973).

2.1.2 Distribution of *Portulacaria afra*

Portulacaria afra is a plant species endemic to South Africa. The plant grows readily in semi-arid areas such as the southern and south-eastern areas of South Africa (Figure 2). Despite the plant predominantly growing in South Africa, the origin can also be linked to neighbouring countries such as eSwatini and Mozambique (Van Jaarsveld and Le Roux, 2021).

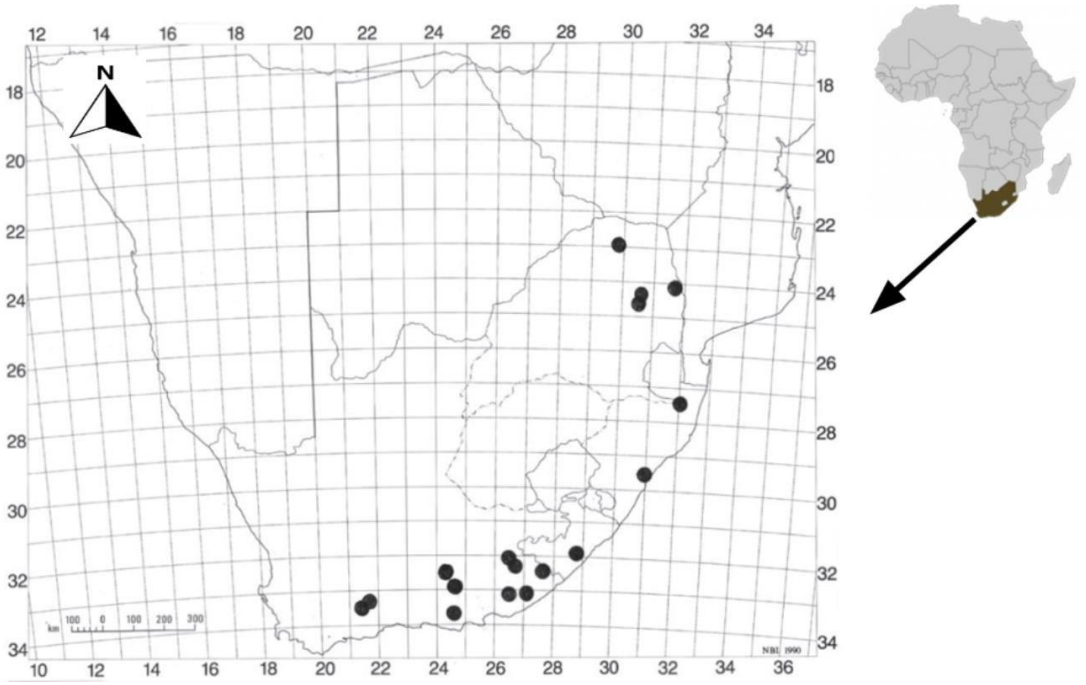


Figure 2: A map indicating the distribution of *Portulacaria afra* (Van Jaarsveld and Le Roux, 2021).

The distribution of a plant plays an important role in identifying the preferred climatic and environmental factors of the plant. This becomes particularly important when identifying the stresses that may be affecting the chemical constituents of the plant (Spalding, 1890).

The semi-arid areas in South Africa are hot and dry and typically receive rainfall of less than 250 mm/year. The preference of the plant for dry areas is also observed by the growth of *P.*

afra

on the dry mountains that border eSwatini and Mozambique (Van Jaarsveld and Le Roux, 2021). This coincides with the drought-tolerant characteristics of *P. afra.*

Portulacaria afra is not a soil specialist and can grow in varying types of soil. However, the plants are most commonly situated on well-drained slopes (Oakes, 1973).

The abundance of *P. afra* in certain areas, such as the Eastern Cape, has led to the labelling of these areas as "spekboomveld". *Portulacaria afra* is an important stem and leaf succulent in South Africa and has been labelled "one of South Africa's most important fodder trees" (Oakes, 1973).

2.1.3 Economic Importance of *Portulacaria afra*

Portulacaria afra is an increasingly important species due to its many uses. Elephants are not the only animals that feed on *P. afra*; the plant is enjoyed by many livestock such as cattle and goats (Kota and Shackleton, 2015). More recently however, humans have been incorporating *P. afra* into their diets. Because of its tart, lemon-like flavour and nutritious component, the plant is an excellent addition to healthy dishes such as salads. *Portulacaria afra* could play an important role in food security. Whilst many food sources are vulnerable to environmental stresses such as drought, *P. afra* thrives in these conditions and hence may become crucial in response to climate change (Gualnick and Ting, 1986).

A leading cause of climate change is the rise in global carbon dioxide (CO_2) emissions. *Portulacaria afra* is well-known for its strong carbon storage capabilities, with *P. afra*-dominated areas (such as spekboomveld) storing more than 200 tonnes of carbon per hectare (Van de Vyer *et al.,* 2013*)*. This characteristic makes the plant a key-player in trying to reduce CO_2 emissions and thereby minimise the effects of global warming (Gualnick and Ting, 1986; Bradfield and Daltry, 2008). *Portulacaria afra* plays an important role in ecological restoration.

The plant improves soil properties through means of altering the litter composition and decomposition, which in turn affects the carbon properties of the soil (Panter and Ruwanza, 2019). In addition, *P. afra* promotes the growth of other plant species as well as improving the biodiversity of the areas it dominates (Van de Vyer *et al.,* 2013). *Portulacaria afra* is a resilient plant with the ability to rejuvenate itself following grazing and destruction. This may contribute to the plant being labelled "least concern (LC)" by the National Red List and thus may be used more readily (Ndhlovu *et al*., 2021).

Portulacaria afra is aesthetically pleasing and easy to care for, making it a popular house plant or used ornamentally, such as for a bonsai (Oakes, 1973).

2.1.4 **Medicinal Uses of *Portulacaria afra***

Plants have the ability to synthesise an infinite number of phytochemicals; as such, they are potential sources for new compounds that may lead to drug discovery. Many phytochemicals have been used as drugs, such as salicin, commonly known as aspirin, which was originally extracted from the bark of a white willow tree (Vickers and Zollman, 1991). *Portulacaria afra* is a medicinal plant species that may provide important resources in drug discovery.

Through interviews conducted by De Wet *et al.* (2013), it was discovered that *P. afra* is used by traditional healers to treat a variety of skin conditions. The leaves are the most commonly used plant part for the treatment of skin conditions, such as, rashes, ringworms, warts and acne (Tabassum *et al.,* 2022). Cutaneous conditions have long been associated with bacteria, viruses, and fungi. The plant possesses anti-inflammatory properties, which may assist in providing relief from skin ailments.

Additionally, chewing the leaves is used to soothe a sore throat and heal mouth infections. This may be a result of the antiseptic properties exhibited by the juice of the leaves (Madhusudan *et*

al., 2018). Sucking on the leaves of *P. afra* has also been shown to treat dehydration and, as a result, exhaustion and dry mouth (de Vos, 2021).

2.1.5 Metabolic strategy of *Portulacaria afra*

Crassulacean acid metabolism (CAM) is a metabolic strategy adopted by some plants in response to environmental stresses such as drought (Males and Griffiths, 2017). *Portulacaria afra* is a facultative CAM plant. Thus, the plant makes use of C3 photosynthesis and performs daytime CO_2 uptake, but the plant can also utilize CAM photosynthesis during droughts. Previous studies have determined that *P. afra* uses CAM photosynthesis in the summer months in South Africa when temperatures are high (Gualnick and Gladsky, 2017). Plants are able to use CAM photosynthesis due to the unique pattern of the stomatal conductance. The stomata primarily open at night, and CO_2 diffuses into the cytoplasm. Essentially, at night, primary carbon assimilation by the enzyme phospho-enol-pyruvate carboxylase (PEPC) produces malic acid which gets stored in the vacuoles. When the sun rises, malate enters the Calvin cycle, where it is decarboxylated to release CO_2 for utilization by Rubisco in the C3 cycle (Gualnick and Gladsky, 2017). Figure 3 is a visual representation of CAM photosynthesis.

The structure of the leaf is closely coordinated with the functional traits of the leaf and the stomatal traits (Gualnick and Gladsky, 2017).

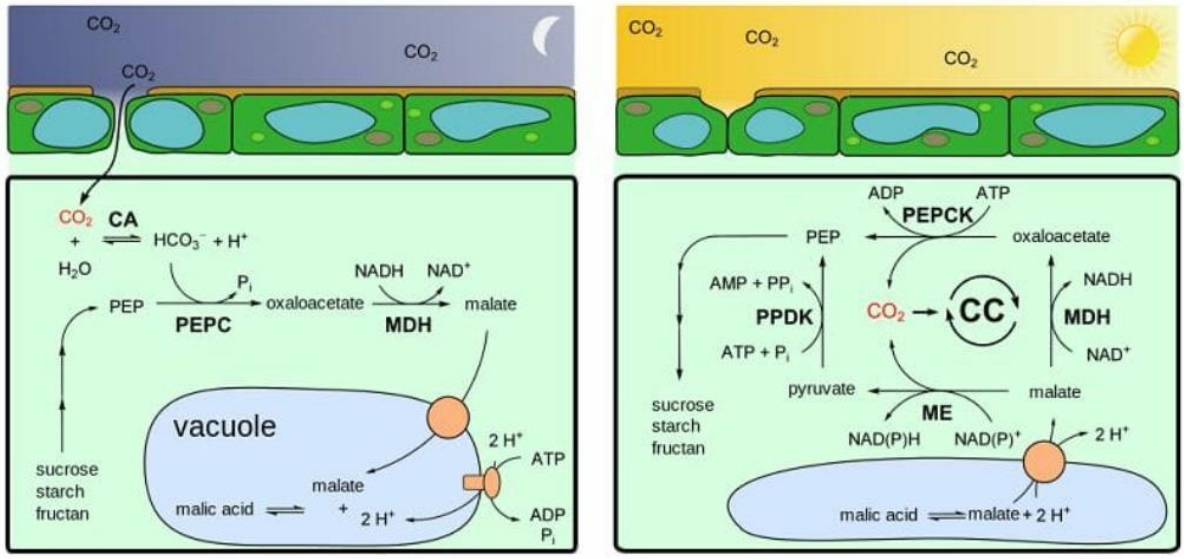


Figure 3: The metabolic pathway of CAM plants (Biology dictionary, 2019).

The utilisation of facultative CAM as a metabolic strategy may affect the high carbon sequestration by *P. afra.*

2.2 Phytochemical Overview

Phytochemicals are defined as bioactive, non-nutrient compounds found in plants. These chemicals are essential for the survival of the plant and can be produced through primary or secondary metabolism. Primary metabolites are important for the growth, photosynthesis and reproduction of the plant; these molecules include amino acids, sugars, and fats. Secondary metabolites are produced by plants in response to stress (Boyer and Liu, 2004). The secondary metabolites are the chemical compounds that are used in pharmaceuticals. Phytochemicals have been used for centuries for the maintenance of chronic-related health care (Tyagi *et al.*, 2010). It is generally the combination of secondary metabolites in plants that elicits a medicinal effect from the plants (Jain and Vijayvergia, 2019). Many developed countries have recognised the merits of using and consuming these natural compounds as opposed to the synthetic chemicals found in many Western medicines (Kadam and Pawar, 2020). Plants have been

identified as having a limitless ability to synthesise aromatic substances, such as phytochemicals, and hence play an important role in drug development and horticulture. The main groups of phytochemical compounds are phenolic acids, flavonoids, stilbenes, and tannins (Boyer and Liu, 2004), the structures can be found in figure 4.

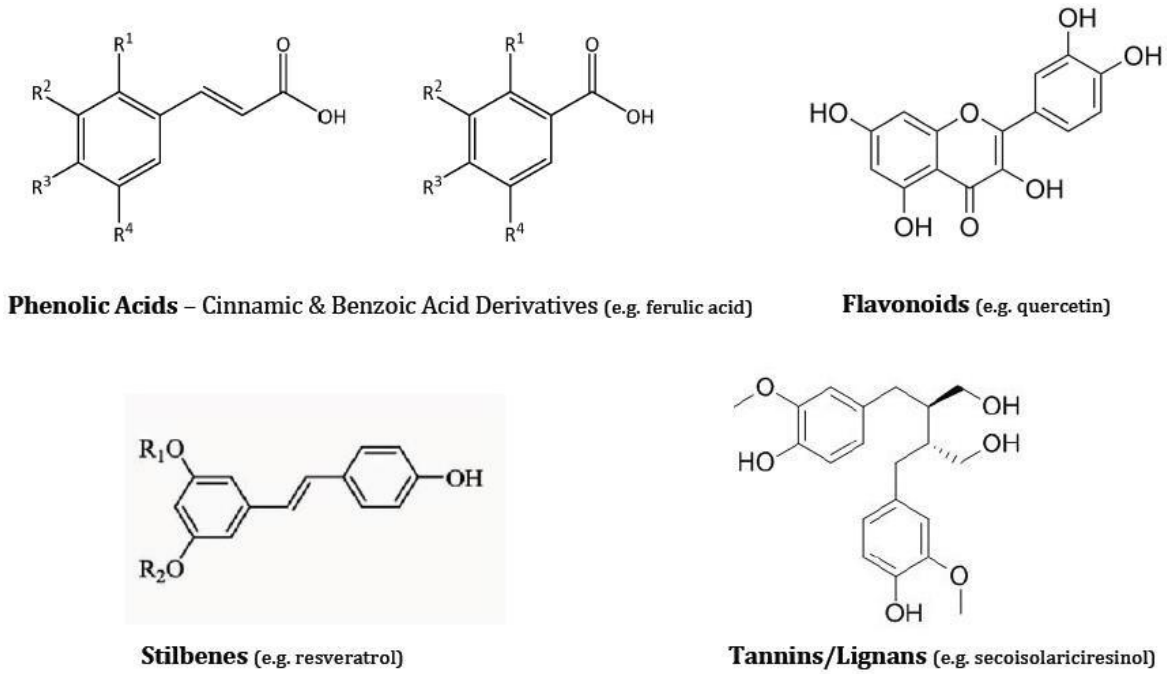


Figure 4: A representation of the structures of different phytochemicals (Goulet, 2018).

2.2.1 Saponins

Saponins are a group of secondary metabolites that consist of many different molecules with different shapes and structures. The complexity of the structures has led to the diverse physicochemical and biological properties exhibited by the chemicals (Güçlü-Üstündağ and Mazza, 2007). Saponins are important in plants as they protect them with their antimicrobial properties and bitter taste, which decreases their palatability among herbivores (Desai *et al*., 2009). These properties have been exploited in natural and industrial applications (Güçlü-Üstündağ and Mazza, 2007). These applications include fish poison, molluscicides, and, in an

industrial setting, foaming agents. Saponins have a foaming ability as a result of their hydrophobic sapogenin and hydrophilic sugar parts (Desai *et al*. 2009). Saponins have a wide range of effects on human health, including anti-inflammatory, anti-fungal, and antimicrobial effects (Desai *et al*. 2009). The wide range of pharmacological benefits allows plants that possess saponins to be used as medicinal plants (Desai *et al*. 2009). Recent research suggests that saponins are the active chemicals in medicinal plants. Some saponins are unable to penetrate cell membranes and are therefore less effective against gram-negative microorganisms.

2.2.2 Phenolics

Phenolic acids are secondary metabolites that provide plants with flavour and health-enhancing benefits. The phenolics are identified by one or more hydroxyl groups on an aromatic ring. Phenolic acid is produced by the plant in response to stress; however, phenolic acid also plays an important role in plant growth and reproduction (Ghasemzadeh, 2011). Many flavours identified in foods are attributed to the presence of phenolics. Phenolics have been identified as possessing both antimicrobial and antioxidant properties. Phenolic compounds extracted from plants have also been shown to possess anti-inflammatory properties (Ghasemzadeh, 2011). Magnolol and honokiol are examples of phenolic compounds that exhibit anti-inflammatory effects (Umar Lu, 2005). The compounds are also applied topically due to the anti-inflammatory effects exhibited without any irritation, and thus have been identified as a potential acne-mitigating solution when applied topically (Umar Lu, 2005).

2.2.3 Flavonoids

Flavonoids are structural derivatives of flavones and are thus water-soluble (Geissman, 1963). Flavonoids are easily recognised by the pigments in flowers; however, they can also be found within the leaves, bark, roots, and fruit of the plant. Flavonoids are important chemicals produced by plants because they attract pollinators by providing the plant with attractive

colours and scents (Harborne and Grayer, 2017). Flavonoids also provide protection for the plant by acting as signal molecules in times of stress (Panche *et al.*, 2016). There are many different groups of flavonoids, and it has been reported that all of the groups have the ability to act as antioxidants (Panche *et al.*, 2016). Additionally, flavonoids have been identified as having the ability to reduce inflammation, prevent platelet aggregation, and kill or stop the growth of microbes (Dillard and German, 2000).

2.2.4 Glycosides

Glycosides are a wide-ranging group of bioactive compounds. The structure of glycosides is presented by the carbohydrate portion of the molecule, consisting of one or more sugar molecules or uronic acid joined to a hydroxy group (Bernhoft, 2010). Glycosides are generally present in the pigment of either fruit or flowers. When ingested by humans, glycosides are hydrolysed in the colon. Studies have shown that glycosides exhibit anti-inflammatory, antioxidant, and anticancer properties (Bernhoft, 2010).

