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Concurrent effects of elevated carbon dioxide and temperatures on the polyphenolics profile, *in-vitro* selected antioxidant and antimicrobial activities in *Carpobrotus edulis* (L.) leaves

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Declaration page

I, Lethabo Sebothoma, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Animal Plant and Environmental Sciences to the University of the Witwatersrand, Johannesburg. It has not been submitted for any other degree or examination in any other University.

The experimental work described in this thesis was carried out in the School of Animal Plant and Environmental Sciences and the School of Chemistry, University of the Witwatersrand, Johannesburg, South Africa, under the supervision of Doctor Ida Risenga (School of Animal Plant and Environmental Sciences, University of the Witwatersrand), Doctor Yannick Nuapia (School of Animal Plant and Environmental Sciences, University of the Witwatersrand), and Doctor Samalesu Mayonde (School of Animal Plant and Environmental Sciences, University of the Witwatersrand).

L. Sebothoma

(Signature of Candidate)

8 day of September 2022 in Johannesburg

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Abstract

Anthropogenic activities have led to the accumulation of carbon dioxide in the atmosphere and consequently the elevation of temperature. Carbon dioxide and temperature influence the physiological and biochemical activities in plants and thus, are important for plant survival, growth, and development. The sessile nature of plants prevents them from physically avoiding environmental factors such as high CO_2 and temperatures, as a result they have developed a physiological response mechanism for protection. This mechanism involves the production of secondary metabolites, which in return have human-health benefits. How then is the production of secondary metabolites impacted by rising atmospheric CO_2 concentrations and temperatures? Moreover, is the pharmaceutical efficacy of medicinal plants impacted as the atmospheric CO_2 concentrations and temperature conditions rise due to climate change? This study therefore, aimed to investigate the possible modifications in the composition of polyphenolic compounds, antioxidant and antibacterial activities in *Carpobrotus edulis* leaves under controlled concurrent elevated atmospheric CO_2 and temperatures. A total of 36 *C. edulis* potted plant samples, constituting 12 pots, divided into 3 pots per treatments were exposed to combined 600 ppm and 35/30°C (day/night), 600 ppm and 45/35°C (day/night), 800 ppm and 35/30°C (day/night), 800 ppm and 45/35 °C (day/night), respectively. The control samples were kept at ambient conditions of combined 400 ppm and 28/25°C (day/night). The plant samples were exposed to these conditions for up to 192 hours, and leave samples were harvested episodically every 48 hours (48, 96, 144 and 192 hours) during the exposure period. All harvested leave samples were air-dried under 40°C and crude extracts were obtained using methanol. Preliminary phytochemical screening was performed to test the presence of tannins, phenolics, flavonoids, steroids, terpenoids,

glycosides, and saponins. The LC-MS/MS method was used to profile the polyphenolic compounds and 2,2-diphenyl-1- picrylhydrazyl (DPPH) used to measure the antioxidant activity of the plant. The antibacterial activity of *C. edulis* was determined by the use of two popular bacterial strains, *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive), using the Agar well diffusion method. The preliminary phytochemical screening results showed the consistent presence of tannins, phenolics, flavonoids, steroids, terpenoids, glycosides, and saponins throughout the treatments as compared with the control, however flavonoids were not detected in the samples under combined 800 ppm and 45/35°C, 600 ppm and 35/30°C as well as 800 ppm and 35/30°C. This indicated that combined elevated CO_2 and temperatures could have impacted on the production of flavonoids. The LC-MS/MS results showed the presence of 24 polyphenolic compounds in *Carpobrotus edulis* leaves and of those only 14 (58.83%) were present in *C. edulis* leaves under ambient/control conditions. Furthermore, the concurrent increment of CO_2 concentrations and temperatures prompted the appearance of 10 (41.67%) more compounds**.** A total of 17 (70.83%) polyphenolic compounds were identified under the 600 ppm and 45/35°C treatment. The presence of these 17 compounds seemed to be influenced by the duration of exposure to these conditions. Polyphenolic compounds profiling showed the disappearance of compounds and appearance of new compounds. The disappearance of some compounds was mainly observed under extreme conditions. Antioxidant activity decreased with increasing combined CO_2 concentration and temperature exposure. Antimicrobial activity showed some inhibition of *S. aureus* and *E. coli,* and the inhibition activity remained constant in all the climatic conditions. This response maybe attributed to the appearance of new polyphenolic compounds. These results suggested that *C. edulis* is a strong antioxidant and antimicrobial agent, owing it to the polyphenolic compounds composition. However, these properties could be negatively

impacted by elevated CO_2 and temperatures, thus influencing the efficacy of *C. edulis*. Future studies could investigate the influences of elevated CO_2 and temperatures independently to assess which factor plays the biggest role in the pharmaceutical efficacy of *C. edulis*.

Keywords

Antibacterial activity; antioxidant activities; *Carpobrotus edulis*; climate change; polyphenolic compound

Symbols and abbreviations

CO_2 – Carbon dioxide

DPPH – 2,2-diphenyl-1- picrylhydrazyl

°C – Degrees Celsius

% – Percentage

mm – Millimeters

mg – Milligrams

ml – Milliliters

LC-MS/MS – Liquid Chromatography with tandem Mass Spectrometry

C. edulis – Carpobrotus edulis

E. coli – Escherichia coli

S. aureus – Staphylococcus aureus

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Appendix A: Bacterial inhibition activity of *Carpobrotus edulis* methanol extracts against *Staphylococcus aureus.* Each plate represents the following treatments control extracts (400 ppm and 28/25°C), TA (600 ppm and 45/35°C), TB (800 ppm and 45/35°C), TC (600 ppm and 35/30°C), and TD (800 ppm and 35/30°C), respectively. Moreover, within each plate, each well was filled with 80% methanol (negative control), control extracts (400 ppm and 28/25°C) and extracts of each treatment at every 48-hour interval.

Appendix B: Bacterial inhibition activity of *Carpobrotus edulis* methanol extracts against *Escherichia coli.* Each plate represents the following treatments control extracts (400 ppm and 28/25°C), TA (600 ppm and 45/35°C), TB (800 ppm and 45/35°C), TC (600 ppm and 35/30°C), and TD (800 ppm and 35/30°C), respectively. Moreover, within each plate, each well was filled with 80% methanol (negative control), control extracts (400 ppm and 28/25°C) and extracts of each treatment at every 48-hour interval.

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CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. General introduction

The use of medicinal plants dates back centuries ago, and it is still expanding worldwide (Salmerón-Manzano *et al.,* 2020). Medicinal plants are most important in developing countries as the primary and stable way to improve human health (Van Andel and Carvalheiro, 2013). The ability of medicinal plants to treat diseases and/or maintain good human health depends on the plants' ability to produce phytochemicals.

Environmental factors such as temperature, drought, salinity, light intensity, are known to induce stress in plants, and are predicted to severely increase and intensify over time (Osakabe *et al.,* 2013). The instability of environmental conditions has catalysed the evolution of several plant adaptive mechanisms (Isah, 2019). This is to counteract the compliant changes caused by environmental stress, to facilitate flexibility without compromising the cellular and physiological developmental process of plants (Isah, 2019). These adaptive mechanisms include the production of secondary metabolites (Isah, 2019).

Secondary metabolite compounds do not necessarily have a significant role in plants' primary life processes such as growth and development but, are responsible for attracting pollinators, seed dispersal, and most importantly, the defence against both biotic and abiotic factors such as pathogens and herbivory, thermal and drought stress respectively (Bennett and Wallsgrove, 1994; Wink, 2003; Mazid *et al.,* 2011; AbdElgawad *et al.,* 2016; Isah, 2019). The composition and concentrations of secondary metabolites in plants are largely influenced by not only biological

factors (i.e., pathogens and herbivory) but also environmental factors (i.e., extremely high temperatures and atmospheric carbon dioxide (CO_2) concentrations) (Akula and Ravishanka, 2011; Isah, 2019). These could be due the fact that the biosynthesis of secondary metabolites acts as an adaptive strategy evolved to maintain survival and fecundity by the plant (Akula and Ravishanka, 2011; Isah, 2019). Therefore, it is predicted that extreme changes in climatic conditions due to global warming could greatly impact on the ability of medicinal plants to produce secondary metabolites (Akula and Ravishanka, 2011).

Carbon dioxide is one of the most essential abundant greenhouse gases as it plays a role in the structural development of organisms (Lindsey, 2020). Its importance stems from the fact that carbon is an essential element for life's building blocks (i.e., carbohydrates, lipids, nucleic acids, proteins and more), thus it sustains life on Earth (Kitadai and Maruyama, 2018; Lindsey, 2020; Garcia *et al.,* 2021). However, scientists have projected a 50% or over increment in atmospheric CO_2 concentrations by the end of the 21st century, because of anthropogenic activities (Van Vuuren *et al.,* 2008; Cassia *et al.,* 2018). Prediction models show a heightened frequency, intensity, and durations of heat waves due to climate change (Carlson, 2008; Arriagada *et al.,* 2020). These increases are expected to significantly impact plant growth, secondary metabolite production, genetic expression, photosynthesis, and influence the efficacy of medicinal plants in treating ailments through the impact on antioxidant and antibacterial activities (Pandey *et al.,* 2019; Alhaithloul *et al.,* 2020). Hence, the growing investigations on the impact of climate change on medicinal plants' therapeutic efficacy to human health.

Previous studies have focused on investigating effects of the projected high climatic conditions on the composition of polyphenolic compounds, the antioxidant activity, and antimicrobial activity in medicinal plants. For instance, Balasooriya *et al.* (2019a) investigated the effect of elevated CO_2 concentrations and temperature on the polyphenol, flavonoid, anthocyanin, antioxidant, and individual phenolic compound content in strawberry fruits (*Fragaria ananassa* Duch.). Another study by Assis *et al.* (2001) was conducted on the impact of elevated CO_2 concentrations on phenolic contents and phenylalanine ammonia-lyase activity in cherimoya fruits (*Annona cherimola*, Mill.). A study was conducted on *Zizania latifolia* Turcz. plants to assess the impacts of temperature and light intensity on the plants' photosynthetic and antioxidant enzyme activities (Yan *et al.,* 2013). Additionally, Chokoe *et al.* (2008) assessed the impact of seasonal variation on the antibacterial and phytochemicals in *Carpobrotus edulis* (L.).

These studies have therefore, influenced the present study, which focused on profiling the polyphenolic compounds of an important native South African medicinal plant, *Carpobrotus edulis* (Omoruyi *et al.,* 2014; Akinyede *et al.,* 2020), when the species was exposed to the projected extremely high atmospheric CO_2 concentrations and temperatures concurrently. Furthermore, this study assessed the influence of above-mentioned conditions on the antioxidant and antimicrobial activities of the species. *Carpobrotus edulis* (Aizoaceae) is commonly used to treat skin-related ailments, respiratory infections, diabetes, gastrointestinal illness, and hypertension (Van Der Watt and Pretorius, 2001; Khattab and El Sherif, 2011; Martins *et al.,* 2011; Ibtissem *et al.,* 2012; Mabona and Van Vuuren, 2013; Mudimba and Nguta, 2019). However, no studies have investigated the effects of projected increased climatic conditions on the medicinal ability of the plant. Therefore, it is essential to determine the responses of the species to combined atmospheric

CO_2 concentrations and temperatures, and the consequent impact on the medicinal properties. Hence, the present study made future implications on the influence of climate change on the phytochemistry and therapeutic efficacy of *C. edulis*.

1.2. Literature review

1.2.1. Phytochemicals in medicinal plants

The sessile nature of plants poses threat to their growth, reproduction and thus survival as external environmental conditions constantly change (Osakabe *et al.,* 2013; Gull *et al.,* 2019). Plants have turned into the main and essential sources of beneficial substances/ compounds to humans through the development of mechanisms to counteract the environmental impacts (Bhaskarachary *et al.,* 2015). One of these is the production of secondary metabolites (Bhaskarachary *et al.,* 2015). Secondary metabolites are by-product compounds of metabolic processes, produced in relatively small quantities but, large varieties (Mera *et al.,* 2019). The distribution of the secondary metabolites varies across the different plant parts as well as between different plant species (Mera *et al.,* 2019). Secondary metabolites have no direct influence on the important processes such as, the differentiation or formation of lipids, proteins, carbohydrates, of protein synthesis, nutrient assimilation, solute transport, respiration, and photosynthesis (Mera *et al.,* 2019). However, these compounds are known to have an indirect influence on the survival, growth, and reproduction of plants because they attract seed pollinators and give the plant the ability to face or adapt to sudden changes in temperature, humidity, light intensity and drought (Yang *et al.,* 2018). Most importantly, they serve as defence mechanisms of plants against pathogens and predators (Yang *et al.,* 2018; Mera *et al.,* 2019).

The study of biological functions and the structure of secondary metabolites is of great importance because this knowledge is used in various industries (Mera *et al.,* 2019). Many secondary metabolites are used as aromas, resins, gums, flavour enhancers, as well as insecticides and herbicides (Mera *et al.,* 2019). In addition, most secondary metabolites have found utility in the pharmaceutical industry, given large number of pharmacological values they possess (Mera *et al.,* 2019). Secondary metabolites may prevent or reduce the damage caused by reactive oxygen species (ROS) in plants. Reactive oxygen species are cytotoxic compounds that cause the death of cells in plants, normally through cell membranes death (Karuppanapandian *et al.,* 2011). The compounds include: alkoxy radical ($RO^{\cdot}$), excited carbonyl (RO*), hydrogen peroxide (H_2O_2), hydroperoxyl radical ($HO_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), peroxy radical ($ROO^{\cdot}$), singlet oxygen ($1O_2$), superoxide radical ($O_2^{\cdot -}$) (Karuppanapandian *et al.,* 2011).

Reactive oxygen species are by-products of cell metabolism that get largely imbalanced in concentration when the plant is under immense biotic or abiotic stresses (Karuppanapandian *et al.,* 2011). Reactive oxygen species rapidly inactivate enzymes, damage vital cellular organelles in plants, and destroy membranes by inducing the degradation of pigments, proteins, lipids, and nucleic acids which ultimately results in cell death (Sharma *et al.,* 2012; Huang *et al.,* 2019). In addition, ROS act as diffusible signals in the signal transduction pathways and as secondary messengers in various developmental pathways within plants (Karuppanapandian *et al.,* 2011). Reactive oxygen species can attack all macromolecules, resulting in serious damage to cellular components, DNA lesions and mutations, and this often leads to irreversible metabolic dysfunction and cell death (Karuppanapandian *et al.,* 2011). Under steady state conditions, the ROS are scavenged by various antioxidative defense systems (Foyer and Noctor, 2005; Navrot *et al*., 2007).

Classes of secondary metabolites in medicinal plants

There are three large groups of secondary metabolites: terpenes, polyphenolic compounds, and alkaloids, which are classified according to the biosynthetic pathway, chemical structure (i.e., presence of sugars or rings), molecular composition (i.e., containing nitrogen or not), and the solubility in organic solvents and water (Mera *et al.,* 2019). Terpenes, known as isoprenoids, are the largest group consisting of over 40 000 different molecules (Mera *et al.,* 2019). They are abundantly found in flowers and fruits as volatile compounds, therefore, have specific and distinct odors (Saxena *et al.,* 2013; Mera *et al.,* 2019). Example of flowers or fruits that contain these odors include eucalyptus, ginger, great basil, lemon, mint (Mera *et al.,* 2019). In the plant primary metabolism, terpenes function as electron carriers (e.g., ubiquinone and plastiquinone); photosynthetic pigments (e.g., carotenes); plant development and growth regulators (e.g., giberilins, strigolactones, brassinosteriods); make up parts of cell membranes (e.g., phytosterols); and they participate in protein glycosylation (Mera *et al.,* 2019). In the secondary metabolism, they function as molecules responsible for attracting pollinators, defense molecules, toxic compounds, and food deterrent for insects (Mera *et al.,* 2019).

Another group involved in the defense mechanism of plants against insects and habitats is the alkaloids group (Mera *et al.,* 2019). Alkaloids are a nitrogen atom containing group, as well as one of the large and diverse groups (Saxena *et al.,* 2013; Mera *et al.,* 2019). Their concentrations in plants normally vary considerably in every part plant, with some possibly containing none of the compounds (Mera *et al.,* 2019). Furthermore, they are involved in plant interspecific competition for specific habitats (Mera *et al.,* 2019). The following are examples of plants

containing alkaloids, black pepper (*Piper nigrum* L.) and long pepper (*Piper longum* L.) (Hussain *et al.,* 2018).

A group sharing a similar function of interspecific competition and plant pathogen defense polyphenolic compounds (Mera *et al.,* 2019). Polyphenolics are benzene ring(s) containing groups with one or more hydroxyl substituents attached to an aromatic hydrocarbon; the simplest form is known as a phenol class (Bennett and Wallagrove, 1994; Lin *et al.,* 2016; Mera *et al.,* 2019). In humans, they are essential for maintaining good health as they contribute to antioxidants and other health beneficial components. They are the key components to the medicinal properties of most medicinal plants, and their therapeutic value is the reason they are widely studied (Mera *et al.,* 2019). In plants they oxidize quickly and act as antioxidants (Mera *et al.,* 2019). They function as plant growth inhibitors, seed germination inhibitors, capture approximately 90% of the UV radiation therefore protecting the plant from UV ray damage (Mera *et al.,* 2019). They influence seed dispersion through conferring appetizing aromas and fruits to herbivores; on the contrary, they can protect the plant from animal herbivory, fungi, and nematodes attacks through the production of bitter flavors or textures (Bennett and Wallagrove, 1994; Demain and Fang, 2000; Kroymann, 2011; Lin *et al.,* 2016; Mera *et al.,* 2019). They are also essential for attracting pollinators (Bennett and Wallagrove, 1994; Lin *et al.,* 2016). Examples of polyphenolics compounds include flavonoids, phenolic acids, complex flavonoids and colored anthocyanins to name a few (Lin *et al.,* 2016; Mera *et al.,* 2019).

1.2.2. Antioxidant and antimicrobial activities in medicinal plants

Antioxidant activity in medicinal plants

Antioxidants are substances that prevent cellular damage through the inhibition of oxidation by ROS, therefore they prevent resulting ailments such as, cancer and cardiovascular diseases (Is and Woodside, 2001; Hamid *et al.,* 2010). The antioxidant defence systems or antioxidant enzymes may be divided into three groups, namely, antioxidant enzymes, chain breaking antioxidants, and transition metal binding proteins (Is and Woodside, 2001). Furthermore, antioxidant enzymes can be subdivided into three categories (Is and Woodside, 2001). The first antioxidant enzyme is catalase, and it is of the catalyst responsible for the conversion stages of hydrogen peroxide to water and oxygen (Is and Woodside, 2001). The second is glutathione peroxides and glutathione reductase, which are known to catalyse the oxidation process of glutathione, converting hydroperoxide into hydrogen peroxide or lipid hydroperoxide (Is and Woodside, 2001; Balla *et al.,* 2007). Lastly, superoxide dismutase, which catalyses the production of hydrogen peroxide through the dismutation of superoxide, then hydrogen peroxide would be eliminated by the above-mentioned catalase or glutathione peroxide (Is and Woodside, 2001). The chain breaking antioxidants are molecules that form stable products through the donation or recipient of an electron to or from a radical, and there are further categorised into lipid and aqueous phases (Is and Woodside, 2001). Lipid phase chain breaking antioxidants scavenge radicals on cell membrane and lipoprotein particles thus preventing lipid peroxidation (Is and Woodside, 2001). Examples include flavonoids, vitamins A and E (Is and Woodside, 2001). Aqueous phase chain breaking antioxidants scavenge radicals found in the aqueous compartment directly (Is and Woodside, 2001). Some examples of compounds with such antioxidant activity properties include,

beta-carotene, lycopene, vitamins A, C, E, flavonoids, ascorbate, phenolic acid, tannins (Figure 1.1; Chokoe *et al.*, 2008; Hamid *et al.*, 2010).

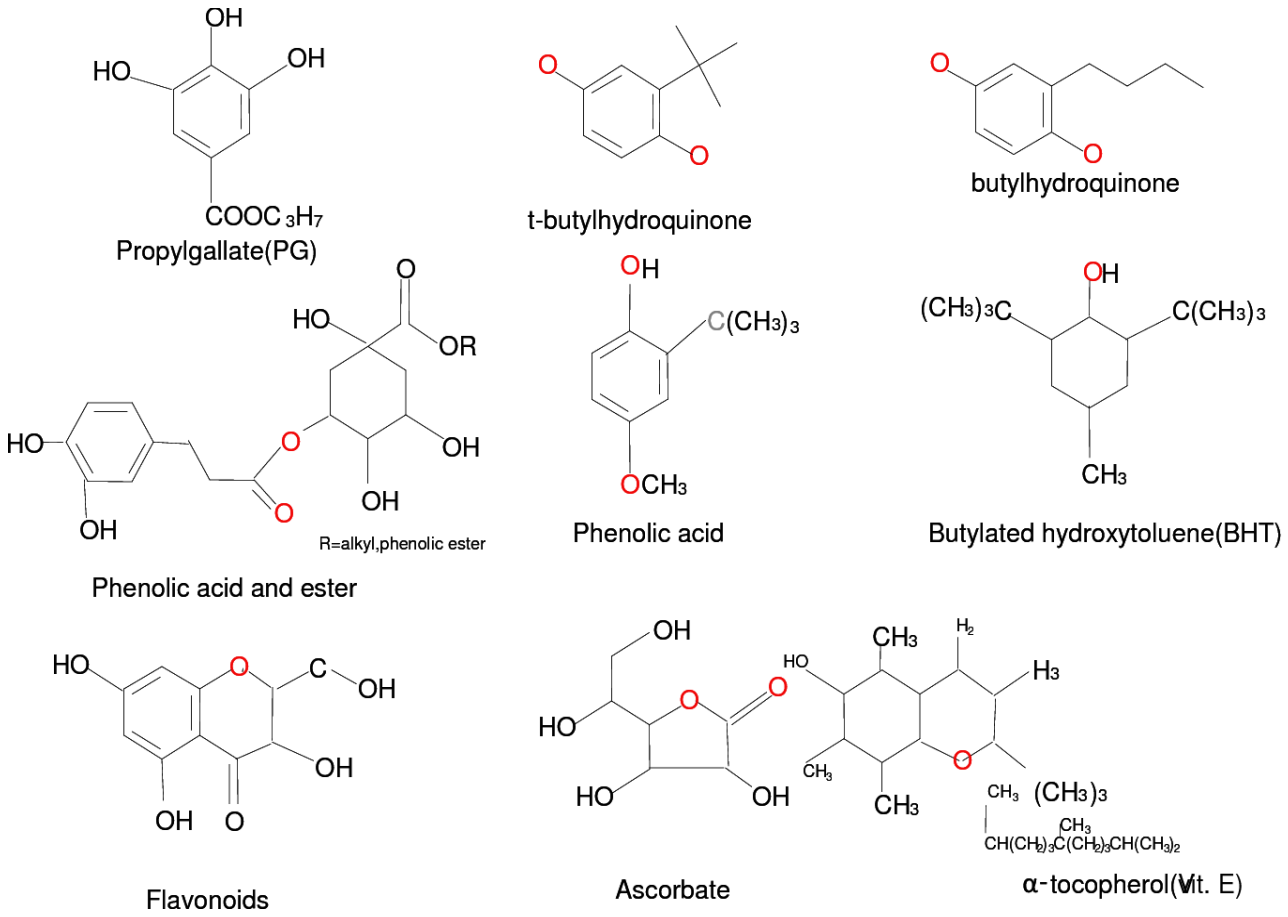


Figure 1.1: Structural examples of some antioxidants found in medicinal plants (Hamid *et al.*, 2010).

The mechanism of action of antioxidants, i.e., the removal of ROS, divided into three stages, the initiation stage, propagation stage and termination stage, was described by Hamid *et al.* (2010); Chavan and Ratnavathi (2016). The initiation stage can be initiated by lipoxygenase, and it involves the production of radicals (Chavan and Ratnavathi, 2016). The propagation and termination stages involve non-radical compounds productions. The whole mechanism is known as the classical route of autoxidation, which follows the autoxidation of lipids (Chavan and Ratnavathi, 2016).

Initiation stage:

(1) $RH \rightarrow R^{\cdot} + H^{\cdot}$

(2) $R^{\cdot} \rightarrow R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$

(3) $2ROOH \rightarrow ROO^{\cdot} + RO^{\cdot} + H_2O$

Propagation stage:

(1) $R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$

(2) $ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$

(3) $RO^{\cdot} + RH \rightarrow ROH + R^{\cdot}$

Termination stage:

(1) $R^{\cdot} + R^{\cdot} \rightarrow R - R$

(2) $R^{\cdot} + ROO^{\cdot} \rightarrow ROOR$

(3) $ROO^{\cdot} + ROO^{\cdot} \rightarrow ROOR + O_2$

(4) Antioxidants + $O_2 \rightarrow$ oxidized antioxidants

Antimicrobial activity in medicinal plants

Antimicrobial agents are important for alleviating infectious diseases (Manandhar *et al.*, 2019). However, the pathogenic bacteria have emerged and disseminated multidrug resistant strains which antimicrobial agents have very little to no effect on (Manandhar *et al.*, 2019). Literature has documented the naturally occurring antimicrobial compounds that are found in medicinal plants such as *Oxalis corniculate* L., *Artemisia vulgaris* L., *Cinnamomum tamala* Buch.-Ham., and

Ageratina Adenophora (Speng.) that are used as alternative treatment to pathogenic/bacterial infections (Manandhar *et al.,* 2019). These plants produce secondary metabolite compounds such as alkaloids, flavonoids, phenolic compounds, and tannins to name a few, that have exhibit antimicrobial activities (Chokoe *et al.,* 2008; Manandhar *et al.,* 2019).

