The effects of concurrent extreme temperature and water deficit on the phytochemical profile and phytopharmacological activities in *Portulacaria afra* Jacq.

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Declaration

I, Oluwafunbi Christianah Adeleye, hereby declare that this thesis is my original work submitted for the degree of Doctor of Philosophy to the University of the Witwatersrand, Johannesburg. It has never been submitted to any other University for any other degree or examination.

Oluwafunbi C. Adeleye

(Signature of candidate)

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Dedication

This thesis is dedicated to God and my dad; Pharm. Frederick Temitope Adeleye.

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Abstract

In nature, all plants are exposed to stress which are mostly biotic and abiotic stress factors. Previous studies have demonstrated the impact of various abiotic stress factors on the production of secondary metabolites in therapeutic plants. Plant responses to stressors brought on by a combination of antagonistic abiotic factors have shown to be phenomenal compared to when plants are exposed to single a factor. According to latest climate change models, it is believed that plants would suffer unique or demanding concurrent abiotic stresses in the years to come. South Africa has been experiencing increasing temperatures over last 40 years and being regarded as a climate change 'hot spot' by the Intergovernmental Panel on Climate Change (IPCC). Therefore, it is critical to conduct research on the impact of climate change on the bioactive compounds in therapeutic plants. The overarching aim of this study was to establish and scientifically document, for the first time to our knowledge, the phytochemical profile, medicinal properties and phytopharmacological attributes of *Portulacaria afra*, a widely renowned medicinal plant used for treating several skin conditions and oral infections which also includes the assessment of the effects of concurrent extreme temperatures and water deficit/drought on species' biological activities.

In this study, South African *Portulacaria afra* plants were selected from healthy parent plants and propagated from cuttings. Samples were allowed to grow and establish a root system in the greenhouse for up to three months. After three months, 180 potted plant samples were exposed to treatments and not watered for up to 144 hours (6 days), and 45 control samples were placed under 25ºC (ambient) and watered every second day with 500ml of water. The control samples were kept at 25°C maximum night-time temperature (7pm to 5am) and 27°C maximum day time temperature. Plants undergoing treatment (coded as treatment A, B, C, D) were treated as follows: A and B were exposed to 0/10ºC (night/day) and 5/15ºC (night/day) respectively,

while C and D were exposed 20/40°C (night/day) and 35/45°C (night/day), respectively. Five potted plants were harvested in each treatment three times for up to 6 days (144hrs). Sample harvesting was scheduled episodically every 48hrs (48, 96, 144) and were then airdried under 40°C for 2 to 3 days.

The aqueous (water) extracts at a temperature of 60°C, alongside methanol, *n*-hexane, and ethyl acetate extracts were derived from the leaves, stems, and roots. The extracts were then used to investigate the phytochemical composition, antibacterial efficacy, antioxidant capacity and antidiabetic potential. The qualitative phytochemical screening encompassed the preliminary assessment of saponins, flavonoids, glycosides, quinones, phenols, terpenoids, steroids, phytosteroids, volatile oil, carbohydrates, amino acids, and coumarins. The quantitative analyses were performed to determine the total phenolic content (TPC), total flavonoid content (TFC), while the antioxidant assays were performed to determine the reducing, scavenging and chelating abilities against DPPH, H_2O_2 and metal (Iron) chelating. The antibacterial activities against gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus*, *Streptomyces griseus* were assessed through agar well diffusion assay. The antidiabetic potential was evaluated using *In vitro* inhibitory α-amylase assay. Chemical profiling of various extracts from the leaves, stems, and roots of *P. afra* was conducted to identify and quantify some secondary metabolites. The methanolic leaf extracts exhibited a notable presence of quinones, phenols, steroids, and coumarins, whereas the aqueous leaf extracts contained moderate presence of saponins, terpenoids, quinones, and coumarins. Ethyl acetate leaf extracts were characterized by a strong presence of tannins and a moderate presence of phytosteroids. Conversely, *n*-hexane leaf extracts showed considerable saponin levels, moderate tannins, and terpenoids. Significantly strong presence of secondary metabolites was observed in methanolic stem extracts, particularly terpenoids, steroids, phenols, and coumarins. Notably, coumarins, known for their anticancer properties, were prominently present in

methanolic leaf and stem extracts, with a moderate presence in root extracts, hinting at potential pharmaceutical applications and future roles in public health. Aqueous stem extracts exhibited strong glycoside presence, while ethyl acetate and *n*-hexane stem extracts exhibited few fewer secondary metabolite groups, ranging from moderate to weak presence.

Distinctive chemical profiles were observed in root extracts, with ethyl acetate extracts showing significantly stronger quinone presence. Methanolic root extracts displayed moderate presence of coumarins and glycosides, whereas aqueous root extracts showed a low glycoside presence. The highest total phenolic contents (TPCs) and total flavonoid contents (TFCs) were found in methanol stem extracts and aqueous root extracts, respectively. Notably, aqueous root extracts exhibited the highest TPC and TFC among all root extracts.

Antibacterial activity assays showed a wide range on inhibitory effects of *n*-hexane extracts from leaf, stem, and root against test microorganisms. Ethyl acetate leaf extracts demonstrated considerable inhibitory efficacy against *Staphylococcus aureus*, while methanolic extracts showed zero zone of inhibition. Aqueous root extracts showed strong antibacterial activity against *Staphylococcus aureus*, whereas other extracts showed no significant activity. Inhibition zones ranged from 13 to 24 mm for the plant extracts. The assessment of antioxidant potential through DPPH, H_2O_2 scavenging, and metal chelating assays showed varied activity among extracts. Ethyl acetate root extracts showed the strongest H_2O_2 scavenging activity, while aqueous stem extracts showed the strongest antioxidant activity against DPPH radicals. Aqueous and *n*-hexane root extracts showed the strongest metal chelating ability. The *in vitro* antidiabetic activity showed that all plant parts were active against α-amylase, with the highest inhibitory action recorded from the methanolic leaf extracts, followed by the methanolic root extracts.

Although herbal medicines are frequently used, they are also widely criticized. One point of disagreement is that plants are known to react to their surroundings and several abiotic factors and as a result, fluctuations, and variations in the accumulations of phytoconstituents inside and between plants parts is expected, which will almost certainly lead to variance in biological activity. Therefore, the phytochemical screening in the treated samples of *P. afra* leaf, stem and root extracts was investigated to better understand the expected variation in the phytopharmacological activities under the concurrent extreme temperatures with water deficit condition exposed to, in this study. Majority of the already identified phytochemicals showed an increasing trend, with methanol and aqueous extracts from all plant parts in both concurrent extreme temperatures and water deficit settings with the most presence of phytochemicals.

A significantly higher total phenolic and total flavonoid content was observed in plant parts when compared to the control, with varying concentrations depending on the extraction solvent, temperature, and treatment period. In the comparative analysis of plant parts, the investigation showed the highest concentrations of total phenolic contents (TPC) and total flavonoids contents (TFC) across all plant parts under extreme hot temperatures (35/45ºC) combined and water deficit conditions. This was observed in the ethyl acetate leaf and methanolic leaf extracts, after a 96-hour treatment period respectively. The antibacterial activity result showed that examined plant extracts under the extreme hot temperatures (35/45ºC) and water deficit had greater inhibitory effect against gram-negative *E. coli* than gram-positive microorganisms. The highest level of inhibitory effect of 21mm was recorded against gram-negative *E. coli*, from the methanolic root extracts after a 48hr-treatment period. However, the inhibitory activities mainly observed in hot temperatures (30/40ºC) were intermediate, which ranged from 11-13mm against gram-positive *Streptomyces griseus* and *Staphylococcus aureus.* In the experimental investigation, the assessment of plant extracts

under the hot temperatures [(30/40ºC), (35/45ºC)] and water deficit showed enhanced antimicrobial efficacy against all microorganisms examined within this study.