2.2.5 Tannins

Tannins are defined as phenolic compounds of high molecular weight ranging from 500 Da (Dalton) to more than 3000 Da (Njeru *et al*., 2013). Tannins are quantitatively abundant in plants and can be found within a plant's leaves, bark, roots, and fruits. Tannins are bitter polyphenols, and hence they are what make plants unpalatable to predators (Farha *et al*., 2020). Tannins have been described as astringent, and as such, they either bind and precipitate or shrink proteins. The structure of tannins contributes to the diverse benefits shown. Tannins have also been associated with an antibacterial function due to their structure containing many phenolic hydroxyl groups (Farha *et al*., 2020). Tannins also exhibit free radical scavenging activity and, as a result, act as an antioxidant. Tannins have been associated with many health benefits, such as balancing blood sugar levels and fighting inflammation (Crozier *et al.*, 2006).

2.2.6 Terpenoids

Terpenoids form a major group of phytochemicals, with over sixty thousand terpenoids identified. The major classifications of terpenoids are monoterpenes, sesquiterpenes, diterpenes, sesterpenes, triterpenes, tetraterpenes, polyterpenes, and meroterpenes. The classification into these groups is based on the number of carbon units as well as the biological activity of the molecule. In plants, terpenoids play a role in defence against predation (Jahangeer *et al*., 2021). The use of terpenoids by humans in medical and therapeutic capacities has occurred for centuries. This may be due to the essential oils, which can be extracted from terpenoids (Jahangeer *et al*., 2021).

2.2.7 Steroids

Plant steroids are characterised by the four carbon rings that make up the steroid nucleus. The addition of different organic molecules at different positions along the steroid nucleus is what creates the large variety of steroidal compounds. The variety of the compound is what makes the molecule so important within plants (Patel and Savjani, 2015). The main steroidal compounds include sex hormones, inflammatory steroids, and anti-inflammatory steroids. The addition of other phytochemicals makes important steroidal compounds. An example is the attachment of saponins to create sapogenins, which have antifungal and antitussive properties (Patel and Savjani, 2015).

2.2.8 Coumarins

Coumarins are organic compounds with a backbone of two organic rings fused together. One ring is a benzene ring, and the other ring has an alkene structure with an ester attached. Coumarins play an important role in a plant's defence against predation because they are secreted when plants are wounded or attacked by predators (Ojala, 2001). Coumarin-rich herbs have shown efficacy in reducing inflammation, and oedemas. Some studies present coumarins as a potential anti-cancer therapy. However, these coumarin-rich herbs pose a small risk for

causing hepatotoxicity and hence patients that lack cytochrome 2A6 (CYP2A6) should be monitored when ingesting coumarins (Yarnell and Abascal, 2009).

2.2.9 Phlobatannins

Phlobatannins are a form of condensed tannin. These compounds are commonly found in herbaceous or woody plant species. They exhibit protective activity against insects and diseases (Ferreira *et al.,* 1999). Phlobatannins are being used in the commercial sector for manufacturing leather. Phlobatannins have been recognised for its medicinal properties in wound healing as well as their anti-inflammatory, antioxidant, and analgesic properties (Ferreira *et al.,* 1999).

2.2.10 Volatile oils

Volatile oils are mixtures of complex organic compounds that are produced and secreted by glandular trichomes. The mixture of hydrocarbons and oxygen derivatives presents itself as having a low molecular weight (Sharifi-Rad *et al*., 2017). Volatile oils are secreted by plants in order to produce a scent that can either attract pollinators or repel predators. Volatile oils are more commonly known as essential oils (Sharifi-Rad *et al*., 2017). Despite the recent scepticism surrounding the efficacy of using essential oils, they have been used since ancient times to treat the symptoms of certain ailments. Many studies have recorded the use of volatile oils for their antimicrobial, anti-inflammatory, and anti-cancer properties (Sharifi-Rad *et al*., 2017).

There are limited available reports on the phytochemical presence in the leaves, stems and roots of *P. afra.* However, in a study conducted by Tabassum *et al.* (2022), it was determined that the leaves of *P. afra* exhibited a high presence of saponins and phenol compounds. The plants leaves and stems have also exhibited a high presence of coumarins (Basson *et al.,* 2023). Phytochemical presence in medicinal plants provides an indication of the efficacy of the plant. Therefore, it is important to determine the presence of phytochemicals in this plant species so that its potential as a medicinal plant may be scientifically recognised.

2.3 Antioxidant activity

Antioxidants are defined as substances that delay or prevent the oxidation of a substrate (Halliwell, 1999). Antioxidants have the ability to donate an electron to a free radical, thus reducing the damage caused by these free radicals. Free radicals are molecules that have an unpaired electron. This structure makes the molecule very unstable and reactive. Free radicals are derived from sources such as environmental pollutants, cigarettes, and exposure to UV or X-ray radiation. However, free radicals are also produced through metabolic processes, which are required for life (Halliwell, 1999). The skin is particularly susceptible to reactive oxygen species (ROS) attacks. This is due to the skin's direct exposure to UV radiation, environmental pollutants, and the high pressure of oxygen molecules (Kruk and Duchnik, 2014). When the ROS overwhelm the skin's natural defence mechanism, oxidative stress occurs. Many chronic human diseases, such as Alzheimer's, arthritis, and heart disease, arise as a result of oxidative stress (Lobo *et al*., 2010). There are many studies that suggest that oxidants play a role in skin diseases. In the skin, a ROS attack occurs through action on biomolecules. The alteration to biomolecules affects gene expression or causes cell death (Kruk and Duchnik, 2014). The chronic inflammation that arises as a result of the alteration of biomolecules leads to collagen fragmentation and disorganisation of collagen fibres. As a result, the skin becomes susceptible to developing diseases such as psoriasis and neutrophilic disorders such as acne (Kruk and Duchnik, 2014).

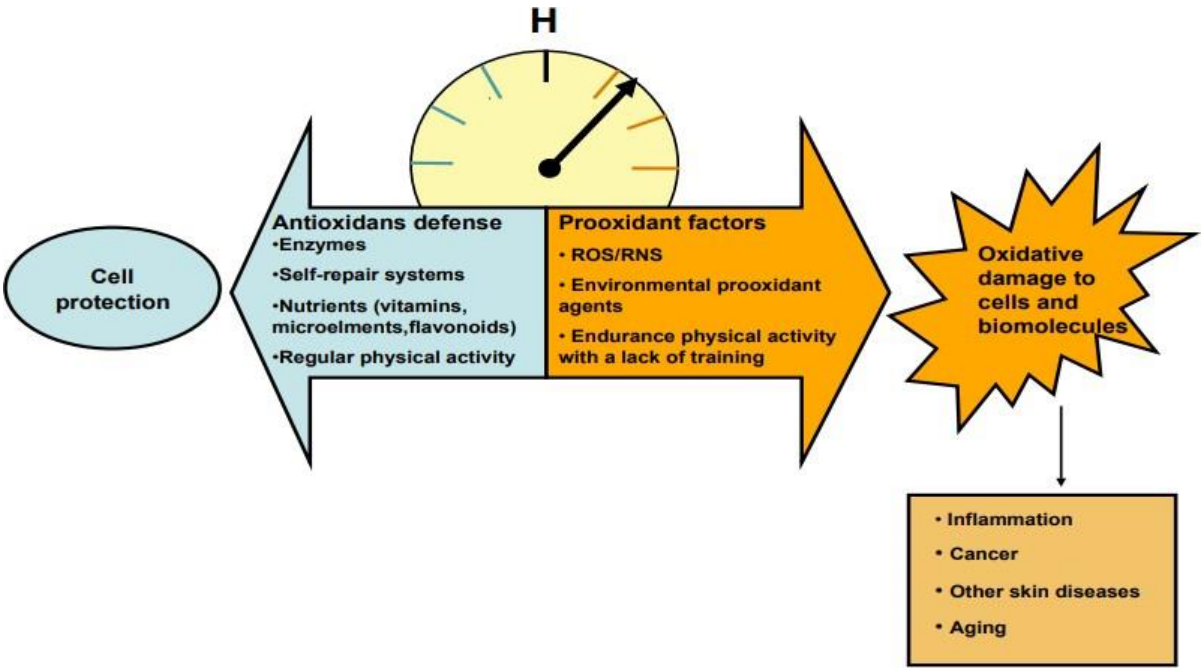


Figure 5: A depiction of the effects of ROS on the skin (Kruk and Duchnik, 2014).

Due to antioxidants' ability to neutralise ROS, they are imperative for reducing the number of free radicals found in the body and hence reducing the risk of chronic illnesses (Pisoschi and Niculescu, 2012). The half-maximal inhibitory concentration (IC_{50}) is a standard measurement of an extract's efficacy and represents the concentration of the extract needed to decrease the activity of the radicals by 50%; thus, a lower IC_{50} value represents a higher antioxidant activity (Abudayeh *et al*., 2019).

Studies suggest that *P. afra* possess a strong affinity for scavenging free radicals. The DPPH scavenging activity of the plant extracts was considered moderate, in comparison to other free radicals (Tabassum *et al.,* 2022; Basson *et al.,* 2023). There are limited studies regarding the antioxidant activity of the leaves, stems, and roots of *P. afra.* However, the studies which have been conducted, suggest that *P. afra* has a strong affinity for reducing oxidative stress in the body and hence provide relief against certain ailments.

2.4 Antimicrobial activity

Microorganisms (microbes) are defined as microscopic life, which includes bacteria, archaea, fungi, and protists (Das *et al.*, 2007). Microbes form an integral part of human health. The colonisation of these microbes forms a microbiome in and on the human body. This microbiome is important for immunity as well as hormonal and metabolic homeostasis in humans. The microbiome also provides a barrier against microbes that are disease-causing, known as pathogens (Wang *et al.,* 2017). Although the majority of microbes are advantageous to human health, there are some that have led to a high mortality rate, such as COVID-19, HIV/AIDS, and tuberculosis (Nathan, 2015).

This research dissertation will focus on bacterial and fungal organisms as they are more commonly associated with skin infections.

2.4.1 Bacterial organisms

Bacterial organisms were the first inhabitants on earth. Bacteria have since adapted to colonise every environmental niche today. As such, bacterial organisms can be found anywhere, from oceanic floors to human respiratory tracts. Bacterial organisms play an important role in human health (Maglica and Ožbolt, 2019).

There are two main types of bacterial organisms: gram-positive bacteria and gram-negative bacteria. The distinction between the two organisms is important when diagnosing and treating an infection. The distinguishing feature is the surface of the structure (Sonohara *et al.*, 1995). The outer membrane of gram-negative bacteria is covered in a liposaccharide. While gram-positive bacteria have an outer membrane that is covered in a peptidoglycan layer, to which teichoic acid, teichuronic acid, and proteins are covalently bonded, the exterior layer between the two bacteria differs, and as such, there is a difference in their electrophoretic behaviours (Sonohara *et al.*, 1995).

2.4.1.1 Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a cocci-shaped bacterium that is generally found in clusters (Taylor and Unakal, 2017). These organisms can grow aerobically or anaerobically and in a relatively large temperature range. *S. aureus* is considered a part of human beings' natural flora, found on the skin and in the upper respiratory tract, and is described as the most common and "almost universal" cause of skin and soft skin diseases (Taylor and Unakal, 2017; McCraig *et al.,* 2007). An infection caused by *S. aureus* can present itself in many clinical manifestations, such as pulmonary infections, gastroenteritis, and urinary tract infections. However, the most common clinical manifestation is in skin and soft tissue infections (Vella *et al.,* 2021).

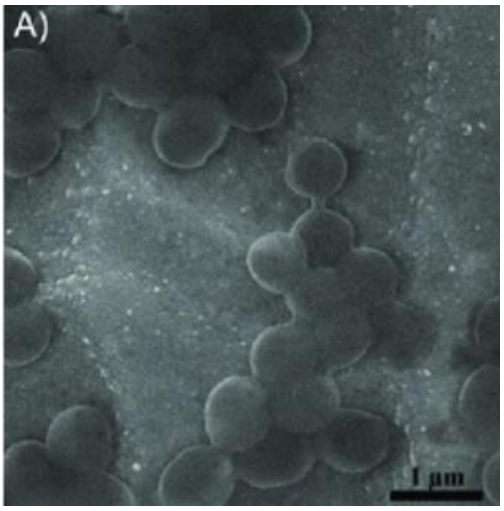


Figure 6: An image of *Staphylococcus aureus* under a scanning electron microscope (Predoi *et al*., 2019).

Treatment of disease caused by *S. aureus* is difficult due to the mutating nature and multi-drug resistant variants of the organism (Taylor and Unakal, 2017).

2.4.1.2 Staphylococcus epidermidis

Staphylococcus epidermidis (*S. epidermidis*) is a gram-positive, cocci-shaped bacterium that forms clusters (Lee and Anjum 2021). This commensal bacterium is commonly found on

human skin and mucosal membranes (Ziebuhr *et al.,* 2006). *Staphylococcus epidermidis* is considered one of the most abundant colonisers of the skin. The bacterium has the ability to inhibit the colonization of *S. aureus* and thus reduce the risk of *S. aureus* infections, among others. Although the bacterium is generally advantageous to the skin's defence, it can also be pathogenic. *Staphylococcus epidermidis* is an opportunistic bacterium that is a common cause of nosocomial skin infections (Claudel *et al.,* 2019).

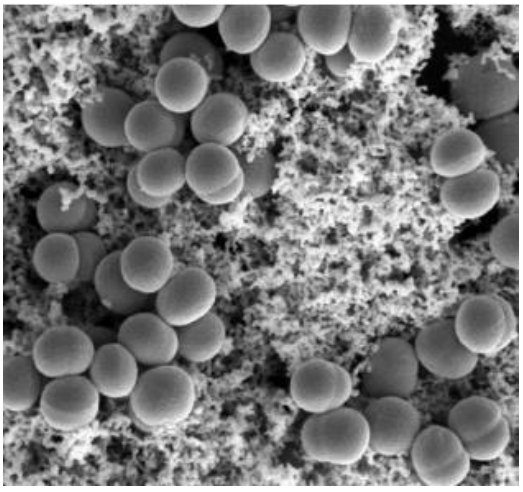


Figure 7: An image of *Staphylococcus epidermidis* under a scanning electron microscope (Bernatova *et al*., 2019).

Similar to *S. aureus,* the treatment of *S. epidermidis* is difficult. The organism produces many molecules, such as phenol-soluble modulins, which assist in immune evasion and biofilm development as protection from the host's defences (Otto, 2012).

2.4.1.3 Pseudomonas aeruginosa

Pseudomonas aeruginosa (*P. aeruginosa*) is a rod-shaped bacterium that is distributed in a wide range of environments, such as water, soil, plants, and animals (Spernovasilis *et al.,* 2021). More recently, *P. aeruginosa has become* increasingly prevalent in hospitals. These gram-negative bacteria are considered opportunistic, as they commonly affect immunocompromised patients (Basseti *et al.,* 2018). *Pseudomonas aeruginosa* causes skin and soft-tissue infections that range from mild cellulitis to life-threatening infections. The

bacterium can be associated with any anatomical location on the human body (Spernovasilis *et al.,* 2021).

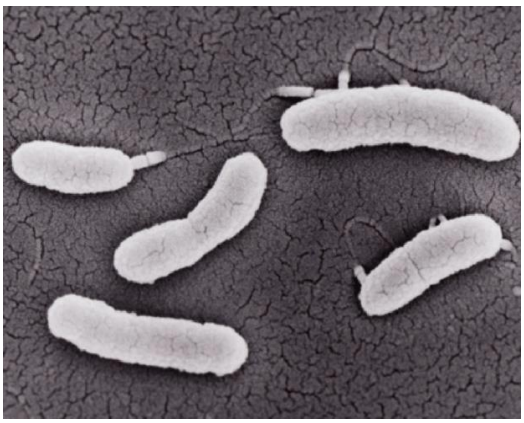


Figure 8: An image of *Pseudomonas aeruginosa* under a scanning electron microscope (Xie *et al*., 2010).

The major concern regarding *P. aeruginosa* is that it is resistant to many antimicrobials (Kelsey, 2014). This microbe produces extracellular enzymes and an external slime layer which causes the antimicrobial resistance (Bennik, 1999)

2.4.1.4 Klebsiella aerogenes

Klebsiella aerogenes is a gram-negative rod-shaped bacterium. This facultative anaerobe causes opportunistic infections in the lower respiratory tract, skin and other soft tissues. *Klebseiella aerogenes* is a nosocomial infection and as such, many of the infections are a result of venous or catheter insertions and surgical procedures (Shantiae *et al.,* 2022).

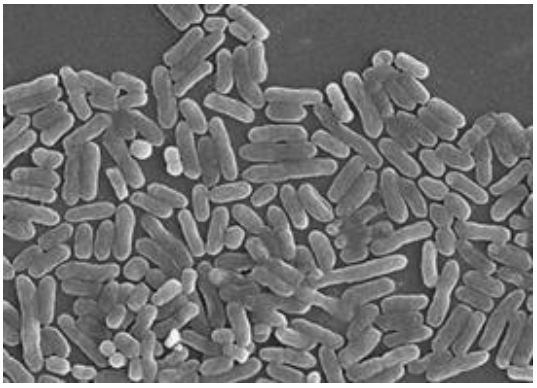


Figure 9: An image of *Klebsiella aerogenes* under a scanning electron microscope (Xie *et al*., 2010).

Many *K. aerogenes* strains are resistant to treatment as a result of biofilm production and antimicrobial resistance (Shantiae *et al.*, 2022).

2.4.1.5 Cutibacterium acnes

Cutibacterium acnes (C. acnes), formerly known as *Propionibacterium acnes,* is a pleomorphic, rod-shaped bacterium. *C. acnes* forms a vital component of the human skin microbiota. This commensal bacterium regulates the skin's homeostasis and prevents other microbes from colonising the skin and causing infection. However, *C. acnes* is an opportunistic pathogen and has been identified as a key component in acne vulgaris (Dréno *et al.*, 2018). This microbe produces enzymes and metabolites that directly damage the host's tissue (Perry and Lambert, 2006).