Previous studies have shown that various medicinal plants, such as *Oxalis corniculate* L., *Artemisia vulgaris* L., *Cinnamomum tamala* Buch.-Ham., and *Ageratina Adenophora* (Speng.) could inhibit various strains of microbials, such as *Escherichia coli, Salmonella Typhi*, MDR *Salmonella Typhi, Klebsiella pneumoniae, Citrobacter koseri, Staphylococcus aureus, Rhizopus* spp, *Pseudomonas aeruginosa*, MDR K. *pneumoniae, Aspergillus niger, Aspergillus flavus, yeast Candida albicans* by extracts from *Oxalis corniculate* L., *Artemisia vulgaris* L., *Cinnamomum tamala* Buch.-Ham., and *Ageratina Adenophora* (Speng.) (Ammer *et al.,* 2016; Manandhar *et al.,* 2019). The effectiveness of *Eucalyptus tereticornis* as an alternative medicine to multi-drug resistant microbial infections such as the bacterial strain *Escherichia coli* was established by Ammer *et al.* (2016).

1.2.3. Environmental impacts on medicinal plants

Climate change results from the accumulation of greenhouse gases such as carbon dioxide (CO_2) in the atmosphere due to human mediated emissions from fossil fuel combustion, deforestation and changes in landforms caused by agricultural practices, hence the term - anthropogenic climate change (Cassia *et al.,* 2018; Bhargava and Mitra, 2021). Projected and already observed alterations in rainfall patterns, deviations in the frequency and distribution in weather events such as droughts, storms, floods, heat waves, rises in sea level just to name a few, are evidence of climate change (Cassia *et al.,* 2018). The most evident change is increases in temperature due to the accumulation of the most important greenhouse gas, CO_2 (Cassia *et al.,* 2018; Bhargava and Mitra, 2021).

Carbon dioxide is an essential gas in the atmosphere as it plays vital roles in the environment include, trapping infrared radiation heat in the atmosphere, promoting the weathering of rocks, as well as a source of plant development and survival through photosynthesis (Wang *et al.,* 2003; Fu *et al.,* 2015; Bhargava and Mitra, 2021). Since CO_2 is a well-mixed gas, its concentration across Earth's surface is only moderately heterogeneous over time and space at any given time primarily because of differences in weather patterns, as well as plant and soil activity. However, an imbalanced rapid accumulation of greenhouse gases in the atmosphere, when the accumulation is far greater than the removal of the gases, may promote excess heat trapped in the atmosphere (Watson *et al.,* 1992). As a result, planet Earth is expected to experience overall increased temperature conditions (Watson *et al.,* 1992; Mbokodo *et al.,* 2020). Past studies have indicated approximately 1-1.5°C temperature increments, globally, by 2017 and beyond (Mbokodo *et al.,* 2020). Other studies have indicated double global rate increments in temperatures in the central and subtropical Africa (Garland *et al.,* 2015; Mbokodo *et al.,* 2020). According to Hansen, *et al.* (2010), rising CO_2 concentrations, along with increasing greenhouse gas concentrations, have contributed to an increase in global temperatures of 0.8°C since 2017. By the end of the century, atmospheric CO_2 concentrations will probably be between 550 and 1000 ppm depending on how aggressively humans reduce CO_2 emissions (and other greenhouse gas emissions) (Ciais *et al.,* 2014). These rises in atmospheric CO_2 concentrations will significantly impact plants as elevated CO_2 concentrations promote resource reallocation within plants, thus impacting the physiological processes of the plant, such as primary and secondary metabolism (Rajashekar, 2018). Indirect effects of increases in CO_2 levels include a rise in air temperature.

Temperature is an essential aspect of biological processes and plants are temperature dependent (Woodward, 1987). Elevated temperatures will not only enhance evaporation from the ground but

also impact plants' microenvironmental conditions, plant distribution (Ficklin and Novick, 2017) and physiological processes. The effects of CO_2 on plants are predominantly seen in their biochemistry and stomatal conductance, but increasing temperatures affect practically every biological process like morphogenesis, membrane lipid composition, and production of secondary metabolites (Pearcy, 1978; Falcone *et al.,* 2004; Begum *et al.,* 2013; Quint *et al.,* 2016). Although warming can affect photosynthesis, photorespiration, and respiration individually, the diverse impacts of higher temperature on other metabolic processes are likely to have a substantial impact on carbon metabolism that we do not yet understand. High temperatures not only negatively impact the photosynthetic capacity of plants by promoting stomatal closure, but also the biomass production, flowering, and fruiting of plants (Ribeiro *et al.,* 2006; Balasooriya *et al.,* 2019a). Consequently, elevations of atmospheric temperature can counteract the positive influence caused by elevated CO_2 concentrations on plants' phytochemical composition, thus affecting the production of the bioactive compounds (Sallas *et al.,* 2003; Balasooriya *et al.,* 2019a).

There is a positive correlation between increases in polyphenolic concentrations as the atmospheric CO_2 and temperature increase. For example, Holopainen *et al.* (2018) reported that elevated CO_2 and temperature increased the phenolic compounds in forest trees leaves. Elevated CO_2 (650 and 950 μmol/mol) and temperature (25 and 30°C) were found to increase the total polyphenolic compounds in strawberries (Balasooriya *et al.,* 2019a). The increased polyphenolic concentrations have been attributed to changes in the production of polyphenolic because of abiotic stresses in the plants (Balasooriya *et al.,* 2019a; Balasooriya *et al.,* 2019b). This positive relationship can be attributed by the stress that elevated CO_2 concentrations and temperature poses on plants thus enhancing the productions of polyphenolic compounds as a counteracting mechanism (Balasooriya

et al., 2019a). Furthermore, elevated CO_2 concentrations and temperature enhances the antioxidant power of plants (Balasooriya *et al.,* 2019a).

Plant metabolism and the production of secondary metabolites are largely impacted by abiotic factors such as altitude, humidity, soil, UV radiation, water availability and more (Isah, 2019; Kabtni *et al.,* 2020). Significant increases in polyphenolic compounds in the annual herbs (*Hordeum murinum* L., *Senecio vulgaris* L., *Bromus madritensis* L., and *Sinapis alba* L.) were when subjected to elevated temperatures and reduced rainfall (Moreira *et al.*, 2020). Cebulak *et al.* (2019) reported an approximately 28% increase in polyphenolic compounds in the skin and flesh of pears when exposed to higher UV-C radiation, higher and lower electromagnetic field, microwaves, and ultrasound.

The production of each specific polyphenolic compounds dependents on the type of response during plant adaptation to the stressful conditions, as the polyphenolic compounds are also species-specific, and differ as per the plant part (Kabtni *et al.,* 2020). For example, phenolic compounds in the leaves of the Northern Hemisphere forest trees increased under elevated CO_2 concentrations while the concentration of terpenoids decreases (Holopainen *et al.,* 2018). Contrary, under high temperatures, the phenolic compounds concentrations of the forest trees' leaves decreased while the concentration of terpenoids increased (Holopainen *et al.,* 2018). An overall increase in polyphenolic compounds in strawberry (*Fragaria ananassa* Duch.) when plants were exposed to both elevated temperatures and CO_2 concentrations (i.e., 650 and 950 μmol mol^{-1} atmospheric CO_2 concentration, and/or 30°C temperature) was reported by Balasooriya *et al.* (2019a). Concentrations of the total polyphenolic compounds such as anthocyanins and flavonoids in strawberry fruits were reported to increase at elevated atmospheric CO_2 (Balasooriya *et al.,* 2019a).

This suggests that elevated CO_2 concentrations and temperatures, and their concurrent effects can significantly influence the nutritional and medicinal quality of fruits and other medicinal plants (Balasooriya *et al.,* 2019a).

Heatwave events in South Africa

Heatwaves can be characterized as extended periods of extremely high day- and night-time temperatures as well as elevated atmospheric humidity (Carlson, 2008). These periods can last for approximately 48 to 72 hours or even longer (Carlson, 2008). Scientists have projected increases in the intensity, frequencies, and duration of heatwaves globally as climate change persists (Carlson, 2008; Luber and McGeehin, 2008; Mbokodo *et al.*, 2020). However, South Africa is suggested to be more prone to such conditions, this is due to the geographical location as well as the socioeconomic development status of southern Africa, where South Africa is located (Mbokodo *et al.,* 2020; Scholes and Engelbrecht, 2021). With the elevated concentrations of greenhouse gases, South Africa might experience 10 times more frequent heat wave events between 2010 and 2039, especially across the eastern parts of the country (Figure 1.2; Mbokodo *et al.*, 2020). The coastal areas of South Africa are projected to experience shorter lasting heatwaves (i.e., average of 3-4 days) whereas the inlands are projected to experience longer lasting events, approximately 2 weeks (Mbokodo *et al.*, 2020). The environmental parameters (i.e., CO_2 concentrations and temperature) used in this study, as well as the maximum duration time of 192 hours, was influenced by the concept of heatwaves in the South African context. Heatwaves pose great threat to the health of the most vulnerable communities around the world (Carlson, 2008; Luber and McGeehin, 2008; Engelbrecht *et al.*, 2015; Mbokodo *et al.*, 2020). They perpetuate illnesses and diseases such as, heat cramps, syncope, exhaustion, stroke and could potentially lead to death (Carlson, 2008; Luber and McGeehin, 2008; Mbokodo *et al.*, 2020).

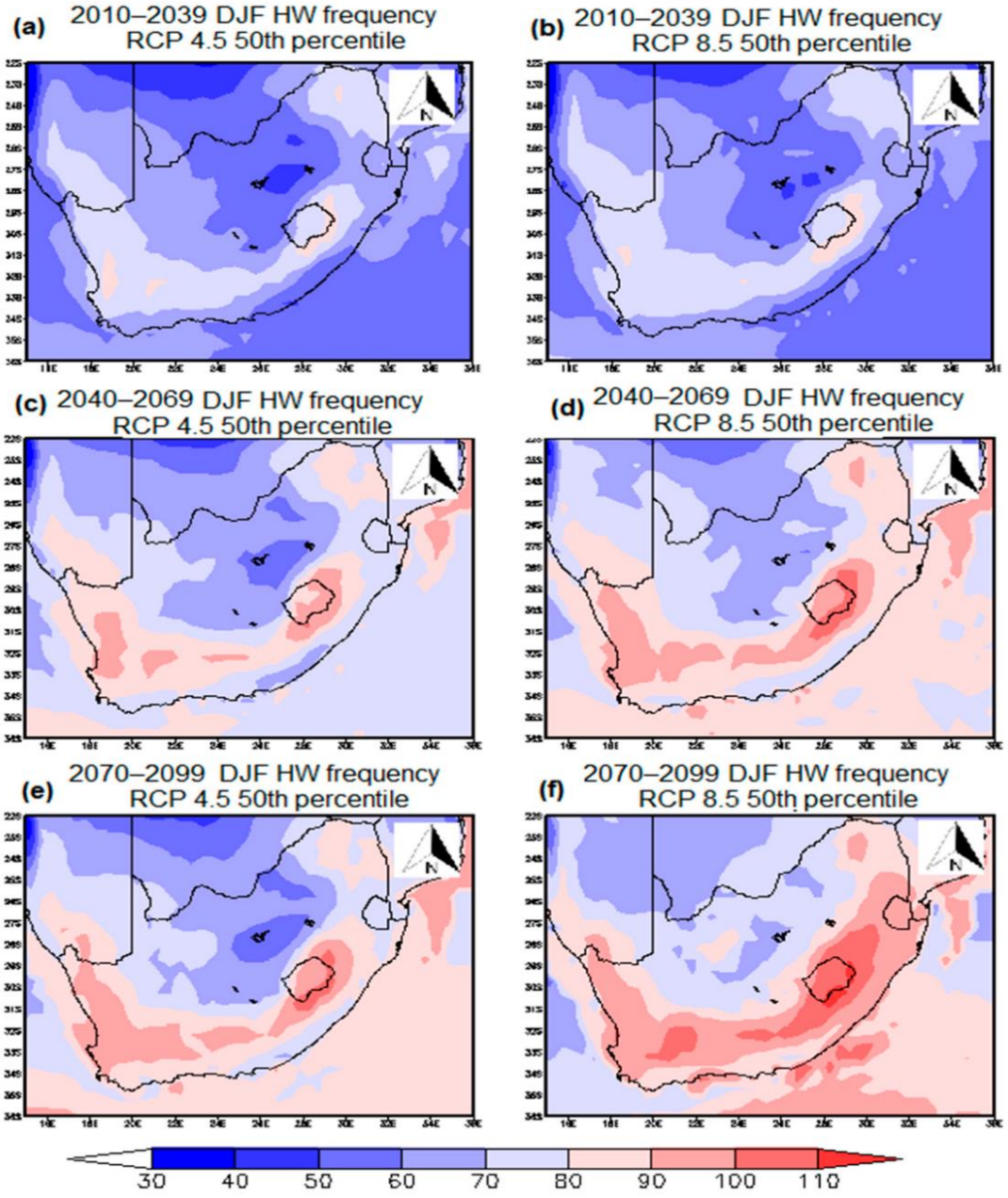


Figure 1.2: Simulations of heat wave (HW) events frequency during the summer season (DJF-December, January, February), between 2010–2039, 2040–2069, and 2070–2099 periods (Mbokodo *et al.*, 2020).

Impacts of temperature and carbon dioxide concentration on antioxidant activity in medicinal plants

Temperature plays an important role in metabolic processes including antioxidant activity. Several studies have demonstrated that heating could result in a linear decrease in plant antioxidant activities (Wang and Zheng, 2001; Balla *et al.,* 2007; Réblová, 2012; Alhaithloul *et al.,* 2021). Tomato seedlings (*Solanum lycopersicum* L.) exposed to cold stress exhibited higher antioxidant activity compared to those exposed to heat stress (Alhaithloul *et al.,* 2021). The antioxidant activity of Earliglow and Kent strawberry fruit (*Fragaria* × *ananassa* Duch) was high in plants grown in high temperatures (30/22°C, day/night) (Wang and Zheng, 2001).

Increase in atmospheric CO_2 concentrations is known to enhance plant growth and development, as well as photosynthesis. Therefore, increase in CO_2 concentrations can enhance production of secondary metabolites (Wang *et al.,* 2003; Fu *et al.,* 2015). Furthermore, elevated CO_2 increases the nutritional quality of medicinal plants such as fruits and vegetables (Fu *et al.,* 2015; Dong *et al.,* 2018). As a result, increases in CO_2 concentrations can be said to enhance the antioxidant activity of plants (Wang *et al.,* 2003; Fu *et al.,* 2015; Dong *et al.,* 2018; Rajashekar, 2018). The oxygen radical scavenging activity of Earliglow and Kent strawberry fruit was higher when exposed to higher CO_2 concentrations (300 $\mu mol.mol^{-1}$ and 600 $\mu mol.mol^{-1}$ above ambient) (Wang *et al.,* 2003). Similarly, Dong *et al.* (2018) reported increases in the antioxidant activities of the widely used vegetables (i.e., lettuce, tomato, and potato) when the CO_2 concentration was increased from 200 μmol^{-1} and 450 μmol^{-1} to 540 μmol^{-1} and 1200 μmol^{-1}. Exposing soybean (*Glycine max* L.) to increased CO_2 concentrations resulted in a decline in antioxidant activity (i.e.,

peroxyl radical-induced oxidations) (Gillespie *et al.,* 2011). Similar observations were made in scallions (Levine and Paré, 2009). These findings were suggested to be due to reduced production of ROS because of elevations in carboxylation rates and, decreases in oxygenation and photorespiration rates (Gillespie *et al.*, 2011). It can therefore be suggested that changes in climatic conditions, such as extreme temperature elevations and changes in atmospheric CO_2 can have various impacts on the antioxidant activity of medicinal plants, depending on the intensity of the changes as well as the response/adaptive mechanism of the plant species.

Impacts of temperature and carbon dioxide concentration on antimicrobial activity in medicinal plants

Environmental stress such as extreme temperature and/or elevated CO_2 concentrations have huge impacts on secondary metabolite production, which could impact the inhibition of microbial activity (Egamberdieva *et al.*, 2017). Temperature is an essential environmental factor that has the potential to influence the secondary metabolite composition as well as the biological activity (Netshiluvhi and Eloff, 2019). Studies have shown that various plants exhibit a variety of antimicrobial activities at varying temperatures. No significant difference was observed in the ability of *Leonotis dysophylla* Benth., *Bulbine frutescens* (L.) Willd. and *Tulbaghia violacea* Hary to inhibit two Gram- positive bacteria (*Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212)) and two Gram-negative bacteria (*Pseudomonas aeruginosa* (ATCC 25922) and *Escherichia coli* (ATCC 27853)) at 15 and 30°C temperature (Netshiluvhi and Eloff, 2019). However, a study by Chokoe *et al.* (2008) against the same bacteria using flavonoids extracted from *Carpobrotus edulis* leaves showed that spring extracts had greater antibacterial activity over autumn extracts. Since the spring season can be said to be slightly warmer than autumn, findings by Chokoe *et al.* (2008) can infer those high temperatures can potentially

promote an increased antibacterial activity in *C. edulis* – the medicinal plant of interest in this study. This can further be justified by the potential increment of antioxidant activity of the plant as temperatures are elevated, and therefore the hypothesis of this study that elevated CO_2 concentrations and temperature would increase the antioxidant and antimicrobial activities of *C. edulis*.

1.2.4. Botany, ecology and distribution overview of *Carpobrotus edulis*

Carpobrotus edulis, formerly known as *Mesembryanthemum edule* L., is from the Domain: Eukaryota; Kingdom: Plantae; Phylum: Spermatophyta; Subphylum: Angiospermae; Class: Dicotyledonae; Order: Caryophyllales; Family: Aizoaceae; Genus: Carpobrotus; Species: Edulis (Campoy *et al.,* 2018; Akinyede *et al.,* 2020). The species is commonly known in English as Hottentot fig, Highway ice plant, Sour fig; in Afrikaans it is known as a 'Ghaukum', 'Ghoenavy', 'Hottentotsvy', 'Kaapsevy', 'Perdevy', 'Rankvy', 'Suurvy' or 'Vyerank'; in isiZulu as 'Ikhambi-lamabulawo' or 'Umgongozi'; and in isiXhosa it is known as 'Igcukuma' (Campoy *et al.,* 2018; Akinyede *et al.,* 2020).

C. edulis is a succulent halophyte native to South Africa where it is most commonly distributed in the Eastern and Western Cape provinces (Campoy *et al.,* 2018; Akinyede *et al.,* 2020). This species is known to be aggressively invasive in the coastal dunes of other continents including, Australia, New Zealand, Southern Europe and USA (Figure 1.3; Wisura and Glen, 1993; Roiloa *et al.,* 2009; Campoy *et al.,* 2018). *C. edulis* was introduced in these continents either as a sand/ soil stabilizer or as an ornamental plant in gardens; however, due to its fast-growing ability, divers dispersers, and low nutrient requirements, the plant easily and quickly spread into local coastal sand dunes ecosystems (D'Antonio, 1990; Roiloa *et al.,* 2009; Novoa and González, 2014).

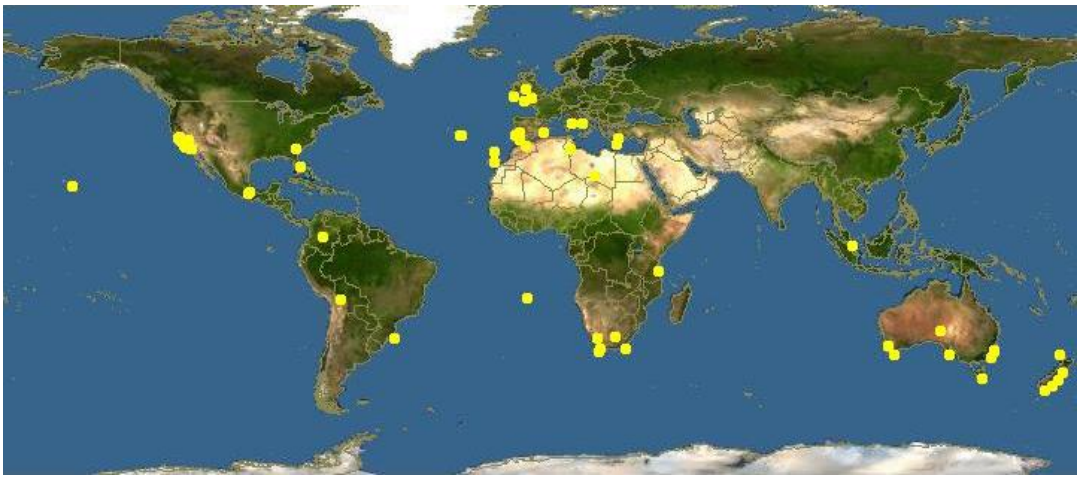


Figure 1.3: Distribution map of *Carpobrotus edulis* (L.) across the globe. Wide distribution within continents owing it to its invasiveness (Discover Life, accessed on the 17th of November 2021).

C. edulis is an evergreen, dense mat-forming succulent plant, that grows and spreads horizontally from its stolon (Figure 1.4) (Roiloa *et al.,* 2010; Campoy *et al.,* 2018). The horizontal spread is facilitated by the production of numerous apical ramets that remain integrated by the stolon connections thus, strengthening the plant's colonization nature (Roiloa *et al.,* 2010). The newly produced ramets have the ability to root and are able to survive even when detached from the parent plant (Roiloa *et al.,* 2010). It has triangular perennial finger-like leaves that grow radially around the nodes of the stem (Wisura and Glen, 1993; Roiloa *et al.,* 2010; Campoy *et al.,* 2018). The species produces yellow flowers which change to pale pink colour when approaching senescence (Figure 1.4) (Roiloa *et al.,* 2010; Campoy *et al.,* 2018). It bears fleshy fruits (Figure 1.4) that contain numerous small seeds (D'Antonio, 1990; Roiloa *et al.,* 2010; Campoy *et al.,* 2018).

Figure 1.4: Morphological features of *Carpobrotus edulis* during the blooming season. (A) Flowers are yellow representing pre-senescence stage (discoverlife.org, accessed on the 18th of February 2022); (B) pink-purple flowers that represent the senescence stage of the flowers (gardengoods.co.za, accessed on the 13th of January 2022); (C) the fruit-bearing plant representing the stage after successful pollination (za.pinterest.com, accessed on the 7th of September 2022).

The flowering season of *C. edulis* begins in August until October, and the fruiting season usually starts in October until March (Pfukwa *et al.*, 2020). The fruits are allegedly sour before maturation, they however get sweeter as they mature and are therefore edible by both humans and other animals (D'Antonio, 1990). The consumption period of the *C. edulis* fruits coincides with the onset of California's rainy season, and the seed germination period coincides with the first autumn rains (D'Antonio, 1990). Therefore, this might be one of the reasons for the successful spread of *C. edulis* making it invasive in its introduced ranges in California.

Seed dispersal of many plants depends on the presence of dispersal vectors (D'Antonio, 1990). The presence, abundance, and behaviour of seed dispersal vectors of plants with animals as specialized agents, negatively affect that ability of plants to colonize new habitats (D'Antonio, 1990). Thus, affecting the distribution range of the plants (D'Antonio, 1990). For example, the reduced distribution range of the gingko tree (*Gingko biloba* L.) in Asia was said to be caused by the extinction of the species' dispersers (Tiffney, 1984; D'Antonio, 1990). The most common seed dispersers of plants are terrestrial mammals (Carlquist, 1974; D'Antonio, 1990). *C. edulis* is no different from these species. *C. edulis* bears indehiscent fleshy fruits which are mainly consumed by herbivores mammals (D'Antonio, 1990; Campoy *et al.,* 2018). In central California, the *C. edulis*' fruits are said to be consumed by mule deer, rabbits (especially brush rabbits and jackrabbits) and ground squirrels (D'Antonio, 1990). Therefore, *C. edulis*' dispersal may depend on endozoochory, which is the dispersion of seeds through the ingestion of the fruits by animals, apart from its vegetative reproduction ability.

1.2.5. Medicinal properties of *Carpobrotus edulis*

For centuries humanity had been dependent on medicinal plants for good health and survival until the introduction of synthetic medicine. Nevertheless, synthetic medicine is now overtaken by the rediscovery of traditional medicine. Reverting to the use of medicinal plants falls on the discovery that synthetic medicine poses negative side-effects on human health, such as increasing the chances of cancer (Friedman *et al.,* 2009; Hussein and El-Anssary, 2019). Research on medicinal plants has since been on the rise. Modern research focuses on the efficacy of medicinal plants, as well as the investigation of phytochemicals which have health benefits. Medicinal plants that are commonly used for traditional medicine include but not limited to *Moringa oleifera* Lam., *Bulbine*

natalensis L.f. and *frutencens* L., *Carpobrotus edulis* (Pather *et al.,* 2011; Matic *et al.,* 2018; Mudimba and Nguta, 2019).

C. edulis is one of the most importantly used traditional medicinal plant in South Africa. Indigenous communities consume the leaves, flowers, fruits of this native plant for medicinal reasons. The leaves are boiled and drank, chewed, or rubbed to treat various ailments (Table 1.1). Moreover, the plant's leaves may be used to manage HIV/AIDS related illnesses (Omoruyi *et al.,* 2012; Mudimba and Nguta, 2019). Although every part of the plant is traditionally consumed/used for medicinal purposes to treat various ailments, only the leaves have been extensively studied. As a result, very little to no studies have been done on the other parts of the plant species. Consequently, because more studies have been conducted on the plant's leaves, their medicinal uses are well understood.

Table 1.1: Medicinal uses and mode of consumption of *Carpobrotus edulis* in relation to the different ailments treated.