In the antioxidant activity results, the most significant DPPH and metal chelating antioxidant activity was found in the ethyl acetate root extracts after a 96-hour treatment period and the methanolic leaf extracts after a 144-hour treatment period respectively after exposure to the hot temperatures (30/40ºC) and water deficit. Overall, *n*-hexane stem extracts under concurrent hot temperatures (35/45ºC) and water deficit showed the strongest hydrogen peroxide scavenging activity after a 144-hour treatment period.

When compared to the control samples, plant extracts showed a significant increase in alpha amylase inhibitory potential. Overall, the strongest inhibitory activity against α-amylase enzyme activity under concurrent hot and cold temperatures [(30/40ºC), (10/15ºC), (35/45ºC), and (0/5ºC)] with water deficit was observed in the aqueous stem extracts under hot temperatures (30/40ºC) after a 48-hour treatment period. The differences in phytopharmacological activities in combination with the various temperature ranges with water deficit condition indicate that secondary metabolite variation can have a profound influence on the bioactivity of medicinal plants.

It is evident that abiotic stress in combination encourages plants to produce secondary metabolites, which increases the output of phytomedicine and enhances the production of phytochemicals, antimicrobial, antioxidant, and antidiabetic activities.

However, variances exist depending on the stress, solvents, plant components, and treatment period. It can be concluded that, in addition to extracting samples with solvents of different polarity, plant part, technique, sample state, and treatment period all play a role in phytochemical extraction and accumulation.

The findings of this investigation illustrated the diverse responses of plant parts to simultaneous environmental stresses, demonstrating alterations in secondary metabolite levels, antioxidant efficacy, as well as antimicrobial and antidiabetic activities. It is evident that abiotic factors in combination may influence the production of secondary metabolites, which could increase the output of phytomedicine and enhance the production of phytochemicals.

Abbreviations and symbols

APES	Animal, Plant and Environmental Sciences
CAM	Crassulacean acid metabolism
NWS	National weather service
SAW	South Africa weather service
IPCC	Intergovernmental panel on climate change
FAO	Food and Agriculture Organisation of the United Nations
TB	Tuberculosis
DPPH	2, 2 diphenylpicrylhydrazyl
DMSO	Dimethyl sulfoxide
TPC	Total Phenolic Content
TFC	Total Flavonoid Content
GAE	Gallic Acid Equivalence
QE	Quercetin Equivalence
WHO	World Health Organisation
ANOVA	Analysis of Variance

AlCl3	Aluminium Chloride
NaNO3	Sodium Nitrate
MeOH	Methanol
mm	Millimetres
cm	Centimetres
ml	Millilitres
L	Litres
%	Percentage
ppm	Parts per million
mg	Milligram
µm	Micrometres
ROS	Reactive Oxygen Species
O_2^-	Superoxide
H_2O_2	Hydrogen peroxide
HO	Hydroxyl radical
$^1O_2$	Singlet oxygen
SM	Secondary Metabolites

UV	Ultraviolet
GSH	Glutathione
GPX	Guaiacol peroxidase
SOD	Superoxide dismutase
APX	Ascorbate peroxidase
CA	Catalase
AsA	Ascorbic acid
MDHA	Monodehydroascorbate
GSSG	Glutathione disulfide
DHA	Dehydroascorbate
ER	Endoplasmic reticulum
VOCs	Volatile organic compounds
DM	Diabetes mellitus
MEP	Methylerythritol phosphate pathway
MEV	Mevolonate pathway
pH	Potential of hydrogen
H_20	Water

O_2	Oxygen
GM^-	Gram-negative
GM^+	Gram-positive
ATCC	American type culture collection
NCTC	National collection of type cultures
FLAE	Flueggea leucopyrus willd
AGEs	Advanced glycation end products
SAAB	South Africa Association of Botanists

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Publication record

Published Papers:

Adeleye OC, Risenga IM (2022) Screening of Phytochemical Profile and Biological Activities in the Leaves, Stems and Roots of South African *Portulacaria afra* using Four Extraction Solvents. Biomedical and Pharmacology Journal 15(3):1561-1572. DOI: https://dx.doi.org/10.13005/bpj/2494

Adeleye O. C, Risenga I. M, The *In Vitro* Assessment of Antidiabetic Activity of the Plant Extracts Obtained from *Portulacaria afra* Jack. Grown under Concurrent Extreme Temperatures and Water-deficit Conditions. Biomed Pharmacol J 2024;17(1). DOI : https://dx.doi.org/10.13005/bpj/2859

Manuscripts resubmitted after review process as requested by the journal editor:

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Conference paper presentation: Adeleye OC, Risenga IM (2022) Screening of Phytochemical Profile and Biological Activities in the Leaves, Stems and Roots of South African *Portulacaria afra* using Four Extraction Solvents. Biomedical and Pharmacology Journal 15(3):1561-1572. Annual South Africa Association of Botanist (SAAB) Postgraduate Symposium, 28th September 2023.

Thesis structure

This thesis comprises of six chapters and was written in the format of scientific publications format. Chapters 3, 4, and 5 are written in scientific paper format or (manuscripts of already published, resubmitted, and accepted papers), which were designed to meet the standards of specific journals. Please be advised that there will be some repetition in aspects such as the introduction and methodology. Furthermore, there are variances in the format of the articles based on the requirements of each journal, which may affect the primary topics, subtopics, and referencing style.

Chapter 1: general introduction of the research which includes the background of the study, the rationale, research questions, aim and objectives.

Chapter 2: literature review, It is followed by three manuscripts (Chapters 3-5) which form the main body of the thesis.

Chapter 3: the published paper provides insight on the medicinal profile and phytopharmacological activities of *Portulacaria afra's* leaf, stem, and root using four extraction solvents with different polarities.

Chapter 4: the resubmitted manuscript provides insight on the influence of concurrent extreme temperatures with water-deficit on the medicinal profile, antimicrobial and antioxidant activities of *Portulacaria afra's* leaf, stem, and root extracts.

Chapter 5: the published paper is based on the effect of concurrent extreme temperatures with water deficit on the antidiabetic activity of *Portulacaria afra's* leaf, stem, and root extracts.

Chapter 6: covers the general discussion, which is followed by closing remarks and recommendations for the future. I performed this research under the supervision of my supervisor; she has been included as second author on paper(s) published submitted to journal

CHAPTER ONE

GENERAL INTRODUCTION

This chapter gives background information to this study and also states the rationale, aims and objectives of the study.

1. Background

Traditional medicine has historically used therapeutic herbs that date back to the Stone Age. To carry out numerous preventive functions, such as defence against pests, diseases, fungus, and herbivores, plants manufacture hundreds of chemical compounds (Ahn, 2017). Mankind also uses them as a type of medical antidote to treat illnesses and protect themselves from infections (Mbuni *et al*., 2020). Many individuals with medical conditions in developed countries utilize both modern treatment and traditional medicine. Traditional medicine is economical and readily available to people in the rural regions (Ozioma & Chinwe, 2019). Eighty percent of people worldwide, according to the World Health Organization (WHO), use traditional herbs for medicinal purposes.

Phytomedicine has been practiced for a very long time in Africa, especially among many tribes (Ozioma & Chinwe, 2019). African civilisation has benefited greatly from the enormous advancements made by medicinal plants scientist over many centuries and beyond. Due to the high demand for medical plants-derived items, the pharmaceutical sector has focused on medicinal plants to develop herbal items including cosmetics, health care formulations, and nutritional supplements. In addition to their health advantages, medicinal plants also have significant economic impact. Significant revenues from the trading of herbal materials are made in both international and local marketplaces (Varshney *et al*., 2021).

There are about 60 million people living in South Africa, and a big portion of them make use of herbal plants for therapeutic purposes. By treating both benign conditions such as the common cold, arthritis, dysmenorrhea, and stomach issues as well as malignant conditions including cancer, diabetes, and tuberculosis (TB), these plants have demonstrated their effectiveness and therapeutic properties (Shedoeva *et al*., 2019).