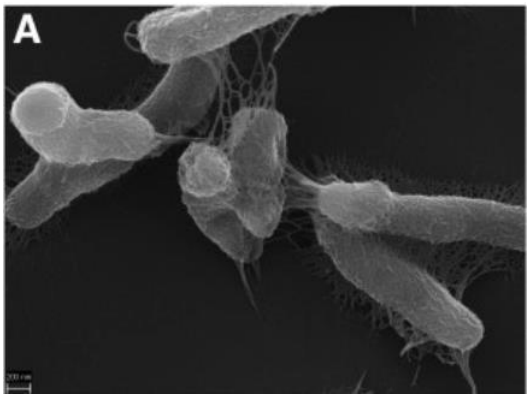


Figure 10: An image of *Cutibacterium acnes* under a scanning electron microscope (Mak *et al*., 2013)

The complex wall structure of *C. acnes* may contribute to treatment failure (Perry and Lambert, 2006), In addition the response of *C. acnes* to antibiotics has also begun to gradually decline. This means that treating *C. acnes* may become increasing difficult (Dréno *et al.*, 2018).

2.4.2 Fungi

Fungi are eukaryotic micro-organisms that are highly variable in morphology, habitat, and even activity. These heterotrophic organisms can be macroscopic or microscopic and present as yeasts, moulds, or a combination (McGinnis and Tyring, 1996). The varying structures of fungi allow them to exist in abundance in almost every environment. It is currently estimated that there are more than 2.2 million different fungi species in the world (Hawksworth and Lücking, 2017). In general, fungi are beneficial to humans, as they are involved in nutrient recycling, the production of antibiotics, and also serve as food, among other things. However, some fungi are pathogenic and cause diseases ranging from superficial infections to subcutaneous infections and even allergic reactions (McGinnis and Tyring, 1996). Commensal fungi become pathogenic when the balance between fungi and immunity is disrupted. Fungal infections range from superficial skin or nail infections to life-threatening infections. *Candida*, which, along with *Aspergillus*, is responsible for more than 800 thousand life-threatening infections each year (Wheeler *et al.,* 2017).

2.4.2.1 Candida albicans

Candida albicans (*C. albicans*) is a polymorphic fungal organism. This means that the structure of *C. albicans* can switch between different yeast and hyphal growth forms (Jacobson *et al.,* 2012). Figure 11 represent different morphologies of *C. albicans*. The polymorphic structure of this fungus allows it to be highly adaptive. *C. albicans* can thrive in a wide range of conditions and colonize areas of varying pH, CO_2 levels, and nutrient availability (da Silva *et al.,* 2016). This contributes to the success of *C. albicans* as a pathogen. While Candida albicans is mostly beneficial to the human microbiome, it is also an opportunistic pathogen (Silva *et al*., 2016). *C. albicans* is responsible for 70% of fungal infections recorded around the world (Talapko *et al.,* 2021).

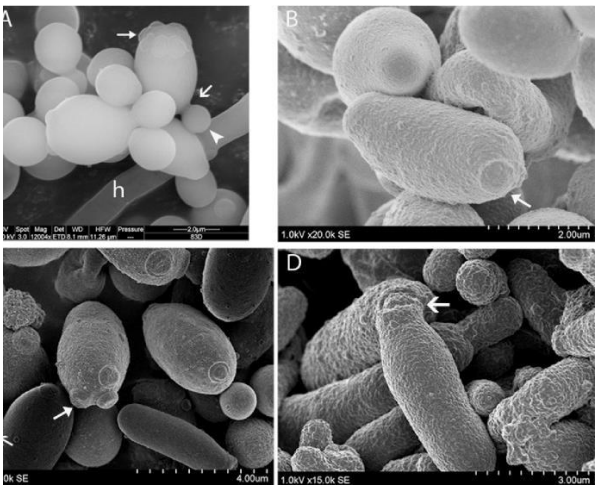


Figure 11: An image of *Candida albicans* under an electron scanning microscope. (Staniszewska, 2013).

Candida albicans colonization and infection occur in many human anatomical regions, including the oral cavity, gastrointestinal tract, and skin. Generally, *C. albicans* infections of the skin are superficial; however, in immune-compromised individuals, systemic candidiasis may occur, which often leads to death (Talapko *et al.,* 2021).

2.5 Elevated Carbon Dioxide

Carbon dioxide is important for the growth, development, and overall function of plants. This is because CO_2 is an important molecule for photosynthesis, which produces sugars and carbohydrates and forms important components of other important organic molecules that are necessary for plant survival (Rajashekar, 2018). Carbon dioxide is a limiting factor in photosynthesis, and hence the rise in atmospheric CO_2 concentration has been of increasing interest to agriculturists (Ibrahim and Jaafar, 2011).

$$\underset{\text{Carbon Dioxide}}{6\,CO_2} + \underset{\text{Water}}{6\,H_2O} \xrightarrow[Chlorophyll]{Sunlight} \underset{\text{Carbohydrate}}{C_6H_{12}O_6} + \underset{\text{Oxygen}}{6\,O_2}$$

Figure 12: Chemical equation of photosynthesis

Over the last few decades, urbanization and industrialisation have grown at an unprecedented rate. These processes are directly associated with a rise in CO_2 and greenhouse gas emissions (Steffen *et al.*, 2007). Prior to the industrialisation era, the atmospheric CO_2 concentration remained at a relatively steady concentration of 280 ± 10 parts per million (ppm) (Ganopolski *et al*, 2016). Geological studies have determined that the last time the atmospheric level reached above 320 ppm was 27 million years ago (Robertson, 2006). In the year 2020, the atmospheric CO_2 concentration was recorded at 412 ppm (Zhongming *et al.*, 2020). This rise in CO_2 levels has been exponential and is expected to continue rising.

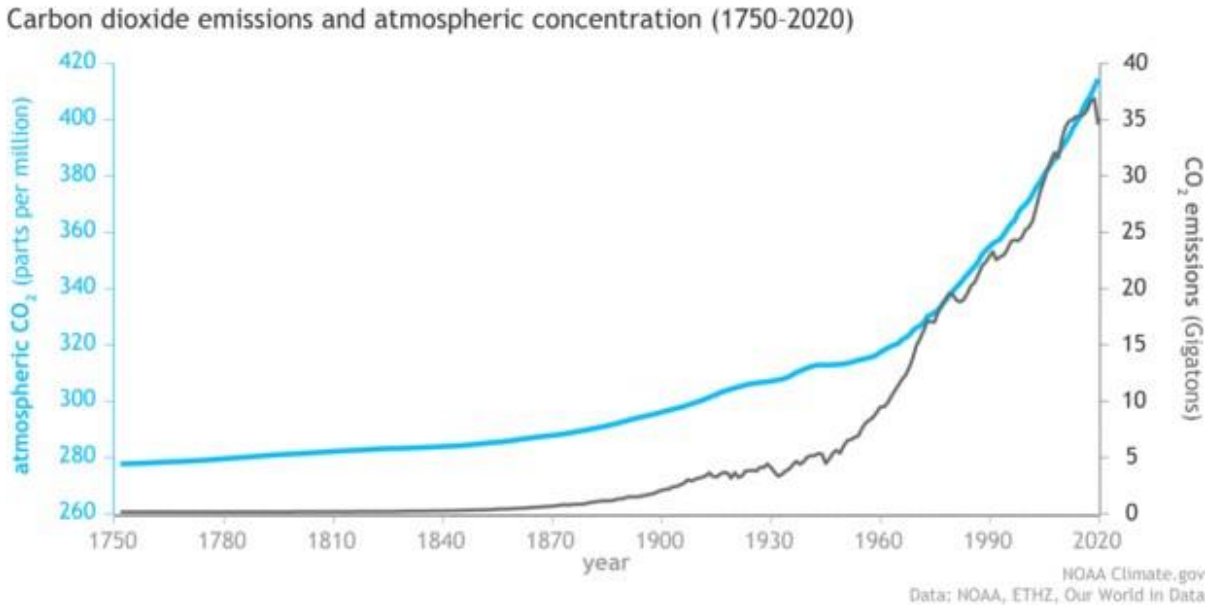


Figure 13: The average atmospheric carbon dioxide levels (ppm) recorded since 1750 at the Mauna Loa Observatory in Hawaii (Lindsey, 2022).

Based on the current energy sector still being heavily reliant on fossil fuels, atmospheric CO_2 levels are expected to reach 600 ppm by 2050 (Tan *et al.*, 2022).

A study performed by Robertson (2006) determined that by the year 2050, the atmospheric CO_2 toxic limit will be reached. At a CO_2 concentration of 600 ppm, occupants began to display symptoms of CO_2 poisoning. The effects of rising CO_2 levels can also be observed in plants.

Due to the importance of CO_2 in the photosynthetic pathway, it is clear that the rising levels of atmospheric CO_2 will have an effect on plants. An increase in CO_2 levels has been shown to increase the photosynthetic rate and lower the stomatal conductance, which in turn stimulates plant growth (Gamage *et al.*, 2018).

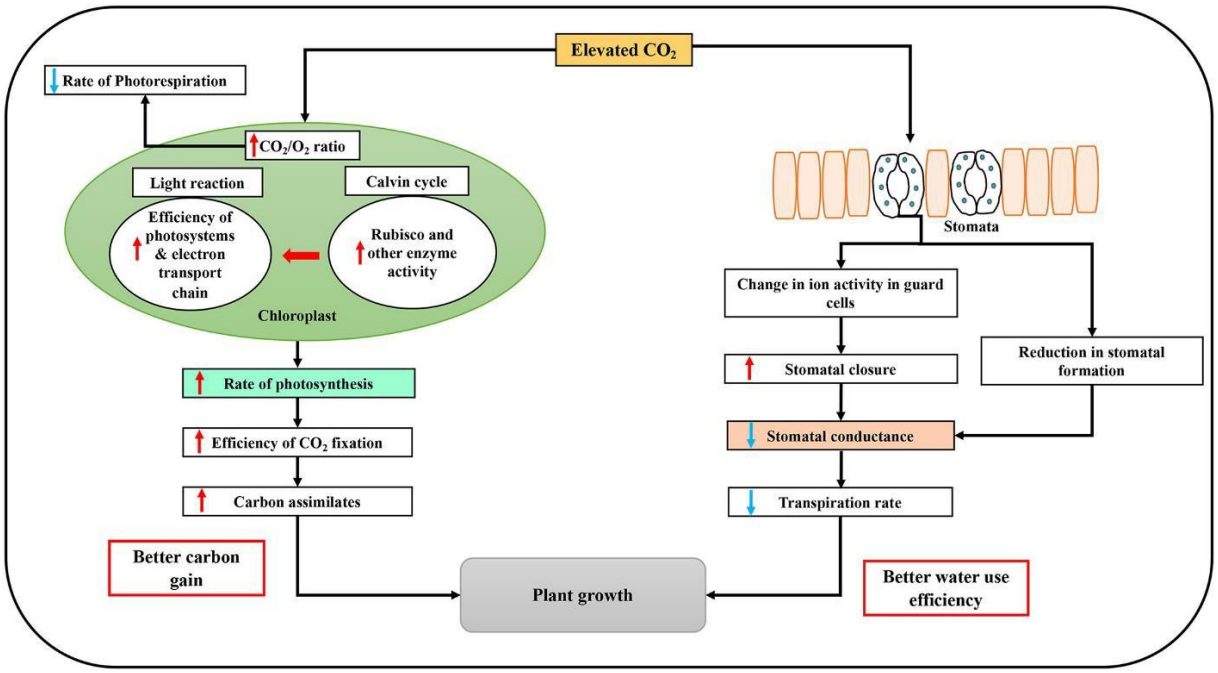


Figure 14: The effects of increased CO_2 on photosynthesis and stomatal conductance leading to an increase in plant growth (Gamage *et al*., 2018).

In a study performed by Drennan and Nobel (2000), all the CAM species investigated, including *P. afra*, displayed an increase in above- and below-ground biomass. This study determined that after CO_2 concentrations of 650–750 ppm, plants exhibited an average of a 35% increase in plant biomass after three months (Drennan and Nobel, 2010). While an increase in CO_2 directly affects the photosynthetic process, the rise in CO_2 can also affect the nutritional profile of the plant (Rajashekar, 2018).

There are studies that suggest that the exposure to increased CO_2 levels can lead to the accumulation of carbon-based phytochemicals, such as phenolics, phlobatannins, and

flavonoids (Rajashekar, 2018). The increase in carbon-based molecules (including carbohydrates) leads to a decrease in the partitioning and allocation of other molecules to plant organs like leaves. This affects the nutrition of the plant as there may be a decrease in the production of phytochemicals that require these limited chemicals, such as nitrogen (Gamage *et al.*, 2018). The reduced nutritional value will affect food security and may change the potency of medicinal plants.

Despite the increasing nutritional value experienced by tomatoes and broccoli, studies performed on rice indicated a decrease in nutritional value (based on the phytochemical profile) (Rajashekar, 2018). Currently, there are limited studies on the effects of increased CO_2 on the phytochemical profile of *P. afra*. The contrasting findings reported by various authors make the effects of rising CO_2 levels an important factor to consider with regards to *P. afra.*

Chapter 3: A physiological, Phytochemical and Antioxidant Assessment of the Leaves, Stems and Roots of *Portulacaria afra* in Response to Elevated CO_2 Concentration.

3.1 Introduction

Global atmospheric CO_2 concentrations have risen exponentially in the last five decades (Walia *et al*., 2022). The current reliance of the energy sector on the burning of fossil fuels suggests that the atmospheric CO_2 concentration is going to continue rising. Carbon Dioxide is essential for the growth, development, and survival of plants (Rajashekar, 2018). Thus, elevated CO_2 levels have been of growing interest to agriculturalists. One of the most consistent findings among studies conducted on plants exposed to elevated CO_2 concentrations (475-600 ppm) is that there is an increase in the photosynthetic rate (Taub, 2010). Increases of 44% and 12% have been recorded in the biomass of C_3 and C_4 plants, respectively (Weiss *et al*., 2010). However, there are fewer studies on how CAM plants (such as *P. afra*) may respond to elevated CO_2 concentrations. Previous research observed plant responses ranging from no response to both an increase and decrease in productivity (Weiss *et al*., 2010). Although CO_2 directly affects the photosynthetic process in plants, it also affects the nutritional profile of plants (Rajashekar, 2018). An increase in the photosynthetic rate, which may arise as a result of elevated CO_2 concentrations, will increase carbohydrate and sugar production in plants. The rise in carbohydrates in plants affects the carbon and nitrogen metabolism in plants, which ultimately leads to the unequal distribution of nutrients throughout the plant (Gamage *et al*., 2018). The unequal distribution may affect the medicinal properties of medicinal plants. A study that subjected *A. curassavica* (milkweed) to elevated CO_2 concentrations witnessed a decrease in lipophilic cardenolides, the chemical compound associated with the medicinal properties of the plant. Ultimately, the plant exhibited a decrease in medicinal properties when

exposed to elevated CO_2 concentrations (Decker *et al.*, 2018).

The rise in CO_2 concentrations may affect the phytochemical profile of plants, as the accumulation of carbon-based secondary metabolites affects the partitioning and allocation of other phytochemicals. Secondary metabolites are essential in the defence against biotic and abiotic stressors. Despite the chloroplasts and mitochondria generating oxidants within the plants' cells, plants are also vulnerable to oxidative stress as a result of environmental stressors such as UV-radiation, drought, and (potentially) the increase in elevated atmospheric CO_2 conditions (Kasote *et al.*, 2015). To avoid oxidative stress, plants react by producing secondary metabolites that display strong scavenging activity or by synthesizing antioxidants. Oxidative stress plays a large role in human diseases such as arthritis, cancer, and skin disorders (Halliwell *et al.*, 1997). Plants with a great phytochemical and antioxidant activity may be essential in treating and preventing skin disorders.

A plant’s ability to withstand or adapt to elevated CO_2 concentrations will make it an important resource in the future, when elevated CO_2 concentrations are continually rising.

3.2 Materials and methods

3.2.1 Plant Material

Portulacaria afra cuttings were collected from the University of the Witwatersrand (26.1929° S, 28.0305° E) in December 2021 through January 2022 (South Africa summer). The plants were then propagated and maintained in the Oppenheimer Life Sciences (OLS) greenhouse.

3.2.2 Plant propagation

Portulacaria afra plants that show no signs of wilting or disease selected to make cuttings. A pair of sterilized secateurs was used to collect 90 cuttings of *P. afra* from the University of the Witwatersrand grounds. Cuttings of 10-20 cm were collected and transferred into pots. A total of 90 pots, with a volume of 2–2.5 litres, were filled with Culterra cutting mix soil (loose and well-draining to allow for adequate air circulation and storage of water and nutrients). The cuttings which were placed in soil and firmed in place, were transferred to the OLS greenhouse. The potted plants are then transferred to the OLS greenhouse for four months, at ambient temperature to allow roots to form.



Figure 15: An image of *P. afra* plants in the OLS greenhouse.

The plants are allowed to grow for a period of three months in the greenhouse. These plants

were watered with 500 ml of water every day.

3.2.3 Treatment

A total of 90 plants were placed in Conviron® climate simulator to undergo treatment. The first 30 plants were placed in a chamber with a CO_2 concentration of 420 ppm as control samples, and all other conditions were ambient (27 ˚C temperature and 18 ˚C -to mimic a day and night-time, relative humidity of 60%).