Plant part	**Form of consumption**	**How is it used?**	**What does it treat?**	**References**
Leaves	Boiled	Taken orally (i.e., drank or chewed)	Tuberculosis; respiratory infections; tooth- and earache; hypertension; diabetes mellitus, analgesia agent and gastrointestinal motility; sore throat and mouth infections; intestinal worms; dysentery; digestive troubles such as diarrhea and stomach aches; chilblains; mouth and vaginal thrush, as well as sinusitis.	Van Der Watt and Pretorius, 2001; Khattab and El Sherif, 2011; Martins *et al.*, 2011; Ibtissem *et al.*, 2012; Mabona and Van Vuuren, 2013; Mudimba and Nguta, 2019; Akinyede *et al.*, 2020
	Leaf gel	Rubbed	Skin conditions such as facial eczema, wounds and burns, soothing itching caused by spider and tick bites.	Akinyede *et al.*, 2020

1.2.6. Biological activity of *Carpobrotus edulis*

The high presence of polyphenolic compounds in *Carpobrotus edulis* leaves give the plant its antimicrobial, antibacterial, antifungal, antioxidant, antiproliferative, anticancer agents and neurological activities (Van Der Watt and Pretorius, 2001; Martins *et al.,* 2011; Ibtissem *et al.,* 2012; Mudimba and Nguta, 2019; Akinyede *et al.,* 2020). Antibacterial compounds from *C. edulis* leaves have been isolated with oleanolic acid showing a greater activity against more bacterial strains (Martins *et al.,* 2011). Triterpene uvaol was seen to exhibit most activity in modulating the efflux activity by Multidrug Resistant Gram-positive strains (Martins *et al.,* 2011). Ibtissem *et al.* (2012) showed that *C. edulis* has relatively greater antioxidant activity compared to the closely related species *Mesembryanthemum crystallinum* (L.), as well as, activity of microorganisms *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

1.2.7. Secondary metabolites in *Carpobrotus edulis* leaves

Previous studies have shown that *Carpobrotus edulis* contain the following polyphenolic compounds; phenolics, proanthocyanidins, alkaloids, tannins, saponin, flavonoids (i.e., rutin, neohesperidin, hyperoside; catechin; ferulic acid; and catechol tannins), flavonols, quercetin, anthraquinones, sulphates, chlorides, coumarins, irodoids, cyanogenic glycosides, cardiac glycosides, glycosides, carbohydrates, triterpenoids and unsaturated sterols (Van Der Watt and Pretorius, 2001; Martins *et al.,* 2011; Ibtissem *et al.,* 2012; Omoruyi *et al.,* 2012; Hafsa *et al.,* 2016; Rocha *et al.,* 2017; Mudimba and Nguta, 2019). Due to these metabolites, *C. edulis* is one of the important medicinal plants in South Africa for treating various ailments and improve human health (Van Andel and Carvalheiro, 2013). Therefore, it is essential to assess the

responses of the species to combined atmospheric CO_2 concentrations and temperatures, and the consequent impact on the medicinal properties and efficacy.

1.3. Study rationale

Carpobrotus edulis is a native South African plant commonly used as an indigenous medicinal plant in traditional medicine. However, climate change may have significant impacts on the ability of the plant to retain its medicinal properties. As literature has shown, the elevation of atmospheric CO_2 concentrations and temperature could potentially compromise the therapeutic efficacy of medicinal plants including *C. edulis*, by altering the secondary metabolites composition. Consequently, this may drastically impact on the livelihoods of indigenous people who are dependent on *C. edulis* for good health. It is, therefore, imperative to understand the extent that climate change can influence the therapeutic efficacy *Carpobrotus edulis*. This study intended to investigate how the therapeutic efficacy of *C. edulis* will be impacted by the concurrent elevated atmospheric CO_2 concentrations and temperatures by assessing the secondary metabolite profile, antioxidant and antimicrobial activities of the species. The medicinal importance of *C. edulis* to South Africa's indigenous communities in conjunction with the effects climate change could have on *C. edulis* efficacy prompted the aim of this study.

1.4. Research aim(s)

The study aimed to investigate the possible modification the in polyphenolic compounds, antioxidant activity, and antibacterial activity in *Carpobrotus edulis* leaves under concurrent elevated CO_2 and temperatures.

1.5. Research objectives

The objectives identified were to:

i. Profile the polyphenolic compounds in *Carpobrotus edulis* leaves under combined elevated CO_2 and temperature, 600 ppm and 35/30°C, 600 ppm and 45/35°C, 800 ppm and 35/30°C, 800 ppm and 45/35°C, respectively. The control samples were kept at ambient conditions of combined 400 ppm and 28/25°C.

ii. Determine the antioxidant activity of *C. edulis* leaves under combined 600 ppm and 35/30°C, 600 ppm and 45/35°C, 800 ppm and 35/30°C, 800 ppm and 45/35°C, respectively.

iii. Determine the antibacterial activity of *C. edulis* leaves under concurrent elevated CO_2 and temperature (600 ppm and 35/30°C, 600 ppm and 45/35°C, 800 ppm and 35/30°C, 800 ppm and 45/35°C, respectively) against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive).

iv. To compare the results of polyphenolic compounds profile, antioxidant and antibacterial activities in the treated samples with those of the control (combined 400 ppm and 28/25°C).

CHAPTER 2: AN ANALYSIS OF THE PHYTOCHEMICAL PROFILE OF *CARPOBROTUS EDULIS* LEAVES UNDER CONCURRENT ELEVATED CARBON DIOXIDE (CO_2) CONCENTRATIONS AND TEMPERATURES

2.1. Introduction

Polyphenolic compounds are phytochemical substances naturally produced by plants to enhance fecundity and survival (Handique and Baruah, 2002). These phytochemicals are produced through secondary metabolism pathways (Oksana *et al.,* 2012; Mera *et al.,* 2019). Polyphenolics are synthesized via shikimic acid and pentose phosphate pathways (Figure 2.1; Oksana *et al.,* 2012). Polyphenols consist of phenolic rings, which are generally classified as phenolic acids and phenolic alcohols. They can be classified by the presence of benzene rings with one or more hydroxyl groups (Oksana *et al.,* 2012; Lin *et al.,* 2016). Polyphenolic compounds range from simple to complex depending on the number of hydroxyl groups present (Lin *et al.,* 2016). There are a wide variety of these polyphenolics compounds present in plants, these include but not limited to, anthocyanins, flavonoids, phenolic acids, quercetin, ferulic acid (Figure 2.2; Lin *et al.,* 2016;).

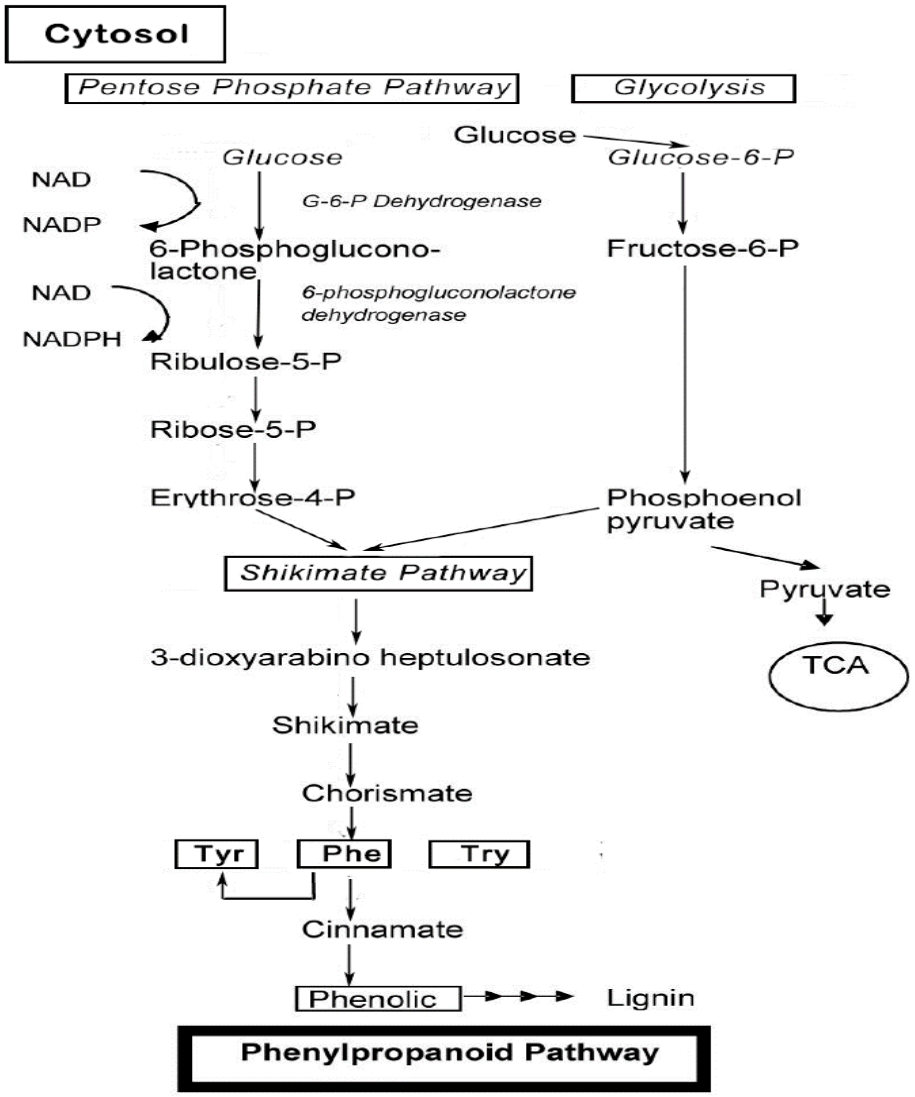


Figure 2.1: Summary pathways of the synthesis of phenolic compounds; shikimate, pentose phosphate and phenylpropanoid pathways (Lin *et al.*, 2016).

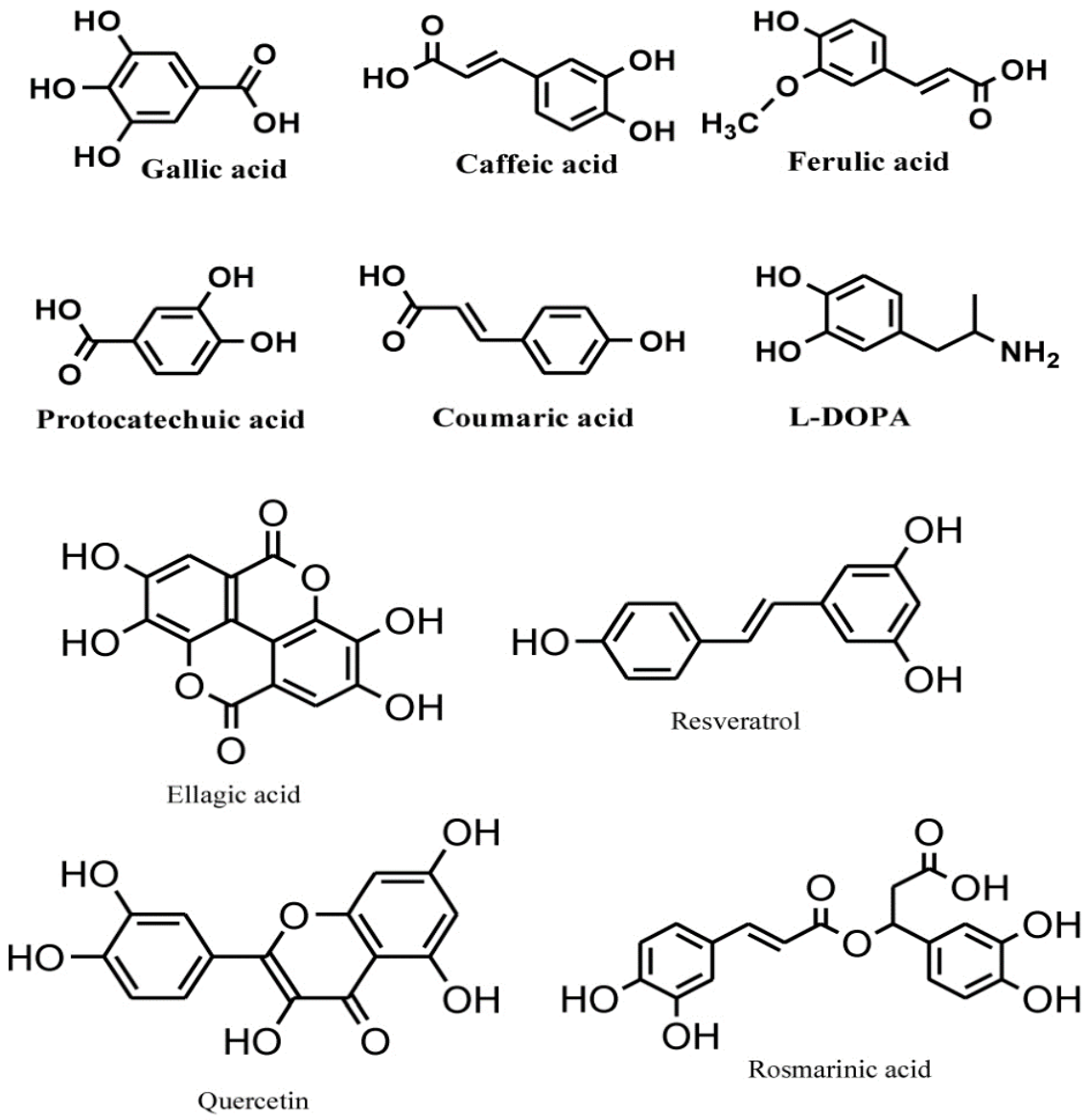


Figure 2.2: Structures of common phenolic compounds ranging from simple to complex compounds (Lin *et al.,* 2016).

Phenolic acids are categorized into two groups: hydroxycinnamic acids and hydroxybenzoic acids (Oksana *et al.,* 2012). The presence of hydroxybenzoic acids in human diets is rare, therefore these compounds are not implicated in human health. Cinnamic acid derivatives have a backbone of C1-6 and benzoic acid derivatives have a C3-6 backbone (Oksana *et al.,* 2012).

Flavonoids are compounds composed of two benzene rings linked with three carbon chains from the nearby pyran ring. Flavonoids are further divided into six classes based on the oxidation state of the central carbon; they include flavanones, flavanols, flavonols, isofalvons, flavones, and anthocyanidins (Oksana *et al.,* 2012; Panche *et al.,* 2016). A total of over 4000 flavonoids have also been identified from plant sources. Among flavonols, a double bond is observed between the carbon atom C3 and its carbon atom C2, and the carbon atom C3 is attached to a hydroxyl group (Panche *et al.,* 2016). Food sources contain primarily flavonols, the most common flavonoid. Such polyphenolics are mainly found in, but are not limited to, onions as well as broccoli and leeks (Proteggente *et al.,* 2002). Flavonoids consist of a backbone structure of C6-C3-C6, and they contain two phenolic units (C6) (Panche *et al.,* 2016). Based on their hydroxylation configuration, flavonoids are divided into four sub-classes: flavonoles, flavones, flavanones, and anthocyanidins (Panche *et al.,* 2016). Flavonoids have structures where the C2 of the C ring attaches to the B ring, but C3 and C4 attachments are also present (Panche *et al.,* 2016). Even though chalcones have no C ring, they are still considered a member of the flavonoid family, and apples and hops are the sources of chalcones (Panche *et al.,* 2016). Plants have such structures as glycosides as well as glycocones.

Polyphenols are classified into many classes depending on the strength of the phenolic ring; however, the main classes are phenolic acids, flavonoids, stiblins, phenolic alcohols, and lignans (Ahmad *et al.,* 2021). Polyphenols are known as bioactive compounds that protect the human body against chronic degenerative ailments and they are embedded in diets (Ahmad *et al.,* 2021). In addition to preventing degenerative diseases, polyphenols also act as antioxidants (Ahmad *et al.,* 2021). Polyphenols have been studied more slowly because of their unique structure. Polyphenols are among the most common antioxidants in our diets. In atherosclerosis, endothelial lesions are

caused by the inhibiting of oxidative change in low density lipoprotein. Research has demonstrated polyphenols' role in treating cardiovascular disease, osteoporosis, neuroinflammatory disease, cancer, and diabetes (Ganesan and Xu, 2017; Leri *et al.,* 2020; Ahmad *et al.,* 2021).

Polyphenolic compounds are the first line of defence against both biological (such as predators and pathogen) and environmental stresses (such as extreme temperatures, UV rays, salinity) (Lin *et al.*, 2016; Ganesan and Xu, 2017). Generally, high level of antioxidant, antimicrobial and nutrients confer to the plant a great value for pharmaceutical and food industries. As a result, the efficacy of a specific plant is dependent to the concentration. As previously mentioned, *Carpobrotus edulis* L. is an essential South African medicinal plant owing it to the plant's phytochemical composition which includes, alkaloids, flavonoids, flavonols, phenolics, proanthocyanidins, saponins, and tannins (Omoruyi *et al.,* 2012). However, no studies have been done on the phytochemical response of *C. edulis* to the concurrent elevation of CO_2 concentrations and temperature.

In this section, a Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS) analysis was used to investigate the presence and/or absence of polyphenolic compounds in *Carpobrotus edulis* leaves before and after treatment. The untargeted metabolomics approach was used to determine the polyphenolic compound profiling of *Carpobrotus edulis* under elevated atmospheric concentrations and temperature. Although untargeted metabolomics analysis deals with a large proportion of unknown metabolic compounds, it also gives a view of the overall chemical state present in the target plant material (Hilgart, 2016). This method is usually used to identify "as many compounds as possible in the sample" and keep track of any chemical changes present in the plant samples (Hilgart, 2016). The limitation of this analysis is the high number of unknown

compounds due to inadequate annotations in the metabolomic databases, thus lowering the number of compounds that can be easily identifiable (Matsuda *et al.,* 2009).

2.2. Materials and methods

2.2.1. Plant propagation and collection

Carpobrotus edulis plants were propagated vegetatively using plant cuttings collected from the ecological garden at the University of the Witwatersrand (26° 11' 23" S 28° 01' 52" E), Johannesburg. The cuttings were potted and grown in the University's greenhouse for approximately two weeks, to allow the plants to establish the root system. Each pot contained three individual plants which were considered replicates. A total of 60 individuals was planted into 20 pots. Each pot was filled halfway with a soil mixture of compost and fertilizer at 1:1 volume ratio.

The potted plants were transferred into the controlled plant growth chambers (CONVIRON) located in the Oppenheimer Life Sciences (OLS) building at the University (26° 11' 29" S; 28° 01' 55" E), where they were exposed to elevated atmospheric CO_2 concentrations and high temperatures. A total of five chambers with the following set atmospheric conditions were used: 1) control/ambient (A) - 400 ppm and 28/25°C (day/night); 2) Treatment A (TA) - 600 ppm and 45/35°C (day/night); 3) Treatment B (TB) - 800 ppm and 45/35°C (day/night); 4) Treatment C (TC) - 600 ppm and 35/30°C (day/night); 5) Treatment D (TD) - 800 ppm and 35/30°C (day/night) (Figure 2.3). A total of 36 plants (12 pots) were placed in each chamber for a total duration of 192 hours, one pot was removed from each treatment settings every 48 hours for further analysis (Figure 2.3).

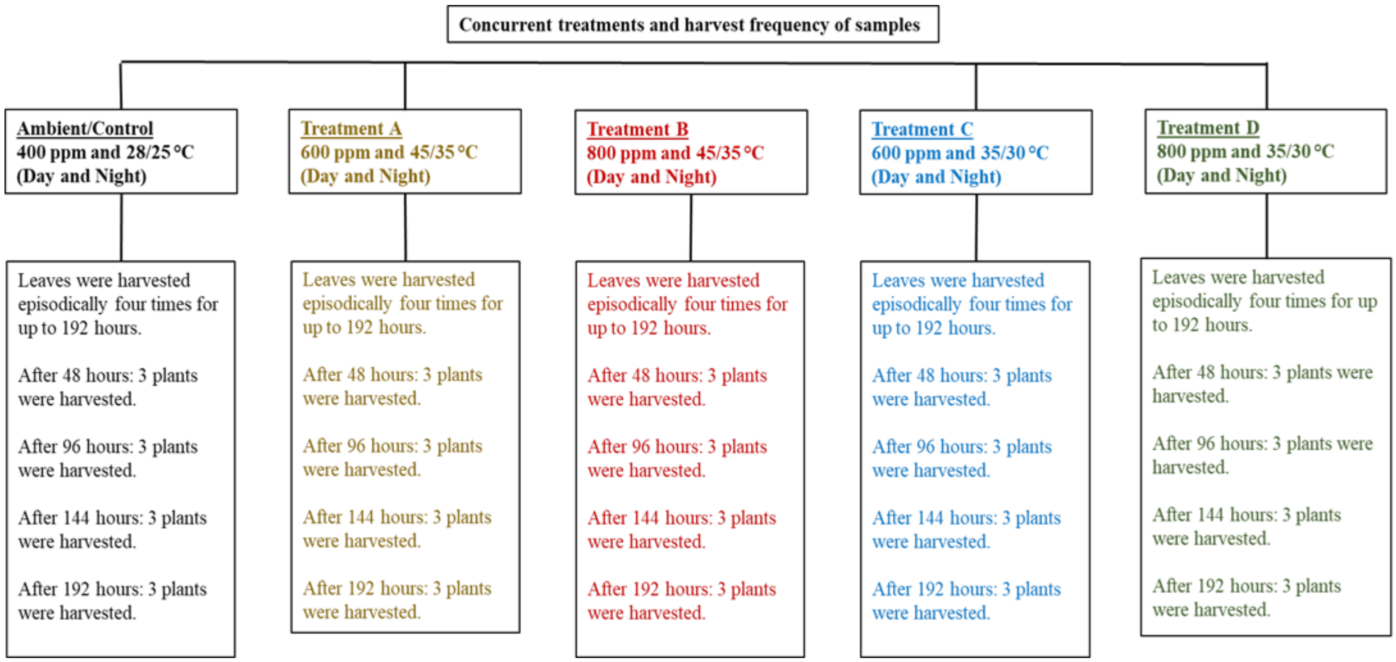


Figure 2.3: Schematic diagram showing the experimental design of concurrently elevated CO_2 concentration (ppm) and temperature (°C), with ambient conditions, as well as the harvest frequency of *Carpobrotus edulis* leaf samples for.

2.2.2. Extract preparation

Analysis of plant leave materials was conducted in the medicinal laboratory in the School of Animal Plant and Environmental Sciences located at the OLS building, at Wits University. Preparation of extracts followed the method by Irawan *et al.* (2018) with some modifications. *Carpobrotus edulis* leaves were collected, oven dried at 45-50°C for 24-36 hours. Thereafter the dried leaves were powdered using an electric blender (Sylvio Jar Blender model; South Africa-Johannesburg; type: 62200 A, 220-240 V ~ 50/60 Hz, 550 W).

Methanol was used as a solvent for the recovery of the secondary metabolites through the assistance of the ultra-sonication extraction technique. The ultra-sonication extraction method is regarded the best technique creating extracts for bioactive compounds related studies (Chokoe *et*

al., 2008 and Shih *et al.,* 2011). The extraction involved the mixing 3 g of the obtained leaf powder with 25 ml of 80% methanol. This solution was then sonicated for 60-90 minutes. Finally, the sonicated solution was filtered with Whatman filter papers and the supernatant was concentrated using a rotary vapour, then kept in brown vials (at 4℃) for further analysis.

2.2.3. Preliminary phytochemical screening

The following seven phytochemical representative groups, tannins, phenolics, flavonoids, steroids, terpenoids, glycosides, and saponins, were screened using qualitative approaches. All the assays were conducted according to Debalke *et al.* (2018) and Owusu *et al.* (2021). The qualitative analysis was done using the crude methanolic extracts. The tests described were used to identify the presence of phytochemicals in the methanolic extract.

Tannins

To conduct the tannins assay, 2 ml of distilled water was added in 2 ml of extract, then 2-3 drops of 5% ferric chloride ($FeCl_3$) were added. The presence of a green precipitate indicated the presence of tannins.

Phenolic compounds

Phenolics were investigated by adding 2-5 drops of 10% ($FeCl_3$) in 1 ml of the extract. A change of colour to violet indicated the presence of phenolics.

Flavonoids

For the flavonoids, 1-5 drops of hydrochloric acid (HCl) were added to 1 ml of extract, and a colour change to red indicated the presence of flavonoids.

Steroids

To investigate the presence of steroids, 2 ml of chloroform and 2 ml of sulfuric acid (H_2SO_4) were carefully added to 2 ml of extract, and the presence of a reddish-brown ring signified the presence of steroids.

Terpenoids

The presence of terpenoids was investigated by adding 0.5 ml of chloroform and 2-5 drops of sulfuric acid into 1 ml of extract. A reddish-brown colour change showed the presence of terpenoids.

Glycosides

Glycosides were determined by adding 2 ml of H_2SO_4 to 0.5 ml of extract, and a change in colour to reddish-brown showed the presence of glycosides.

Saponins

The presence of saponins was checked by adding 5 ml of distilled water to 0.5 ml of extract, then shaking the solution vigorously. This was followed by the addition of 2-5 drops of olive oil, then shaken vigorously again; foam-forming indicated the presence of saponins.

In the assays, the greater the intensity of colour, precipitation, ring and/or foam indicated a greater presence of the compound in the extract. The presence/absence and intensity of colour, precipitation, ring and/or foam data obtained was tabulated using excel.

2.2.4. Polyphenolic analysis of *Carpobrotus edulis* leave extracts

Untargeted analysis of polyphenolic compound in *Carpobrotus edulis* leaves

The phenolic profiles were comprehensively investigated using an targeted metabolomic approach based on liquid chromatography-mass spectrometry (LC-MS/MS). Subsequent to extract preparation, LC-MS/MS profiles of the plant leaves were generated according to Hilgart (2016). However, prior to the LC-MS/MS assay, the crude extracts were reduced in volume to increase the concentration using a rotary vapor, where about 50% of the methanol solvent was evaporated. The concentrated extract was further filtered using 0.45 µm syringe filters. These were then stored in LC-MS/MS glass bottles for further analysis.