In nature, all plants are typically exposed to significant amount of stress. Abiotic and biotic factors are two categories for these stress factors. As explained by Sulmom *et al*. (2015), biotic factors include all kinds of life and how they interact with other organisms in their habitat, without the exemption of predators and prey. Human invasions, hostile microbes, and interactions between fauna and flora in a habitat are all examples of biotic components. The physico-chemical aspect of the ecosystem and the overall effect biologically on living organisms are referred to as the living and non-living components (Mohuiddin, 2019). Extreme temperature, salt, and drought are a few environmental conditions that have an impact on the growth and production of medicinal plants as well as the biosynthesis of secondary metabolites (Pant *et al*., 2021).

In South Africa, where temperatures have been rising for the past 40 years, the average frequency and temperature of heatwaves is currently (35°C), whereas that of cold waves is (0°C) (Mbokodo *et al*., 2020). By 2039, more frequent heat waves are expected, particularly in east and north of South Africa (Wright *et al*., 2021). According to forecasts, there will be an increase in drought and extremely hot temperatures, which will result in fewer cold front incidents. This might then have a negative effect on the growth, production, and especially the biosynthesis of secondary metabolites and phytochemical profile of medicinal plants in South Africa. Therefore, it is crucial to conduct this research with *Portulacaria afra* Jacq. given its indigenous status in South Africa and its documented therapeutic properties.

In this study, the impact of concurrent extreme temperatures and water deficit on the phytochemical and pharmacological activities within the leaf, stem, and root of *Portulacaria afra* Jacq was determined and assessed. The productivity and physiological responses of several succulent plants to different antagonistic abiotic factors have been studied (Pant *et al*., 2021),

however, all plant species do not respond to abiotic factors the same manner or possess the same coping mechanism.

Consequently, *P. afra* was exposed to concurrent extreme temperatures with water deficit settings. This was carried out to further explore the medicinal properties, response, and prospects of *P. afra* under these stresses. The results will consequently contribute towards better predictions of potential impacts on the secondary metabolites' accumulation, productivity, and pharmacological activities of succulent medicinal plants under climate change. Also, to enhance the accuracy of correlative distribution models.

1.2 Rationale

As previously stated, medicinal plants are extensively employed in therapeutic applications and function as primary sources for drug synthesis and formulation. The therapeutic efficacy of these plants is predominantly attributed to their secondary metabolites (Khanyile *et al*., 2021).

Variations in abiotic factors influence the synthesis and accumulation of secondary metabolites**.** The reproduction of medicinal plants is severely hampered by abiotic factors like temperature and drought/water-deficit, which affect the biosynthesis, production of secondary metabolites, and consequently causing irregularities in plant pharmacological activity (Soni *et al*., 2015). Therefore, studies on the phytochemical profiles and identification of secondary metabolites of medicinal plants such as *Portulacaria afra* and, more importantly, their responses to these various abiotic factors are needed to ensure their efficacy even under stress conditions of extreme climate conditions as predicted for South Africa in the next future. In addition, there is need to find out the influence of these abiotic factors on the medicinal values that may benefit the pharmaceutical industry's future role in drug production and public health.

Studies have investigated the effects of environmental factors and seasonal fluctuations on the physiological aspects including growth, development, reproductive processes, and secondary metabolite production of diverse medicinal plant species. Comprehensive scientific documentation on the simultaneous occurrence of extreme temperatures (high and low) with the influence of water deficit on the phytochemistry, antioxidant, antidiabetic, and antimicrobial activity in *P. afra*, using extracts from the leaf, stem, and root is completely lacking.

1.3 Novelty of research

To date, a comprehensive scientific investigation into the medicinal attributes of *Portulacaria afra* remains limited in literature. Hence, this current study aims to contribute novel insights on the phytochemical profile and biological activities of the species. This will be done by conducting an in-depth exploration of phytochemical profiles and associated biological activities in the leaf, stem, and root extracts, utilizing four solvents varying in polarity.

Moreover, limited investigations exist regarding the simultaneous impact of extreme temperatures and drought/water deficit on *Portulacaria afra*. This study was carried out to further explore the medicinal properties, response, and prospects of *P. afra* under these stresses. The results will consequently contribute wards better predictions of potential impacts on the secondary metabolites' accumulation, productivity and phytopharmacological activities of succulent medicinal plants under climate change. Also, to enhance the accuracy of correlative distribution models**.**

1.4 Aim and Objectives

1.4.1 Aim

The overarching aim of this study was to establish scientifically the phytochemical profile, medicinal properties, phytopharmacological attributes of *Portulacaria afra* as well as to assess the effects of concurrent extreme temperatures and water deficit on its biological activities.

1.4.2 Objectives

1. To determine the phytochemical profile on the leaf, stem, and root extracts of *P. afra* under normal and concurrent extreme temperatures and water deficit.
2. To determine the antimicrobial activity of *P. afra* exposed to concurrent extreme temperatures and water deficit against *Staphylococcus aureus*, *Streptomyces griseus,* and *Escherichia coli*.
3. To determine the antioxidant activity responses in the leaf, stem, and root extracts of *P. afra* under concurrent extreme temperatures and water deficit using three different assays.
4. To determine the antidiabetic response of *P. afra* exposed to concurrent extreme temperatures and water deficit using *in vitro* α-Amylase Inhibitory Activity method.

1.5 Research Questions

1. How will concurrent extreme temperatures and water deficit impact the phytochemical profile of *Portulacaria afra* plant parts?
2. Will concurrent extreme temperatures and water deficit improve the antimicrobial activity of *P. afra* plant parts?

3. Will concurrent extreme temperatures and water deficit improve the antioxidant activity of *P. afra* plant parts?
4. What impact will concurrent extreme temperatures and water deficit have on the antidiabetic properties in *P. afra* plant parts?
5. In relation to the expectations as noted in points 1-4 above, can any conclusions be drawn from the impact of the concurrent abiotic factors and the phytochemical accumulation and performance of the biological activities in the plant part samples?

1.6 Research Design and Approach

In July 2021, specimens of *Portulacaria afra* were gathered from the University of the Witwatersrand in Johannesburg (26° 10' 50.412" S and 28° 01' 46.236" E), East Campus (26.19092,28.03134) Gauteng, South Africa and were further grown from cuttings. The plants were identified by a botanist (IMR) and my supervisor. A voucher specimen was deposited in the institute's herbarium (IMR001).

Healthy cuttings with desirable characteristics, such as vibrant green foliage, absence of drooping, and sustained leaf coloration, were selectively obtained from a disease-free parent *P. afra* plant. Cuttings measuring 40–45 cm in length and 4 cm in diameter were collected using sterile secateurs and subsequently transplanted into the greenhouse for cultivation.

A total of 225 pots, each having a volumetric capacity ranging between 2 and 2.5 litres, were utilized for containing stem cuttings aimed at root formation. These pots were filled with professional cutting mix soil sourced from Culterrean Potting, chosen for its superior drainage, optimal moisture retention, and absence of pathogenic agents potentially detrimental to the initial rooting phase of the cuttings.

The cuttings were carefully inserted and pressed into position within the partially filled potting mix soil, and subsequently secured by additional potting mix soil.

The plants underwent a three-month acclimatization period within the University of Witwatersrand's greenhouse to facilitate adaptation, root system development, and the emergence of new leaves along their stems. Maintenance involved monitoring and administering 500 ml of water per pot every 48 hours due to their low water requirements.

Table1. 1 Showing the experimental treatments and harvest durations.