The 420 ppm CO_2 concentration was chosen to simulate the current average global atmospheric CO_2 concentration at the time of this experiment (2022) according to the Intergovernmental Panel on Climate Change (IPCC).

The remaining 30 plants were placed in a separate chamber which had a CO_2 concentration of 600 ppm and ambient other conditions (27 ˚C temperature and 18 ˚C temperature to mimic day and night-time, and a relative humidity of 60%). The 600 ppm CO_2 concentration was used as literature predicts that the atmospheric CO_2 will reach 600 ppm by the year 2050 (Tan *et al.*, 2022).



Figure 16: An image of *P. afra* plants in a Conviron® chamber.

The plants were watered everyday. Each month, 10 plants were harvested, and each plant part

was subjected to analysis. This process continued for a period of three months (Harvest 1: harvest after 1 month, Harvest 2: harvest after 2 months and Harvest 3: harvest after 3 months). The remaining samples were left in the greenhouse, 30 samples were harvested once after 3 months. See schematic diagram of experimental treatment and harvesting frequency below (Figure 17).

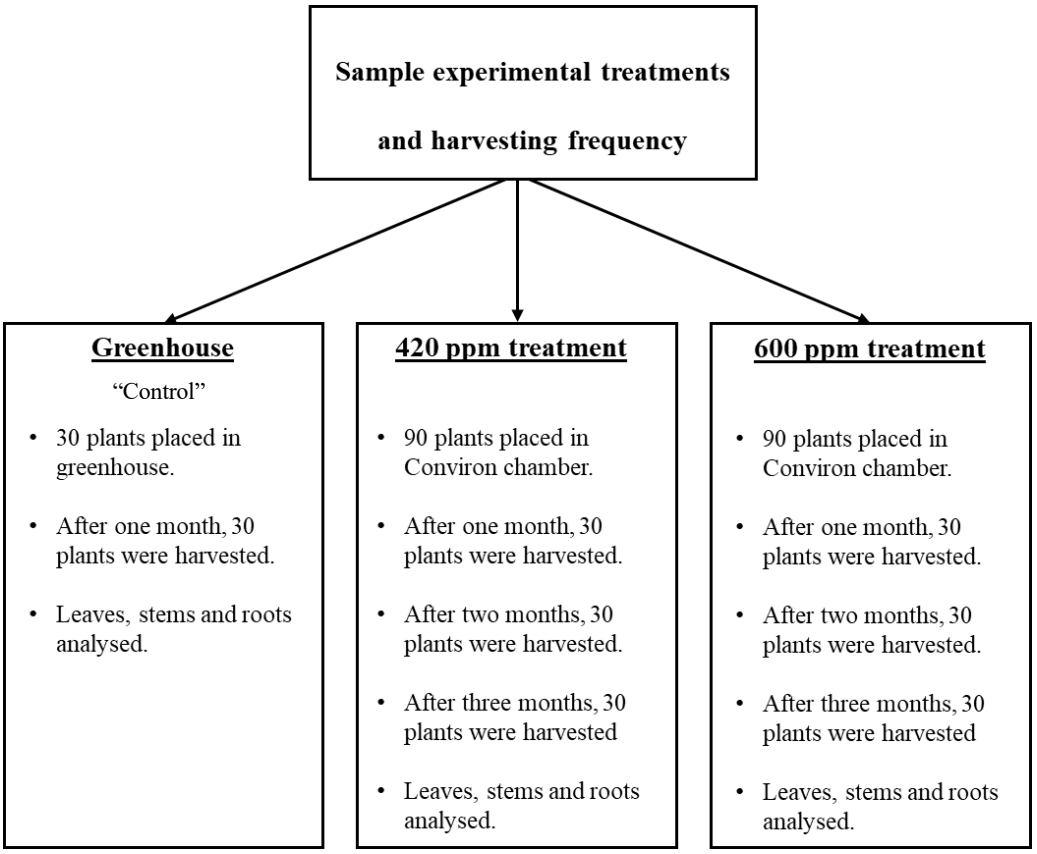


Figure 17: A schematic diagram of experimental treatment and harvesting frequency.

3.2.4 Physical parameters

In order to assess the effect of carbon dioxide on the physical aspects of *P. afra,* the following was calculated and then compared:

- The average weight of the fresh roots
- The average weight of 10 fresh leaves from 10 plants
- The average weight of the stems.

3.2.5 Chlorophyll Content

The fresh leaves (3 g) were mixed with10 ml of 80% (v/v) methanol and incubated in a dark, ambient temperature cupboard for 24 hours. The supernatant was then collected and placed in a cuvette. These cuvettes were placed in a spectrophotometer (Genesys 10S UV-VIS) at 645 nm and 663 nm to obtain the absorbance values. The chlorophyll content was

calculated using the following:

$$\text{Chlorophyll content} = 20.21 \times A_{645} + 8{,}02 \times A_{663}$$

Equation 1: Chlorophyll content

This method followed the procedure outlined by Wang *et al* (2017).

3.2.6 Crude Plant Extract

A crude plant extract was prepared using solvents in order to perform phytochemical and biological analyses (Pandey and Tripathi, 2014). The four solvents were used in this experiment were 100% distilled water at 40°C, 80% methanol (v/v), dichloromethane and hexane. These solvents were chosen due to the varying polarity.

Table 1: A list of solvents and their respective polarity adapted from Abubakar *et al*. (2020).

	Solvent	Polarity
1	*n*-Hexane	0.009
2	Dichloromethane	0.309
3	Methanol	0.762
4	Water	1.000

The plant material was placed in an air dryer at 40°C for the duration of a week, or until sufficiently dried. The dried plant samples were chosen over fresh samples due to the duration of the experiment. Experiments using fresh samples are often limited to a few hours. There was also no significant difference in the in the extraction of flavonoids and phenolics (Azwanida,

2015). Once sufficiently dried, the plant material was placed into a mechanical grinder to create powdered plant material. The decreased particle size allows for greater surface contact between the particles and the solvent, thus allowing greater extraction (Azwanida, 2015).

The crude plant extract was prepared by placing one gram of powdered root, stem, or leaf material into separate bottles with 25 ml of the respective solvents added into each bottle. The mixtures were placed on shaker for 48 hours. The hot water extracts were placed on a hot plate set at 40°C and stirred with a magnetic stirrer. The leaf, stem and root extracts created for all solvents were placed in the centrifuge for five minutes at 3000 rpm. All extracts were then filtered through a filter paper into a resealable vial. The pellets which remained were discarded whilst the vials were stored in the refrigerator at 4°C until the tests commenced.

3.2.7 Preliminary Phytochemical Screening

A qualitative analysis was conducted in order to determine the presence or absence of ten phytochemical groups in the stems, leaves and roots of *P. afra* using standard colour test methods according to Pandey and Tripathi (2014) and Ahmed *et al.* (2020).

3.2.7.1 Froth test for saponins

In a test tube, 0.5 ml of the crude plant extract was mixed with 5 ml of distilled water. The test tube was then vigorously shaken. Three drops of olive oil were placed inside the test tube and shaken vigorously. The presence of a stable foam indicated the presence of saponins.

3.2.7.2 Ferric chloride test for phenolics

In a test tube, 1 ml of the crude plant extract was mixed with five to ten drops of 10% ferric chloride ($FeCl_3$). The appearance of a green blue or violet colour was an indication of the presence phenolic compounds.

3.2.7.3 Hydrochloric acid test for flavonoids

In a test tube, 1 ml of the crude plant extract was mixed with three drops of hydrochloric acid (HCl). After a yellow colour was observed, a few drops of a dilute acid were then added. A colourless solution indicated presence of flavonoids.

2.2.4.4 Salkowski's test for glycosides

In a test tube, 0.5 ml of the crude plant extract was mixed with 2 ml of concentrated HCl. A reddish-brown colour indicated the presence of the steroidal aglycone part of the glycoside.

2.2.4.5 Bromine water test for tannins

In a test tube, 0.5 ml of the crude plant extract was mixed with 10 ml of bromine water. Discolouration of bromine water indicated the presence of tannins.

2.2.4.6 Test for terpenoids

In a test tube, 1 ml crude plant extract was mixed with 0.5 ml of chloroform ($CHCl_3$). A few drops of concentrated sulphuric acid (H_2SO_4) were added to the mixture. The formation of a reddish-brown precipitate indicated the presence of terpenoids.

2.2.4.7 Test for steroids

In a test tube, 2 ml of the crude plant extract was mixed with 2 ml of $CHCl_3$ and 2 ml of concentrated HCl. A reddish-brown ring at the junction indicated the presence of steroids.

2.2.4.8 Test for coumarins

In a test tube, 1 ml of crude plant extract was reacted with 1 ml of 10% NaOH in a test tube. The formation of a yellow colour indicated the presence of coumarins.

2.2.4.9 Test for phlobatannins

In a test tube, 1 ml crude plant extract was mixed with a few drops of concentrated HCl. The appearance of red colour indicated presence of phlobatannins.

2.2.4.10 Test for volatile oils

In a test tube, 1 ml of the crude plant extract was added to 0.2 ml solution of 1% NaOH. The presence of a precipitate indicated presence of volatile oils.

3.2.8 Quantitative Phytochemical Screening

3.2.8.1 Total flavonoid content

An aluminium chloride colorimetric assay was used to determine the total flavonoid content in the leaves stems and roots of *P. afra* (Pakade *et al.,* 2013). In a test tube, 3 ml of the crude plant extracts was mixed with 4 ml of 5% sodium nitrate. The mixture was incubated for five minutes. Following the incubation period, 3 ml of 10% aluminium chloride was added to the test tube and incubated for an additional six minutes. A sodium hydroxide solution with a volume of 2 ml was then added to the test tube. The test tube was topped with 0.7 ml of distilled water to create a mixture of 10 ml. The vials were then placed in a dark cupboard and allowed to incubate for an hour at room temperature. The absorbance of each mixture was measured at 510 nm using the Genesys 10S UV-VIS spectrophotometer. The test was conducted in triplicates.

A calibration curve was created using a Quercetin standard. The total flavonoid content was calculated using the following calculation obtained from the calibration curve.

$$Y=0.2388x-0.0019$$

Equation 2: Total flavonoid content

3.2.9 Antioxidant Assay

3.2.9.1 1, 1-diphenyl-2-picrylhydrazyl scavenging assay

The stable radical DPPH was used to determine the scavenging activity and hence, antioxidant activity of the plant parts of *P. afra* (de Torre *et al.,* 2019). The crude plant extract was placed

in capped test tubes at varying concentrations (10-50 µl) and mixed with 700 µl of the DPPH work solution. In addition, 80% methanol (v/v) was used to create a mixture of 1 ml. The test tubes were incubated in a dark cupboard, at room temperature for 45 minutes. Following the incubation period, the mixtures were placed into cuvettes which were placed within a spectrophotometer (Genesys 10S UV-VIS). The absorbance was measured at 517 nm against a blank. The test was then triplicated while the percentage of inhibition was calculated using the following formular below.

$$\% \text{ Inhibition of DPPH radical} = ([A_{br} - A_{ar}]/A_{br}) \times 100$$

Equation 3: Percentage inhibition of DPPH radical

Where A_{br} is the absorbance of the control and A_{ar} is the absorbance of the sample.

The concentrations were then plotted against percentage inhibition values, in order to calculate the IC_{50}.

3.2.9.1.2 Hydrogen peroxide assay

A 40mM solution of hydrogen was prepared in a phosphate buffer (pH 7.4). The crude plant extracts (10-50 µL) were placed in vials and mixed with 600 µL of the hydrogen peroxide solution. The vials were then place in a dark cabinet and incubated for 10 minutes. The absorbance of the mixtures was determined using a spectrometer (Genesys 10S UV-VIS) at 230 nm. A phosphate buffer without the hydrogen peroxide solution was used as the blank. This test was completed three times, to have three replicates. The different absorbances were recorded. The percentage of hydrogen peroxide scavenging activities was calculated using:

$$\% \text{ Scavenged } [H_2O_2] = ([A_{br} - A_{ar}]/A_{br}) \times 100$$

Equation 4: Percentage inhibition of H_2O_2 radical.

Where A_{br} is the absorbance of the control and A_{ar} is the absorbance of the sample.

The concentrations were then plotted against percentage inhibition values (from the equation above) from which IC_{50} values were calculated.

3.2.10 Data analyses

A qualitative and quantitative analyses was performed of the phytochemical presence of the phytochemical's presence in the study. The qualitative study involved observing the vials for physical changes in the appearance and comparing it to the expected outcomes suggested by literature.

The data obtained from the antioxidant assays were stored and manipulated using Microsoft Excel (Microsoft Corporation, 2018). The scavenging activity (%) was plotted in a scatter plot against the relative concentrations used. A linear regression analysis was performed to determine the IC_{50} value. These values were plotted as a line graph with markers. R-studio was used to perform a repeated measures ANOVA test to determine whether there was a significance between the treatments. This was repeated for all plant parts. This was followed by a Tukey post-hoc test to determine where the significant differences were. All statistical tests were conducted at a significant level of 0.05.

3.3 Results

3.3.1 Physical parameters and physiological analysis

Portulacaria afra plant samples which were exposed to 420 ppm and 600 ppm treatments were monitored for any significant visual changes between the harvests (Table 2). There is an increase in leaf foliage in both treatments, however, samples which were exposed to 600 ppm exhibited a much greater increase in leaf foliage. The leaf foliage is denser in the plant which was exposed to 600 ppm after harvest 3. The height of the plants did not exhibit a significant

change in either treatment. The visual clues correlate with the weight changes measured of the plants.

Table 2: A table displaying images of *P. afra plants* exposed to 420 ppm and 600 ppm after harvest 1 and harvest 3.

	Harvest 1*	**Harvest 3***
420 ppm		
600 ppm		

**Harvest 1: harvest after 1 months*

**Harvest 3: harvest after 3 months*

The visual changes observed in table 2 correlate with the quantitative changes exhibited in table 3.

The weight of the leaves increased in the plant samples which were exposed to both 420 ppm and 600 ppm (Table 3). The leaves exposed to 420 ppm exhibited a weight increase of 23.6%

($P>0.05$), whereas the weight of the leaves exposed to 600 ppm CO_2 exhibited a 65.7% increase between harvest 1 (harvest after one month) and harvest 3 (harvest after three-months) ($P<0.05$). The increase in the weight of leaves of plants exposed to 420 ppm was not statistically significant, whereas the weight of the leaves exposed to 600 ppm exhibited a significant increase in weight in harvest 2 and 3, when compared to the control sample.

No significant increase was detected in the weight of either - 420 ppm or 600 ppm, stem sample. The plants showed no clear increase in height in either sample, which correlates to insignificant increase in the average weight of the stems.

The roots of *P. afra* exhibited the next highest change in biomass. The samples exposed to elevated CO_2 concentration exhibited a 39% increase ($P<0.05$). Despite the almost 40% increase in weight in the samples which were exposed to 600 ppm, no statistical significance was determined in either harvest.

The chlorophyll content of the leaves remained relatively constant in both treatments across all three harvests ($P>0.05$).

Table 3: The recorded weight of the leaves, stem, and root of *P. afra* after each harves

	Greenhouse	**Harvest 1***		**Harvest 2***		**Harvest 3***	
	Control	420 ppm	600 ppm	420 ppm	600 ppm	420 ppm	600 ppm
Weight of wet leaves (mean± SD)	0.43±0.62	0.38±0.43	1.08±0.63	0.88±0.03	1.69±0.71*	1.25±0.07	1.79±0.14*
Weight of stems (mean± SD)	23.32±0.30	23.02±1.20	27.92±12.43	27.63±5.34	30.24±12.21	27.01±3.98	32.12±2.22
Weight of roots (mean± SD)	20.9±0.43	22.9±7.78	25.9±5.37	24.99±5.24	32.91±12.03*	25.35±1.35	36.25±3.46*
Chlorophyll Content (mg/g ± SD)	5.57±0.43	5.68±0.60	5.99±0.31	5.61±0.42	6.88±0.76	6.44±0.34	6.90±0.53

"*" indicates a statistical significance against the greenhouse extracts determined using Tukey ad-hoc test.

**Harvest 1: harvest after 1 months*

**Harvest 2: harvest after 2 months*

**Harvest 3: harvest after 3 months*

3.3.2 Phytochemical analysis

A qualitative phytochemical analysis was used to determine the presence or absence of ten phytochemical groups, namely: saponins, flavonoids, glycosides, phenolics, tannins, terpenoids, steroids, coumarins, phlobatannins and volatile oils. The intensity of the change was used to preliminarily determine whether the phytochemical group was absent, present, moderately present or exhibited high presence. An example of change in intensity can be seen in figure 18 below, with intensity increasing from right to left.

A qualitative test was used to determine the significance of the change in flavonoid content in the plant parts of *P. afra*.

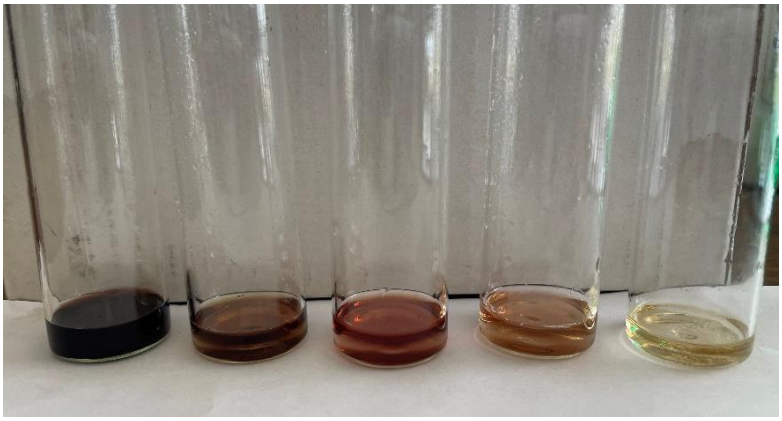


Figure 18: An image depicting the difference in intensity among the varying crude plant extracts against the glycoside test.