Processing Liquid Chromatography-Mass Spectrometry data

The obtained data was pre-processed in the open source, MZmine 2.53 version, following the guideline by Hilgart (2016). The outline of the LC-MS/MS raw data pre-processing is shown in Figure 2.4. The mass detection stage started after importing the obtained LC-MS/MS raw data onto the MZmine software. In this stage, a list of the compounds in each extract was compiled. The centroid mass detector was utilized, and the noise level (used to filter the chromatogram) was set to the intensity of $1e^4$ or $1e^3$ depending on the visual noise level of the chromatogram. This stage was then followed by the chromatogram builder stage, which created chromatograms using the list of compounds obtained in the mass detection stage. The chromatograms were created "based on the continuous appearance of compound masses over consecutive scans" (Hilgart, 2016). The following parameters were used in the chromatogram builder stage: min group size in # of scans – 5 (number of sequential scans); group intensity threshold – $3e^6$ ("threshold by which scans are compared against to count spectra as part of a group"); min highest intensity – $3e^6$ (peak

of intensity); m/z tolerance – 0.02m/z or 10 ppm ("tolerance of the difference between data points in consecutive scans to be counted"). In the chromatographic deconvolution stage, "the process of separating various mass spec data from the measured chromatographic information", the following parameters were used: min peak height – $1e^5$; peak duration – 0 to 3 minutes; baseline level – $1e^4$. Following this stage, compounds with different peaks but were isotopes were identified and grouped together, in the deisotoping stage. The parameters included in the deisotoping stage were as follows: m/z tolerance – 0.02 or 10 ppm; RT tolerance – 0.25 [absolute (min)]; max charge – 2; representative isotope – most intense. In the alignment stage, the peaks of the compounds were aligned, including the isotopes, resulting in a single list, and the following parameters were used: m/z tolerance – 0.02 m/z or 10 ppm; weight for m/z – 75; retention time tolerance – 0.25 [absolute (min)]; weight for RT – 25. Prior to the gap filling stage, peak filtering was applied to allow selection of nodes/compounds that will later be analysed. Over 22 000 compounds were identified, therefore, the peak filtering stage was done in order to keep only the peaks with desired parameters. Filtering relied on the visualizing networks within the GNPS environment, therefore the following parameters were used: min peak in row – 5; m/z range – 75-1500; reset peak number ID – TRUE. This was followed by the gap-filling stage, which "searched the original raw data to find spectra that may have been mis-aligned during the alignment step" or spectra that were filtered out due to low peak intensity or important peaks that might have been accidentally removed. The parameters used in the gap-filling stage were: intensity tolerance – 10%; m/z tolerance – 0.02 m/z or 10 ppm; RT tolerance – 0.25. The obtained data was then exported into an excel spreadsheet for the identification stage. In the identification stage an open-source repository called PubChem, was used to identify compounds obtained in the extracts. The data was tabulated using Microsoft excel spreadsheet.

A Principal Component Analysis (PCA) was conducted in Soft independent modelling by class analogy (The SIMCA® 15) following the manufacturer's guide to assess the similarities for the presence of metabolites in the leaves of *C. edulis* from the different treatment groups.

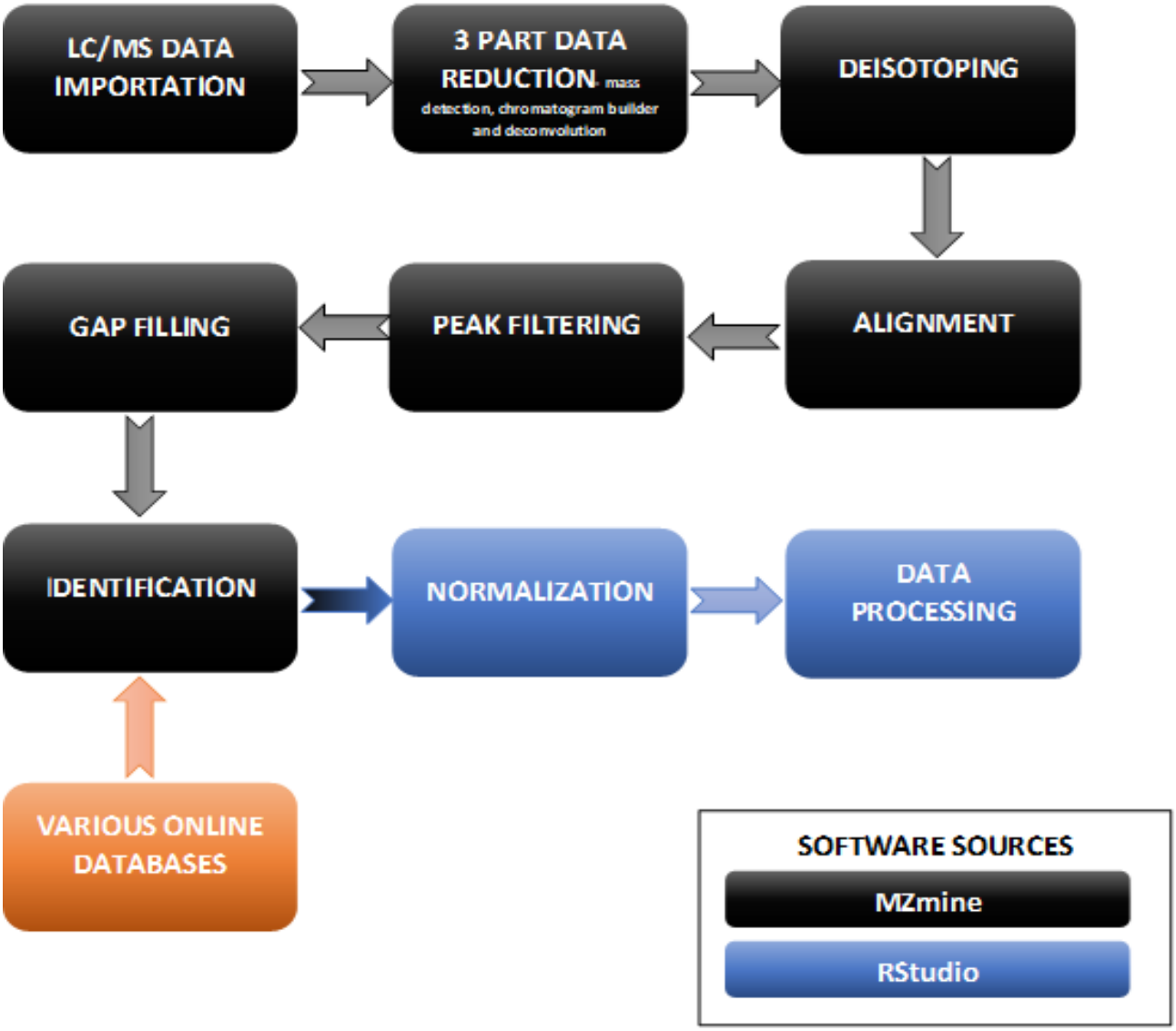


Figure 2.4: Summary workflow of the LC-MS/MS data processing of polyphenolic compounds in *Carpobrotus edulis* leaves. Diagram was adopted from the methods by Hilgart (2016) and MZmine guideline.

2.3. Results

2.3.1. Preliminary phytochemical screening of *Carpobrotus edulis* leaves under elevated CO_2 and temperature

The preliminary phytochemical screening conducted on *Carpobrotus edulis* methanolic extracts showed the presence of tannins, phenolics, flavonoids, steroids, terpenoids, glycosides, and saponins in varying amounts (Table 2.1). The presence of the phytochemical groups was interpreted as follows: the plus (+) symbol denoted the qualitative presence of the phytochemical compounds with very strong presence (+++); moderately strong presence (++); moderately low presence (+); and not detected (-). The qualitative results indicated the moderately low presence of flavonoids and saponins, moderately strong presence of tannins and terpenoids, and very strong presence of phenolics, steroids, and glycosides (Table 2.1).

The presences of tannins, phenolics, steroids, and glycosides ranged from extremely to moderately strong throughout all treatment conditions while, terpenoids were present in very strong to moderate amounts throughout the treatment conditions (Table 2.1). In contrast, the presence of the flavonoids group seemed to fade when both CO_2 concentrations and temperatures are elevated concurrently as compared to the control (Table 2.1). Interestingly, flavonoids were not detected in extremely high CO_2 concentration and moderately high temperatures (800 ppm and 35/30°C) (Table 2.1). At extremely high CO_2 concentration and moderately low temperatures, (800 ppm and 35/30°C), the flavonoids were initially absent, then reappeared when temperatures were extremely elevated (800 ppm and 45/35°C) (Table 2.1). Under 600 ppm and 35/30°C, the presence of flavonoids was indicated by a red colour of the extract, then the colour intensified under 600ppm and 45/35°C, indicating a stronger concentration of flavonoids (Table 2.1). This showed that

increasing temperature can promote the production of flavonoids. This study also showed presence of saponins in control extracts was moderately low, then it presence increased to moderately strong when CO_2 concentrations and temperatures were elevated concurrently (Table 2.1).

The presence of all the phytochemical compounds was potentially not influenced by the duration of exposure to the specific atmospheric conditions. Meaning that, the presence of these compounds did not change regardless of how long they have been exposed to elevated CO_2 concentrations and temperatures (Table 2.1). For instance, flavonoids were present in *C. edulis* leaves at moderately low level in TA conditions, and this persists for the whole 192 hours (Table 2.1). This trend was consistent for all other treatments as well as all the other compounds (Table 2.1). The observed trend was with the exception of terpenoids, where the decline in presence to very low level was observed between 96 hours and 144 hours depending on the atmospheric conditions, however, the presence appeared to be stronger either before or at 192 hours of exposure (Table 2.1).

Table 2.1: Qualitative overview of the polyphenolic compounds found in methanolic extracts of *Carpobrotus edulis* leaves at different atmospheric conditions at different time intervals. Ambient (400 ppm and 28/25°C), TA (600 ppm and 45/35°C), TB (800 ppm and 45/35°C), TC (600 ppm and 35/30°C), TD (800 ppm and 35/30°C).

		48 Hours				96 Hours				144 Hours				192 Hours			
	Ambient	**TA**	**TB**	**TC**	**TD**	**TA**	**TB**	**TC**	**TD**	**TA**	**TB**	**TC**	**TD**	**TA**	**TB**	**TC**	**TD**
Tannins	++	++	+++	+++	+++	++	+++	+++	+++	++	+++	+++	+++	++	+++	+++	+++
Phenolics	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Flavonoids	+	+	-	-		+	-	-		+	-	-		+	-	-	
Steroids	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Terpenoids	++	++	++	++	+	++	+	++	++	+	+	+	++	++	++	+	++
Glycosides	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Saponins	+	++	++	++	++	++	++	++	++	++	++	+	++	++	++	+	++

Notes: the plus (+) symbol denotes the qualitative presence of the phytochemical compounds with very strong presence (+++); moderately strong presence (++); moderately low presence (+); not detected (-)

2.3.2. LC-MS/MS polyphenolic compounds analysis of *Carpobrotus edulis* under high CO_2 and temperature

The LC-MS/MS revealed the presence of 24 polyphenolic compounds in *Carpobrotus edulis* leaves and of those only 14 (58.83%) were present in *C. edulis* leaves under ambient conditions (Table 2.2). Furthermore, the concurrent increment of CO_2 concentrations and temperatures prompted the appearance of 10 (41.67%) more compounds (Table 2.2). A total of 17 (70.83%) polyphenolic compounds were identified under TA conditions. The presence of these 17 compounds seemed to be influenced by the duration of exposure to these conditions. For example, the 11 (64.71%) compounds were present throughout the 192 hours of exposure to TA conditions, namely: p-coumaric acid, citric acid, palmitic acid, oleic acid, cianidanol, protocatechuic acid-O-glucoside, phlorizin, oleanolic acid, β-type procyanidin dimer, isorhamnetin glucosyl-rhamnoside, and chlorogenic acid (Table 2.2). While the rest of the six (35.29%) compounds disappear before the 192 hours is reached (Table 2.2). Moreover, of all the 17 compounds, four (23.53%) were not found under ambient conditions, these included dibutyl phthalate, linoleic acid, docosanoic acid, and oleanolic acid (Table 2.2).

Only one (4.17%) polyphenolic compound out of the identified 24 compounds, protocatechuic acid-O-glucoside, was present in *C. edulis* leaves under TB climatic conditions (Table 2.2). This compound was only present from 48 to 96 hours of exposure to these conditions (Table 2.2).

Most of the identified polyphenolic compounds (22 out of 24 ~ 91.67%) found in *C. edulis* leaves were observed when the plants were exposed to TC conditions. Out of the 22 compounds, 10 (45.45%) only were observed under TC conditions and not under ambient conditions, these include myristic acid, palmitoleic acid, linoleic acid, stearic acid, cis-11-Eicosenoic acid, arachidic acid,

docosanoic acid, oleanolic acid, quercetin 3-rhamninoside (Table 2.2). Moreover, out of the 22 compounds, nine (40.91%) of them were present throughout the whole 192 hours, while four (18.18%) compounds only appear from 96 hours of exposure, and the other nine (40.91%) compounds disappeared from 96 hours of exposure (Table 2.2).

Furthermore, LC-MS/MS results also show the presence of 22 (91.67%) out of the 24 identified compounds under TD conditions (Table 2.2). Out of these 23 compounds, only eight (36.36%) of them were strictly observed in under TD conditions and not in ambient conditions, these are myristic acid, palmitoleic acid, linoleic acid, stearic acid, cis-11-Eicosenoic acid, arachidic acid, docosanoic acid, oleanolic acid, quercetin 3-rhamninoside (Table 2.2). Moreover, only seven (31.82%) of the 23 compounds were present for the whole 192 hours of exposure, while six (27.27%) and nine (40.91%) compounds appear and disappear from 96 hours, respectively (Table 2.2).

Table 2.2: LC-MS/MS polyphenolic compound profiling of *Carpobrotus edulis* leaves, at different atmospheric conditions at different time intervals. Ambient, TA, TB, TC, TD.

			Ambient condition	TA				TB				TC				TD		
			Time of exposure (hours)															
cture	Mass (m/z)			48	96	144	192	48	96	144	192	48	96	144	192	48	96	144
8O3	163.04	p-Coumaric acid	*	*	*	*	*					*	*	*	*	*	*	*
8O7	191.019	Citric acid	*	*	*	*	*					*	*	*	*	*	*	*
12O6	191.055	Quinic acid	*	*	*									*	*	*	*	*
H10O4	193.05	Ferulic acid	*	*	*	*						*	*		*	*	*	*
H28O2	227.201	Myristic acid										*	*		*	*	*	*
H30O2	253.216	Palmitoleic acid										*	*	*		*	*	
H32O2	255.232	Palmitic acid	*	*	*	*	*						*	*	*			*
H22O4	277.144	Dibutyl phthalate		*		*	*											
H30O2	277.216	α-Linolenic acid	*		*							*	*	*	*	*	*	
H32O2	279.232	Linoleic acid		*		*						*	*				*	
H34O2	281.248	Oleic acid	*	*	*	*	*					*	*	*	*	*		*
H36O2	283.263	Stearic acid											*	*	*		*	
H14O6	289.071	Cianidanol	*	*	*	*	*					*	*	*	*	*	*	*
H38O2	309.278	cis-11-Eicosenoic acid										*		*		*		
H40O2	311.294	Arachidic acid										*	*	*	*		*	*
H16O9	315.072	Protocatechuic acid-O-glucoside	*	*	*	*	*	*	*			*	*	*	*	*	*	*
H44O2	339.326	Docosanoic acid			*		*					*					*	
H24O10	435.128	Phlorizin	*	*	*	*	*					*				*		
H48O3	455.351	Oleanolic acid		*	*	*	*					*	*		*	*	*	
H26O12	577.133	B-type procyanidin dimer	*	*	*	*	*					*	*	*	*	*	*	*
H32O16	623.16	Isorhamnetin glucosyl-rhamnoside	*	*	*	*	*						*		*		*	*
H40O20	755.201	Quercetin 3-rhamninoside										*				*		
H42O21	785.212	Isorhamnetin-3-O-rutinoside-4'-O-glucoside	*	*	*	*												

H18O9	353.087	Chlorogenic acid	*		*	*	*	*		*	*	*	*	*	*	*	

Chemometric assessment of LC-MS/MS data

Polyphenolic compounds variation and identification

The principal component analysis (PCA) and hierarchical cluster analysis (HCA) techniques are used to identify similarities in the chemical composition of samples, and in doing so, they identify differences that can assist in identifying chemical markers within the dataset (Granato *et al.,* 2018). These are unsupervised techniques, meaning that no class information is provided to the model. A PCA model can provide an overview of the chemistry of a sample, and resulting scores plots can identify trends, patterns, and groupings within a dataset (Galindo-Prieto, 2017; Granato *et al.*, 2018). Hierarchical Cluster Analysis can highlight groups that may be present in samples (Granato *et al.,* 2018). PCA models were constructed using aligned LC-MS/MS data for each treatment to investigate polyphenolic compounds variation in *Carpobrotus edulis* under concurrently elevated CO_2 and temperature variations. The model statistics for each treatment are listed in Table 2.3. All three work sets were found to be most suitable for the pareto-scaled model. According to the scores plot, all samples fell within Hotelling's T2 ellipse (Figure 2.5). As evidenced by the score plot, there was substantial chemical variation, as indicated by a scattering of samples across all four quadrants (Figure 2.5).

In order to construct the PCA models, three scaling methods were applied to the dataset: univariate, pareto and center. In order to determine the best model to use, the number of principal components (PCs), cumulative variation within X (R^2X_{cum}) and predictability of the model (Q^2 cum) were all taken into consideration. As a result, a Pareto-scaled model consisting of six principal components (PCs) was chosen for further investigation since it is characterized by the best model statistics.

On the scores plot (Figure 2.5), all samples fell within the Hotelling's T2 ellipse, suggesting that there were no strong outliers, so all data were included. Q^2 cume was 0.631, indicating 46.89% variation across the matrix while R^2Xcume was 0.4689. The models are considered valid when both values are greater than 0.5, however, this may not always be the case, while the difference between two values must be less than 0.2. Q^2 cumulative value was below the value set for a good predictive model and only had a moderate prediction accuracy of 63.1%. From Figure 2.5, PC1 was strongly associated with PC2, and that five treatments are clustered in three distinct group. PC1 ($R^2X = 0.4689$) showed the largest variation in the samples (46.89%). There was less variation observed by PC2, with a variation of 29.78% ($R^2X = 0.2978$). The PCA score plot indicated an overlap in the control and TA clusters thus showing some similarities in chemical composition for both treatments and forming one cluster (Figure 2.5). In addition, TC and TD were clustered together, indicating similarities in the compound's composition by the *C. edulis* leaves under these climatic conditions. Particularly, TB points created its own cluster, which can be explained by a transition acclimatization of the plants.

Table 2.3: Statistical Parameters for PCA and OPLS-DA using *Carpobrotus edulis* methanolic extracts.

Number of PC's	3
R^2X_{cum}	0329
Q^2_{cum}	0.631
% Variation PC1	56.89
% Variation PC2	29.78
OPLS-DA models	
Predictive Components (P)	**1**
Orthogonal Components (O)	**1**
R^2X (P1)	**0.489**
R^2X (O1)	**0.0679**
R^2Y	**0.962**
Q^2Y	**0.789**

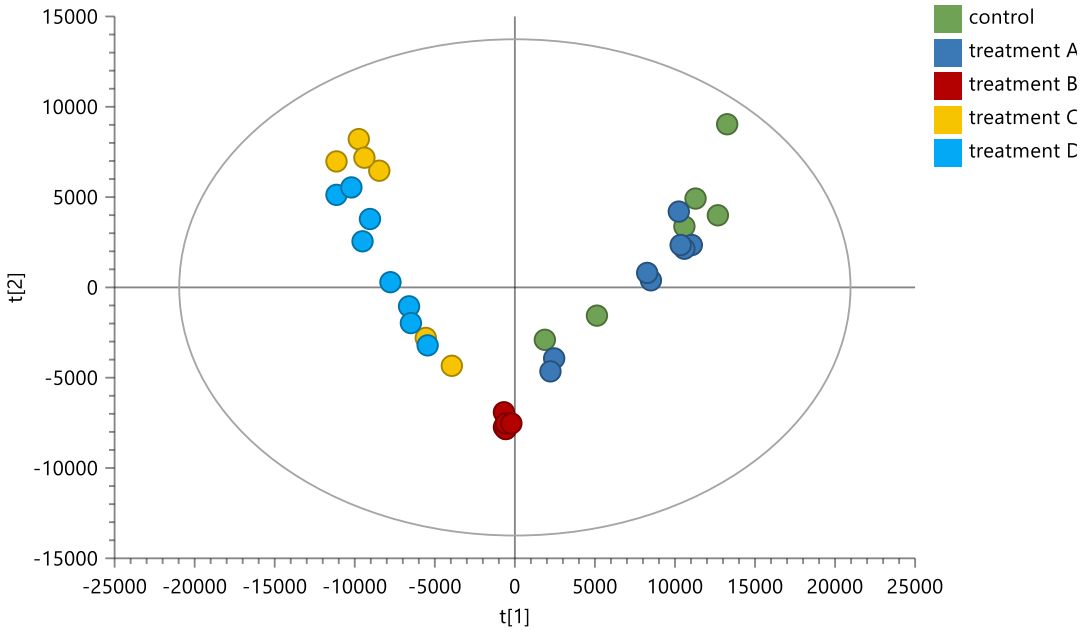


Figure 2.5: Score plot from a PCA of 2475 detected secondary metabolites in *Carpobrotus edulis* leaves, at different atmospheric conditions for 192 hours. Control [400 ppm and 28/25°C (day/night)], TA [600 ppm and 45/35°C (day/night)], TB [800 ppm and 45/35°C (day/night)], TC [600 ppm and 35/30°C (day/night)], TD [800 ppm and 35/30°C (day/night)].

The dendrograms derived from the HCA analysis showed distinct chemical differences within the treatments (Figure 2.6). The significance of this separation lied in the fact that treated and control samples were clearly separated into two chemically distinct groups. The dendrogram (Figure 2.6) confirmed the separation within treatments, showing two major branches for the five treatments. The blue branch represented samples exposed to treatments D, B and A which was clearly separated from the green branch which clustered samples exposed to treatments A and control (Figure 2.6). Dendrograms showed that there was also variation within the treatments based on the branching within each group. According to Figure 2.6 the polyphenolic profiles in the treatments and control samples are not relatively similar.

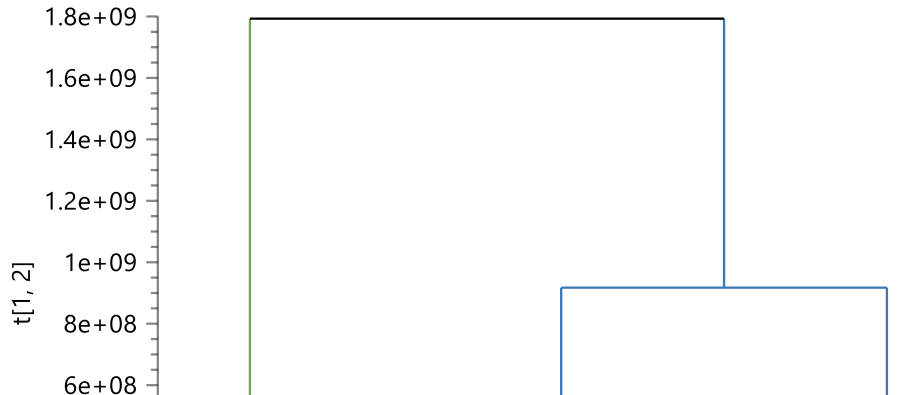


Figure 2.6: An HCA dendrogram of the 2475 detected secondary metabolites in *Carpobrotus edulis* leaves, at different atmospheric conditions for 192 hours, TA [600 ppm and 45/35°C (day/night)], TB [800 ppm and 45/35°C (day/night)], TC [600 ppm and 35/30°C (day/night)], TD [800 ppm and 35/30°C (day/night)], and one control [400 ppm and 28/25°C (day/night)].

As a supervised multivariate method, orthogonal projection to latent structure discriminant analysis (OPLS-DA) uses information in the Y-matrix to decompose the X-matrix into predictor components (correlated) and orthogonal components (uncorrelated) variations, depending on Y (Galindo-Prieto, 2017). The method can be used to identify the source of variation among samples in relation to a defined Y (Galindo-Prieto, 2017). Data from each treatment and control was used to construct an OPLS-DA model. The Pareto-scaled models provided the best statistics (Table 2.3). A score plot is presented in Figure 2.8. The obtained plot confirmed chemical separation of treated and control samples into two groups. Chemical compounds from different groups were shown in S-plots (Figure 2.7). Quantitative differences rather than qualitative differences were revealed by these compounds.

According to the OPLS-DA score scatter and S-plot, the first group comprised of plants under treatment A and control (Figures 2.7 and 2.8). The second group consisted of samples exposed to

treatment B and the third group contained samples under treatments C and D. For the first group, p-coumaric acid and ferulic acid were found and identified as distinctive compounds, respectively (Figure 2.7). In the second group, no distinctive compounds were found (Figure 2.7). However, in third group, chlorogenic acid, catechin, quercetin 3-rhamninoside and isorhamnetin-3-O-rutinoside-4'-O-glucoside were revealed as distinctive compounds, respectively (Figure 2.7).

The OPLS-DA was used to identify the active compounds responsible for the differences between five treatments. An OPLS-DA model, constructed from the pareto-scaled work set used for PCA, consisted of three PCs. Two orthogonal and two predictive components were used (R^2X for O1 = 0.489, R^2X for O2 = 0.419). P2 = 0.428, R^2X for O1 = 0.0679, R^2X for O2 = 0.194). As a result, The OPLS-DA loadings plot (Figure 2.8) was constructed. Clusters of variables in the centre suggest that there are several compounds common to all treatments and control. Alternatively, the variables furthest from the centre on the arms at the corresponding points in the scores plot indicate the retention times (Rt) / exact mass pair of marker compounds that is distinctive within each group.