	Control	**Treatment A**	**Treatment B**	**Treatment C**	**Treatment D**
Water deficit	**500ml of water every 2nd day.**	**No water**	**No water**	**No water**	**No water**
Temperatures (˚C night/day)	**25/27**	**0/5**	**10/15**	**30/40**	**35/45**
Episodic Harvest frequency (hrs)	**Once off**	**48, 96, 144**	**48, 96, 144**	**48, 96, 144**	**48, 96, 144**

Sample size per harvest: n= 15/harvest

The conviron simulations settings used for the treatments were deduced from the predictions made by the South African Department of Environmental Affairs (2019), IPCC (2018), and Mbokodo *et al* (2020) records about alterations in the current and future climatic conditions. The treatments were selected in terms of temperature ranges and the span of heat and cold waves. The varying temperature settings used are as follows: control (25/27ºC), (0/5ºC), (10/15ºC), (30/40ºC), and (35/45ºC). Each plant was placed in the controlled climate simulation chamber (Conviron chambers, PGW40) with the high and low temperatures set concurrently with water deficit while other parameters like CO_2 (400 ppm), humidity (60%), light (160

nits/level 1) pH, salinity, were kept at ambient. The lights were set to come on for 12 hours per day (6 am to 6pm). Control temperature ranges were based on records from the South African Weather Service (2020).

The plants were exposed to treatments and not watered for up to 144 hours (6 days), and the control samples were placed under 25ºC (ambient) and watered every second day with 500ml of water. The controls were kept at 25°C maximum night-time temperature (7pm to 5am) and 27°C maximum day time temperature (5am to 7pm) based on South African weather service 2020 records. Plants undergoing treatment (A, B, C, D) received no water. For treatments A and B: samples were exposed to cold temperatures of 0 and 10ºC maximum night-time temperature and 5 and 15ºC maximum daytime temperature. For the C and D treatments: samples were also exposed to hot temperatures of 30 and 40°C maximum night-time and 35 and 45°C maximum daytime.

Five plants were harvested for all plant parts episodically 3 times for up to 6 days (144hrs), where plants were harvested every 48hrs (48, 96, 144) (Table 2. 3). Harvested samples were oven airdried under 40°C for 2 to 3 days (Poorter *et al*., 2012). This procedure was repeated three times for each of the treatments. Where, n=45 control (25°), n=45 for (0/5°C), n=45 (10/15°C), n=45 (30/40°C), n=45 (35/45°C) with a total sample size of 225.

1.6.1 Choice of experimental design

With case studies and experimental investigations, this study combined qualitative and quantitative research approaches. The crude plant extracts of *P. afra* leaf, stem, and root were obtained via extraction with the following solvents: 80% methanol, *n*-hexane, ethyl acetate and 100% distilled water at temperature (60°C). The extraction process with the solvents was done at 48-hour intervals (48, 96, and 144 hours) to identify any potential variations in the various

groups of phytochemicals under concurrent extreme temperatures and water deficit, as well as to potentially suggest the best extraction solvent.

The techniques were also applied in the assessment and comparison of *P. afra*'s phytochemical constituents and therapeutic properties (antimicrobial, antioxidant, antidiabetic) in the leaf, stem, and root extracts of the solvents, at both high and low temperatures with water deficit. Literature has shown that various compounds will dissolve in different solvents based on their degree of polarity or polarity index (Tables 1 and 2). As a result, fractionation was selected as an appropriate method for this experimental design.

Table1. 2 Showing the polarity index of the selected solvents

Solvent	**Polarity index**
Aqueous (water)	10.2
Methanol	5.1
Hexane	0.1
Ethyl acetate	4.4

Source: Abubakar and Haque, (2020).

1.6.2 Rationale for case studies and experimental studies approach

Primary data are obtained through experimental investigations and case studies using both qualitative and quantitative research methods (Kumatongo & Muzata, 2021). The techniques were chosen because they give room for conditions that could be too difficult to notice over time, such as climate change effects.

The exposure of *Portulacaria afra* to concurrent extreme low and high temperatures with water deficit can be simulated, allowing for the observation of potential changes in the plant's phytochemicals, antimicrobial, antioxidant, and antidiabetic activities. All the objectives were accomplished by exposing the species' samples to concurrent extreme low and high

temperatures and water deficit for up to six days and analysing the therapeutic characteristics of the species.

The chosen approach allowed for the results to be substantiated through replicability, allowing for the detection of any flaws. The experiments were repeated three times since they could produce statistically significant results (Creswell, 2013). The benefit of using both qualitative and quantitative research methods in conjunction with case and experimental studies is that the data can be statistically analysed to provide a thorough understanding of the research statement and enable extrapolations (Creswell, 2014). Throughout the experiment, the findings were compared to the control samples using statistical analysis on the objectives. It is crucial that the experimental research design be valid and reliable ((Kumatongo & Muzata, 2021). Validity is the suitability of the experimental investigation, while reliability is the ability of the experiment to remain consistent. This study's investigations, measurements, analytical tests, and instruments were consistent throughout, demonstrating reliability. A pilot study on each objective was done to increase the research's validity and eliminate experimental bias. The pilot research eliminated the possibility of making experimental adjustments while the experiment was still running. It also allowed for mastery of the instruments.

The use of case studies in conjunction with experimental studies has the advantage of focusing on a single species and creating conditions that are not quick to observe, such as climate change related studies. The results were not used as a generalization for combined temperature and water deficit stress on plants because plant responses are species dependent. Furthermore, the quantitative data from the study were subjected to statistical analysis where the concentration values were graphed against the % inhibition values using Microsoft Excel to derive a trendline equation necessary for calculating the IC50. A one-way analysis of variance (ANOVA) was used to assess significant variations. All experiments were conducted in triplicate, and results

were presented as mean ± standard error (SE) when n = 3. Statistical significance was considered at $P < 0.05$. Tukey (HSD) test in RStudio was used to ascertain specific significant differences among groups.

The objectives of this study were achieved, addressing the research problem with emphasis on the novelty.

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CHAPTER TWO

Literature review

This chapter contains a detailed review on the phytochemistry, biological activities of *Portulacaria afra* Jacq. as well as aspects of medicinal plants and climate change.

2. Botanical overview of *Portulacaria afra*

Within the classification system and taxonomically, *Portulacaria afra* is categorized under the Family: *Portulacaceae* or more recently *Didiereaceae*, Genus: *Portulacaria*, Species: *afra*. The genus *Portulacaria* encompasses a total of 7 species, namely *P. armiana, P. carrissoana, P. fruticulosa, P. longipedunculata, P. namaquensis, P. pygmaea*, with *P. afra* and *P. namaquensis* being the sole native tree species found in South Africa among these (Bruyns *et al*., 2014).

It is an evergreen succulent widely utilized all over the world and native to South Africa, Kenya, and Mozambique. It goes by several names, including spekboom and elephant bush in English. This plant is effective for carbon sequestration, while in arid and desertic zones, it is helpful for landfill projects (Panter & Ruwanza, 2019).

2.1 Physical characteristics

Portulacaria afra Jacq. classified as an evergreen, soft-wooded shrub, exhibits succulent, glossy, broadly ovate leaves (Figure 2.1), typically measuring 1-2.5 cm in length (Mahlanza *et al*., 2023). This densely branched shrub attains a height ranging from 2-4 m, adorned with pink, small star-shaped flowers (Figure 2.1) serving as a significant nectar source for numerous insects, thereby enhancing biodiversity through the attraction of insectivorous birds (Mahlanza *et al*., 2023). It bears resemblance to the Jade plant (*Crassula ovata*) from the Crassulaceae family, often co-planted; however, its erect upper branches and less firm, dissimilar succulent leaves distinguish it from the Jade plant. In contrast to *Crassula ovata,* which blooms in the winter from April to August, it typically has crimson stems that stand out against the green leaves and blooms from September to November (Panter & Ruwanza, 2019).

Figure 2. 1 *Portulacaria afra* leaves, stems, and flowers

Source: Beckings (2016).