3.3.3 Preliminary phytochemical screening

A great phytochemical presence was detected in the leaves (Table 3), stems (Table 4) and roots (Table 5) of *P. afra.*

The leaves extracts exhibited the highest phytochemical presence among all three plant parts, with eight out of ten of the phytochemical groups being recorded as present in the control sample (greenhouse). The control sample experienced the highest phytochemical presence in the coumarins group. In contrast, the flavonoids and phlobatannins were recorded as absent

among all four greenhouse extracts. The leaves exposed to the 420-ppm treatment exhibit a phytochemical presence similar to the leaves in the control group.

The samples which were exposed to 420 ppm treatment experienced a general increase in phytochemical presence among the saponins, phenolics, and coumarins after harvest 1. However, after harvest 2 and harvest 3, the phytochemical presence returned to a presence similar to/ or less than the greenhouse samples. Like the greenhouse samples, the flavonoids and phlobatannins group was recorded as absent among all three harvests of the 420 ppm samples. The samples which were exposed to 420 ppm ~~samples~~ experienced a lower phytochemical presence than the samples exposed to 600 ppm, in five out of the ten phytochemical groups examined.

The leaves extracts which were exposed to 600 ppm exhibited an overall, higher phytochemical presence than samples exposed to the greenhouse and 420 ppm samples. Terpenoids are the only phytochemical group that decreased from “present” in the control to “absent” in harvests 2 and 3. Phytochemical groups, such as, saponins, glycosides, tannins, coumarins, and volatile oils, all increased after exposure elevated CO_2 concentration. The most notable increase in phytochemical presence was in the flavonoid group.

Flavonoids were recorded as absent in the greenhouse samples, as well as the samples exposed to 420 ppm. However, in samples exposed to 600 ppm, the flavonoid group was recorded as present after 60 days (Harvest 2) and reached “moderate presence” after 60 days (Harvest 3).

Table 4: A heat map representing the presence of the different phytochemical groups absent or present in the leaves in the green house and the 420 ppm and 600 ppm treatment.

		Leaves							
Phytochemical		**420 ppm**				**600 ppm**			
		Hex	**Di**	**Meth**	**HW**	**Hex**	**Di**	**Meth**	**HW**
Saponins	**Greenhouse**								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Flavonoids	**Greenhouse**								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Glycosides	**Greenhouse**								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Phenolics	**Greenhouse**								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Tannins	**Greenhouse**								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Terpenoids	**Greenhouse**								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Steroids	**Greenhouse**								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Coumarins	**Greenhouse**								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Phlobatannins	**Greenhouse**								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Volatile oils	**Greenhouse**								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								

Key:



Absent **Present** **Moderate Presence** **High presence**

*(Hex- Hexane; Di: Dichloromethane; Meth- Methanol; HW- Hot water)

**Harvest 1: harvest after 1 months*
**Harvest 2: harvest after 2 months*
**Harvest 3: harvest after 3 months*

A great phytochemical presence was detected in the methanolic and hot water stem extracts, with eight out of the ten phytochemical groups being marked as present in the control samples. The hexane, and dichloromethane extracts only detected a presence of four and five phytochemical groups, respectively. The differences between the greenhouse extracts and those exposed to 420 ppm and 600 ppm are less pronounced in the stems than they were in the leaves. A slight increase in presence can be seen in the tannins and coumarins.

Similar to the leaves' extracts, a notable difference occurred in the flavonoids group. In the greenhouse and 420 ppm-samples, the flavonoids were marked as absent, however the flavonoids were detected in the methanolic, 600 ppm samples in harvest 2 and 3, and in the hot water, 600 ppm samples in harvest 3.

Table 5: A heat map representing the presence of the different phytochemical groups absent or present in the stems in the green house and the 420 ppm and 600 ppm treatment.

		Stems							
		420 ppm				600 ppm			
		Hex	Di	Meth	HW	Hex	Di	Meth	HW
Saponins	Greenhouse								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Flavonoids	Greenhouse								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Glycosides	Greenhouse								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Phenolics	Greenhouse								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Tannins	Greenhouse								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Terpenoids	Greenhouse								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Steroids	Greenhouse								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Coumarins	Greenhouse								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Phlobatannins	Greenhouse								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								
Volatile oils	Greenhouse								
	Harvest 1*								
	Harvest 2*								
	Harvest 3*								

Key:

Absent **Present** **Moderate Presence** **High presence**



(Hex- Hexane; Di: Dichloromethane; Meth- Methanol; HW- Hot water)

**Harvest 1: harvest after 1 months*
**Harvest 2: harvest after 2 months*
**Harvest 3: harvest after 3 months*

The root extracts exhibited the weakest phytochemical presence among all three plant parts. A lower number of phytochemicals were recorded as present, and there is a lower intensity of the phytochemicals which were present in the root extracts.

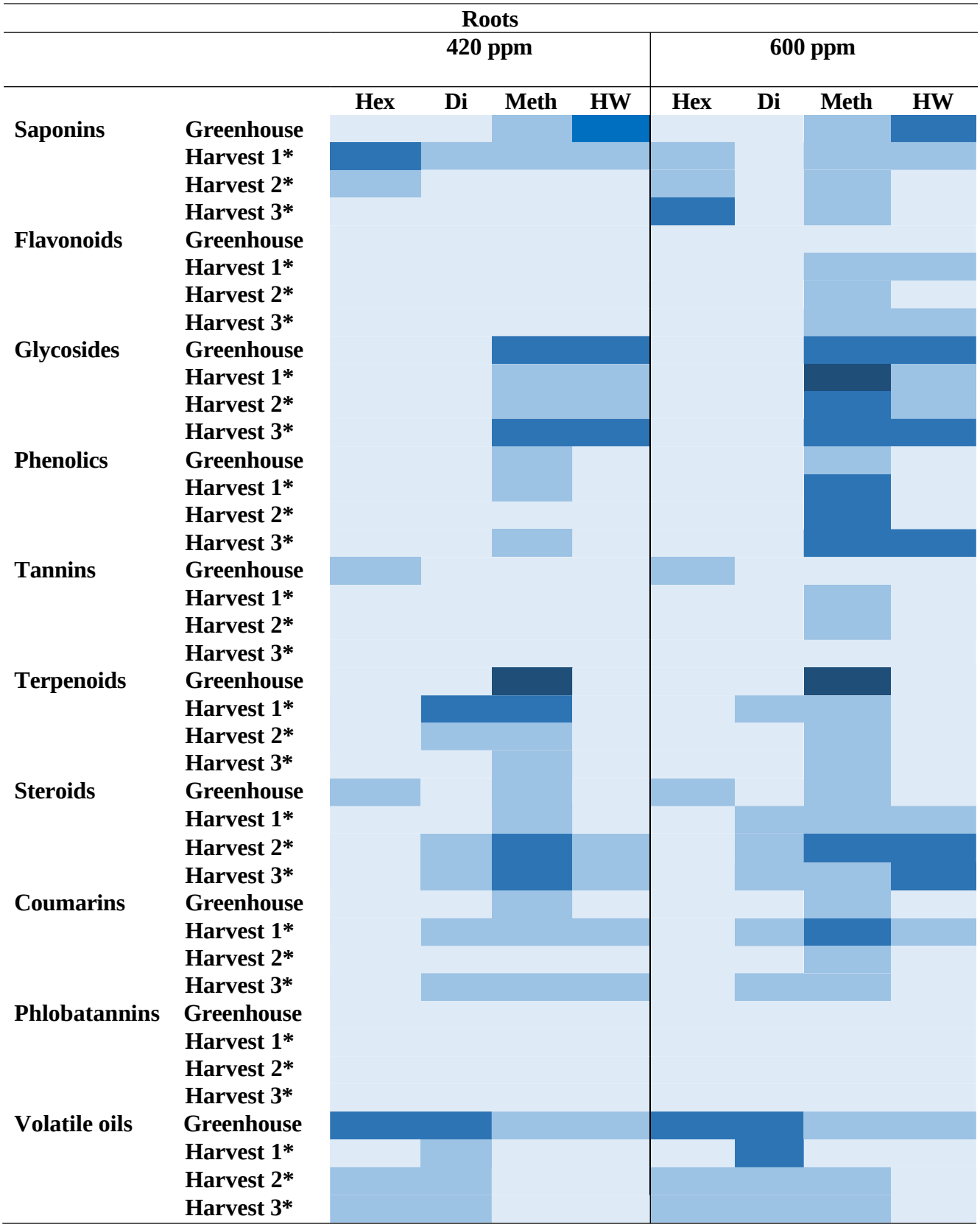
The methanolic greenhouse samples, exhibited the highest phytochemical presence in the root extracts, with seven out of the ten phytochemical groups being recorded as present. The terpenoids were in "high presence" in the control sample. The coumarins, which recorded as "high presence" in the leaves and stems were only marked as present in the roots.

The samples which were exposed to 420 ppm experienced a decrease in the phytochemical presence. The phytochemical groups saponins, terpenoids and tannins were recorded as present in the greenhouse extracts, however, after exposure to 420 ppm the samples were recorded as absent.

Despite the phytochemical presence in the roots being relatively weak, after exposure to elevated CO_2 concentrations, the presence stayed the same or increased. The phenolics and flavonoids group exhibited the greatest increase in the roots extracts which were exposed to 600 ppm.

Similar to the leaves and stems, the flavonoids were recorded as absent the greenhouse and 420 ppm extracts. However, after 30 days (harvest 1) of exposure to 600 ppm, flavonoids were present in the methanolic and hot water extracts. The flavonoids remained present throughout harvest 2 and harvest 3.

Table 6: A heat map representing the presence of the different phytochemical groups absent or present in the roots in the greenhouse and the 420 ppm and 600 ppm treatment.



Key:



Absent **Present** **Moderate Presence** **High presence**

*(Hex- Hexane; Di: Dichloromethane; Meth- Methanol; HW- Hot water)

**Harvest 1: harvest after 1 months*
**Harvest 2: harvest after 2 months*
**Harvest 3: harvest after 3 months*

3.3.4 Total flavonoid Content

The preliminary phytochemical screening recorded the flavonoids as being absent across all three plant parts in the greenhouse extracts, as well as those exposed to the 420 ppm. The qualitative study of flavonoids determined that the flavonoids were not absent, but in a quantity which may have been in too low of a quantity to appear in the qualitative analysis. In the control samples, the methanolic leaves exhibited the highest flavonoid content of 6,48 ± 0,32 (table 6). However, these samples were still marked as absent in the qualitative analysis. The samples which were exposed to 420 ppm, which were also noted as absent, were in a low quantity. All four extracts (Methanol, dichloromethane, hexane, and hot water) which were exposed to 420 ppm, exhibited an initial decrease in flavonoid content in harvest 1. However, after harvests 2 and 3, the flavonoid content returned to a content similar to those of the greenhouse samples. A similar trend was observed in the qualitative study among the saponins, phenolics and coumarins which exhibited an increase in presence after harvest 1, before returning to a presence similar to the greenhouse samples (table 3). The changes exhibited during harvest 1 may allude to an adaptation period for the plant.

In the samples which were exposed to 600 ppm, a significant increase in flavonoid content was observed in the samples which appeared “present” in the qualitative analysis. A significant increase in flavonoid content was observed in the methanolic, hexane and hot water leaf extracts. The hot water extracts exhibited a significant increase after harvest 1. The highest

flavonoid content was recorded in the methanolic leaf extracts which had been exposed to 600 ppm (19.27 ± 0,10 mg QE/g).

The stems also exhibited a significant increase in flavonoid content in two of the four extracts exposed to elevated CO_2 concentrations. The methanolic extracts exhibited a significant increase in flavonoid content after harvest 2 and 3, compared to the greenhouse extracts. The dichloromethane extracts which were exposed to 600 ppm exhibited a significant increase in flavonoid content, however, after harvest 3 the value decreased again.

The roots displayed the lowest flavonoid content among all three plant parts. The root extracts which were exposed to 420 ppm displayed no significant change in flavonoid content compared to the greenhouse extracts. The extracts which were exposed to 600 ppm did exhibit a significant increase in the methanolic and hot water extracts. The hot water extracts exhibited a significant increase after harvest 2, whereas the methanolic extracts only exhibited an increase after harvest 3.

Table 7: The total flavonoid content in the extracts of *P. afra* leaves which were exposed to 420 ppm (A) and 600 ppm treatment (B) in comparison to the control (Greenhouse).

	Total flavonoid content (mg QE/g)							
Leaves	**Methanol**		**Dichloromethane**		**Hexane**		**Hot water**	
Greenhouse	6.48 ± 0.32		2.12 ± 0.87		1.24 ± 0.54		2.51 ± 0.43	
	420 ppm	**600 ppm**	**420 ppm**	**600 ppm**	**420 ppm**	**600 ppm**	**420 ppm**	**600 ppm**
Harvest 1*	3.79 ± 0.19	9.38 ± 0.45	1.62 ±0.39	6.33 ± 0.77	1.09 ± 0.37	2.54 ± 0.72	2.20 ± 0.67	12.65 ± 0.19*
Harvest 2*	4.88 ± 0.43	13.80 ± 0.29*	3.45 ± 0.67	6.87 ± 0.68	1.92 ± 0.34	4.68 ± 0.69	3.79 ±0.12	14.28 ± 0.31*
Harvest 3*	4.79 ± 0.11	19.27 ± 0.10*	3.69 ± 0.34	4.65 ± 0.61	3.51 ± 0.39	7.20 ± 0.83*	4.31 ± 0.19	17.81 ± 0.18*

"*" indicates a statistical signifance against the greenhouse extracts determined using Tukey ad-hoc test.

**Harvest 1: harvest after 1 months, *Harvest 2: harvest after 2 months, *Harvest 3: harvest after 3 months*

Table 8: The total flavonoid content in the stems extracts of *P. afra* which were exposed to 420 ppm and 600 ppm treatment in comparison to the control (Greenhouse).

	Total flavonoid content (mg QE/g)							
Stems	**Methanol**		**Dichloromethane**		**Hexane**		**Hot water**	
Greenhouse	3.79 ± 0.13		3.15 ± 0.79		1.12 ± 0.54		2.69 ± 0.32	
	420 ppm	**600 ppm**	**420 ppm**	**600 ppm**	**420 ppm**	**600 ppm**	**420 ppm**	**600 ppm**
Harvest 1*	4.02 ± 0.23	6.04 ± 0.22	1.43 ± 0.68	2.87 ± 0.18	1.19 ± 0.48	3.24 ± 0.43	3.79 ± 0.65	6.78 ± 0.54
Harvest 2*	5.66 ±0.22	12.13 ± 0.61*	2.98 ± 0.69	9.32 ± 0.87*	2.36 ±0.54	4.17 ± 0.38	4.21 ± 0.34	7.06 ± 0.32
Harvest 3*	5.78 ±0.39	17.88 ± 0.32*	3.14 ± 0.45	8.61 ± 0.69	3.78 ± 0.65	6.89 ± 0.31	3.99 ± 0.21	16.78 ± 0.17*

"*" indicates a statistical signifance against the greenhouse extracts determined using Tukey ad-hoc test.

**Harvest 1: harvest after 1 months, *Harvest 2: harvest after 2 months, *Harvest 3: harvest after 3 months*

Table 9: The total flavonoid content in the roots extracts of *P. afra* which were exposed to 420 ppm (A) and 600 ppm treatment (B) in comparison to the control (Greenhouse).

	Total flavonoid content (mg QE/g)							
Roots	**Methanol**		**Dichloromethane**		**Hexane**		**Hot water**	
Greenhouse	4.99 ± 0.56		2.50 ± 0.32		1.78 ± 0.43		4.57± 0.54	
	420 ppm	**600 ppm**	**420 ppm**	**600 ppm**	**420 ppm**	**600 ppm**	**420 ppm**	**600 ppm**
Harvest 1*	5.01 ± 0.34	9.07 ± 0.41*	2.13 ± 0.41	2.78 ± 0.27	3.22 ± 0.62	1.93 ± 0.42	5.69 ± 0.87	8.67 ± 0.65
Harvest 2*	4.32 ± 0.40	8.19 ± 0.12	4.32 ± 0.62	4.67 ± 0.54	4.67 ± 0.33	3.43 ± 0.37	5.87 ± 0.53	9.86 ± 0.42*
Harvest 3*	6.37 ± 0.13	11.63 ± 0.23*	5.65 ± 0.68	6.12 ± 0.34	5.02 ± 0.28	4.60 ± 0.87	4.12 ± 0.51	11.92 ± 0.49*

"*" indicates a statistical signifance against the greenhouse extracts determined using Tukey ad-hoc test.

**Harvest 1: harvest after 1 months, *Harvest 2: harvest after 2 months, *Harvest 3: harvest after 3 months*

3.3.3 Antioxidant activity

A strong antioxidant activity was detected in the leaves of *P. afra* (Figure 19; Figure 20). The methanolic and hot water extracts consistently exhibit the strongest scavenging activity, with IC_{50} values consistently below the upper limit of 10 mg/ml. This trend can be seen in the samples exposed to 420 ppm and 600 ppm. The methanolic and hot water extracts exposed to 600 ppm, exhibited a stronger antioxidant activity than the 420 ppm counterparts. The hexane and dichloromethane extracts which were exposed to the elevated CO_2 concentration exhibited a respective decrease and increase in scavenging activity against DPPH.