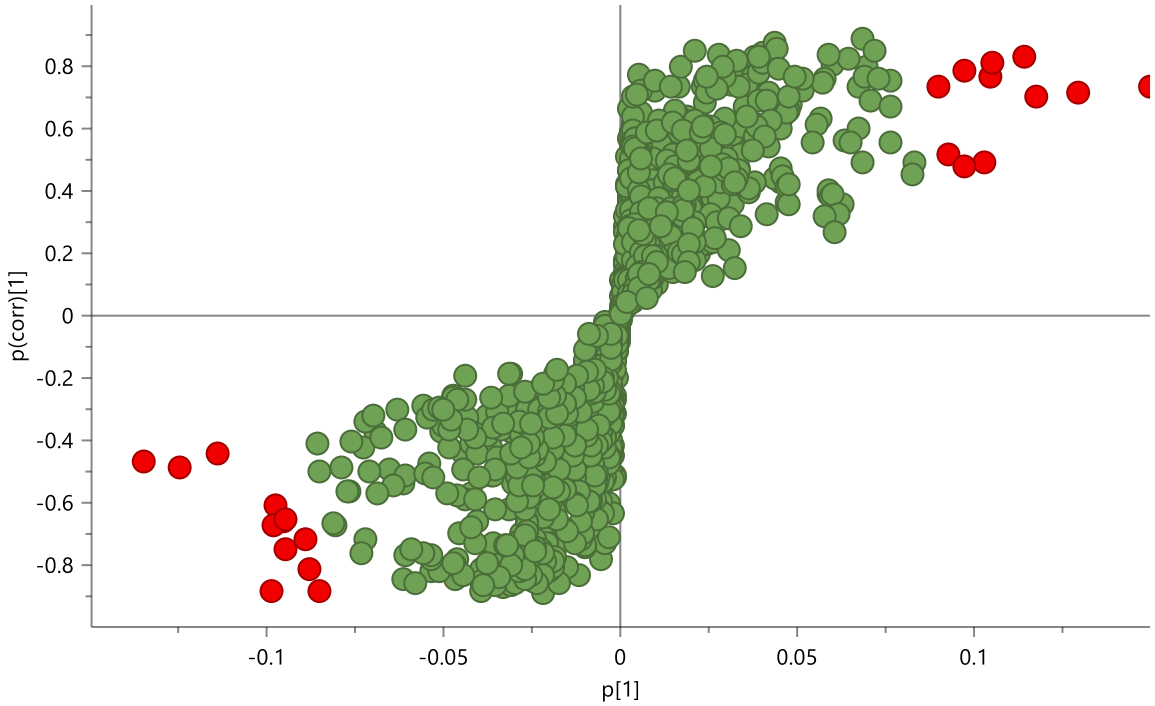


Figure 2.7: An S-plot of the 2475 detected secondary metabolites in *Carpobrotus edulis* leaves, at different atmospheric conditions for 192 hours, TA [600 ppm and 45/35°C (day/night)], TB [800 ppm and 45/35°C (day/night)], TC [600 ppm and 35/30°C (day/night)], TD [800 ppm and 35/30°C (day/night)], and one control [400 ppm and 28/25°C (day/night)].

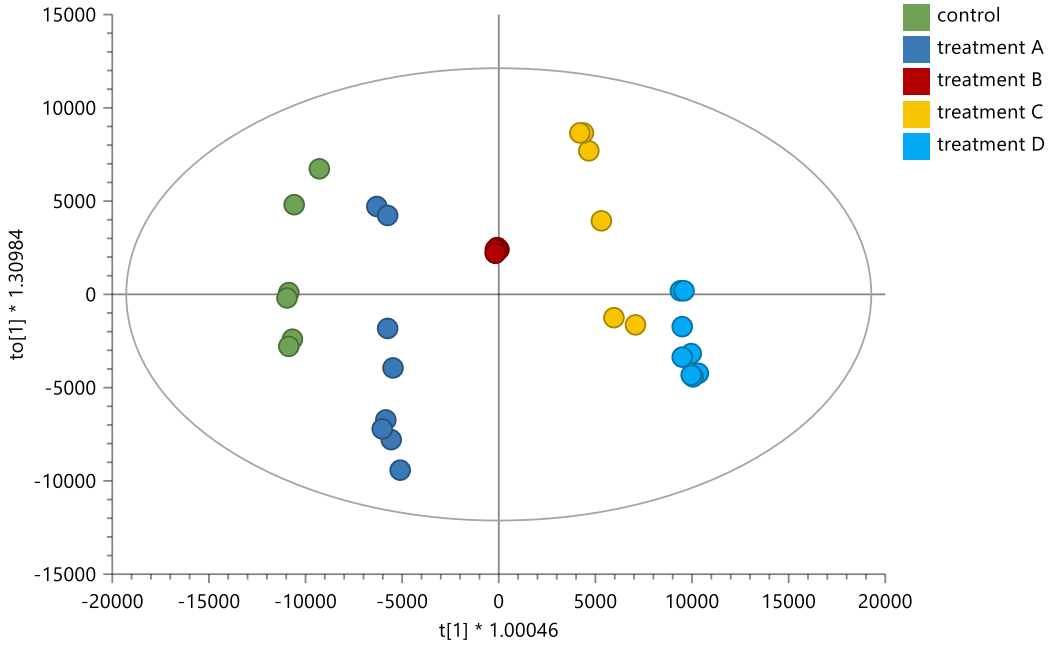


Figure 2.8: An OPLS-DA score plot of the 2475 detected secondary metabolites in *Carpobrotus edulis* leaves, at different atmospheric conditions for 192 hours, TA [600 ppm and 45/35°C (day/night)], TB [800 ppm and 45/35°C (day/night)], TC [600 ppm and 35/30°C (day/night)], TD [800 ppm and 35/30°C (day/night)], and one control [400 ppm and 28/25°C (day/night)].

2.4. Discussion and Conclusion

Phytochemical analyses studies are of great importance especially to pharmaceutical and food industries as they inform the production of new drugs and food ingredients (Kumar *et al.*, 2017). It is well known that the production of phytochemicals can be exacerbated by stress, including environmental stress (Kumar *et al.*, 2017). The present study observed the presence of seven medicinally important phytochemical groups in the leaves of *Carpobrotus edulis* (i.e., tannins, phenolics, flavonoids, steroids, terpenoids, glycosides, and saponins), which persisted under concurrently elevated atmospheric CO_2 concentrations and temperatures, apart from flavonoids. The preliminary phytochemical screening obtained in this study was consistent with previous

studies on *C. edulis*. For example, Omoruyi *et al.* (2012) and Akinyede *et al.* (2020) reported the presence of phenolics, flavonoids, flavonols, proanthocyanidins, tannins, saponins, and alkaloids, in *C. edulis* leaves. In addition to these compounds, both Alam (2011); Mudimba and Nguta (2019) detected the presence of chlorides, sulphates, coumarins, anthraquinones, cyanogenic glycosides, cardiac glycosides, carbohydrates and/or glycosides, unsaturated sterols and/or triterpenoids, in the leaves, stems and flowers of *C. edulis*. A study on the climate change effect of the phytochemical profiling of *Aloe vera* showed that under six different agro-climatic conditions of India the following compounds were present: alkaloids, flavonoids, phenols, saponins, and terpenes (Kumar *et al.,* 2017). Similarly, Omoruyi *et al.* (2012) observed a high presence of phenolics and tannins, a moderate presence of saponins, and low presence of flavonoids in *C. edulis* leave extracts. Another study by Lyubchyk *et al.* (2019) reported 50-60% of the phenolics family, with flavonoids being the abundant sub-family, hence suggesting a strong presence of phenolics and moderately low presence of flavonoids in *C. edulis*. Moreover, Kumar *et al.* (2017) indicated the production of phenolic compounds from highest to least as follows: flavonoids, anthocyanins and mucilaginous substances in *A. vera*. Maghsoudlou *et al.* (2019) reported the presence of the following compounds when quince fruits are put under heat stress: chlorogenic acid (5-O-caffeoylquinic acid), quercetin, catechin, rutin, and p-coumaric acid; in descending order of concentration.

The presence of polyphenolic compounds in *C. edulis* leaves was supported by various studies (Hilgart, 2016; Lyubchyk *et al.,* 2019; Mudimba and Nguta, 2019; Akinyede *et al.,* 2020). The results showed similarities in the *C. edulis* polyphenolic compound profiling of extracts under ambient and TA climatic conditions as well as similarities in extracts under TC and TD conditions. The obtained results also illustrated that the production of polyphenolic compounds, therefore the

composition polyphenolic, can be altered by environmental stress induced by concurrently elevated atmospheric CO_2 and temperature. These results were consistent with the knowledge that abiotic stress enhances the synthesis of polyphenolic compounds. A supporting statement to these results by AbdElgawad *et al.* (2014), states that under extreme climatic conditions, plants tend to have an adaptation and protection strategy of increasing their production of polyphenols.

In support with the obtained results, previous studies have shown that total phenolic compounds, total flavonoids (especially anthocyanins, flavonols, and flavanols), tend to increase in concentration under atmospheric temperature, CO_2 stress (Balasoorya *et al.,* 2017; Balasoorya *et al.,* 2019). Blancquaert *et al.* (2019) reported that the concentration of proanthocyanidins can either increase or decrease when grapevine fruits are exposed to high temperatures. In a study on *Zingiber officinale* Roscoe by Ghasemzadeh *et al.* (2010), results indicated that elevating CO_2 concentrations from 400 to 800 µmol mol^{-1}, enhances the concentrations of quercetin, catechin, kaempferol, fisetin and naringenin. However, decreases in concentrations of rutin, epicatechin and morin were observed. Balasoorya *et al.* (2019) reported that strawberry fruits are rich in polyphenols under elevated CO_2 and temperature conditions.

Temperature influences optimal physiological processes responsible for plant growth, development, and survival (Hatfield and Prueger, 2015; Nievola *et al.,* 2017; Jamloki *et al.,* 2021; Moore *et al.,* 2021), thus explaining the results obtained in this study. For example, plant photosynthetic rate is largely dependent on optimal temperature and CO_2 concentration (Nievola *et al.,* 2017; Jamloki *et al.,* 2021; Moore *et al.,* 2021). Generally, photosynthesis process is known to correlate to with the production of secondary metabolites, including polyphenolic compounds (Jamloki *et al.,* 2021). Therefore, the production of secondary metabolite is in return influenced by both temperature and CO_2 (Nievola *et al.,* 2017; Jamloki *et al.,* 2021). In addition, plant enzyme

activity is reliant on an optimal temperature range and any anomalies can cause enzyme denaturation and the inactivation of enzymatic machinery, thus disrupting physiological and biochemical processes (Nievola *et al.,* 2017; Jamloki *et al.,* 2021; Moore *et al.,* 2021). Thermal stress also damages cell membranes and causes the denaturation of lipids (Nievola *et al.,* 2017; Jamloki *et al.,* 2021). All these will as a result alter the concentration and composition of secondary metabolites within plants (Nievola *et al.,* 2017; Jamloki *et al.,* 2021). It can be said that thermal stress might have a positive influence on secondary metabolite production as a way of counteracting the stress on the plant. All these alterations due to the sudden rise in temperature occur rapidly, thus any sudden changes in ambient temperature could enhance the production and/or reduction of certain polyphenolic compounds as indicated by the results of this study (Nievola *et al.,* 2017).

Atmospheric CO_2 concentration also has an influence of secondary metabolite production, thus explaining the enhance production of phytochemical compounds in *C. edulis* leaves in this study. Jamloki *et al.* (2021), noted that increases in CO_2 concentrations enhanced the phenolic content in plant leaves. This can be explained by the important part that CO_2 play on photosynthesis. With this knowledge it can be expected that the interaction of both factors would largely enhance secondary metabolite production in plants, and this could potentially explain the presence of phytochemicals screened in this study and the polyphenolic screening of *C. edulis* in concurrently elevated temperature and CO_2 concentrations. This was parallel with results by Goicoechea *et al.* (2021) who found an accumulation of phenolic compounds in red grapefruit, *Tinto Velasco* and *Pasera,* under elevated temperature and CO_2 concentrations.

It must be noted however, that the above-mentioned influences are usually when the factor act independently on plants, but temperature and CO_2 act concomitant as they occur concurrently in

the atmosphere. Various studies on the concurrent influence of high temperature and CO_2 on phytochemistry of plants indicated that when both factors act simultaneously on plants, a negative relationship is observed (Veteli *et al.,* 2007). This could explain the observed disappearance of certain phytochemicals, such as flavonoids, and certain polyphenolic compounds when temperatures and CO_2 concentrations were highly elevated. Moreover, this can also be explained by the opposing effects of temperature to CO_2 influences, which causes cell damage thus limiting physiological processes (Veteli *et al.,* 2007; Jamloki *et al.,* 2021). However, the appearance of other polyphenolic compounds can be explained by Veteli *et al.* (2007) who reported that elevated CO_2 concentrations increases the concentration of secondary metabolites because the carbon molecule from CO_2 is an important component of constructing the metabolic compounds. These appearances and disappearances of phytochemicals, such as the individual polyphenolic compounds, can be a way of attempting to achieve homeostasis by *C. edulis* plants while under environmental stress (Nievola *et al.*, 2017). Moreover, because thermal stress usually induces short term plant responses such as after minutes or a few hours of stress exposure, the sudden appearances and/or disappearances of polyphenolics just after very few hours of exposure can be attributed (Nievola *et al.,* 2017).

CHAPTER 3: ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *CARPOBROTUS EDULIS* (L.) LEAVES

3.1. Introduction

3.1.1. Antioxidant activity

As previously mentioned in chapter 1, human activities, such as fossil fuel combustions and land use changes, elevate atmospheric CO_2 concentrations, which in return cause rises in temperatures (Cassia *et al.,* 2018; Bhargava and Mitra, 2021). Climate change as a result of rising atmospheric CO_2 concentrations and temperatures employ environmental stress upon plants as sessile organisms. Environmental stress such as extreme temperatures and high CO_2, induces an excessive production of cellular integrity toxic compounds known as Reactive Oxygen Species (ROS) (Balla *et al.,* 2007). Neutralization of these compounds requires antioxidant agents (Balla *et al.,* 2007). The production of antioxidants does not only benefit the plants but also has a significant importance to human health (Cui *et al.,* 2020). Antioxidants in humans reduce the risks of illnesses such as cancer, diabetes, tuberculosis, cardiovascular diseases to name a few (Is and Woodside, 2001; Hajhashemi *et al.,* 2010; Hamid *et al.,* 2010).

Antioxidant activity is directly related to amount and type of antioxidant compounds present in the plant (Santos-Sánchez *et al.,* 2019). Furthermore, the amount of antioxidant compounds produced can be said to be directly proportional to the intensity and type of stress the plant undergoes at a certain period (Irshad *et al.,* 2012; Srividya *et al.,* 2012). Different studies have documented both positive and negative correlations between antioxidant activity and polyphenolic concentration, independently (Srividya *et al.,* 2012). Irshad *et al.* (2012) reported a high antioxidant activity in methanolic extracts of *Cassia fistula* (L.), which correlated with the high phenolic contents. On

the other hand, Srividya *et al.* (2012) reported significantly high total phenolic and flavonoid contents, but lower antioxidant activity in *Abutilon indicum* (L.) ethanolic extracts. However, in both cases, polyphenolic compound content does in some way influence antioxidant activity, and the relationship between polyphenolic compound concentrations and antioxidant activity varies across plant species (Srividya *et al.,* 2012). Considering this, the current study assessed the antioxidant activity of *Carpobrotus edulis* (L.) leaves, since the plant's leaves do show a presence of polyphenolic compounds.

The antioxidant activity of *Carpobrotus edulis* leaves was estimated using a common technique, the 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) scavenging activity method (Fitriana *et al.,* 2016). Scavenging activity can be defined as the ability and rate at which ROS are stabilized by antioxidants (Pavithra and Vadivukkarasi, 2015). DPPH scavenging activity assay is among the more accurate and frequently techniques of assessing antioxidant activity (Irshad *et al.,* 2012). The amalgamation of DPPH (1,1-diphenyl-2-picrylhydrazyl) free radicals and an antioxidant result in a discoloration of the solution from purple-violet to yellow (Chauke *et al.,* 2012; Ibtissem *et al.* 2012; Omoruyi *et al.,* 2012). This colour change is due to the acceptance of electrons by the DPPH radicals from the antioxidant compound, and it is this decolorization that is measured spectrophotometrically at 517 nm absorbance (Chauke *et al.,* 2012; Omoruyi *et al.,* 2012). The colour change indicates that DPPH radicals have been paired with hydrogen ions from the antioxidant to form DPPH-H 'pairs', therefore the DPPH radicals can be said to have been inhibited or scavenged (Ibtissem *et al.* 2012; Omoruyi *et al.,* 2012). Interpretation of the obtained results is commonly through an "efficient concentration" parameter termed the IC_{50} value (Proestos *et al.,* 2013). This parameter can be defined as the concentration of the extract or sample required to scavenge/inhibit 50% of the DPPH radicals (Proestos *et al.,* 2013; Jadid *et al.,* 2017).

A general rule is that a lower IC_{50} value indicates a higher antioxidant activity of the extract, as very little of the extract would be required to inhibit 50% of the DPPH radical (Jadid *et al.*, 2017).

3.1.2. Antimicrobial activity

Conventionally, antibiotics have been successfully used against bacterial infections in humans, to lower morbidity and mortality rates (Owusu *et al*., 2021). However, the general overuse of antibiotics has increased microbial resistance, and thus posing a great health threat (Owusu *et al*., 2021). This has promoted the investigation of alternatives antimicrobial agent sources, such as medicinal plants (Owusu *et al*., 2021). Medicinal plants such as A*lchornea cordifolia* (Schumach. and Thonn.) Müll.Arg., *Justicia flava* (Forssk.) Vahl., *Psidium guajava* L., *Myrianthus arboreus* P. Beauv, and *Momordica charantia* L., are commonly used globally to manage acute and chronic illnesses or diseases (Owusu *et al*., 2021). The antimicrobial activity of medicinal plants is due to the presence of secondary metabolites (Martins *et al.,* 2011). Therefore, the effective inhibition of microorganisms is suggested to be directly dependent on the presence and/or concentration of the secondary metabolites.

Carpobrotus edulis is popular for its antimicrobial activity, commonly against *Mycobacterium tuberculosis*, *Staphylococcus aureus*, meticillin-resistant *S. aureus* (Martins *et al.*, 2011). Although *C. edulis* extracts were not active against Gram-negative bacteria, they were active against some Gram-positive and mycobacteria; owing it to the presence of certain polyphenolic compounds such as oleanolic acid, uvoal, catechin and epicatechin, flavonoids (Martins *et al.,* 2011). For this reason, *C. edulis* could potentially reduce the reliance on antibiotics, and therefore, it is the focus species for the present study. Therefore, this chapter also aimed at investigating the antimicrobial activity of *Carpobrotus edulis* against clinical important and common bacteria, *Staphylococcus aureus* and *Escherichia coli* to assess the plant's ability to act against diseases.

The antimicrobial activity of a plant is dependent on the polyphenolic compounds within the plant (AbdElgawad *et al.,* 2021). The presence of polyphenolic compounds is influenced by environmental stress (Akula and Ravishanka, 2011; Isah, 2019), due to climate change. Environmental stress such as temperature and CO_2 increase oxidative stress in plants, thus promoting the production polyphenolic compounds as a counteracting mechanism. However, enhanced polyphenolic compounds production enhances the antioxidant activity of plants (AbdElgawad *et al.,* 2021). Therefore, we also assessed the impacts that climate change had on the antimicrobial activity of the plant– *Carpobrotus edulis*.

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive bacterium that is known to infect surgical sites in patients. Infection by the bacteria can originate from health care workers and/or carrier patients (Keele, 2014). These bacteria can also cause other infections on the skin and/or soft tissue, pneumonia, as well as bone and joint infections (Keele, 2014). The most concerning aspect is that infections by *S. aureus* can lead to cancer, renal diseases and even mortality in patients (Keele, 2014).

Escherichia coli

Contrary to the Gram-positive bacterium, *Escherichia coli* is a Gram-negative bacterium that lives in the intestinal environment of both humans and other animals (Keele, 2014). Although it is known to be harmless in the gut, when found in other parts of the body it can cause various ailments (Keele, 2014). *Escherichia coli* can cause urinary tract infections, bacteremia, meningitis, as well as acute diarrhea (Keele, 2014).

3.2. Materials and methods

A total of 40 pots, each with three plant replicates (i.e., a total of 120 individual plants) were divided equally and used for both antioxidant and antibacterial activity analyses. The plant collection, propagation, combined elevated CO_2 and temperatures (Figure 2.3 in Chapter 2), and extract preparation methods followed in this chapter were similar to those of Chapter 2 (The phytochemistry analysis of *Carpobrotus edulis* leaves under concurrently elevated CO_2 concentrations and temperature).

3.2.1. Antioxidant activity estimated using the DPPH scavenging method

A 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) scavenging activity assay by Chu *et al.* (2000); Garcia *et al.* (2012) was used with slight modifications. Initially, 50 mg of DPPH was mixed with 100 ml of 80% methanol to produce a Stock solution. A Work solution was then produced by diluting the Stock solution at the 1:5 ratio. The Work solution was freshly prepared every day and for each replicate (i.e., three replicates for each concentration). To prepare a reaction solution, 10 – 50 µl of the extracts from each set of climatic conditions was mixed with 700 µl of the Work solution. Each solution was then equated to 1 ml by topping up with 80% methanol. The resulting solution was incubated in the dark for 45 minutes. Thereafter, the absorbance of each solution was measured using a spectrophotometer at 517 nm respectively. Analysis of the scavenging activities of the extracts were calculated using the following equation:

$$\% \text{ Scavenging/ Inhibition Activity} = [(Abs_{sample} - Abs_{blank}) / Abs_{control}] * 100$$

Where Abs_{sample} is the absorbance of the reaction solution; Abs_{blank} is the absorbance of the blank (i.e., 80% methanol only); and $Abs_{control}$ is the absorbance of the control solution (i.e., Work solution mixed with 80% methanol only). To interpret the obtained results, the percentage

scavenging activity values were converted into IC_{50} values as described by Proestos *et al.* (2013); Jadid *et al.* (2017). These IC_{50} values were averaged and reported as mean ± standard error in Microsoft Excel® (2021). Following this, a one-way ANOVA statistical test was conducted using the averages from each investigated treatment. Furthermore, a post-hoc test was manually conducted also in excel to further assess where the significant difference lies. Results were tabulated and comparison bar graphs were also created.

3.2.2. Antimicrobial activity determination of *Carpobrotus edulis* by the Agar Well Diffusion method

Two important nosocomial bacteria namely, *Staphylococcus aureus* ATCC 25923 (Gram-negative) and *Escherichia coli* ATCC 25922 (Gram-positive) (Netshiluvhi and Eloff, 2019) were used in this study. The nosocomial bacteria were obtained from Thermo-Fisher Laboratory specialties (Pty) Ltd, Johannesburg, South Africa, together with the Mueller-Hinton and Baird Parker Agars.

To determine the antibacterial activity of *Carpobrotus edulis* leaves, the Agar well diffusion method (Appendix A and B) by Sen and Batra (2012); Koohsari *et al.* (2015); Gonelimali *et al.* (2018) was followed with slight modifications. Two types of cooled agar plates (10*90 mm plates) were inoculated each with different bacterium, Mueller-Hinton Agar was inoculated with Gram-positive bacterium, *Escherichia coli*, while the Baird Parker Agar was inoculated with Gram-negative bacterium, *Staphylococcus aureus*. The differences in the agar media stems from the knowledge that each bacteria grows better in their respective agar. In addition, *S. aureus* is known to form grey-black colonies/mats when growing in its respective agar thus making the results collection step much easier (Rosa *et al.*, 2001; Kim and Oh, 2010). Though *S. aureus* can be best

enumerated in different agar media, Baird Parker is one of the few that gives the best readings (Rosa *et al.,* 2001; Kim and Oh, 2010).

The inoculation process as well as the rest of the experiment was conducted under a sterile fumigator that was sanitized with 80% ethanol. For the inoculation process, a total of eight the plates (four plates for each bacterium) were swabbed with their respectively bacteria using sterile cotton swabs, sealed with parafilm then incubated in the oven at 37°C. Each investigated treatment was allocated a single plate, and each plate was divided into six equal compartments within which 6 mm wells were perforated using a sterile micropipette head. Each compartment was labelled and was assigned a solution as follows: C/Control – samples under ambient climatic conditions; M – 80% methanol (served as a negative control); 48, 96, 144 and 192 hours respectively. Solutions as per the compartment labelling were carefully dispensed (i.e., 5-10 µl, depending on the thickness of the agar in the plate) respectively using a micropipette. The plates, with extracts, were then sealed with parafilm and incubated in the oven at 37°C for 24-36 hours, depending on the observed clearance of the bacteria around the well. At the end, the inhibition zones were measured using a ruler in millimetres. Inhibition zone refers to the observed clearance of the bacteria around the hole, caused by the presence of the extract/sample. These measurements were taken as the diameters.

Using in Microsoft Excel® (2021), inhibition zones were averaged and reported as mean ± standard error. A one-way ANOVA statistical test was conducted using the averages from each investigated treatment. This was followed by, a post-hoc test was manually conducted also in excel to further assess where the significant difference lies. Results were tabulated and comparison bar graphs were also created.

3.3. Results

3.3.1. Antioxidant activity of *Carpobrotus edulis* leaves under elevated CO_2 and temperature conditions

A discoloration from purple-violet to yellow of the DPPH (1,1-diphenyl-2-picrylhydrazyl) and the methanolic extract solutions was observed after 45 minutes incubation in the dark, thus indicating antioxidant activity of the extracts against DPPH. Although there was no significant difference in averaged IC_{50} values of the control (i.e., ambient conditions) and treatment samples ($F_{(4,15)} = 1.3937$, $p = 0.2834$), the following antioxidant activity pattern from highest activity to lowest was observed: TD>Control>TC>TA>TB, where TD has the lowest IC_{50} value (1.4794 mg/ml) and TB has the highest IC_{50} value (10.1207 mg/ml) (Table 3.1).

Table 3.1: Antioxidant activity expressed as averaged IC_{50} values (mg/ml) of leaf methanol extracts of *Carpobrotus edulis*. Lower IC_{50} value indicates a higher scavenging activity. Control samples were exposed to 400 ppm and 28/25°C (day/night); TA, TB, TC, and TD denotes samples exposed to concurrent 600 ppm and 45/35°C (day/night), 800 ppm and 45/35°C (day/night), 600 ppm and 35/30°C (day/night), and 800 ppm and 35/30°C (day/night), respectively.

Treatment	**IC_{50} value (mg/ml)**
Control	4.8327 ± 1.5753^{a}
Treatment A (TA)	9.9439 ± 2.2499^{a}
Treatment B (TB)	10.1207 ± 2.8105^{a}
Treatment C (TC)	9.7051 ± 6.2126^{a}
Treatment D (TD)	1.4794 ± 0.606^{a}

Values (mean ± standard error; n = 4) with similar superscripts showing no statistical difference at 5% confidence level; ($F_{(4,15)} = 1.3937$, $p = 0.2834$).