2.2 Distribution

P. afra exhibits a wide distribution across various regions within South Africa (Becker, 2013). Its optimal growth occurs in warm climatic conditions, and it is notably present in geographical areas spanning from the Eastern Cape of South Africa to KwaZulu-Natal, Mpumalanga, and Limpopo Province. Particularly, its prevalence is prominent in Sekhukhuneland, situated in the southern part of Limpopo Province (Becker, 2013).

It grows on rocky hillsides up to 1400 m in the succulent karoo scrub, thicket, bushveld, and dry river valleys (Figure 2. 2). Aside from South Africa, it also flourishes in Namibia,

Swaziland, Kenya, Mozambique, Madagascar, and East Africa (Arnold *et al*., 1993)

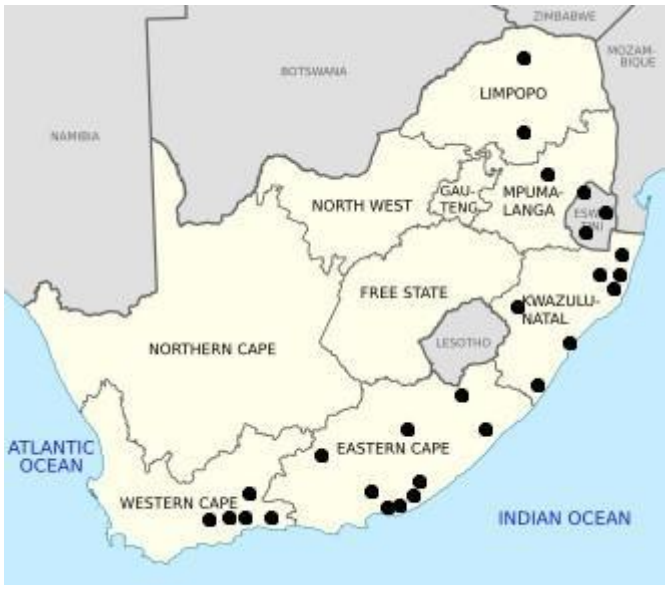

Figure 2. 2 South African map showing *Portulacaria afra's* geographical locations https://en.wikipedia.org/wiki/File:Map_of_South_Africa_with_English_labels.svg

2.3 Ethnobotany and therapeutic uses of *Portulacaria afra*

Portulacaria afra leaves are eaten raw and are renowned for having a sour flavour, especially if collected before dawn. As a result of the dissolved carbon dioxide that was overnight gathered by the Crassulacean acid metabolism (CAM) process, the flavour is slightly acidic; however, as the carbon dioxide is consumed by photosynthesis throughout the day, it reduces the acidity of the taste (Prisa, 2020). The leaves can be used in many ways, including making salads as well as alleviating thirst by sucking on them. This helps to prevent weariness, dehydration, and heat stroke. Ingesting the leaves can also cure mouth and throat infections. The sap is used for curing skin disorders such as acne, rashes, and insect bites, while the crushed forms are used on the foot to cure corns and blisters (Table 2. 1). Rural populations with little or no access to urban healthcare frequently adopt it as a remedy for epidermal infections (De wet *et al*., 2013).

The dried-powder forms are applied as snuffs, while the sap can be used to heal sunburn. Thatched roofs are constructed with the branch in the Eastern Cape region (Roberts, 1990). Livestock including cattle, goats, wild African elephants, kudus, and black rhinoceroses all eat the leaves. It is a beneficial honey plant that is cultivated all over the world as horticultural crop to help stop soil erosion. Additionally, it makes a wonderful bonsai model plant and an excellent source of paper pulp (Roberts, 1990).

Furthermore, the South African government intends to restore a million hectares of *P. afra* thicket in partnership with the ecosystem programme. Panter and Ruwanza (2019) believe that native species of plants that have been removed from their natural habitat, can be restored by cultivating with *P. afra*. This enhances the establishment of young plants that were displaced from their environment owing to overgrazing and desertification by providing protection and biological material for sandy soil (du Toit *et al*., 2023).

Table 2. 1 Therapeutic uses of *P. afra*

Parts used	**Treatment**	**References**
Leaf juice	Rashes, bug bites, several skin disorders are treated with it.	De Wet *et al*. (2013)
Chewed leaf	It helps to remedy oral infections and strep throat.	De Wet *et al*. (2013)
Leaf	On the foot, it helps to cure blisters and corns.	Shearing (1994) and Venter and Venter (2002**).**
Leaf juice	It is applied to soothe sunburn.	Roberts (1990)
Leaf	Chronic wounds and dermatitis are both cured with it.	Dlova and Ollengo (2018).

2.4 Phytochemical constituents

In response to challenges posed by several biotic and abiotic factors, plants synthesize a diverse array of compounds termed phytochemicals through primary and secondary metabolic pathways, contributing significantly to their defence mechanisms (Saboon *et al*., 2019). They are found in plant-based foods such as nuts, seeds, whole grains, beans, fruits, and vegetables. Since they are consumed to prevent major illnesses and heart disease conditions, plant-based natural foods with potent phytochemical qualities have been reported to have significant health benefits (Saboon *et al*., 2019).

The primary and secondary metabolites are the two major classes used to classify the natural substances produced by plants (Hussein & El-Anssary, 2019). Although secondary metabolites are not essential for the direct plant growth, development, and reproduction, they mediate hostile ecological interactions existing between plant species and surrounding habitat. Due to their therapeutic properties, they also help in the protection of plants from pathogens (Bhatla & Lal, 2023). Based on their chemical makeup, they fall in four primary groups: terpenes, phenylpropanoids (phenolics), polyketides, and alkaloids. Examples include phytoestrogens, terpenoids, carotenoids, limonoids, flavonoids, isoflavonoids, anthocyanidins, phytosterols, glucosinolates and polyphenols.

Phytochemicals are divided into 6 major classes based on their chemical structures and characteristics (Figure 2. 3). According to Campos-Vega and Oomah (2013), these classifications include carbohydrates, lipids, phenolics, terpenoids, alkaloids, and chemicals containing nitrogen.

Primary metabolites are distinct from secondary metabolites. They are classified as major metabolites that primarily contribute to the maintenance of common physiological processes

that are intrinsic functions. Lactic acid, certain amino acids, and alcohols such as ethanol are examples of primary metabolites (Hussein & El-Anssary, 2019).