The leaves extracts exhibited a stronger antioxidant activity against H_2O_2 than DPPH. The scavenging activity of the leaves remained relatively strong against H_2O_2, after exposure to elevated CO_2, with all extracts exhibiting an IC_{50} value below the acceptable upper limit. The dichloromethane extracts showed a significant increase in scavenging activity against H_2O_2.

The stems of *P. afra* performed similarly to the leaves against DPPH and H_2O_2 (Figure 21; Figure 22). The dichloromethane extracts, exposed to the 600-ppm treatment exhibited a significant increase in scavenging activity against DPPH. The stems extracts exhibited a stronger antioxidant activity against H_2O_2 than DPPH. Three of the four extracts remained below the upper limit in the extracts which were exposed to elevated CO_2 concentrations.

The root extracts exhibited the most varying antioxidant activity between the 420 ppm treatment and 600 ppm treatment of all the plant parts (Figure 23; Figure 24). The extracts ~~which were~~ exposed to 420 ppm exhibited IC_{50} values which were less consistent between harvests than those exposed to 600 ppm. The extracts which were exposed to elevated CO_2 concentration exhibited a stronger scavenging activity against both DPPPH and H_2O_2. The methanolic root extracts were consistently strong and below the upper limit.

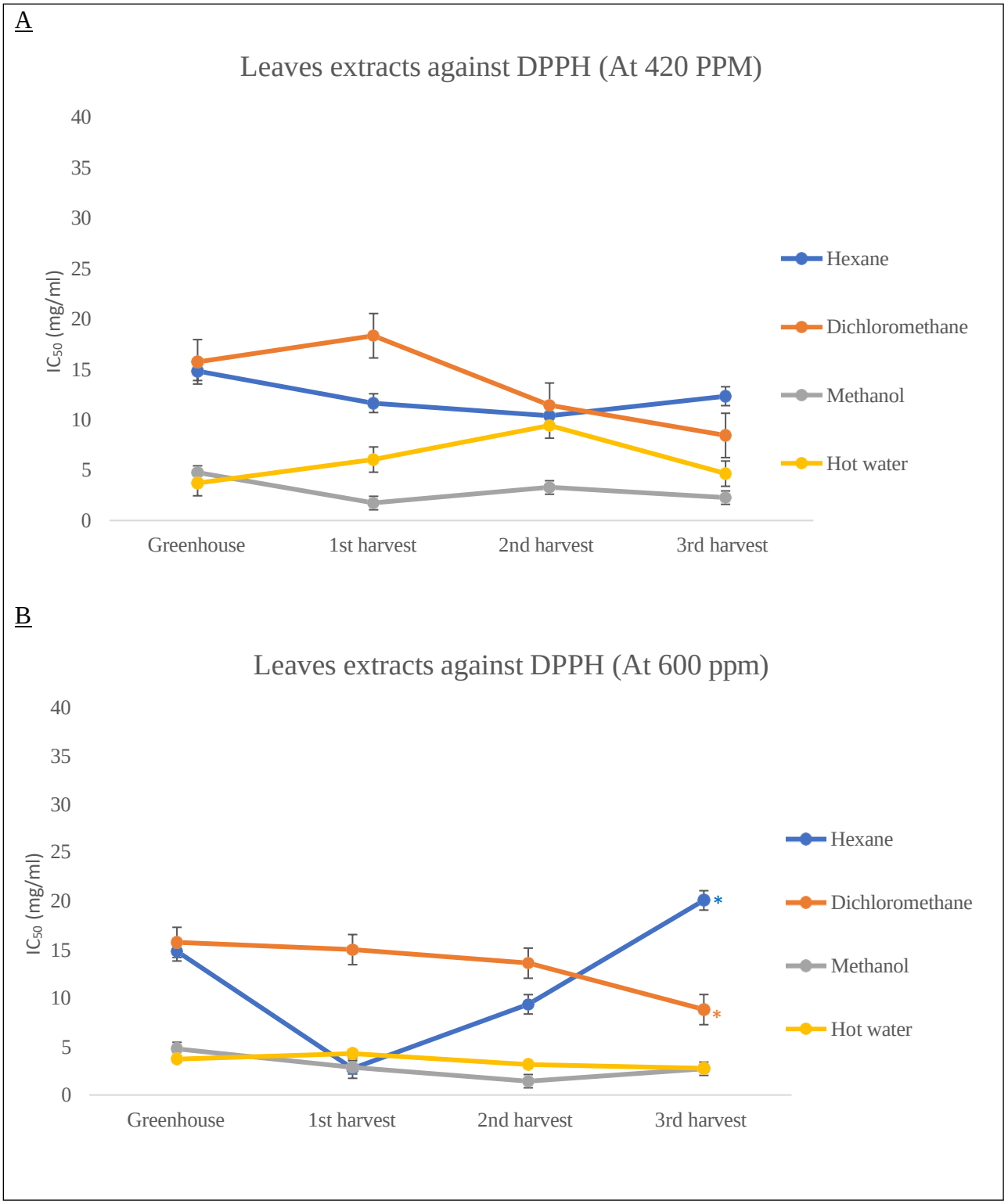


Figure 19: A graph representing the IC_{50} values of the leaves' extracts at 420 ppm (A) and 600 ppm (B) against DPPH ($P<0.05$). "*" represents a statistical signific result against greenhouse extracts (Control). Harvest 1: harvest after 1 months, Harvest 2: harvest after 2 months, Harvest 3: harvest after 3 months.

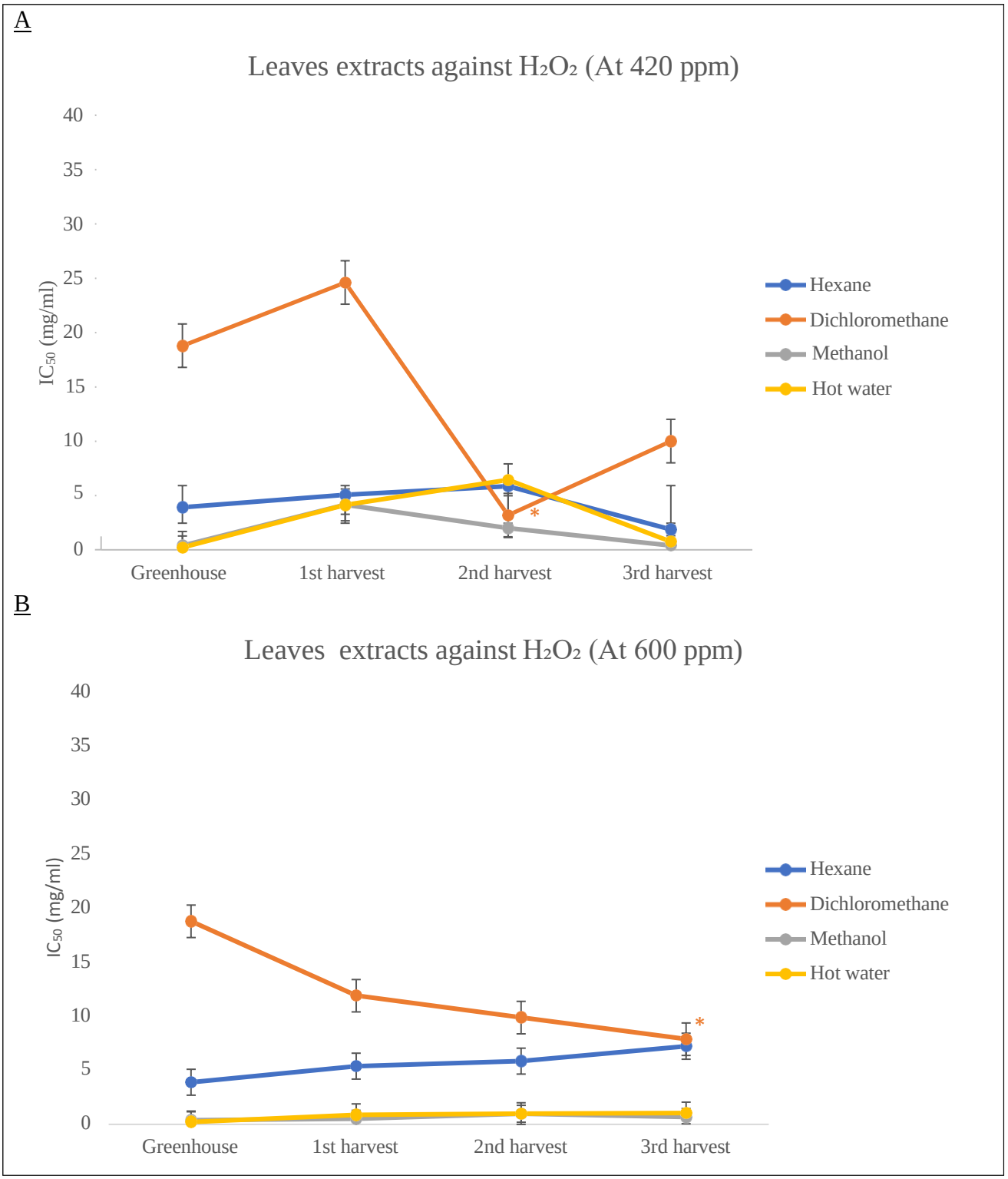


Figure 20: A graph representing the IC_{50} values of the leaves' extracts at 420 ppm (A) and 600 ppm (B) against H_2O_2 ($P<0.05$). "*" represents a statistical signific result against greenhouse extracts (Control). Harvest 1: harvest after 1 months, Harvest 2: harvest after 2 months

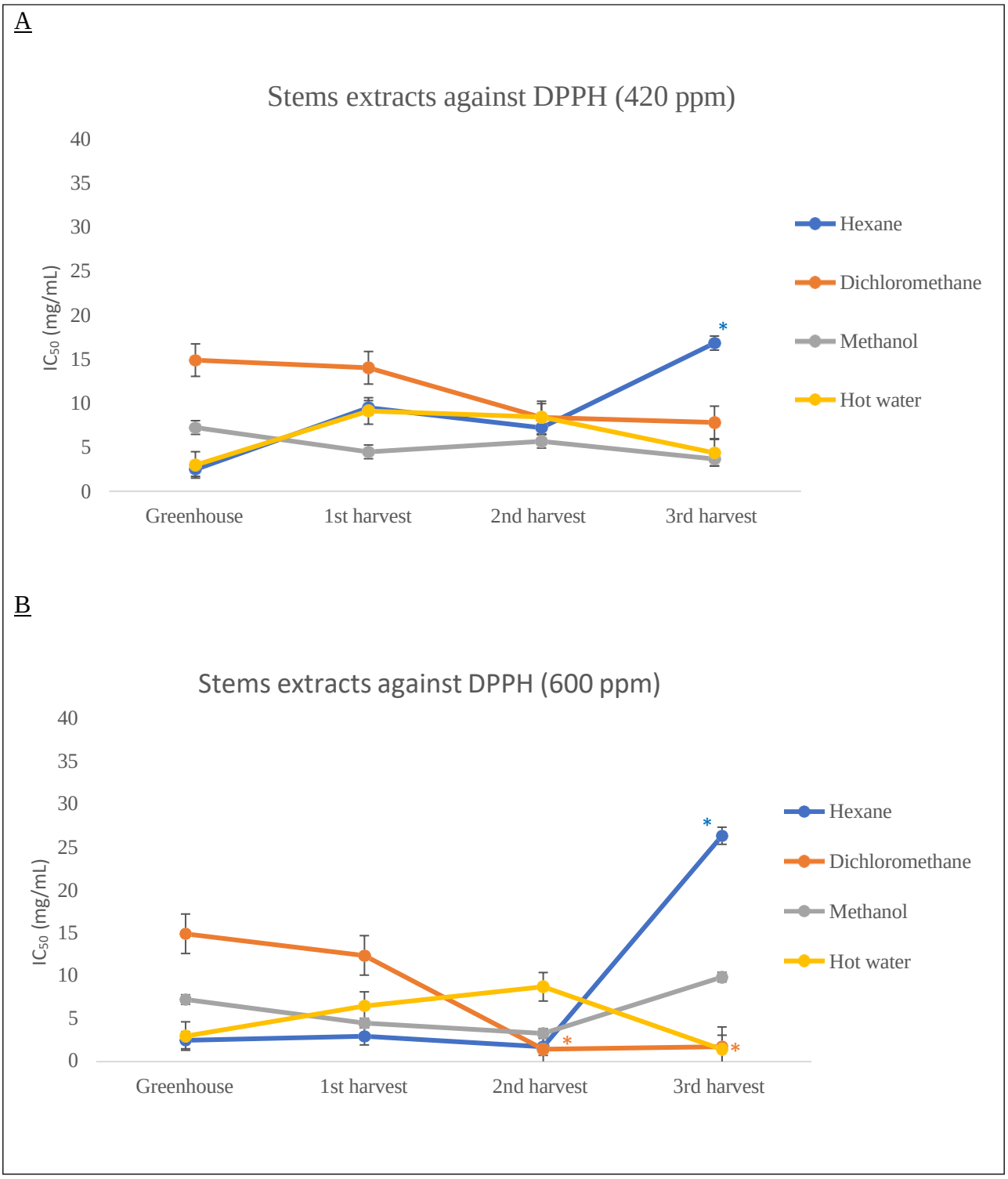


Figure 21: A graph representing the IC_{50} values of the stems extracts at 420 ppm (A) and 600 ppm (B) against DPPH ($P<0.05$). "*" represents a statistical signific result against greenhouse extracts (Control). Harvest 1: harvest after 1 months, Harvest 2: harvest after 2 months, Harvest 3: harvest after 3 months.

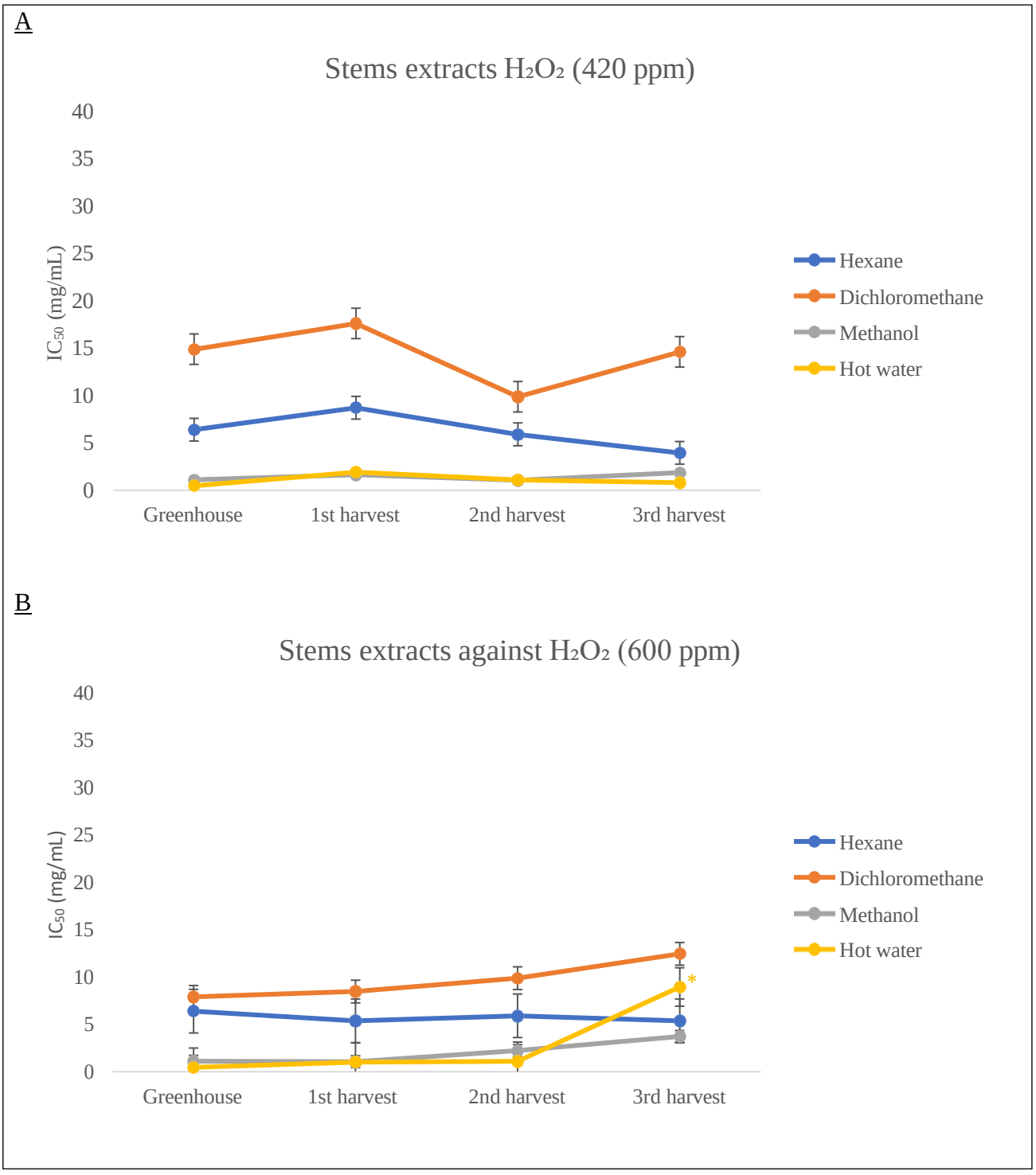


Figure 22: A graph representing the IC_{50} values of the stems extracts at 420 ppm (A) and 600 ppm (B) against H_2O_2 ($P<0.05$). "*" represents a statistical signific result against greenhouse extracts (control). Harvest 1: harvest after 1 months, Harvest 2: harvest after 2 months, Harvest 3: harvest after 3 months.