Results indicated no significant difference between the antioxidant activity of control samples (those exposed to ambient conditions) and all treatment conditions ($F_{(4,15)} = 1.3937$, $p = 0.2834$; Figure 3.2). The period of exposure to elevated CO_2 and temperature had no significant impact on

the antioxidant activity of *C. edulis* under TA and TD ($F_{(3,16)} = 4.8538$, $p = 0.0138$ and $F_{(3,16)} = 14.1337$, $p = 9.21*10^{-05}$, respectively; Figures 3.2 A and D). However, when exposed to TB and TC conditions, the antioxidant activity significantly decreased by 17.67% at 192 hours ($F_{(3,16)} = 2.7975$, $p = 0.0737$) and increased by 84.25% at 144 hours then drastically decreased from 144 to 192 hours by 91.91% ($F_{(3,16)} = 1.3814$, $p = 0.2844$), respectively (Figures 3.2 C and B).

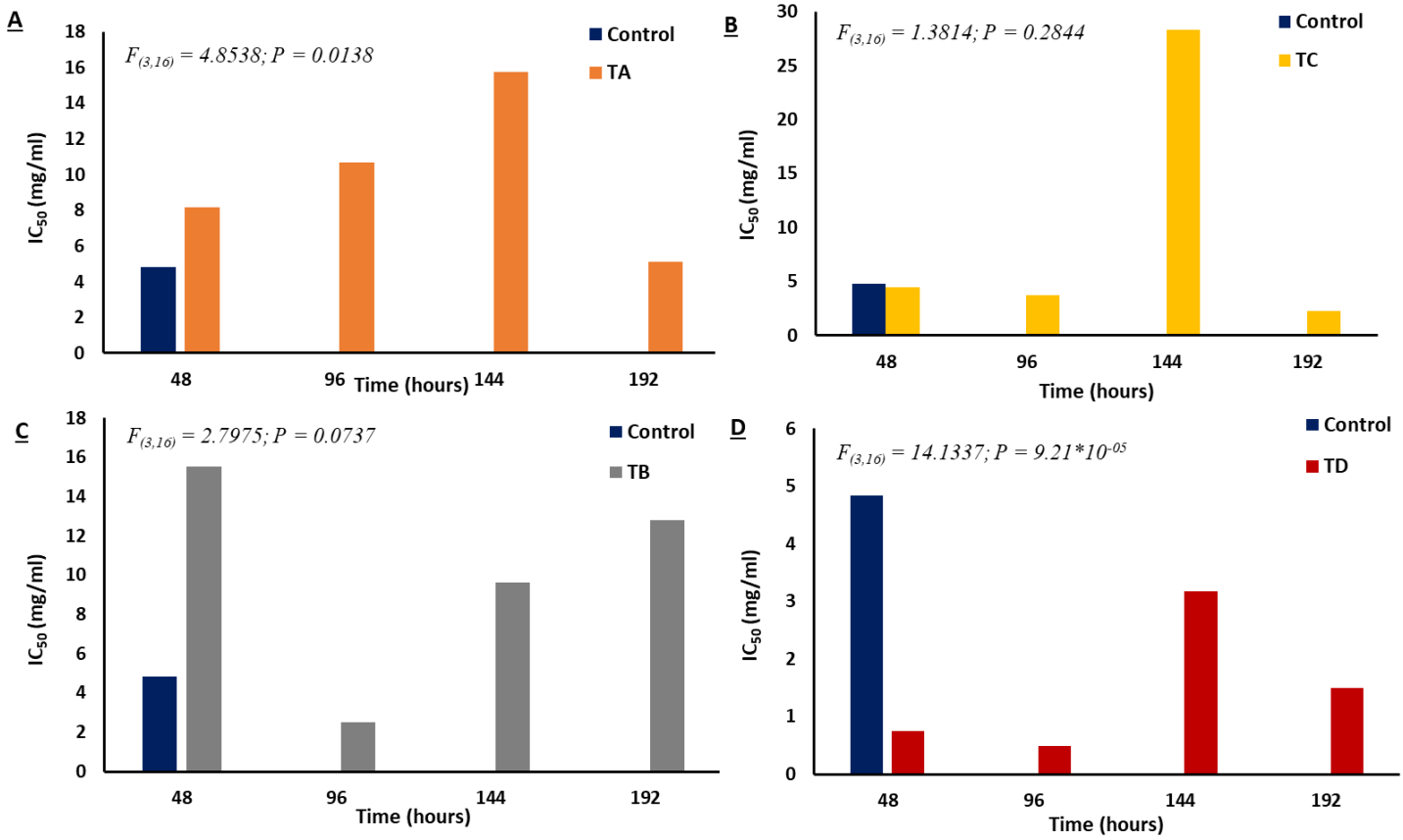


Figure 3.2: DPPH scavenging activity expressed as IC_{50} values (mg/ml) of leaf methanol extracts of *Carpobrotus edulis*. Sub-figures A, B, C and D, illustrate changes in antioxidant activity from ambient conditions [400 ppm and 28/25°C (day/night)] to treatments TA [600 ppm and 45/35°C (day/night)], TC [600 ppm and 35/30°C (day/night)], TB [800 ppm and 45/35°C (day/night)] and TD [800 ppm and 35/30°C (day/night)], respectively. As well as changes over 192 hours of exposure. The antioxidant activity of control extracts was not significantly different from that of the treatment extracts ($F_{(4,15)} = 1.2904$, $p = 0.3178$).

3.3.2. Antimicrobial activity of *Carpobrotus edulis* leaves under elevated CO_2 and temperature conditions

The concurrent elevation of CO_2 and temperature from ambient conditions did not significantly change the antimicrobial activity of *Carpobrotus edulis* against *Staphylococcus aureus* as there was no significant difference in inhibition activity between the control samples and all the treatments ($F_{(4,15)} = 1.2904$, $p = 0.3178$; Table 3.2). However, a significant difference was observed in the inhibition activity of the plant extracts against *Escherichia coli* between the control extracts and all the treatments ($F_{(4,15)} = 3.186$, $p = 0.0441$; Table 3.2). The inhibition zone diameters against *S. aureus* ranged from 17.75 to 20.5 mm across the treatments, whereas the inhibition zone diameters against *E. coli* ranged from 14.75 to 21.25 mm across the treatments (Table 3.2). These results suggest that *C. edulis* leaves do inhibit the activity of both *S. aureus* and *E. coli*, however they also suggest that the plant species keep the same activity against *S. aureus* even when under elevated CO_2 and temperature stress. However, the activity of *C. edulis* leaves against *E. coli* seems to be enhanced when the plant was under elevated CO_2 and temperature stress. The antimicrobial activities observed in the leaves of *C. edulis* were ranked according to the following magnitude in each treatment group: against *S. aureus*; TD > TC > TB > TA > Control; and against *E. coli*; TD > TA > TB > TC > Control. Looking at control samples, *C. edulis* methanolic extracts were more actives against *S. aureus* (17.75±0.25 mm) than they were against *E. coli*, 14.75±2.50 mm (Table 3.2).

Table 3.2: Inhibition zones indicating the antibacterial activity of *Carpobrotus edulis* under elevated CO_2 concentrations and temperatures. Values (means ± standard error; n = 4) with similar superscripts in the same row showing no statistical difference at 5% confidence level. *Staphylococcus aureus* (F (4,15) = 1.2904, p = 0.3178) and *Escherichia coli* (F (4,15) = 3.186, p = 0.0441).

		Zones of inhibition (mm)				
	Treatments	**Control**	**TA**	**TB**	**TC**	**TD**
Bacteria	***Staphylococcus aureus***	17.75±0.25*	18±1.08*	19.25±0.85*	19.25±0.63*	20.5±1.55*
	Escherichia coli	14.75±2.50^{a}	20.25±1.18^{b}	20±1.47^{b}	19.25±0.25^{b}	21.25±0.48^{b}

The inhibition activity of *Carpobrotus edulis* against *Staphylococcus aureus* when exposed to all treatments slightly fluctuated throughout the 192 hours of exposure (Figure 3.3). Results show that the inhibition zones of the methanolic extracts declined by 15.59% - TA, 4.23% - TB and TC each, from before exposure to elevated CO_2 and temperatures (i.e., ambient conditions) to 192 hours, while TD extracts slightly increased by 6.58% (Figure 3.3). However, the inhibition zones of the extracts in each treatment slightly decreased from 48 to 192 hours of exposure to elevated conditions by 25% -TA, 19.05% -TB, 9.52% - TC, and 22.73% - TD (Figure 3.3).

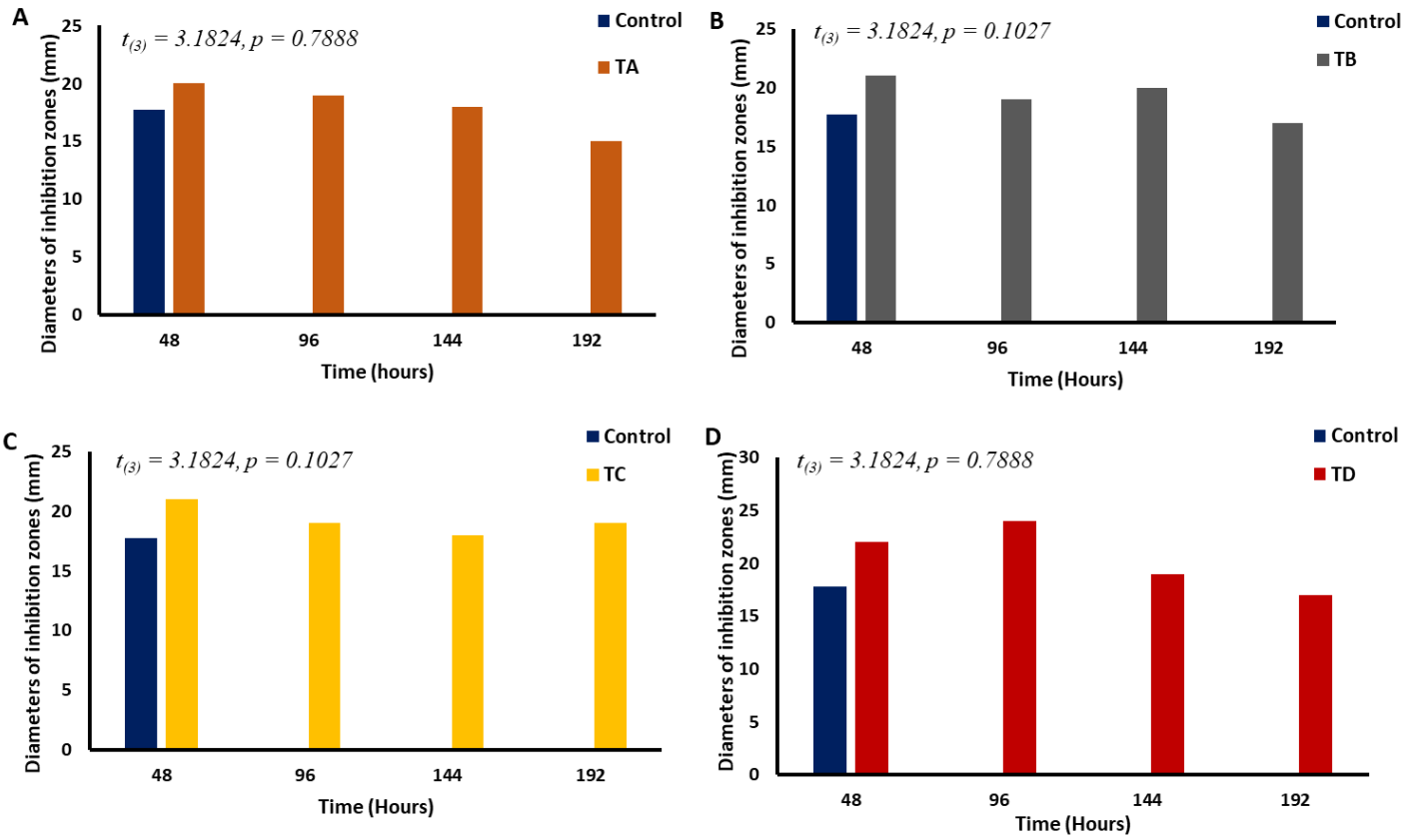


Figure 3.3: Zones of inhibition in diameter (mm) exhibited by *Carpobrotus edulis* leaf extracts against *Staphylococcus aureus*. *C. edulis* plants were exposed to different temperatures and CO_2 concentrations: Control (400 ppm and 28/25°C), Treatment A (600 ppm and 45/35°C), Treatment B (800 ppm and 45/35°C), Treatment C (600 ppm and 35/30°C), and Treatment D (800 ppm and 35/30°C).

The inhibition activity of *Carpobrotus edulis* leave extracts against *Escherichia coli* from before the elevation of CO_2 and temperature (i.e., ambient conditions) to 192 hours of exposure was decreased by 22.73% and 5.56% in TA and TB conditions respectively, whereas TC extracts showed no change and TD extracts significantly increased by 9.09% ($t_{(3)} = 3.1824$, $p = 0.0536$; Figure 3.4). The inhibition activity from 48 to 192 hours of exposure increased by 13.24% -TA and TB each, 22.37% and 32.95% - TC and TD respectively (Figure 3.4).

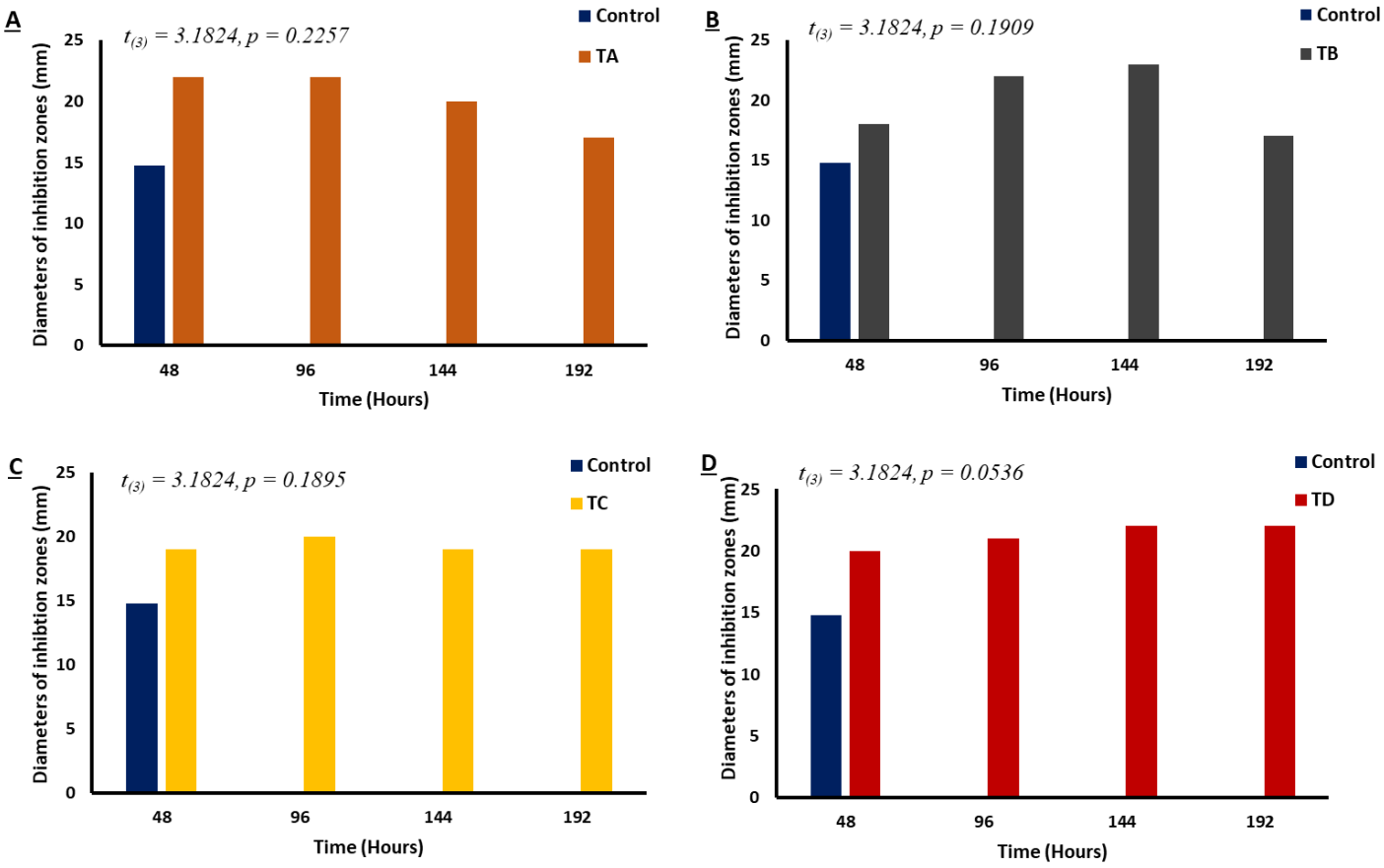


Figure 3.4: Zones of inhibition in diameter (mm) exhibited by *Carpobrotus edulis* leaf extracts against *Escherichia coli*. *C. edulis* plants were exposed to different temperatures and CO_2 concentrations: Control (400 ppm and 28/25°C), Treatment A (600 ppm and 45/35°C), Treatment B (800 ppm and 45/35°C), Treatment C (600 ppm and 35/30°C), and Treatment D (800 ppm and 35/30°C).

3.4. Discussion and Conclusion

3.4.1. Antioxidant activity of *Carpobrotus edulis* leaves under concurrent elevated CO_2 and temperature.

Environmental stress such as elevated CO_2 and temperature prompt a protection mechanism in plants such as the production of secondary metabolites. These secondary metabolites have capabilities of scavenging Reactive Oxygen Species (ROS) which originate through stress and cause damage plant cells (Das and Roychoudhury, 2014). One of the characteristics of medicinal plants is the antioxidative ability, determined by the presence of secondary metabolites, and this

is either enhanced or limited by environmental stress (Das and Roychoudhury, 2014). The increase in ROS concentration, thus oxidative stress, in plants can be observed once the environmental temperature surpasses the functional physiological threshold (Soengas *et al.,* 2018). However, this oxidative stress can be overcome by enhancing the production of antioxidant defence mechanisms such as phenolic compounds (Soengas *et al.,* 2018). In this study, the antioxidant activity of an indigenous South African plant, *Carpobrotus edulis* was established through the ability of the methanolic leave extracts of the plant to scavenge DPPH free radicals. Plants are considered to have strong antioxidant activity if the efficient concentration (IC_{50}) values are within the 10-50 mg/ml range (Jadid *et al.,* 2017). The general rule is that the lower the IC_{50} value, the stronger the antioxidant activity of the extract since lesser extract would be required to scavenge 50% of ROS free radicals (Jadid *et al.,* 2017). Therefore, because the reported IC_{50} range of *C. edulis* was 1-11 mg/ml, the plant can be said to possess extremely strong antioxidant activity. This suggest that *C. edulis* is potentially a great natural antioxidant.

In support to with the obtained results, previous studies have reported higher DPPH scavenging activities of both aqueous and ethanol *C. edulis* extracts relative to standard antioxidant drugs such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and rutin (Ibtissem *et al.,* 2012; Omoruyi *et al.,* 2012). Similarly, Falleh *et al.* (2011) investigated the antioxidant activity of the leaves, stem, and roots of *C. edulis*, and reported greater antioxidant activity by the leaves and stem. Moreover, the plant exhibited greater antioxidant activity compared to BHT and BHA (Falleh *et al.,* 2011).

The intensity of environmental stress and period of exposure to the stress could either enhance the production of antioxidants to strengthen the protection mechanism or, further damage the enzymatic system leading to the halting or lowering the production of antioxidants in plants.

Moreover, the concomitant occurrence nature of environmental factors intensifies the stress impact on plants. The concurrent elevation of CO_2 and temperature could have both negative and positive impacts on the antioxidant activity of plant species, but elevating temperature further could potentially encourage the decline of antioxidant activity in plants (Sun *et al.,* 2012). Thermal stress enhances ROS production through the catalysis ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), e.g., hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), singlet oxygen ($^1O_2$), and superoxide anion ($O_2^{\bullet-}$) (Hasanuzzaman *et al.,* 2013; Cassia *et al.,* 2018; Mohi-Ud-Din *et al.,* 2021). These compounds adversely impact membrane lipids and pigments, membrane permeability and functions (Mohi-Ud-Din *et al.,* 2021). As a counteracting mechanism, plants tend to synthesize various compounds such as antioxidative enzymes e.g., superoxide dismutase (SOD) and ascorbate peroxidase (APX) to name a few; heat shock proteins; osmo-protectants such as glycine betaine and proline (Cassia *et al.,* 2018; Mohi-Ud-Din *et al.,* 2021). This counteracting mechanism enhances the antioxidant activity to bring about homeostasis within plants (Nievola *et al.,* 2017), this can explain the results observed in this study that not only does *C. edulis* maintain its extremely strong antioxidant activity under elevated CO_2 and temperature, but also the duration of exposure to the elevated conditions does not change the plant's antioxidants activity. The result also suggested that if antioxidant activity of *C. edulis* does increase, the increase might however not be significant.

The results further suggested that if the climatic conditions rise to the projected conditions or even halfway, *C. edulis* would experience a continuous decline in antioxidant activity, and this might worsen as the exposure duration is prolonged. A decline in antioxidant activity in plants can be attributed by the decline in the concentration of bioactive compounds with high antioxidant activity such as catechins, total phenolics and total flavonoids; as well as the oxidation of fatty acids,

proteins and nucleic acids on cell membranes due to heat stress (Soengas *et al.,* 2018; Kim *et al.,* 2020). Amongst the two investigated factors–CO_2 and temperature–temperature may have the most contribution to the decline in antioxidant activity of *C. edulis*. Temperature is essential for both plant development and growth, and drastic elevations or drops could damage the productivity and metabolic process of a plant, thus plummeting plant yield and/or quality (Yan *et al.,* 2013).

Temperature influences the function of metabolic enzymes and therefore, high temperature can cause damage to biochemical and physiological processes (Hasanuzzaman *et al.,* 2013; Mohi-Ud-Din *et al.,* 2021). Elevated temperatures could lead to leaf senescence prematurely due to major influence that temperature has on the metabolic activities of plants Jamloki *et al.* (2021). This influence is based on the functioning of enzymes under an optimal temperature range, thus anything beyond that could lead to enzyme denaturation and/or inactivation thus disrupting physiological and biochemical processes (Hasanuzzaman *et al.,* 2013; Soengas *et al.,* 2018; Jamloki *et al.,* 2021). High temperature can lead to a decrease in the activity of phenylalanine ammonia-lyase, enzymes responsible for the shikimic acid pathway which is essential to produce phenolic compounds, thus, this decrease would lead to antioxidant synthesis and activity; although the opposite is true (Jamloki *et al.,* 2021). Moreover, high temperature enhances the production of a cytotoxic and genotoxic-αβ-dicarbonyl aldehyde compound known as MG (Mohi-Ud-Din *et al.,* 2021). This is a greatly reactive mutagenic compound, and Magnesium accumulation signals for a stress response (Mohi-Ud-Din *et al.,* 2021). How it works is that it could configure both DNA and proteins thus resulting in the formation of advanced glycation end products (AGEs) (Mohi-Ud-Din *et al.,* 2021). The intracellular accumulation of MG inhibits cell proliferation, and this can lead to protein degradation and the inactivation of the antioxidant defence system (Mohi-Ud-Din

et al., 2021). This could have contributed to the overall decline in the antioxidant activity of *C. edulis* under elevated temperatures in the present study.

Elevations of CO_2 concentrations have various impacts on plants, these include plant growth, photosynthesis rate enhancement, as well as alterations in the biochemical compositions and processes (Ghasemzadeh and Jaafar, 2011; Cassia *et al.,* 2018). Exposure of plants to elevated CO_2 concentrations is known to alter the distribution of secondary metabolites across different plant parts (Ghasemzadeh and Jaafar, 2011), therefore alter the antioxidant activity of the plants. The enhanced antioxidant capacity in various vegetables such as lettuce because of elevated atmospheric CO_2 concentration can be explained by the rise in soluble sugar precursors in the plants, thus promoting antioxidant synthesis and accumulation (Dong *et al.,* 2018). In addition, the synthesis of NADPH can be enhanced by elevated atmospheric CO_2 concentration, this then retains the high antioxidant (e.g., ascorbate and glutathione) concentration in plants (Cassia *et al.,* 2018; Dong *et al.,* 2018). Therefore, CO_2 concentration elevations increases the antioxidant activity of plants, this can also be explained by the observed increase in photosynthesis rate (Ghasemzadeh and Jaafar, 2011; Cassia *et al.,* 2018). Elevated CO_2 concentration increases the activity of the ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) enzyme, therefore enhance photosynthesis (Ghasemzadeh and Jaafar, 2011; Cassia *et al.,* 2018). As photosynthesis results in the production of secondary metabolites it can be said that an increase in photosynthesis enhances antioxidant activity. The insignificant increase in antioxidant activity in *C. edulis* may be a result of the counteraction of the negative impacts of thermal stress on enzymatic activity and the positive impact of CO_2 concentration on photosynthesis. Moreover, as a facultative C3-CAM, the C3 nature of *C. edulis* allows enhanced photosynthesis initially due to increased CO_2 concentrations, however the further accumulation of chemicals and ROS in the plant cause an imbalance and

therefore negatively impact photosynthesis rates (Cassia *et al.,* 2018; Sarmati *et al.,* 2019). This then suggests that continual exposure of *C. edulis* to elevated CO_2 can further negatively impact the antioxidant activity of the plant.