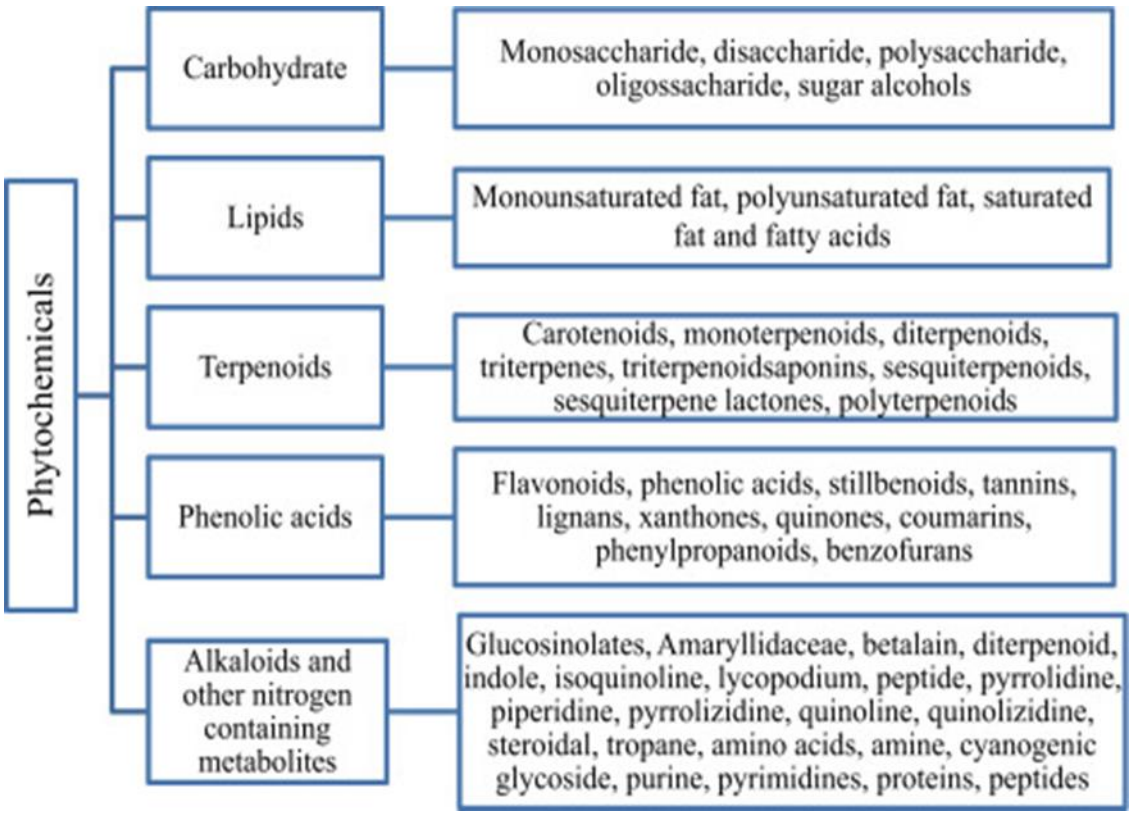

Figure 2. 3 The classes of Phytochemicals

Source: Campos-Oomah (2013).

There is relatively little scientific information available on *P. afra's* plant parts and phytochemical composition across the parts. Most of the research has focused on physiological elements such as leaves and sap that are used to cure oral and skin infections (De Wet *et al*., 2013). The following are some of the secondary metabolites examined in this study due to the common biosynthetic pathways that they demonstrate on overlapping functionalities.

1. Coumarins: The name ‘coumarin’ originated from ‘coumarou’ (Dighe *et al*., 2010). They are members of the Benzopyrone family which consists of simple coumarins, triscoumarins, furanocoumarins, biscoumarins, coumarinoligans and pyranocoumarins (Sharifi-Rad *et al*., 2021). Coumarins exhibit broad distribution within vascular tissues

of plant organisms and are biosynthesized via phenylpropanoid pathway (Küpeli Akkol *et al*., 2020). As free radicals' scavengers, they are essential for plants' adjustment to their environment (Robe *et al*., 2021).

2. Volatile organic compounds (essential oils; VOCs): Volatile organic molecules are defined by their low boiling point, high vapour pressure at room temperature, and small molecular weight (less than 300 Da) (Dhifi *et al.*, 2016). The methylerythritol phosphate pathway (MEP) and mevalonate pathway (MEV) pathways additionally can generate VOCs (Dhifi *et al*., 2016). All aromatic plant parts, including the flowers, leaves, seeds, barks, wood, and rhizomes, contain these chemicals (Lange *et al*., 2000). VOCs are essential for maintaining hormonal balance and signalling in organisms (Santoro *et al*., 2011). Furthermore, they operate as allelopathic agents, cytotoxicants, cancer chemoprotectants, antioxidants, anti-inflammatory agents, repellents, and insecticides (Santoro *et al*., 2011).

3. Tannins: Barks, leaves, fruits, wood, and roots of plants contain phenolic chemicals called tannins (Tong *et al*., 2022). Tannins can combine with proteins to create complexes and are present in a wide variety of plant species, including both flowering and non-flowering species (Pizzi, 2021). Tannins can be divided into two primary categories: condensed (which consists of flavonoid units) and hydrolysed (which is made up of phenolic and carbohydrate groups (Pizzi, 2021). In addition, they have several therapeutic properties like antioxidant, anti-inflammatory, antidiarrheal, antibacterial, antiparasitic, and antiviral (Tong *et al.*, 2022).

4. Terpenoids: With about 15 000 structures identified, terpenoids make up a significant subclass of secondary metabolites (Tholl, 2015). Terpenoids are found mostly in vegetative structures, such as leaves, roots, and flowers, and are also common in higher

plants (Yang *et al*., 2020). Paclitaxes, artemisinin, and ginsenoside are some of the varieties of terpenoids (Yang *et al*., 2020). With defence structures like thorns and trichomes containing terpenoids, plants use terpenoids to interact with other plants, insects, and pathogens (Booth *et al*., 2017). They are biosynthesized in the cytosol through the mevalonate pathway (MEV) and in the plastidium via methylerythritol phosphate pathway (MEP) (Cheng *et al*., 2007). Terpenoids have anti-inflammatory, antibacterial, anxiolytic, and sedative characteristics (Yang *et al*., 2020).

5. Saponins: As reported by Hussein and El-Anssary (2019), saponins are steroidal triterpene molecules that are extremely amphipathic. They are referred to as saponins because of their close resemblance to soap (Hussein & El-Anssary, 2019). They play a role in signal transduction, serve as a plant's defence against herbivores, and are spread across various plant tissues (Thimmappa *et al*., 2014). In addition, they perform several biological activities like anticancer, anti-inflammatory, antibacterial, anti-parasitic, and antifungal (Thimmappa *et al*., 2014).

6. Flavonoids: Comprising more than 8000 metabolites, flavonoids constitute one of the biggest classes of polyphenols and are present in every component of plants (Tohge *et al*., 2017). According to Liu *et al*. (2021), flavonoids are classified as flavonols, proanthocyanidins, anthocyanidins, flavones, chalcones, isoflavones, and flavanones. They are plant pigments and are biosynthesized through the phenylpropanoid pathway (Tohge *et al*., 2017). Flavonoids play a role in phototropic responses, pollen development, auxin, and iron (Fe) transport, signalling during nodulation, and defence against disease-causing organisms, ultraviolet (UV) radiation, high carbon, low nitrogen, herbivores, and cold stress (Liu *et al*., 2021).

7. Steroids: Plant steroids act as parts of membranes. They often are biosynthesized in the mevalonate pathway in the same manner as cardiac glycosides and are made from triterpenes similar to squalene. Their therapeutic properties include anti-inflammatory, anti-tumour, anti-fungal, and antiparasitic (Hussein & El-Anssary, 2019). Most plant tissue cells composed of steroids exhibit these therapeutic actions**,** especially those containing nitrogenous steroids (Valitova *et al*., 2016). Plant steroids significantly contributes to plant growth by promoting cell division and elongation, reproductive development, vascular differentiation, and changes in gene expression (Hussein & El-Anssary, 2019). They also help plants survive biotic and abiotic stress conditions.

8. Glycosides: Cardiac glycosides are triterpenes that undergo a minimal loss of three methyl groups during production in the mevalonic pathway (Bartnik & Facey, 2024). Generally, they are often present in the bark, seeds, flowers, leaves, and roots (Tomilova *et al*., 2022). Although the function of cardiac glycosides in plants is still unknown, it has been shown to have an impact on the central nervous system, smooth muscles, and cardiac muscles (Ainembabazi *et al*., 2022). Consequently, these compounds are present in many cardiotonic drugs.

9. Carbohydrates: Carbohydrates consist of a variety of sugars, including monosaccharides and disaccharides, polymers like cellulose and starch, and sugar alcohols, which are essential substances (Hussein & El-Anssary, 2019). In all living things, including plants, carbohydrates play a critical role in the production of metabolic energy. Glycogenesis, gluconeogenesis, and the Calvin-cycle triose-phosphate are the three pathways that generate carbohydrate during photosynthesis (Arnao *et al*., 2021). Furthermore, carbohydrates play a variety of roles in plant growth and development, photorespiration, seed germination, plant hormonal balance, ROS scavenging, root

formation, and osmoregulation. They act as precursors of a wide range of secondary metabolites, including simple phenols, carotenoids, flavonoids, and various terpenoids (Hussein & El-Anssary, 2019).