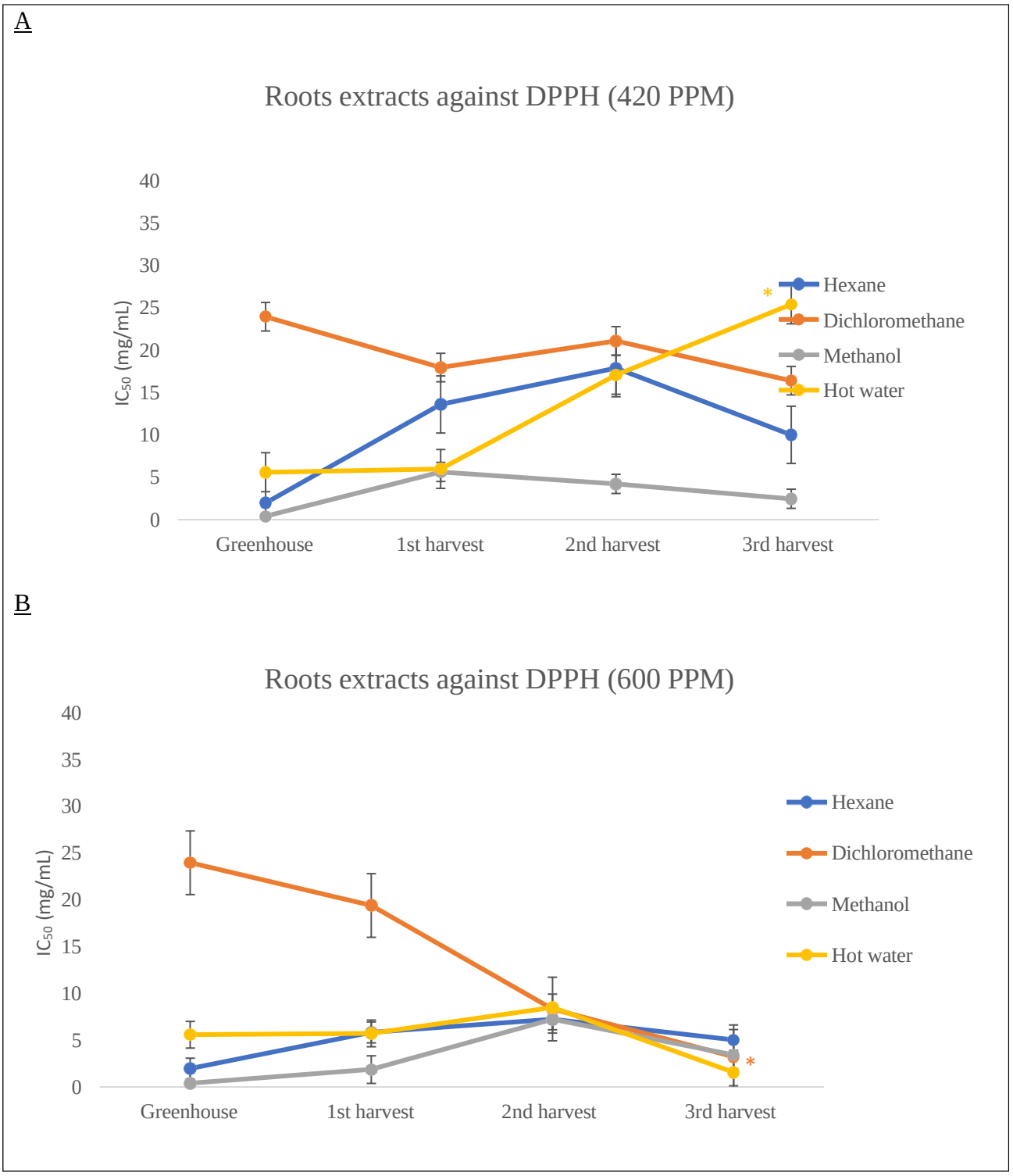


Figure 23: A graph representing the IC_{50} values of the leaves' extracts at 420 ppm (A) and 600 ppm (B) against DPPH ($P<0.05$). "*" represents a statistical signific result against greenhouse extracts (control). Harvest 1: harvest after 1 months, Harvest 2: harvest after 2 months, Harvest 3: harvest after 3 months.

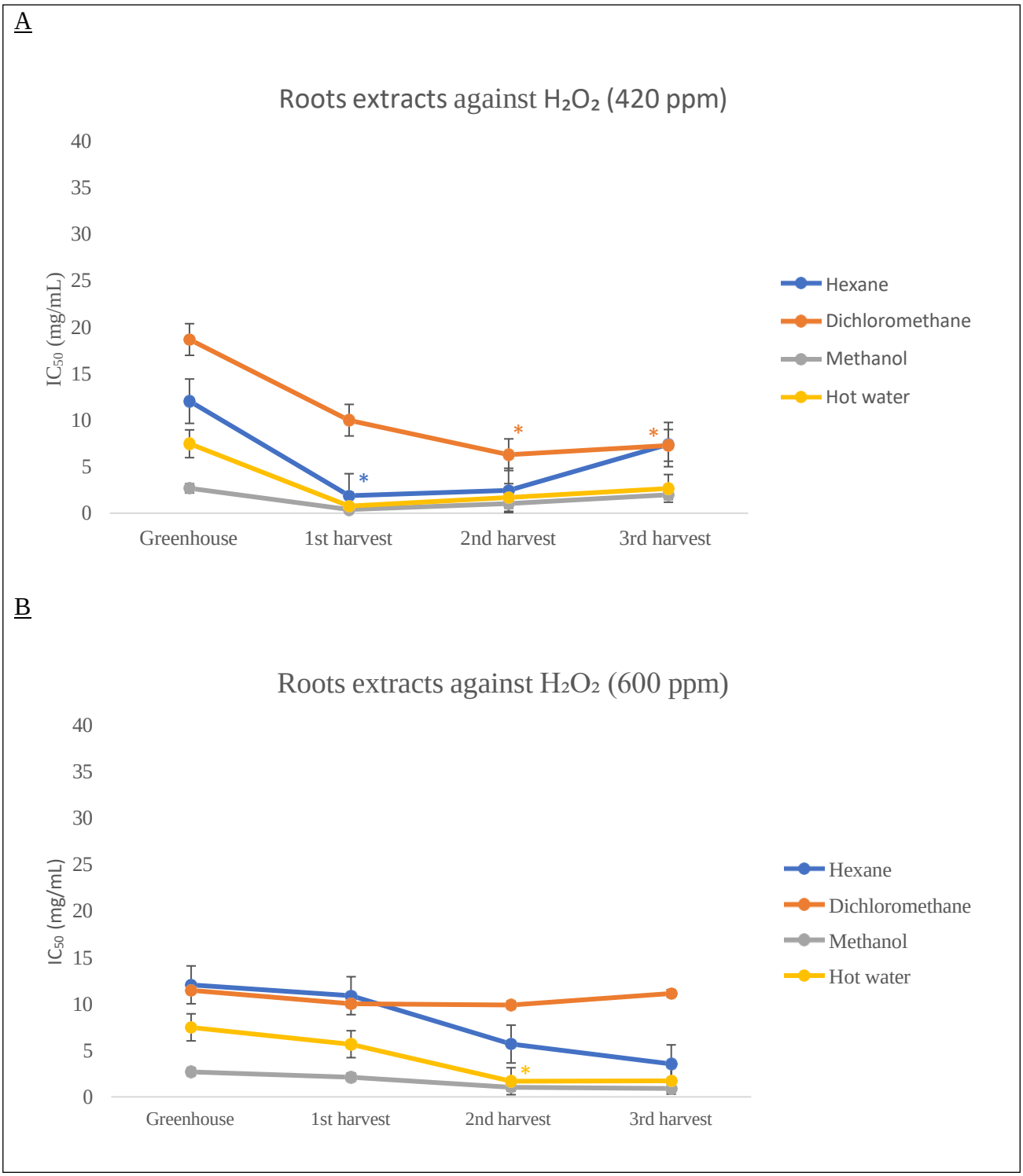


Figure 24: A graph representing the IC_{50} values of the roots extracts at 420 ppm (A) and 600 ppm (B) against H_2O_2 ($P<0.05$). "*" represents a statistical signific result against greenhouse extracts (control). Harvest 1: harvest after 1 months, Harvest 2: harvest after 2 months, Harvest 3: harvest after 3 months.

3.4 Discussion

3.4.3 Physical parameters and physiological analysis

The leaves exhibited the highest increase in weight in samples exposed to 600 ppm among all three plant parts. The leaf foliage also increased significantly in the samples exposed to 600 ppm. This corresponds with current literature, which suggests that when plants are exposed to elevated CO_2, they exhibit an increase in their photosynthetic rate and, as a result, experience an increase in above-ground plant biomass. The increase in weight mass could indicate that the leaves are thickening in response to elevated CO_2 concentrations, which is a common trend in literature (Zhongming *et al.*, 2018). The reason for this is that the rise in CO_2 concentrations leads to an increase in the photosynthetic rate, and as a result, there is an increase in the mesophyll and other vascular tissue in the leaves (Pritchard *et al.*, 1999).

Stems contain vascular tissue known as xylem and phloem, which acts as a transport system for water and nutrients. The thickening of this vascular tissue may also account for the slight increase in weight exhibited in the stems exposed to 600 ppm.

The increase in vascular tissue may also account for the slight increase in weight in stems exposed to 600 ppm.

The roots that were exposed to elevated CO_2 concentrations exhibited an increase in weight, similar to the 35% increase exhibited by other CAM plant species in the literature (Drennan and Nobel, 2000; Weis *et al.*, 2010). The increase in CO_2 concentration leads to an increase in the photosynthetic rate. In order to accommodate the increased photosynthetic rate, the root shoots increase to accommodate a faster and/or more efficient water and nutrient uptake (Rogers *et al.*, 1999).

In a study performed by Kim *et al. (*2017) *Phalaenopsis amabilis* (a CAM plant species) was exposed to elevated CO_2 concentrations. The plant experienced a significant increase in the weight of the leaves which were exposed to elevated to CO_2, whilst the chlorophyll content remained relatively unchanged. These results coincide with the results of the current study.

Current literature suggests that chlorophyll content generally decreases in plant species exposed to elevated CO_2. However, the majority of the studies that did exhibit a decrease in chlorophyll content simultaneously experienced a decrease in nitrogen content (Gardner *et al.*, 2022). This suggests that the decrease in chlorophyll content experienced by other plant species is a result of limiting nutrients rather than the direct elevation of CO_2 concentrations.

The results of this study are similar to those in the literature. This confirms the general trend that increasing CO_2 may have on the physiological properties of this plant.

3.4.4 Phytochemical analysis

A strong phytochemical presence was determined in the leaves, stems, and roots of the control *P. afra*. The leaves exhibited the greatest presence of phytochemicals over the stems and root extracts. The leaves are the commonly used plant part in traditional medicine. The greenhouse sample exhibited the presence of eight out of the ten phytochemicals tested. Phytochemical presence gives an indication of the medicinal value of the plant (Agbafor and Nwachukwu, 2011). In a control setting, the leaves exhibit a high phytochemical presence, which may contribute to the medicinal properties witnessed by traditional healers. The leaves in the study conducted by Tabassum *et al*. (2022) also exhibited a phytochemical presence, with a particularly large presence of tannins, glycosides, saponins, and phenols.

The leaves samples that were exposed to 420 ppm exhibited a phytochemical profile similar to the samples maintained in the greenhouse. The increase in saponins, phenolics, and coumarins after the first harvest indicates the plant may have been acclimating to the new environment. Because the following harvests (harvest 2 and harvest 3) experience a phytochemical presence similar to the greenhouse extracts. The samples exposed to 600 ppm exhibited an overall increase in phytochemical presence across the three harvests.

The leaves that were exposed to elevated CO_2 exhibited an increase in saponins, glycosides, coumarins, and volatile oils. These chemicals either exhibit antioxidant, anti-inflammatory, or antibacterial activity and hence the increase of these phytochemicals may strengthen the medicinal properties exhibited by the plant. Coumarins were the phytochemical group that exhibited the highest presence among all of the phytochemical groups tested. And yet, after exposure to elevated CO_2, the coumarin's presence increased further. This suggests that coumarins are an important phytochemical within the leaves of *P. afra*.

The stems and roots exhibit a lower phytochemical presence than the leaves, which may justify the use of the leaves among traditional healers.

The most notable change observed in the preliminary phytochemical screening is an increase in the presence of flavonoids. Analysis of control samples revealed the absence of flavonoids in all three plant parts. This absence was also observed in samples exposed to 420 ppm of CO_2. However, the presence of flavonoids was observed in samples exposed to 600 ppm of CO_2. The highest presence was determined in the leaves which exhibited a moderate presence in the third harvest.

Flavonoids are a group of secondary metabolites that play a variety of roles in plant physiology (including pigmentation), defence, and growth regulation (Mathesius, 2018). They also function as signalling molecules in response to environmental stress (Panche *et al*., 2016).

Thus, the significant increase in flavonoids may suggest that the plant was still under stress, even after harvests 2 and 3. The appearance of flavonoids in the samples exposed to 600 ppm prompted further investigations into this phytochemical group.

The leaves of *P. afra* exhibited the highest flavonoid content among all three plant parts; however, all three exhibited a significant increase in the methanolic and/or water extracts in samples exposed to 600 ppm. The increase in flavonoid content after exposure to elevated CO_2 concentrations is a common occurrence in literature (Patni and Bhattacharyya, 2021).

Flavonoids have great therapeutic potential. The phytochemical group has been identified as a great anti-inflammatory and antimicrobial chemical (Dillard and German, 2000).

The increase in phytochemical presence, such as that exhibited by flavonoids, indicates that the plant may have enhanced therapeutic potential when exposed to elevated CO_2 concentrations.

3.4.5 Antioxidant activity

The leaves, stems and roots exhibit a high antioxidant activity. In general, the plant exhibited a strong scavenging activity toward H_2O_2 than DPPH. A similar trend was exhibited in the study by Tabassum *et al.* (2021), where the extracts exhibited a moderate scavenging activity against DPPH in comparison to other free radicals which exhibited a strong scavenging activity. The strong presence of phenolics in the plant may contribute to the strong scavenging activity against H_2O_2. Phenolic compounds readily donate an electron to H_2O_2 when the plant is undergoing stress (Kasote *et al.*, 2015).

The methanolic and hot water extracts exhibited the overall strongest antioxidant activity in all three plant parts. These extracts consistently remained below the upper limit, with the exception of the hot water 420 ppm root extract, which significantly decreased in its ability to scavenge DPPH after harvest 3.

In general, the leaves, stems and root extracts which were exposed to 600 ppm exhibited a lower IC_{50} value and hence a stronger affinity for scavenging oxidants. As seen above, the rising CO_2 concentration leads to an increase in phytochemical presence which as a result, may contribute to the strong antioxidant activity which was exhibited in samples exposed to 600 ppm. The relationship between primary and secondary metabolism in plants suggests that changes in primary metabolism, such as those caused by elevated CO_2, may also affect secondary metabolism (Ibrahim and Jaafar, 2012). The proposed mechanism for this relationship is that elevated CO_2 leads to an increase in non-structural carbohydrates, which in turn stimulates the synthesis of secondary metabolites, which possess antioxidant properties. Oxidative stress is a major factor contributing to skin diseases and infections. The strong antioxidant activity may provide relief against certain skin disorders.

The relatively steady antioxidant activity exhibited by the leaves stems and roots after exposure to 600 ppm, indicates that plant may retain its antioxidant activity in the future, despite the rising atmospheric CO_2 concentration.

3.5 Conclusion

This study found that the leaves, stems, and roots of *P. afra* demonstrate resilience in response to elevated CO_2 concentrations. Analysis of samples exposed to elevated CO_2 revealed a significant increase in the presence of phytochemicals, specifically flavonoids. This increase in flavonoids suggests a potential enhancement of the medicinal properties of *P. afra*. Additionally, antioxidant activity was found to be consistently high among methanolic and hot water extracts, with the plant displaying stronger scavenging activity towards H_2O_2 compared to DPPH in samples exposed to both ambient and elevated CO_2 conditions. These findings suggest that *P. afra* may be a valuable resource in the future, despite the rising CO_2 concentratons.

Chapter 4: An antimicrobial analysis of the Leaves, Stems and Roots of *Portulacaria afra*.

4.1 Introduction

In recent years, the use of natural products, specifically medicinal plants, as a treatment for various skin conditions has become increasingly popular (Saisang *et al*., 2022). This trend is a result of the numerous advantages offered by these plant-based treatments, including ease of accessibility, cost-effectiveness, and a lower incidence of side effects. Phytochemicals, such as flavonoids and phenolic compounds, present in these plants have displayed in-vitro antimicrobial activity (Vaou *et al*., 2021). Additionally, medicinal plants have proven to be valuable resources in the fight against multi-drug-resistant microorganisms. Bacteria and fungi have not been able to develop resistance to the chemically complex phytochemicals (Anand *et al.,* 2019). This, along with minimal side effects, makes medicinal plants a desirable treatment for many skin conditions. Approximately one-third of the medicinal properties exhibited by plants are used to treat wounds and skin disorders (Mantle *et al*., 2001).