The combination of the independent cellular and metabolic activities within plant cells due to temperature and CO_2 individually have a counteracting overall influence on the antioxidant activity of *C. edulis*. This counteraction could explain the insignificant change in antioxidant activity of *Carpobrotus edulis* leaves, allowing the plant to maintain its extremely strong antioxidant activity even under the concurrent elevation of atmospheric CO_2 concentrations and temperatures. Many studies have reported that antioxidant activity of plants is enhanced by environmental stress especially elevated CO_2 concentrations and temperatures, and the concurrent effects of the two (Dong *et al.,* 2018). Yan *et al.* (2013) found that *Zizania latifolia* Turcz. plants exhibit some form of protection against both low and high atmospheric temperatures, by increasing their antioxidant enzyme and antioxidant activities, shown by an increased hydrogen peroxide scavenging activity. Effects of elevated CO_2 and temperature on the oxidative stress in *Lolium perenne* L. and *Medicago sativa* L. resulted in an increased scavenging activity of lipid peroxidation in *Medicago sativa* (Farfan-Vignolo and Asard, 2012). On the contrary, the quality and yield of strawberries was enhanced under elevated CO_2 concentrations but lessened under elevated temperatures (Balasooriya *et al.,* 2017), this suggest a decline in the antioxidative property of the plants as the quality of medicinal plants is proportional to the production of polyphenolic compounds (Balasooriya *et al.,* 2019a).

3.4.2. Antimicrobial activity

The antimicrobial property of *Carpobrtus edulis* can be attributed by the plant's phytochemical profiling (AbdElgawad *et al.,* 2021). Increment in polyphenolic compounds enhance the antimicrobial activity of plants (AbdElgawad *et al.,* 2021). The antimicrobial activity of *C. edulis* leaves was established by the results of this study. Samples with inhibition zones ranging from 14.00 mm to 19.00 mm and above 19.00 mm are active to very active, respectively, against microorganisms (Ruiz-Ruiz *et al.,* 2017). Therefore, *C. edulis* falls under the very active group since the inhibition zones of the plant against *Staphylococcus aureus* was 17.75-20.5 mm and against *Escherichia coli* was 14.75-21.25 mm. Interestingly, previous studies have reported a good antimicrobial activity of *C. edulis* against *S. aureus* but, poor to no activity against *E. coli*. Ibtissem *et al.* (2012) observed an inhibition zone with a diameter of 17 mm against *S. aureus* but no inhibition zone against *E. coli*. Van der Watt and Pretorius (2001) reported activity against *S. aureus* and none against *E. coli*. Buwa and Afolayan (2009) reported a strong inhibition activity of *C. edulis* ethanolic leaf extracts against Gram-positive bacteria such as *S. aureus* and weak inhibition activity against Gram-positive bacteria such as *E. coli*. Following the isolation of six essential compounds (i.e., β-amyrin, catechin, epicatechin, monogalactosyldiacylglycerol, oleanolic acid, and uvaol) from *C. edulis* extract, Martins *et al.* (2011) tested their inhibition activity against amongst others *S. aureus* and *E. coli* and found that the compounds obtained from *C. edulis* extract inhibited the activity of *S. aureus* and not *E. coli*. A similar by Meddeb *et al.* (2017) also showed activity of phenolic *C. edulis* extract against *S. aureus* and not *E. coli*. In a study focused on the peel and flesh of *C. edulis* fruit, Castañeda-Loaiza *et al.* (2020) found that the fruit peels were more active against Gram-positive bacteria than they were against Gram-negative bacteria. Aqueous extracts of the fruit flesh showed activity against *E. coli*, whereas both aqueous and ethanolic extracts of the fruit flesh were active against *S. aureus* (Castañeda-Loaiza

et al., 2020). Elevated CO_2 enhances photosynthesis in plants which in return enhance the production polyphenolic compounds (Jamloki *et al.,* 2021), this could explain the good antimicrobial activity against *E. coli*.

Environmental stress such as high atmospheric CO_2 and temperature does influence the polyphenolic compound profiling of plant material through alterations in ROS production, hence influencing the antimicrobial activity of the plants (Balasooriya *et al.,* 2019a; AbdElgawad *et al.,* 2021). *Carpobrotus edulis* maintained its antimicrobial activity under elevated atmospheric CO_2 and temperature. The maintenance of antimicrobial activity by *C. edulis* could mean that the plant possesses thermo-tolerant polyphenolic compounds (Ginovyan, 2017).

The concomitant occurrence of both high atmospheric CO_2 and temperature does not significantly change the antioxidant activity of *Carpobrotus edulis* leaves but allows the plant to maintain its extremely strong antioxidant activity. The concomitant occurrence of these environmental conditions also resulted in an insignificant decrease of antioxidant activity over time. This can be explained by various metabolic and biochemical processes. High temperature alone is known to increase antioxidant activity by enhancing the shikimic acid pathway which results in increased production of antioxidant compounds. It also enhances the activity and production of the stress-responsive signal MG which in turn, can inactivate the antioxidant defence system. High CO_2 concentration alone enhance antioxidant activity through the increase of antioxidant compound synthesis, and NADPH synthesis. Moreover, increased CO_2 concentration enhance photosynthesis rate, a process that gives rise to secondary metabolites (responsible for antioxidant activity). Therefore, the combination of the enhanced MG production and activity together with the C3 nature of *Carpobrotus edulis* explain overall decline in antioxidant activity.

CHAPTER 4: GENERAL DISCUSSION, CONCLUSION AND FUTURE RECOMMENDATIONS

The concomitant increase in atmospheric CO_2 concentrations and temperatures, globally, over the years have impacted plants both physiologically and morphologically (Morison and Lawlor, 1999). The effect of the rising CO_2 and temperatures on plants may influence the pharmaceutical efficacy of medicinal plants. Therefore, the aim of this study was to investigate the effect of concurrently elevated CO_2 concentrations and temperature on the therapeutic efficacy of *Carpobrotus edulis* by assessing the antioxidant and antimicrobial activities of the plant's leaves. Results from the DPPH scavenging activity assays showed that the antioxidant activity of *C. edulis* leaves decreased as a result of the concurrent elevation of CO_2 concentrations and temperatures. The results suggested that a typical heat wave event in the South African context could potentially negatively impact the antioxidant activity of *C. edulis*. Additionally, if the elevated conditions are prolonged in accordance with the predicted climatic changes, the decrease in antioxidant activity might worsen. *C. edulis* showed a strong ability to inhibit *S. aureus* and *E. coli*. However, the activity against *S. aureus* only did not change regardless of environmental stress. This was attributed by the decrease in antioxidative compounds such as flavonoids within the plant, as the results suggested, thus, reducing the antioxidant activity. The modifications in the antioxidant and antimicrobial activities of *C. edulis* can be attributed to a phytochemical screening that showed the presence of the seven important groups namely, tannins, phenolics, flavonoids, steroids, terpenoids, glycosides, and saponins. The presence of the phytochemical groups persisted throughout the concurrently elevated CO_2 concentration and temperatures except for flavonoids which were not detected as CO_2 concentration and temperature were increased. The results also showed the presence of 24

polyphenolic compounds. The presence of these compounds differed throughout the conditions with more compounds being found in control samples. The polyphenolic profile of *C. edulis* leaves differed across the climatic conditions, with TB standing alone, TC and TD showing similar results, Control and TA showing similar results. The study, therefore, suggested that abiotic factors such as elevated CO_2 and temperatures could alter the phytochemical profiling of *C. edulis* due to changes in physiological processes.

The sudden and prolonged exposure to abiotic stress, rather than a gradual exposure, might have a significant impact on the antioxidant activity of *C. edulis*. The decline might also be due to a decline antioxidant enzymes and metabolic activities, denatured enzymes, inactivated physiological and biochemical processes due to high temperature. Moreover, high temperatures alter the DNA and protein structures of the antioxidant defence system (Hasanuzzaman *et al.,* 2013; Soengas *et al.,* 2018; Cassia *et al.,* 2018; Kim *et al.,* 2020; Jamloki *et al.,* 2021; Mohi-Ud-Din *et al.,* 2021). Increases in CO_2 concentrations alter the photosynthesis rate, biochemical composition and processes (Ghasemzadeh and Jaafar, 2011; Cassia *et al.,* 2018). The C3-CAM facultative nature of *C. edulis* could have contributed to the decline, as increasing CO_2 concentration can negatively affect photosynthetic rates of C3 plants after prolonged exposure thus, negatively affecting the production of polyphenolic compounds in the plants' leaves and consequently the antioxidant activity.

In conclusion, the study has demonstrated the effects of concurrently elevated CO_2 and temperatures on *C. edulis* by showing the changes and fluctuations of phytochemicals and compared to the control. Concentration of flavonoids is inconsistent and extremely low. Polyphenolic compounds profiling show disappearance of compounds and appearance of new ones, but mainly disappearance under extreme conditions. Further, the study demonstrated that the

antioxidant activity decreases with increasing CO_2 concentration and temperature. Antimicrobial activity inhibits *S. aureus* and *E. coli*, and the inhibition activity remains constant throughout the treatments due to the appearance of possibly new polyphenolic compounds and the consistent occurrence of phytochemical groups. These results suggested that *C. edulis* is a strong antioxidant and antimicrobial agent, owing it to the polyphenolic compounds composition. However, these properties could be negatively impacted by elevated CO_2 and temperature, thus influencing the efficacy of *C. edulis*.

Future studies can focus in greater detail on the influence the of individual environmental factors, i.e., elevated CO_2 concentration and temperature separately, tracking how each factor independently influences the antioxidant and antimicrobial activities of the plants. Studies can also isolate and track the changes in the production of individual polyphenolic compounds responsible for the specific medicinal properties of *C. edulis*. Further, studies could include the influence of climate change on the plants' photosynthetic and respiration mechanisms, as well as track changes in the genetic expression of the *C. edulis* leaves.

REFERENCES

AbdElgawad, H., Okla, M. K., Al-Amri, S. S., Al-Hashimi, A., Al-Qahtani, W. H., Al-Qahtani, S. M., and Abdel-Mawgoud, M. (2021). Effect of elevated CO_2 on biomolecules' accumulation in caraway (*Carum carvi* L.) plants at different developmental stages. *Plants*, *10*(11), 2434.

AbdElgawad, H., Peshev, D., Zinta, G., Van den Ende, W., Janssens, I. A., and Asard, H. (2014). Climate extreme effects on the chemical composition of temperate grassland species under ambient and elevated CO_2: a comparison of fructan and non-fructan accumulators. *PLoS One*, *9*(3), e92044.

AbdElgawad, H., Zinta, G., Beemster, G. T., Janssens, I. A., and Asard, H. (2016). Future climate CO_2 levels mitigate stress impact on plants: increased defense or decreased challenge?. *Frontiers in plant science*, *7*, 556.

Ahmad, A., Ahmad, V., Zamzami, M. A., Chaudhary, H., Baothman, O. A., Hosawi, S., and Khan, M. J. (2021). Introduction and Classification of Natural Polyphenols. In *Polyphenols-based Nanotherapeutics for Cancer Management* (pp. 1-16). Springer, Singapore.

Akinyede, K. A., Ekpo, O. E., and Oguntibeju, O. O. (2020). Ethnopharmacology, therapeutic properties and nutritional potentials of *Carpobrotus edulis*: A comprehensive review. *Scientia Pharmaceutica*, *88*(3), 39.

Akula, R., and Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant signaling & behavior*, *6*(11), 1720-1731.

Alam, E. A. (2011). Phytochemical screening on different plant parts of some succulent plants of Egypt. *New York Sci J*, *4*, 15-18.

Alhaithloul, H. A., Galal, F. H., and Seufi, A. M. (2021). Effect of extreme temperature changes on phenolic, flavonoid contents and antioxidant activity of tomato seedlings (*Solanum lycopersicum* L.). *PeerJ*, *9*, e11193.

Alhaithloul, H. A., Soliman, M. H., Ameta, K. L., El-Esawi, M. A., and Elkelish, A. (2020). Changes in ecophysiology, osmolytes, and secondary metabolites of the medicinal plants of *Mentha piperita* and *Catharanthus roseus* subjected to drought and heat stress. *Biomolecules*, *10*(1), 43.

Ammer, M. R., Zaman, S., Khalid, M., Bilal, M., Erum, S., Huang, D., and Che, S. (2016). Optimization of antibacterial activity of *Eucalyptus tereticornis* leaf extracts against *Escherichia coli* through response surface methodology. *Journal of Radiation Research and Applied Sciences*, *9*(4), 376-385.

Arriagada, N. B., Bowman, D. M., Palmer, A. J., and Johnston, F. H. (2020). Climate change, wildfires, heatwaves and health impacts in Australia. In *Extreme Weather Events and Human Health* (pp. 99-116). Springer, Cham.

Assis, J. S., Maldonado, R., Muñoz, T., Escribano, M. I., and Merodio, C. (2001). Effect of high carbon dioxide concentration on PAL activity and phenolic contents in ripening cherimoya fruit. *Postharvest Biology and Technology*, *23*(1), 33-39.

Balla, K., Bedő, Z., and Veisz, O. (2007). Heat stress induced changes in the activity of antioxidant enzymes in wheat. *Cereal Research Communications*, *35*(2), 197-200.

Balasooriya, H. N., Dassanayake, K. B., and Ajlouni, S. (2019a). The Impact of Elevated CO_2 and High Temperature on the Nutritional Quality of Fruits-A Short Review. *American Journal of Agricultural Research*, *4*(26), 1-9.

Balasooriya, H. N., Dassanayake, K. B., Seneweera, S., and Ajlouni, S. (2019b). Impact of elevated carbon dioxide and temperature on strawberry polyphenols. *Journal of the Science of Food and Agriculture*, *99*(10), 4659-4669.

Balasooriya, H. N., Dassanayake, K. B., Tomkins, B., Seneweera, S., and Ajlouni, S. (2017). Impacts of elevated carbon dioxide and temperature on physicochemical and nutrient properties in strawberries. *Scientific Pages Hortic*, *1*(1), 19-29.

Begum, S., Nakaba, S., Yamagishi, Y., Oribe, Y., and Funada, R. (2013). Regulation of cambial activity in relation to environmental conditions: understanding the role of temperature in wood formation of trees. *Physiologia plantarum*, *147*(1), 46-54.

Bennett, R. N., and Wallsgrove, R. M. (1994). Secondary metabolites in plant defence mechanisms. *New phytologist*, *127*(4), 617-633.

Bhargava, S., and Mitra, S. (2021). Elevated atmospheric CO_2 and the future of crop plants. *Plant Breeding*, *140*(1), 1-11.

Bhaskarachary, K., Naveena, N., and Polasa, K. (2015). Potential benefits of plant metabolites for human health. *The Indian Journal of Nutrition and Dietetics*, *52*(2), 213-225.

Blancquaert, E. H., Oberholster, A., Ricardo-da-Silva, J. M., and Deloire, A. J. (2019). Effects of abiotic factors on phenolic compounds in the Grape Nerry-a review. *South African Journal of Enology and Viticulture*, *40*(1), 1-14.

Buwa, L. V., and Afolayan, A. J. (2009). Antimicrobial activity of some medicinal plants used for the treatment of tuberculosis in the Eastern Cape Province, South Africa. *African Journal of Biotechnology*, *8*(23).

Campoy, J. G., Acosta, A. T., Affre, L., Barreiro, R., Brundu, G., Buisson, E., and Roiloa, S. R. (2018). Monographs of invasive plants in Europe: *Carpobrotus*. *Botany Letters*, *165*(3-4), 440-475.

Carlquist, S. (1974). Island biology. Columbia Univ, Press, New York. 1981. *Chance dispersal. American Scientist*, *69*, 509-516.

Carlson, A. E. (2008). Heat waves, global warming, and mitigation. *UCLA J. Envtl. L. & Pol'y*, *26*, 169.

Cassia, R., Nocioni, M., Correa-Aragunde, N., and Lamattina, L. (2018). Climate change and the impact of greenhouse gasses: CO_2 and NO, friends and foes of plant oxidative stress. *Frontiers in plant science*, *9*, 273.

Castañeda-Loaiza, V., Placines, C., Rodrigues, M. J., Pereira, C., Zengin, G., Uysal, A., and Custódio, L. (2020). If you cannot beat them, join them: Exploring the fruits of the invasive species *Carpobrotus edulis* (L.) NE Br as a source of bioactive products. *Industrial Crops and Products*, *144*, 112005.

Cebulak, T., Oszmiański, J., Kapusta, I., and Lachowicz, S. (2019). Effect of abiotic stress factors on polyphenolic content in the skin and flesh of pear by UPLC-PDA-Q/TOF-MS. *European Food Research and Technology*, *245*(12), 2715-2725.

Chauke, A. M., Shai, L. J., Mphahlele, P. M., and Mogale, M. A. (2012). Radical scavenging activity of selected medicinal plants from Limpopo province of South Africa. *African Journal of Traditional, Complementary and Alternative Medicines*, *9*(3), 426-430.

Chavan, U. D., and Ratnavathi, C. V. (2016). Role of Polyphenols in Nutrition and Prevention of Diseases. *Red*, *1*(1.3), 6.

Chokoe, P. K., Masoko, P., Mokgotho, M. P., Howard, R. L., and Mampuru, L. J. (2008). Does seasonal variation influence the phytochemical and antibacterial properties of *Carpobrotus edulis*?. *African Journal of Biotechnology*, *7*(22).

Chu, Y. H., Chang, C. L., and Hsu, H. F. (2000). Flavonoid content of several vegetables and their antioxidant activity. *Journal of the Science of Food and Agriculture*, *80*(5), 561-566.

Ciais, P., Sabine, C., Bala, G., Bopp, L., Brovkin, V., Canadell, J., and Thornton, P. (2014). Carbon and other biogeochemical cycles. In *Climate change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (pp. 465-570). Cambridge University Press.

Cui, X., Lin, Q., and Liang, Y. (2020). Plant-derived antioxidants protect the nervous system from aging by inhibiting oxidative stress. *Frontiers in Aging Neuroscience*, *12*, 209.

D'Antonio, C. M. (1990). Seed production and dispersal in the non-native, invasive succulent *Carpobrotus edulis* (Aizoaceae) in coastal strand communities of central California. *Journal of Applied Ecology*, 693-702.

Das, K., and Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in*

environmental science, *2*, 53.Debalke, D., Birhan, M., Kinubeh, A., and Yayeh, M. (2018). Assessments of antibacterial effects of aqueous-ethanolic extracts of *Sida rhombifolia*'s aerial part. *The Scientific World Journal*, *2018*.

Demain, A. L., and Fang, A. (2000). The natural functions of secondary metabolites. In History of modern biotechnology I, (pp. 1-39). Springer, Berlin, Heidelberg.

Dong, J., Gruda, N., Lam, S. K., Li, X., and Duan, Z. (2018). Effects of elevated CO_2 on nutritional quality of vegetables: a review. *Frontiers in plant science*, *9*, 924.

Egamberdieva, D., Wirth, S., Behrendt, U., Ahmad, P., and Berg, G. (2017). Antimicrobial activity of medicinal plants correlates with the proportion of antagonistic endophytes. *Frontiers in microbiology*, *8*, 199.

Engelbrecht, F., Adegoke, J., Bopape, M. J., Naidoo, M., Garland, R., Thatcher, M., and Gatebe, C. (2015). Projections of rapidly rising surface temperatures over Africa under low mitigation. *Environmental Research Letters*, *10*(8), 085004.

Falcone, D. L., Ogas, J. P., and Somerville, C. R. (2004). Regulation of membrane fatty acid composition by temperature in mutants of Arabidopsis with alterations in membrane lipid composition. *BMC plant biology*, *4*(1), 1-15.

Falleh, H., Ksouri, R., Medini, F., Guyot, S., Abdelly, C., and Magné, C. (2011). Antioxidant activity and phenolic composition of the medicinal and edible halophyte *Mesembryanthemum edule* L. *Industrial Crops and Products*, *34*(1), 1066-1071.

Farfan-Vignolo, E. R., and Asard, H. (2012). Effect of elevated CO_2 and temperature on the oxidative stress response to drought in *Lolium perenne* L. and *Medicago sativa* L. *Plant physiology and biochemistry*, *59*, 55-62.

Ficklin, D. L., and Novick, K. A. (2017). Historic and projected changes in vapor pressure deficit suggest a continental-scale drying of the United States atmosphere. *Journal of Geophysical Research: Atmospheres*, *122*(4), 2061-2079.

Fitriana, W. D., Ersam, T., Shimizu, K., and Fatmawati, S. (2016). Antioxidant activity of *Moringa oleifera* extracts. *Indonesian Journal of Chemistry*, *16*(3), 297-301.

Foyer, C. H., and Noctor, G. (2005). Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. *Plant, Cell & Environment*, *28*(8), 1056-1071.

Friedman, G. D., Udaltsova, N., Chan, J., Quesenberry, C. P., and Habel, L. A. (2009). Screening pharmaceuticals for possible carcinogenic effects: initial positive results for drugs not previously screened. *Cancer Causes & Control*, *20*(10), 1821-1835.

Fu, Y., Shao, L., Liu, H., Li, H., Zhao, Z., Ye, P., and Liu, H. (2015). Unexpected decrease in yield and antioxidants in vegetable at very high CO_2 levels. *Environmental chemistry letters*, *13*(4), 473-479.

Galindo-Prieto, B. (2017). *Novel variable influence on projection (VIP) methods in OPLS, O2PLS, and OnPLS models for single-and multi-block variable selection: VIPOPLS, VIPO2PLS, and MB-VIOP methods* (Doctoral dissertation, Umeå University).

Ganesan, K., and Xu, B. (2017). A critical review on polyphenols and health benefits of black soybeans. *Nutrients*, *9*(5), 455.

Garcia, A. K., Cavanaugh, C. M., and Kacar, B. (2021). The curious consistency of carbon biosignatures over billions of years of Earth-life coevolution. *The ISME Journal*, 1-12.

Garcia, E. J., Oldoni, T. L. C., Alencar, S. M. D., Reis, A., Loguercio, A. D., and Grande, R. H. M. (2012). Antioxidant activity by DPPH assay of potential solutions to be applied on bleached teeth. *Brazilian dental journal*, *23*, 22-27.

Garland, R. M., Matooane, M., Engelbrecht, F. A., Bopape, M. J. M., Landman, W. A., Naidoo, M., and Wright, C. Y. (2015). Regional projections of extreme apparent temperature days in Africa and the related potential risk to human health. *International journal of environmental research and public health*, *12*(10), 12577-12604.

Gillespie, K. M., Rogers, A., and Ainsworth, E. A. (2011). Growth at elevated ozone or elevated carbon dioxide concentration alters antioxidant capacity and response to acute oxidative stress in soybean (*Glycine max*). *Journal of experimental botany*, *62*(8), 2667-2678.

Ginovyan, M. M. (2017). Effect of heat treatment on antimicrobial activity of crude extracts of some Armenian herbs. *EPA Scientific Information and Biology*, *51*(2), 113-117.

Ghasemzadeh, A., and Jaafar, H. Z. (2011). Effect of CO_2 enrichment on synthesis of some primary and secondary metabolites in ginger (*Zingiber officinale* Roscoe). *International Journal of Molecular Sciences*, *12*(2), 1101-1114.

Ghasemzadeh, A., Jaafar, H. Z., and Rahmat, A. (2010). Elevated carbon dioxide increases contents of flavonoids and phenolic compounds, and antioxidant activities in Malaysian young ginger (*Zingiber officinale* Roscoe.) varieties. *Molecules*, *15*(11), 7907-7922.

Goicoechea, N., Jiménez, L., Prieto, E., Gogorcena, Y., Pascual, I., Irigoyen, J. J., and Antolín, M. C. (2021). Assessment of Nutritional and Quality Properties of Leaves and Musts in Three Local Spanish Grapevine Varieties Undergoing Controlled Climate Change Scenarios. *Plants*, *10*(6), 1198.

Gonelimali, F. D., Lin, J., Miao, W., Xuan, J., Charles, F., Chen, M., and Hatab, S. R. (2018). Antimicrobial properties and mechanism of action of some plant extracts against food pathogens and spoilage microorganisms. *Frontiers in microbiology*, *9*, 1639.

Goufo, P., Pereira, J., Figueiredo, N., Oliveira, M. B. P., Carranca, C., Rosa, E. A., and Trindade, H. (2014). Effect of elevated carbon dioxide (CO_2) on phenolic acids, flavonoids, tocopherols, tocotrienols, γ-oryzanol and antioxidant capacities of rice (*Oryza sativa* L.). *Journal of Cereal Science*, *59*(1), 15-24.

Granato, D., Santos, J. S., Escher, G. B., Ferreira, B. L., and Maggio, R. M. (2018). Use of principal component analysis (PCA) and hierarchical cluster analysis (HCA) for multivariate association between bioactive compounds and functional properties in foods: A critical perspective. *Trends in Food Science & Technology*, *72*, 83-90.

Gull, A., Lone, A. A., and Wani, N. U. I. (2019). Biotic and abiotic stresses in plants. *Abiotic and biotic stress in plants*, 1-19.

Hafsa, J., Hammi, K. M., Khedher, M. R. B., Smach, M. A., Charfeddine, B., Limem, K., and Majdoub, H. (2016). Inhibition of protein glycation, antioxidant and antiproliferative

activities of *Carpobrotus edulis* extracts. *Biomedicine & Pharmacotherapy*, *84*, 1496-1503.

Hajhashemi, V., Vaseghi, G., Pourfarzam, M., and Abdollahi, A. (2010). Are antioxidants helpful for disease prevention?. *Research in pharmaceutical sciences*, *5*(1), 1.

Hamid, A. A., Aiyelaagbe, O. O., Usman, L. A., Ameen, O. M., and Lawal, A. (2010). Antioxidants: Its medicinal and pharmacological applications. *African Journal of pure and applied chemistry*, *4*(8), 142-151.

Handique, J. G., and Baruah, J. B. (2002). Polyphenolic compounds: an overview. *Reactive and Functional Polymers*, *52*(3), 163-188.

Hansen, J., Ruedy, R., Sato, M., and Lo, K. (2010). Global surface temperature change. *Reviews of Geophysics*, *48*(4).

Hasanuzzaman, M., Nahar, K., and Fujita, M. (2013). Extreme temperature responses, oxidative stress and antioxidant defense in plants. *Abiotic stress-plant responses and applications in agriculture*, *13*, 169-205.

Hatfield, J. L., and Prueger, J. H. (2015). Temperature extremes: Effect on plant growth and development. *Weather and climate extremes*, *10*, 4-10.

Hilgart, A. (2016). Determination of a robust metabolic barcoding model for chemotaxonomy in Aizoaceae species: expanding morphological and genetic understanding.