10. Amino acids: Amino acids and proteins, called osmolytes, are within the tissues and cellular structures of plants (Hou *et al*., 2019). The most prevalent kinds in plants include isoleucine, leucine, phenylalanine, methionine, valine, histidine, arginine, tryptophan, lysine, alanine, cysteine, proline, glutamine, asparagine, aspartic acid, serine, glycine, tyrosine, and threonine (Concato *et al.*, 2022). In plants, amino acids (which are the building blocks of proteins) serve a variety of purposes, like functioning as substitute substrates for respiration when there is a decrease in carbon dioxide (CO_2) uptake by the stomata (Batista-Silva *et al*., 2019). They operate as precursors for secondary metabolites, plant hormones, intracellular pH regulators, signalling molecules, metabolic energy production during biotic and abiotic stress, and plant growth and development (Hou *et al*., 2019). Proteins and amino acids break down under adverse conditions to produce a surge of chemicals that are employed in a variety of biological processes and physiological defences (Hussein & El-Anssary, 2019).

2.5 Antioxidants

Antioxidants are naturally occurring or synthetically derived compounds that exhibit the capacity to retard the progression of oxidative reactions. They are molecules capable of giving a free radical an electron without becoming unstable. They stabilize free radicals and reduce their reactivity (Zeb, 2020). Furthermore, these compounds exhibit the capacity to counteract free radicals and mitigate the effects of unstable chemicals, thereby preventing cellular damage.

As stated by Parcheta (2021), flavonoids and other phenolic components are the chemical compounds that produce the antioxidant impact, and their antioxidant activity is an outcome of

their redox properties. Free radicals are absorbed and neutralized by these redox properties, it destroys decaying peroxides as well as singlet and triplet oxygen.

Researchers have conducted extensive research on the antioxidant components found in plant-based meals and materials. Due to their therapeutic characteristics in the avoidance and treatment of cancer and chronic heart illnesses brought on by oxidative stress, they have attracted increased recognition from end users and food production firms (Neha *et al*., 2019). These compounds further contribute to the enhancement of both the nutritional content and overall quality of food (Neha *et al*., 2019). Since they do not have side effects like synthetic antioxidants, natural antioxidants are the most advantageous for usage or intake. Food, fruits, vegetables, whole foods, and other plant-based food, all contain natural antioxidants. Beneficial antioxidants include several vitamins, including vitamins E and C, selenium, and carotenoids including beta-carotene, lycopene, lutein, and zeaxanthin (Neha *et al*., 2019). Fundamentally, secondary metabolites slow down or stop oxidative stress, which impacts on the antioxidant activity (Pisoschi *et al*., 2021). There are, however, only a few scientific records of this up to date.

It is essential for plants to maintain redox homeostasis because excessive tissue oxidation is harmful to the plant's ability to function effectively. Plants' sessile nature subjects them to a variety of environmental factors that potentially impede their growth and developmental processes (Santoyo, 2022). Enzymatic and non-enzymatic antioxidant defence systems are developed by plants for adaptation, for them to thrive and reproduce in their dynamic environment (Dumanovi *et al*., 2021). The non-enzymatic system contains low molecular weight antioxidants like glutathione, ascorbic acid (AsA), carotenoids, proline, flavonoids, phenolic acids, and high molecular weight secondary metabolites such as tannins. The enzymatic system consists of antioxidant enzymes like glutathione (GSH), guaiacol peroxidase (GPX), superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT)

(Pisoschi *et al*., 2021). As a defence mechanism against biotic and abiotic factors and for neutralization of excessive generation of ROS, both defence systems are essential for plants (Neha *et al*., 2019)

2.5.1 Non-enzymatic defence system

Plant secondary metabolites (SM), also referred to as specialised metabolites, serve as mediators between plants and the antagonistic ecological factors in the environment to increase their viability and fecundity (Hussein & El-Anssary, 2019). According to Yeshi *et al*. (2022), these various classes of chemicals are produced due to the influence of different variables, including a variety of plants, environmental factors, and wild or farmed plants. SM have a wide range of advantageous biological properties, including potent antioxidant, anticancer, and anti-inflammatory properties, etc. The investigation of their biosynthetic processes, enzymes, and biological and ecological activities has drawn a lot of research attention (Khare *et al*., 2020).

Secondary Metabolites (SM) from research findings, are now known for their triple action in plants. SM are involved in growth and development, communication, and regulation, they also function as primary metabolites (promoting basic plant growth with the supply of the resources needed for key processes like photosynthesis), as well as hormones in plants (Pang *et al*., 2021).

2.5.2 Enzymatic defence system

Superoxide dismutase (SOD) is essential for scavenging hydroxyl radicals and preserving healthy physiology, and as such, it is a vital enzyme in the mitigation of ROS-induced oxidative damage (Pisoschi *et al*., 2021). The primary redox buffers are ascorbic acid (AsA) and glutathione (GSH), which are transformed to MDHA (monodehydroascorbate), DHA (dehydroascorbate), and GSSG (glutathione disulfide) (Dumanovi *et al.*, 2021). Due to their overlapping actions, a vast majority of these antioxidant systems can work to make up for one

another (Dumanovi *et al*., 2021). O- is changed into hydrogen peroxide (H_2O_2) by SOD, while ascorbate peroxidase (APX) and catalase (CAT) then detoxify it (Irato & Santovito, 2021). The mitochondria, chloroplast, cytoplasm, peroxisomes, endoplasmic reticulum (ER), and glyoxysomes are among the tissues where the isoforms (variants) of APX are concentrated (Dumanovi *et al*., 2021). CAT is in peroxisomes, where it detoxifies H_2O_2 into oxygen (O_2) and water (H_2O) (Ahmad *et al*., 2009).

2.6 Extraction solvents

For efficient and precise extraction of diverse bioactive compounds, literature suggests the use of a variety of solvents with varying polarity (Truong *et al*., 2019). The degree of polarity of the desired solute should be considered when choosing a solvent since a solvent with a close polarity will dissolve the solute more accurately (Lefebvre *et al*., 2021). The polarity of a few frequently used solvents is listed below, from least polar to most polar: hexane, chloroform, ethyl acetate, acetone, methanol, and water (Lefebvre *et al*., 2021). To lower the proportion of similar compounds in the yield, various solvents will be applied in succession (Table 2. 2).

Table 2. 2 Some solvents used for phytochemical extraction

Polarity	Solvents	Chemicals extracted
Low	*n*-hexane	Waxes fats fixed oils volatile oils
Medium	Ethyl acetate	Alkaloids aglycones glycosides
	Methanol	Sugars amino acids glycosides
High	Water	Sugars amino acids glycosides

Source: Houghton & Raman (1998)

2.7 Biological activities in *P. afra* examined in this study

2.7.1 Microorganisms

Microorganisms, commonly referred to as microbes, are microscopic living things present in virtually every setting (Horve *et al*., 2020). Microbes are ubiquitous because they can survive and even flourish under harsh conditions. They are the most diverse class of organisms on earth and encompass a wide range of species including bacteria, protists, viruses, archaea, fungus, and prions. The presence of pathogens and the increasing number of drug-resistant strains have increased the need for bacterial research over time (Mancuso *et al*., 2021). Drug-resistant bacteria has become a menace to human health and endangers the progress of modern medicine. Although some bacteria, such as *Staphylococcus aureus*, *Streptomyces albulus*, *Escherichia coli*, *Listeria*, and *Salmonella*, are potentially dangerous to people. While many other bacteria, including *Bifidobacterium* and *Lactobacillus*, can be of use (Schuchat *et al*., 1997). The Agar or broth dilution method, antimicrobial gradient method (Etest), and the disk-diffusion method have all been devised to test if plant extracts have antibacterial potential (Eloff, 2019). *Portulacaria afra* has shown to have antibacterial potential in some research, the leaf extracts were used in studies by Khanyile *et al*. (2021) to ascertain the inhibitory efficacy against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The outcomes indicated a negligible

antimicrobial activity with the tested microorganisms. Additionally, earlier research by De Wet *et al*. (2013) noted *P. afra's* antibacterial efficacy against *S. aureus* and *P. aeruginosa.*