Portulacaria afra is a medicinal plant species that has been traditionally used to treat skin-related disorders. The use of this plant for this purpose was first reported in an ethnobotanical interview conducted by de Wet, Nckiki, and van Vuuren in 2013. Thus, the present study aimed to evaluate the antimicrobial properties of the leaf, stem, and root extracts of this plant against microorganisms commonly associated with skin infections.

It is important to note that the antimicrobial activity of plants is closely linked to the phytochemical profile of the plant. Therefore, changes in environmental factors such as rising CO_2 concentrations, which can affect the phytochemical composition of plants, may also impact the antimicrobial activity exhibited by these plants (Rajashekar, 2018).

4.2 Materials and Methods

4.2.1 Plant material and propagation

The collection and propagation of the plant material was performed as described in Chapter 3- in section 3.2.1 and 3.2.2, page 41 and 42.

4.2.2 Treatment

The CO_2 treatments surrounding the *P. afra* plants were performed as described in Chapter 3- in section 3.2.3, page 42, 43 and 44.

4.2.3 Crude plant extract

The crude plant extracts used to determine the antimicrobial activity of *P. afra* was created following the method used by Hassan (2018).

An Erlenmeyer flask (conical flask) was labelled and weighed. The powdered plant material (100 g) was placed into the Erlenmeyer flask and the weight was recorded. An organic solvent was added to the flask. The amount of solvent placed in the flask was in a ratio of 2:1 with the powdered plant material. The flask was then sealed with autoclaved cottonwool and incubated at 37°C for 24 hours. Following the incubation period, autoclaved cotton wool was added to the flask and a pipette was used to withdraw the supernatant fluid. The supernatant fluid was transferred into a beaker and weighed. The organic solvent (dichloromethane: methanol) was added into the conical flask until the organic matter was covered. The supernatant fluid was placed in a fume-hood and left to evaporate for three days. Once evaporation was completed, the mass was dissolved in acetone. The mixture was vortexed to ensure adequate mixture and placed in the fridge until the tests commence.

4.2.4 Culture preparations

Bacterial micro-organisms which were selected for the study include: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 1228), *Cutibacterium acnes* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella aerogenes* (ATCC 11397). Whereas the fungal micro-organisms used include *Candida albicans* (ATCC 10231). The microorganisms and the strains are all cultures used as reference strains of American Type Culture Collection from the Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg.

4.2.5 Minimum inhibitory Concentration antimicrobial assay

The two-fold serial dilution microdilution technique was used to determine the lowest concentration of the plant extracts which will inhibit the growth of the selected micro-organisms. A 96-well micro-titre plate was prepared by adding 100 µl of the respective broth into the wells.

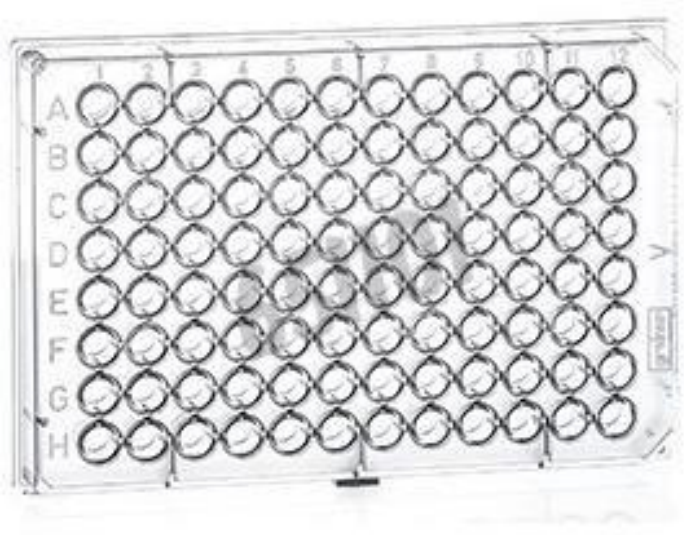


Figure 25: An image of a 96-well microtiter plate.

A volume of 100 µl of the plant extract was placed into the first well (A) using an aseptic technique. A positive control (Ciprofloxacin or Nystatin), negative control (dichloromethane:

methanol) and culture control (TSB or TGB) was placed in the last three wells. The positive control was used to detect microbial susceptibility of the pathogens. The negative control was used to detect whether the pathogen was reacting to the plant extract or to the solvent. The culture-control was to ensure that microbial growth does in fact occur.

A serial dilution was conducted down the plate at concentrations of 8, 4, 2, 0.5, 0.25, 0.125 and 0.0625 mg/ml. A McFarland standard was created by mixing the bacterial culture to the broth at a ratio of 1:100. A volume of 100 µl of the McFarland standard was then placed in each well. The micro-titre plates were sealed with a sterile adhesive film and incubated. A summary of the incubation conditions can be found in table 9 below. A streak plate was prepared using the McFarland standard of each microbe. The streak plate and McFarland standard were placed in the respective incubations.

Table 10: A summary of the MIC conditions for the various microbes.

	S. aureus	*S. epidermidis*	*P. aeruginosa*	*K. aerogenes*	*C. acnes*	*C. albicans*
Broth	Tryptone Soya broth	Tryptone Soya broth	Tryptone Soya broth	Tryptone Soya broth	Tryptone Soya broth	Thioglycolate broth
Positive control	Ciprofloxacin (0.01 mg/ml)	Ciprofloxacin (0.01 mg/ml)	Ciprofloxacin (0.01 mg/ml)	Ciprofloxacin (0.01 mg/ml)	Ciprofloxacin (0.01 mg/ml)	Nystatin (0.01 mg/ml)
Incubate	24 hours (37°C)	24 hours (37°C)	24 hours (37°C)	24 hours (37°C)	CO_2 incubator 96 hours (37°C)	48 hours (37°C)

Following the incubations periods, 400 µl of iodonitrotetrazolium violet solution (INT) was added to all the wells in the micro-titre plate. The wells which exhibited microbial growth (shown by a purple-pink colour) were recorded.

4.3 Results

The leaves, stem, and root extracts of *P. afra* were screened for antimicrobial activity to determine the effect of elevated CO_2 concentrations on the antimicrobial activity of the plant. Extracts from the greenhouse and those treated with 420 ppm and 600 ppm were screened against six microorganisms: three gram-positive bacteria, two gram-negative bacteria, and one fungal microbe, all of which are commonly associated with skin infections. The antimicrobial activity of an extract is considered significant if the minimum inhibitory concentration (MIC) value is less than 1000 µg/ml (Akhalwaya *et al*., 2018). The results for the MIC values are represented in tables 11 (leaves), 12 (stems), 13 (roots).

The leaves, stem, and root extracts of *P. afra* exhibited weak antimicrobial activity against the various microorganisms. The most noteworthy result was exhibited by the leaves extracts against *C. acne*s. The control leaves extracts (greenhouse) exhibited a MIC value of 8 mg/ml. However, the extracts that were exposed to 600 ppm concentrations displayed a stronger antibacterial effect of 2 mg/ml. These values are still considered weak when compared to ciprofloxacin, which requires 1.25 µg/ml to inhibit microbial growth. However, the increase in antimicrobial activity is considered significant.

The leaves extract also showed an increase in antibacterial activity against *S. aureus* and *C. albicans*. However, these changes were a difference of one serial dilution and hence were not considered significant.

Table 11: The MIC results of the leaves extracts of the control (greenhouse) and those which were subjected to CO_2 concentration (420 ppm and 600ppm).

Plant Extract	**Mean MIC values (µg/ml)**					
	Gram positive bacteria			**Gram negative bacteria**		**Fungi**
Sample	***S. aureus***	***S. epidermidis***	***C. acnes***	***P. aeruginosa***	***K. aerogenes***	***C. albicans***
Greenhouse	4000	4000	8000	6000	2000	8000
420 (1)	4000	4000	8000	4000	2000	8000
420 (2)	4000	4000	4000	4000	2000	8000
420 (3)	4000	4000	4000	4000	2000	8000
600 (1)	4000	4000	4000	4000	2000	8000
600 (2)	3000	4000	2000	4000	4000	8000
600 (3)	2000	4000	2000	4000	2000	4000
Ciprofloxacin (+ve control)	0.625	0.625	1.25	1.25	0.625	N/A
Nystatin (+ve control)	N/A	N/A	N/A	N/A	N/A	2.50
-ve control	8000	>8000	8000	>8000	8000	>8000
Culture control	>8000	>8000	>8000	>8000	>8000	>8000

420 (1) – harvest after 1 month, 420 (2) - harvest after 2 months, 420 (3) - harvest after 3 months

600 (1) – harvest after 1 month, 600 (2) - harvest after 2 months, 600 (3) - harvest after 3 months

The stems exhibited a weak antimicrobial activity against all six microorganisms. The increase in antimicrobial activity in the extracts exposed to 600 ppm treatment against, *P. aeruginosa*, *K. aerogenes* and *C. albicans* are considered insignificant.

Table 12: The MIC results of the stems extracts of the control (greenhouse) and those which were subjected to CO_2 concentration (420 ppm and 600ppm).

Plant Extract	**Mean MIC values (µg/ml)**					
	Gram positive bacteria			**Gram negative bacteria**		**Fungi**
Sample	***S. aureus***	***S. epidermidis***	***C. acne***	***P. aeruginosa***	***K. aerogenes***	***C. albicans***
Greenhouse	4000	4000	4000	4000	4000	>8000
420 (1)	4000	4000	8000	2000	8000	8000
420 (2)	4000	4000	8000	2000	4000	8000
420 (3)	4000	4000	8000	2000	4000	8000
600 (1)	4000	4000	4000	2000	2000	8000
600 (2)	4000	4000	4000	2000	2000	8000
600 (3)	4000	4000	4000	2000	2000	4000
Ciprofloxacin (+ve control)	0.625	0.625	1.25	0.625	N/A	2.50
Nystatin (+ve control)	N/A	N/A	N/A	N/A	N/A	2.50
-ve control	8000	>8000	>8000	8000	8000	>8000
Culture control	>8000	>8000	>8000	>8000	>8000	>8000

420 (1) – harvest after 1 month, 420 (2) - harvest after 2 months, 420 (3) - harvest after 3 months

600 (1) – harvest after 1 month, 600 (2) - harvest after 2 months, 600 (3) - harvest after 3 months

The roots exhibited an overall weaker antimicrobial activity than the stems and leaves. The increase in antimicrobial activity in the extracts exposed to 600 ppm treatment against *C. acne* is considered insignificant.

Table 13: The MIC results of the roots extracts of the control (greenhouse) and those which were subjected to CO_2 concentration (420 ppm and 600ppm).

Plant Extract	**Mean MIC values (µg/ml)**					
	Gram positive bacteria			**Gram negative bacteria**		**Fungi**
Sample	***S. aureus***	***S. epidermidis***	***C. acne***	***P. aeruginosa***	***K. aerogenes***	***C. albicans***
Greenhouse	4000	8000	4000	8000	4000	8000
420 (1)	6000	4000	4000	8000	4000	8000
420 (2)	4000	4000	4000	8000	4000	8000
420 (3)	4000	4000	4000	8000	4000	8000
600 (1)	4000	4000	4000	8000	4000	8000
600 (2)	4000	4000	4000	8000	4000	8000
600 (3)	4000	4000	2000	8000	4000	8000
Ciprofloxacin (+ve control)	0.625	0.625	1.25	1.25	0.625	2.50
Nystatin (+ve control)	N/A	N/A	2.50	N/A	N/A	2.50
-ve control	8000	>8000	8000	>8000	8000	>8000
Culture control	>8000	>8000	>8000	>8000	>8000	>8000

420 (1) – harvest after 1 month, 420 (2) - harvest after 2 months, 420 (3) - harvest after 3 months

600 (1) – harvest after 1 month, 600 (2) - harvest after 2 months, 600 (3) - harvest after 3 months

4.4 Discussion

The antimicrobial activity of the leaves, stems, and roots of *P. afra* is relatively weak, as there is no MIC value below 1000 µg/ml, against either bacterium (Akhalwaya *et al*., 2018). This result is similar to a study conducted by Salaheldin *et al*. (2019), in which the leaf extracts were tested against *P. aeruginosa*, *S. aureus*, and four other bacteria, and the activity was negligible. A similar result was observed in the study conducted by Nciki *et al*. (2016), in which the leaf extracts were tested against 12 microorganisms using the MIC assay. The leaves of *P. afra* exhibited a weak antimicrobial activity against eight out of the 12 microorganisms tested.
Despite the weak antimicrobial activity of the *P. afra* extracts, there was a significant increase in antibacterial activity against *C. acnes* in the leaves samples, which were exposed to 600 ppm.

The increase in bacteria activity in the leaves that were exposed to *C. acnes* indicates that the potency of the plant might increase when exposed to elevated CO_2.
Cutibacterium acnes is an opportunistic pathogen that has been identified as being a key component in acne vulgaris (Dréno *et al*., 2018). Acne is listed as one of the skin conditions that *P. afra* is commonly used to treat. Thus, the plant may exhibit a more potent effect against skin conditions such as those caused by *C. acnes* in the future when atmospheric CO_2 concentrations reach 600 ppm.

The weak antimicrobial activity of the various plant parts of *P. afra* suggests that the plant may provide relief against skin ailments through other mechanisms, such as pain relief, antioxidants, or anti-inflammation.

4.5 Conclusion

The leaves, stems, and root extracts of *P. afra* exhibit a weak antimicrobial activity against the six microorganisms tested in this study. The activity remained relatively constant between the greenhouse extracts and those subjected to 420 ppm and 600 ppm treatments. Thus, the rise in atmospheric CO_2 may cause the antimicrobial activity of *P. afra* to increase slightly or, more likely, stay the same. The significant increase in activity against *C. acnes* in leaves samples exposed to 600 ppm alludes to the plant still possessing therapeutic properties in patients with acne, despite the rising CO_2 concentration.

Chapter 5: Summary, future recommendations and Concluding remarks

5.1 Summary

Medicinal plants are a globally significant, serving as primary healthcare to a large population, as well as a major resource in drug discovery. Factors such as over-utilisation and climate change threaten the state of these resources.

Portulacaria afra is a medicinal plant used to treat a variety of skin diseases, with its first mention as a medicinal plant in 2013 (de Wet *et al*., 2013). Despite its medicinal properties, there remains limited research on *P. afra*, specifically regarding its potential state with rising CO_2 levels. The purpose of this study is to determine the impact of elevated CO_2 concentrations on the physiological, phytochemical, and biological properties of the leaves stems and roots of *P. afra.*

The study found that above and below ground biomass of the plant increased in accordance with current literature. The significant increase in weight of the plant parts suggests that the efficiency and photosynthetic rate of the plant were affected by the increase in CO_2. Studies suggest that changes in the photosynthetic rate may alter the biochemical composition of the plant and affect its phytochemical profile. Understanding the chemical profile of a plant can provide insight into its medicinal properties (Hussein *et al*. 2019). The study found a high presence of phytochemicals in the leaves, stems, and roots of *P. afra*, with the methanolic leaves extract exhibiting the greatest presence. All three plant parts also demonstrated an increase in phytochemical presence in extracts exposed to 600 ppm CO_2. The significant increase in flavonoids in particular, may indicate an increase in medicinal properties of the leaves. Other phytochemicals, such as coumarins, also demonstrated an increase in presence. Many phytochemical groups have been shown to elicit biological properties, such as antioxidant and antimicrobial properties. The plant parts which were exposed to 420 ppm

displayed a strong scavenging activity of DPPH and did not significantly differ from those exposed to 600 ppm CO_2. The leaves also performed stronger than their stem and root counterparts in this test. The antibacterial activity of the various plant parts of *P. afra* remained mostly unchanged in extracts exposed to elevated CO_2 concentrations. The antibacterial activity of the leaves was slightly stronger than the other two plant parts, although neither extract exhibited strong antimicrobial activity. However, there was a significant increase in the antibacterial activity against *C. acne*s. Overall, the leaves of *P. afra* outperformed their stem and root counterparts in each test conducted in this study. Therefore, in order to sustain these resources, it is not necessary to fully harvest the plant to reap its benefits. Instead, only the leaves should be harvested to promote sustainable harvesting.

5.2 Future recommendations

This study exposed the plant parts to a short-term exposure of CO_2. While short-term studies provide a useful model for understanding potential changes in the plant, it is important to note plants begin to acclimate to their environment, which may subsequently affect the phytochemical profile and biological activity of the plant. Future studies should expose the plant to a long-term treatment and determine whether the acclimation of the plant affects the phytochemical and biological activity of the plant.

Additionally, it should be noted that this study is an in-vitro study, and the results may not always translate to clinical studies (Vaou *et al*., 2021). Therefore, it is recommended to further test the plant in a clinical setting to better understand the effects observed by traditional healers. It is important to note that the plants were examined in CO_2 chambers and not in their natural habitat, which may have influenced the phytochemical profile of the plant.

Lastly, the toxicity of the plant parts should also be studied as it is used therapeutically for human treatment.

5.3 Concluding remarks

This study determined that the leaves, stems, and roots of *P. afra* demonstrated resilience to elevated CO_2 concentrations. Analysis revealed a strong presence of phytochemicals in the leaves, stems, and roots of *P. afra*. Exposure to elevated CO_2 concentrations resulted in an increase in the presence and quantity of important phytochemicals, such as flavonoids. The antioxidant activity, while strong, remained relatively constant between the control samples and those exposed to the treatment. Notably, the leaves displayed a significant improvement in antimicrobial activity against *C. acne*. The phytochemical and biological properties displayed either no change or an increase, suggesting that *P. afra* may continue to provide relief against certain ailments in the future, despite rising global CO_2 concentrations.

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Appendices



Appendix 1: Certificate of attendance at SAAB postgraduate symposium

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