Holopainen, J. K., Virjamo, V., Ghimire, R. P., Blande, J. D., Julkunen-Tiitto, R., and Kivimäenpää, M. (2018). Climate change effects on secondary compounds of forest trees in the northern hemisphere. *Frontiers in plant science*, *9*, 1445.

Huang, H., Ullah, F., Zhou, D. X., Yi, M., and Zhao, Y. (2019). Mechanisms of ROS regulation of plant development and stress responses. *Frontiers in Plant Science*, *10*, 800.

Hussain, G., Rasul, A., Anwar, H., Aziz, N., Razzaq, A., Wei, W., and Li, X. (2018). Role of plant derived alkaloids and their mechanism in neurodegenerative disorders. *International journal of biological sciences*, *14*(3), 341.

Hussein, R. A., and El-Anssary, A. A. (2019). Plants secondary metabolites: the key drivers of the pharmacological actions of medicinal plants. *Herbal medicine*, *1*, 13.

Ibtissem, B., Abdelly, C., and Sfar, S. (2012). Antioxidant and antibacterial properties of *Mesembryanthemum crystallinum* and *Carpobrotus edulis* extracts.

Irawan, C., Rochaeni, H., Sulistiawaty, L., and Roziafanto, A. N. (2018). Phytochemical Screening, LC-MS Studies and Antidiabetic Potential of Methanol Extracts of Seed Shells of *Archidendron bubalinum* (Jack) IC Nielson (*Julang Jaling*) from Lampung, Indonesia. *Pharmacognosy Journal*, *10*(6s).

Irshad, M., Zafaryab, M., Singh, M., and Rizvi, M. (2012). Comparative analysis of the antioxidant activity of *Cassia fistula* extracts. *International journal of medicinal chemistry*, *2012*.

Is, Y., and Woodside, J. V. (2001). Antioxidant in health and disease. *J Clin Pathol*, *54*(3), 176-186.

Isah, T. (2019). Stress and defense responses in plant secondary metabolites production. *Biological research*, *52*.

Jadid, N., Hidayati, D., Hartanti, S. R., Arraniry, B. A., Rachman, R. Y., and Wikanta, W. (2017, June). Antioxidant activities of different solvent extracts of *Piper retrofractum* Vahl. using

DPPH assay. *In AIP conference proceedings* (Vol. 1854, No. 1, p. 020019). AIP Publishing LLC.

Jamloki, A., Bhattacharyya, M., Nautiyal, M. C., and Patni, B. (2021). Elucidating the relevance of high temperature and elevated CO_2 in plant secondary metabolites (PSMs) production. *Heliyon*.

Kabtni, S., Sdouga, D., Bettaib Rebey, I., Save, M., Trifi-Farah, N., Fauconnier, M. L., and Marghali, S. (2020). Influence of climate variation on phenolic composition and antioxidant capacity of *Medicago minima* populations. *Scientific reports*, *10*(1), 1-15.

Karuppanapandian, T., Moon, J. C., Kim, C., Manoharan, K., and Kim, W. (2011). Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms. *Australian Journal of Crop Science*, *5*(6), 709-725.

Keele, M. Z. (2014). *Development of a Bulbine Frutescens and Carpobrotus Edulis Cream in Collaboration with African Traditional Healers of the Nelson Mandela Metropole* (Doctoral dissertation, Nelson Mandela Metropolitan University).

Khattab, S., and El Sherif, F. (2011). Effect of growth regulators on *Carpobrotus edulis* rapid micropropagation and molecular analysis. *Journal of American Science*, *7*, 511-520.

Kim, H. J., and Oh, S. W. (2010). Performance comparison of 5 selective media used to detect *Staphylococcus aureus* in foods. *Food Science and Biotechnology*, *19*(4), 1097-1101.

Kim, J. M., Kang, J. Y., Park, S. K., Han, H. J., Lee, K. Y., Kim, A. N., and Heo, H. J. (2020). Effect of storage temperature on the antioxidant activity and catechins stability of Matcha (*Camellia sinensis*). *Food science and biotechnology*, *29*(9), 1261-1271.

Kitadai, N., and Maruyama, S. (2018). Origins of building blocks of life: A review. *Geoscience Frontiers, 9*(4), 1117-1153.

Koohsari, H., Ghaemi, E. A., Sheshpoli, M. S., Jahedi, M., and Zahiri, M. (2015). The investigation of antibacterial activity of selected native plants from North of Iran. *Journal of medicine and life, 8*(Spec Iss 2), 38.

Kroymann, J. (2011). Natural diversity and adaptation in plant secondary metabolism. Current opinion in plant biology, 14(3), 246-251.

Kumar, S., Yadav, A., Yadav, M., and Yadav, J. P. (2017). Effect of climate change on phytochemical diversity, total phenolic content and in vitro antioxidant activity of *Aloe vera* (L.) Burm. f. *BMC research notes, 10*(1), 1-12.

Leri, M., Scuto, M., Ontario, M. L., Calabrese, V., Calabrese, E. J., Bucciantini, M., and Stefani, M. (2020). Healthy effects of plant polyphenols: molecular mechanisms. *International journal of molecular sciences, 21*(4), 1250.

Levine, L. H., and Paré, P. W. (2009). Antioxidant capacity reduced in scallions grown under elevated CO_2 independent of assayed light intensity. *Advances in space research, 44*(8), 887-894.

Lin, D., Xiao, M., Zhao, J., Li, Z., Xing, B., Li, X., and Chen, S. (2016). An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes. *Molecules,* 21(10), 1374.

Lindsey, R. (2020). Climate change: atmospheric carbon dioxide. *NOAA Climate. gov, Maryland, News and Features, Understanding Climate*, *14*.

Luber, G., and McGeehin, M. (2008). Climate change and extreme heat events. *American journal of preventive medicine*, *35*(5), 429-435.

Lyubchyk, S., Shapovalova, O., Lygina, O., Oliveira, M. C., Appazov, N., Lyubchyk, A., and Pombeiro, A. J. (2019). Integrated Green Chemical Approach to the Medicinal Plant *Carpobrotus edulis* Processing. *Scientific reports*, *9*(1), 1-12.

Mabona, U., and Van Vuuren, S. F. (2013). Southern African medicinal plants used to treat skin diseases. *South African Journal of Botany*, *87*, 175-193.

Maghsoudlou, Y., Asghari Ghajari, M., and Tavasoli, S. (2019). Effects of heat treatment on the phenolic compounds and antioxidant capacity of quince fruit and its tisane's sensory properties. *Journal of food science and technology*, *56*(5), 2365-2372.

Manandhar, S., Luitel, S., and Dahal, R. K. (2019). In vitro antimicrobial activity of some medicinal plants against human pathogenic bacteria. *Journal of tropical medicine*, *2019*.

Martins, A., Vasas, A., Viveiros, M., Molnár, J., Hohmann, J., and Amaral, L. (2011). Antibacterial properties of compounds isolated from *Carpobrotus edulis*. *International journal of antimicrobial agents*, 37(5), 438-444.

Matic, I., Guidi, A., Kenzo, M., Mattei, M., and Galgani, A. (2018). Investigation of medicinal plants traditionally used as dietary supplements: A review on *Moringa oleifera*. *Journal of public health in Africa*, *9*(3).

Matsuda, F., Shinbo, Y., Oikawa, A., Hirai, M. Y., Fiehn, O., Kanaya, S., and Saito, K. (2009). Assessment of metabolome annotation quality: a method for evaluating the false discovery rate of elemental composition searches. *PLoS One*, *4*(10), e7490.

Mazid, M., Khan, T. A., and Mohammad, F. (2011). Role of secondary metabolites in defense mechanisms of plants. *Biology and medicine*, *3*(2), 232-249.

Mbokodo, I., Bopape, M. J., Chikoore, H., Engelbrecht, F., and Nethengwe, N. (2020). Heatwaves in the future warmer climate of South Africa. *Atmosphere*, *11*(7), 712.

Meddeb, E., Charni, M., Ghazouani, T., Cozzolino, A., Fratianni, F., Raboudi, F., and Fattouch, S. (2017). Biochemical and molecular study of *Carpobrotus edulis* bioactive properties and their effects on *Dugesia sicula* (turbellaria, tricladida) regeneration. *Applied biochemistry and biotechnology*, *182*(3), 1131-1143.

Mera, I. F. G., Falconí, D. E. G., and Córdova, V. M. (2019). Secondary metabolites in plants: main classes, phytochemical analysis and pharmacological activities. *Rev Bionatura*, *4*, 1000-9.

Mohi-Ud-Din, M., Siddiqui, M., Rohman, M., Jagadish, S. V., Ahmed, J. U., Hassan, M. M., and Islam, T. (2021). Physiological and Biochemical Dissection Reveals a Trade-Off between Antioxidant Capacity and Heat Tolerance in Bread Wheat (*Triticum aestivum* L.). *Antioxidants*, *10*(3), 351.

Moore, C. E., Meacham-Hensold, K., Lemonnier, P., Slattery, R. A., Benjamin, C., Bernacchi, C. J., and Cavanagh, A. P. (2021). The effect of increasing temperature on crop photosynthesis: from enzymes to ecosystems. *Journal of experimental botany*, *72*(8), 2822-2844.

Morison, J. I. L., and Lawlor, D. W. (1999). Interactions between increasing CO_2 concentration and temperature on plant growth. *Plant, Cell & Environment*, *22*(6), 659-682.

Mudimba, T. N., and Nguta, J. M. (2019). Traditional uses, phytochemistry and pharmacological activity of *Carpobrotus edulis*: A global perspective. *J. Phytopharmacol*., *8*, 111-116.

Navrot, N., Rouhier, N., Gelhaye, E., and Jacquot, J. P. (2007). Reactive oxygen species generation and antioxidant systems in plant mitochondria. *Physiologia Plantarum*, *129*(1), 185-195.

Netshiluvhi, T. R., and Eloff, J. N. (2019). Temperature stress does not affect antimicrobial activity of some South African medicinal plants. *South African Journal of Botany*, *123*, 93-97.

Nievola, C. C., Carvalho, C. P., Carvalho, V., and Rodrigues, E. (2017). Rapid responses of plants to temperature changes. *Temperature*, *4*(4), 371-405.

Oksana, S., Marian, B., Mahendra, R., and Bo, S. H. (2012). Plant phenolic compounds for food, pharmaceutical and cosmetics production. *Journal of Medicinal Plants Research*, *6*(13), 2526-2539.

Omoruyi, B. E., Bradley, G., and Afolayan, A. J. (2012). Antioxidant and phytochemical properties of *Carpobrotus edulis* (L.) bolus leaf used for the management of common infections in HIV/AIDS patients in Eastern Cape Province. *BMC Complementary and Alternative Medicine*, *12*(1), 1-9.

Osakabe, Y., Osakabe, K., and Shinozaki, K. (2013). Plant environmental stress responses for survival and biomass enhancement. *Climate change and plant abiotic stress tolerance*, 79-108.

Owusu, E., Ahorlu, M. M., Afutu, E., Akumwena, A., and Asare, G. A. (2021). Antimicrobial Activity of Selected Medicinal Plants from a Sub-Saharan African Country against Bacterial Pathogens from Post-Operative Wound Infections. *Medical Sciences*, 9(2), 23.

Panche, A. N., Diwan, A. D., and Chandra, S. R. (2016). Flavonoids: an overview. *Journal of nutritional science, 5*.

Pandey, V., Bhatt, I. D., and Nandi, S. K. (2019). Environmental stresses in Himalayan medicinal plants: research needs and future priorities. *Biodiversity and Conservation*, *28*(8), 2431-2455.

Pather, N., Viljoen, A. M., and Kramer, B. (2011). A biochemical comparison of the in vivo effects of *Bulbine frutescens* and *Bulbine natalensis* on cutaneous wound healing. *Journal of ethnopharmacology*, *133*(2), 364-370.

Pearcy, R. W. (1978). Effect of growth temperature on the fatty acid composition of the leaf lipids in *Atriplex lentiformis* (Torr.) wats. *Plant Physiology*, *61*(4), 484-486.

Peñuelas, J., and Estiarte, M. (1998). Can elevated CO_2 affect secondary metabolism and ecosystem function?. *Trends in Ecology & Evolution*, *13*(1), 20-24.

Pfukwa, T. M., Chikwanha, O. C., Katiyatiya, C. L., Fawole, O. A., Manley, M., and Mapiye, C. (2020). Southern African indigenous fruits and their byproducts: Prospects as food antioxidants. *Journal of Functional Foods, 75,* 104220.

Proestos, C., Lytoudi, K., Mavromelanidou, O. K., Zoumpoulakis, P., and Sinanoglou, V. J. (2013). Antioxidant capacity of selected plant extracts and their essential oils. *Antioxidants, 2*(1), 11-22.

Proteggente, A. R., Pannala, A. S., Paganga, G., Buren, L. V., Wagner, E., Wiseman, S., and Rice-Evans, C. A. (2002). The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. *Free radical research*, *36*(2), 217-233.

Quint, M., Delker, C., Franklin, K. A., Wigge, P. A., Halliday, K. J., and Van Zanten, M. (2016). Molecular and genetic control of plant thermomorphogenesis. *Nature plants, 2*(1), 1-9.

Rajashekar, C. B. (2018). Elevated CO_2 levels affect phytochemicals and nutritional quality of food crops. *American Journal of Plant Sciences*, *9*(2), 150-162.

Réblová, Z. (2012). Effect of temperature on the antioxidant activity of phenolic acids. *Czech Journal of Food Sciences*, *30*(2), 171-175.

Ribeiro, R. V., Machado, E. C., and Oliveira, R. F. D. (2006). Temperature response of photosynthesis and its interaction with light intensity in sweet orange leaf discs under non-photorespiratory condition. *Ciência e Agrotecnologia*, *30*(4), 670-678.

Rocha, M. I., Rodrigues, M. J., Pereira, C., Pereira, H., Da Silva, M. M., da Rosa Neng, N., and Custódio, L. (2017). Biochemical profile and in vitro neuroprotective properties of *Carpobrotus edulis* L., a medicinal and edible halophyte native to the coast of South Africa. *South African journal of botany*, *111*, 222-231.

Roiloa, S. R., Rodríguez-Echeverría, S., de la Pena, E., and Freitas, H. (2010). Physiological integration increases the survival and growth of the clonal invader *Carpobrotus edulis*. *Biological Invasions*, *12*(6), 1815-1823.

Rosa, C., Calleja, C. A., and Benito, M. (2001). Assessment of Baried-Parker Agar as Screening Test for Determination of *Staphylococcus aureus* in Poultry Meat. *Journal of Microbiology*, *39*(4), 321-325.

Ruiz-Ruiz, C. J., Ramón-Sierra, J., Arias-Argaez, C., Magaña-Ortiz, D., and Ortiz-Vázquez, E. (2017). Antibacterial activity of proteins extracted from the pulp of wild edible fruit of *Bromelia pinguin* L. *International Journal of Food Properties*, *20*(1), 220-230.

Sallas, L., Luomala, E. M., Utriainen, J., Kainulainen, P., and Holopainen, J. K. (2003). Contrasting effects of elevated carbon dioxide concentration and temperature on Rubisco activity, chlorophyll fluorescence, needle ultrastructure and secondary metabolites in conifer seedlings. *Tree Physiology*, *23*(2), 97-108.

Salmerón-Manzano, E., Garrido-Cardenas, J. A., and Manzano-Agugliaro, F. (2020). Worldwide research trends on medicinal plants. *International journal of environmental research and public health*, *17*(10), 3376.

Santos-Sánchez, N. F., Salas-Coronado, R., Villanueva-Cañongo, C., and Hernández-Carlos, B. (2019). *Antioxidant compounds and their antioxidant mechanism* (pp. 1-28). London, UK: IntechOpen.

Sarmati, S., Conti, L., and Acosta, A. T. (2019). *Carpobrotus acinaciformis* vs *Carpobrotus edulis*: Are there any differences in their impact on coastal dune plant biodiversity?. *Flora*, *257*, 151422.

Sartorius Stedim Data Analytics AB, all rights reserved. (2020). SIMCA® [SIMCA® 15 User Guide]. https://www.sartorius.com/download/544940/simca-15-user-guide-en-b-00076-sartorius-data.pdf

Saxena, M., Saxena, J., Nema, R., Singh, D., and Gupta, A. (2013). Phytochemistry of medicinal plants. *Journal of pharmacognosy and phytochemistry*, *1*(6).

Scholes, R., and Engelbrecht, F. (2021). Climate impacts in southern Africa during the 21st Century. *Report for Earthjustice and the Centre for Envrionmental Rights. Global Change Instiute, University of Witwatersrand*.

Sen, A., and Batra, A. (2012). Evaluation of antimicrobial activity of different solvent extracts of medicinal plant: *Melia azedarach* L. *Int J Curr Pharm Res*, *4*(2), 67-73.

Sharma, P., Jha, A. B., Dubey, R. S., and Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of botany*, *2012*.

Shih, C. J., Vijayaraghavan, A., Krishnan, R., Sharma, R., Han, J. H., Ham, M. H., and Strano, M. S. (2011). Bi-and trilayer graphene solutions. *Nature Nanotechnology*, *6*(7), 439-445.

Soengas, P., Rodríguez, V. M., Velasco, P., and Cartea, M. E. (2018). Effect of temperature stress on antioxidant defenses in *Brassica oleracea*. *Acs Omega*, *3*(5), 5237-5243.

Sun, P., Mantri, N., Lou, H., Hu, Y., Sun, D., Zhu, Y., and Lu, H. (2012). Effects of elevated CO_2 and temperature on yield and fruit quality of strawberry (*Fragaria× ananassa* Duch.) at two levels of nitrogen application. *PloS one*, *7*(7), e41000.

Tiffney, B. H. (1984). Seed size, dispersal syndromes, and the rise of the angiosperms: evidence and hypothesis. *Annals of the Missouri Botanical Garden*, *71*(2), 551-576.

Van Andel, T., and Carvalheiro, L. G. (2013). Why urban citizens in developing countries use traditional medicines: the case of Suriname. *Evidence-Based Complementary and Alternative Medicine*, *2013*.

Van Der Watt, E., and Pretorius, J. C. (2001). Purification and identification of active antibacterial components in *Carpobrotus edulis* L. *Journal of ethnopharmacology*, *76*(1), 87-91.

Van Vuuren, D. P., Meinshausen, M., Plattner, G. K., Joos, F., Strassmann, K. M., Smith, S. J., and Reilly, J. M. (2008). Temperature increase of 21st century mitigation scenarios. *Proceedings of the National Academy of Sciences*, *105*(40), 15258-15262.

Veteli, T. O., Mattson, W. J., Niemelä, P., Julkunen-Tiitto, R., Kellomäki, S., Kuokkanen, K., and Lavola, A. (2007). Do elevated temperature and CO_2 generally have counteracting effects on phenolic phytochemistry of boreal trees?. *Journal of Chemical Ecology*, *33*(2), 287-296.

Wang, S. Y., and Zheng, W. (2001). Effect of plant growth temperature on antioxidant capacity in strawberry. *Journal of agricultural and food chemistry*, *49*(10), 4977-4982.

Wang, S. Y., Bunce, J. A., and Maas, J. L. (2003). Elevated carbon dioxide increases contents of antioxidant compounds in field-grown strawberries. *Journal of Agricultural and Food Chemistry*, *51*(15), 4315-4320.

Watson, R. T., Meira Filho, L. G., Sanhueza, E., and Janetos, A. (1992). Greenhouse gases: sources and sinks. *Climate change*, *92*, 25-46.

Wink, M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry*, *64*(1), 3-19.

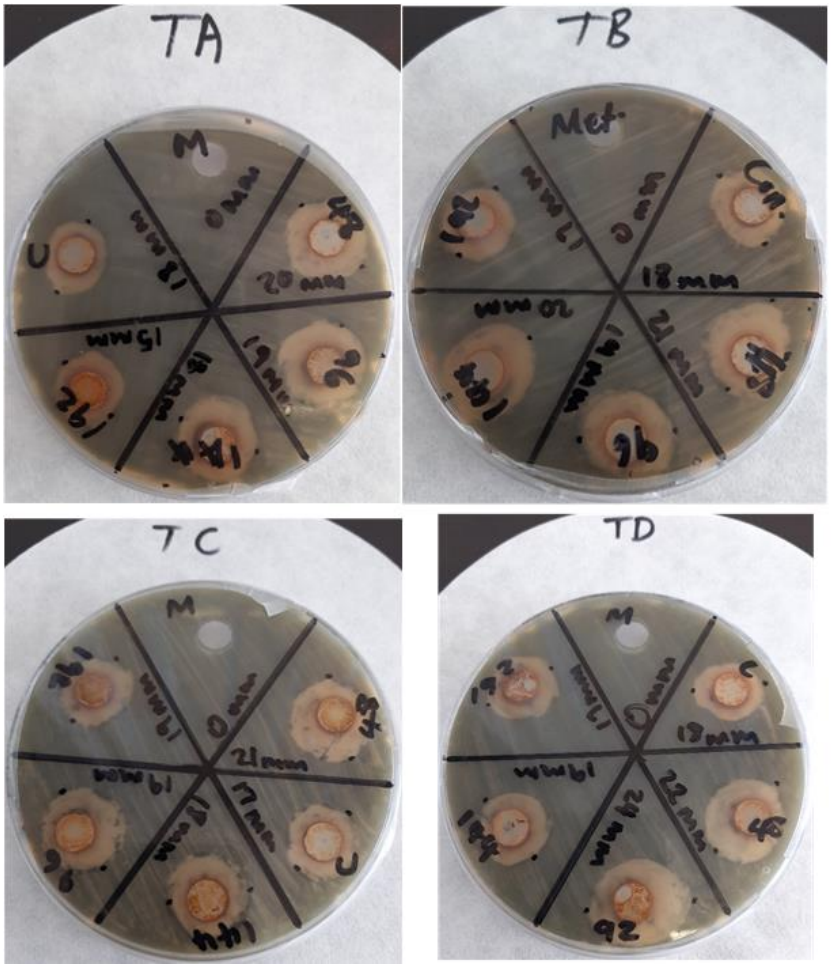
Wisura, W., and Glen, H. F. (1993). The South African species of *Carpobrotus* (Mesembryanthema–Aizoaceae). *Contributions to the Bolus Herbarium*, *15*, 76-107.

Woodward, F. I., and Woodward, F. I. (1987). *Climate and plant distribution*. Cambridge University Press.

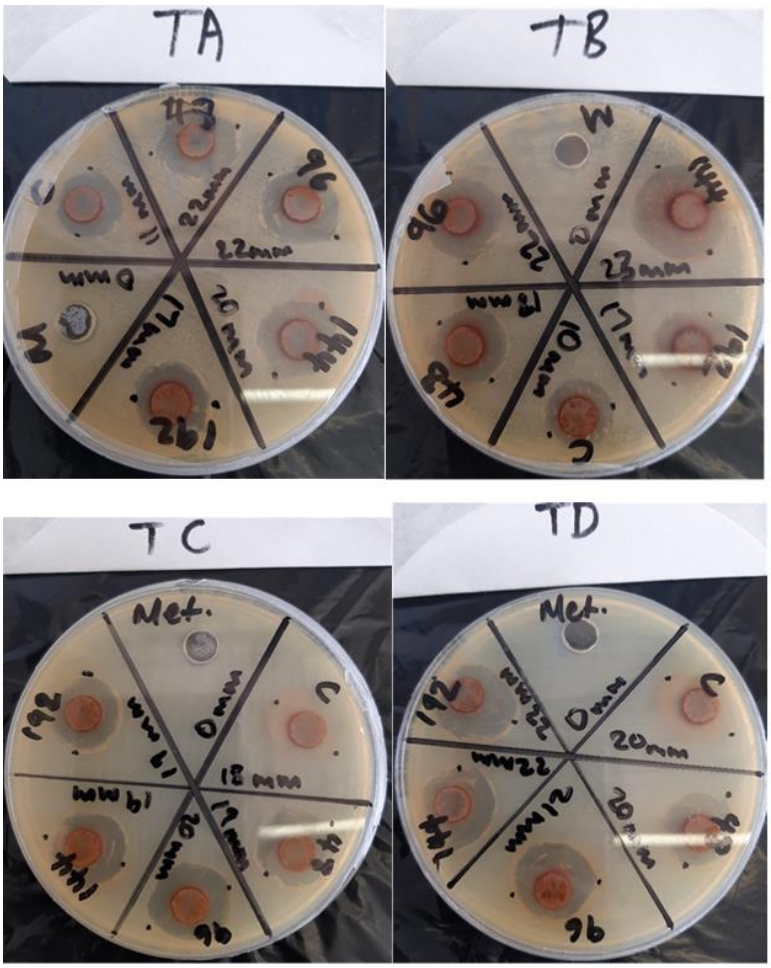
Yan, N., Xu, X. F., Wang, Z. D., Huang, J. Z., and Guo, D. P. (2013). Interactive effects of temperature and light intensity on photosynthesis and antioxidant enzyme activity in *Zizania latifolia* Turcz. plants. *Photosynthetica*, *51*(1), 127-138.

Yang, L., Wen, K. S., Ruan, X., Zhao, Y. X., Wei, F., and Wang, Q. (2018). Response of plant secondary metabolites to environmental factors. *Molecules*, *23*(4), 762.

APPENDICES



Appendix A: Bacterial inhibition activity of *Carpobrotus edulis* methanol extracts against *Staphylococcus aureus.* Each plate represents the following treatments control extracts (400 ppm and 28/25°C), TA (600 ppm and 45/35°C), TB (800 ppm and 45/35°C), TC (600 ppm and 35/30°C), and TD (800 ppm and 35/30°C), respectively. Moreover, within each plate, each well was filled with 80% methanol (negative control), control extracts (400 ppm and 28/25°C) and extracts of each treatment at every 48-hour interval.



Appendix B: Bacterial inhibition activity of *Carpobrotus edulis* methanol extracts against *Escherichia coli*. Each plate represents the following treatments control extracts (400 ppm and 28/25°C), TA (600 ppm and 45/35°C), TB (800 ppm and 45/35°C), TC (600 ppm and 35/30°C), and TD (800 ppm and 35/30°C), respectively. Moreover, within each plate, each well was filled with 80% methanol (negative control), control extracts (400 ppm and 28/25°C) and extracts of each treatment at every 48-hour interval.