Gram-negative and Gram-positive bacteria are divided into two groups by their cell envelopes, which are protective, complex layered structures that are selectively permeable to enable the flow of nutrients into and out of an organism (Fischetti *et al*., 2019). Gram-negative (GM-) bacteria do not appear as crystal violet when stained with gram stain because they have a thin peptidoglycan cell wall that is encased in an outer lipopolysaccharide membrane (Fischetti *et al*., 2019). *Chlamydia trachomatis*, *Escherichia coli*, and *Pseudomonas aeruginosa* are examples of Gram-negative bacteria. Gram-positive (GM+) bacteria have surface receptors and can hold the violet stain because they have a thick peptidoglycan cell wall and no outer membrane (Fischetti *et al*., 2019). *Staphylococcus aureus*, *Streptomyces albulus*, *Hay bacillus*, and *Listeria monocytogenes* are examples of gram-positive bacteria. Over the past century, several bacterial strains are found to be crucial in the production of antibiotics and new pharmaceuticals. Bacterial strains are gathered, cultured, and used as a benchmark for comparison (Mortier *et al*., 2021). They are typically identified by their initials followed by a number, such as ATCC (American Type Culture Collection) or NCTC (National Collection of Type Cultures). Even when strains are from a single species, they often exhibit varying degrees of pathogenicity, allowing different antibacterial assays to be performed quickly, which saves time and resources (Mortier *et al*., 2021). In this study, the antibacterial potential of plant extracts was evaluated against gram+ *Staphylococcus aureus* (ATCC 25923), gram- *Escherichia coli* (ATCC 25922), and gram+ *Streptomyces griseus.*

Staphylococcus aureus

Staphylococcus aureus is a gram-positive bacterium that can grow at temperatures between 18° C and 40° C under aerobic or anaerobic conditions (Ouedrhiri *et al*., 2016). *S. aureus* is commonly found on mucous membranes and skin surfaces. It causes many infections in humans, including skin and soft tissue infections (cellulitis, anthrax), infective endocarditis, and urinary tract infections. urology, meningitis, and gastroenteritis toxic shock syndrome (Ouedrhiri *et al*., 2016).

Streptomyces griseus

Streptomyces griseus is a common strain of bacteria in the soil. It belongs to the genus *Streptomyces*. Deep-water sediments also contain a few strains (Arslan, 2022). The bacterium is gram-positive and has a high guanine-cytosine content. Like most other *streptomycetes*, strains of *S. griseus* are well-known producers of antibiotics and other commercially important secondary metabolites (Zaid *et al*., 2021). Thirty-two structurally distinct types of bioactive compounds are produced by these strains. *S. griseus* strains are the source of streptomycin, the first antibiotic ever identified in a bacterium. The ability of these strains to produce streptomycin, a substance that has shown significant bactericidal activity against organisms like *Yersinia pestis* (the plague-pathogen) and *Mycobacterium tuberculosis* that causes tuberculosis, has sparked interest in them (Arslan, 2022).

Escherichia coli

E. coli is a sizable (and frequently unharmful) group of bacteria that inhabits both human and animal intestines and is crucial to normal intestinal function. Many *E. coli* strains are not harmful to humans, but some of them can result in serious conditions like respiratory disease, diarrheal, and urinary tract infections (Ali *et al*., 2020).

2.7.2 Diabetes mellitus (DM)

Diabetes mellitus (DM) comprises a spectrum of health conditions characterized with the side effects and symptoms of insulin deficiency (Salehi *et al*., 2019). As indicated in World Health Organization (WHO, 2004) data, there are a total of 177 million people with diabetes worldwide. Global diabetes prevalence and incidence are projected to increase from 8.6% in 2012 to 9.8% in 2030, a remarkable rise from 285 million to 435 million people. Among nations, India stands out as having the highest diabetic population. While According to data from Statistics South Africa, non-communicable diseases accounted for 58% of the country's increase in mortality rates between 1997 and 2018. Of these, 12% of adult patients had diabetes, making the condition the second leading cause of death in South Africa. These predicted increases in global diabetes prevalence represent a significant burden on the health system.

Type 2 diabetes is a disease resulting insulin deficiency caused by insulin resistance present in responsive cells combined with pancreatic beta-cell dysfunction. It occurs when there is an enormous build-up of fat in the body (Zhang, *et al*., 2021). Obesity occurs when adipose tissue largely accumulates, liberating free fatty acids and adipokines in excess which then triggers inflammatory signalling pathways (Zhang, *et al*., 2021). Research findings indicate that oxidative stress induced by excessive free radical build-up and destruction of endogenous antioxidant defences associated with diabetes occurs due to hyperglycemia (Pisoschi *et al*., 2021). A study by Olaokun *et al.* (2017) on *Curtisia dentata*, *Portulacaria afra,* and *Pittosporum viridiflorum* plant species for antidiabetic activity and additional phytopharmacological activities on acetone leaf extracts, demonstrated the potential therapeutic properties of the plant parts for curing lethal diseases.

Due to the life-threatening impacts of orthodox medicine and gastrointestinal disturbances associated with some of these medications such as acarbose, numerous studies have been

undertaken to investigate the therapeutic potential of organic plant materials in the treatment of diabetes (Thrikawala *et al*., 2018). According to Thrikawala *et al*. (2018), *Flueggea leucopyrus* Willd (FLAE) is just as potent as antidiabetic medications because of its inhibitory effect against α-amylase, which turns starch into glucose. Advanced glycation end products (AGEs) generation is also prevented. However, there is no scientific evidence on how concurrent abiotic factors such as extreme temperatures and water deficit affect the phytochemical profile and antidiabetic activity of *Portulacaria afra*. Therefore, there is a need for evaluation of the impact of these abiotic factors on the phytopharmacological attributes exhibited by *P. afra* plant parts.

2.8 Temperature and drought effects

2.8.1 Temperature stress

One of the main abiotic factors influencing plant development and growth is temperature. A rise in temperature leads to heat stress, and a decrease in temperature causes cold stress. According to Marx *et al*. (2021), a heatwave is an interval during which three consecutive calendar days have temperatures that are higher than the 90th percentile. It is an adverse two-day weather pattern which both the daytime heat index and night-time temperature decline, and never fall below the National Weather Service (NWS) heat stress limits of 27 °C and 41 °C (Van der Walt *et al*., 2012). In addition, it is a period of severe rise in temperatures for an unspecified duration (Mbokodo *et al*., 2020). In contrast, cold waves are experienced usually when air temperature constantly decreases, exceeding the minimum limit consecutively for a 48hour-period (Van der walt *et al*., 2021).

A heat wave according to the South Africa Weather Service (SAW) is a severe weather event with temperatures greater than 5°C above the average daily maximum temperature in the hottest month and lasting no less than 72 hours (Figure 2. 4-2. 5) (Mbokodo *et al*., 2020).

Temperatures in South Africa are expected to rise prior to the wind up of the 21st century (Department of Environmental Affairs, 2017). The Eastern Cape recently experienced record-high average winter temperatures in June, July, and August, with average daily temperatures exceeding 20.9°C (as of 2019). Additionally, in September 2019 there was a record of severe high temperatures exceeding 35 °C in Cape Town (Van der Walt & Fitchett, 2021). Therefore, South Africa's average annual temperature has increased by at least 1.5 times the world's average over the past 50 years, an estimated 0.65 °C (Mbokodo *et al*., 2020).

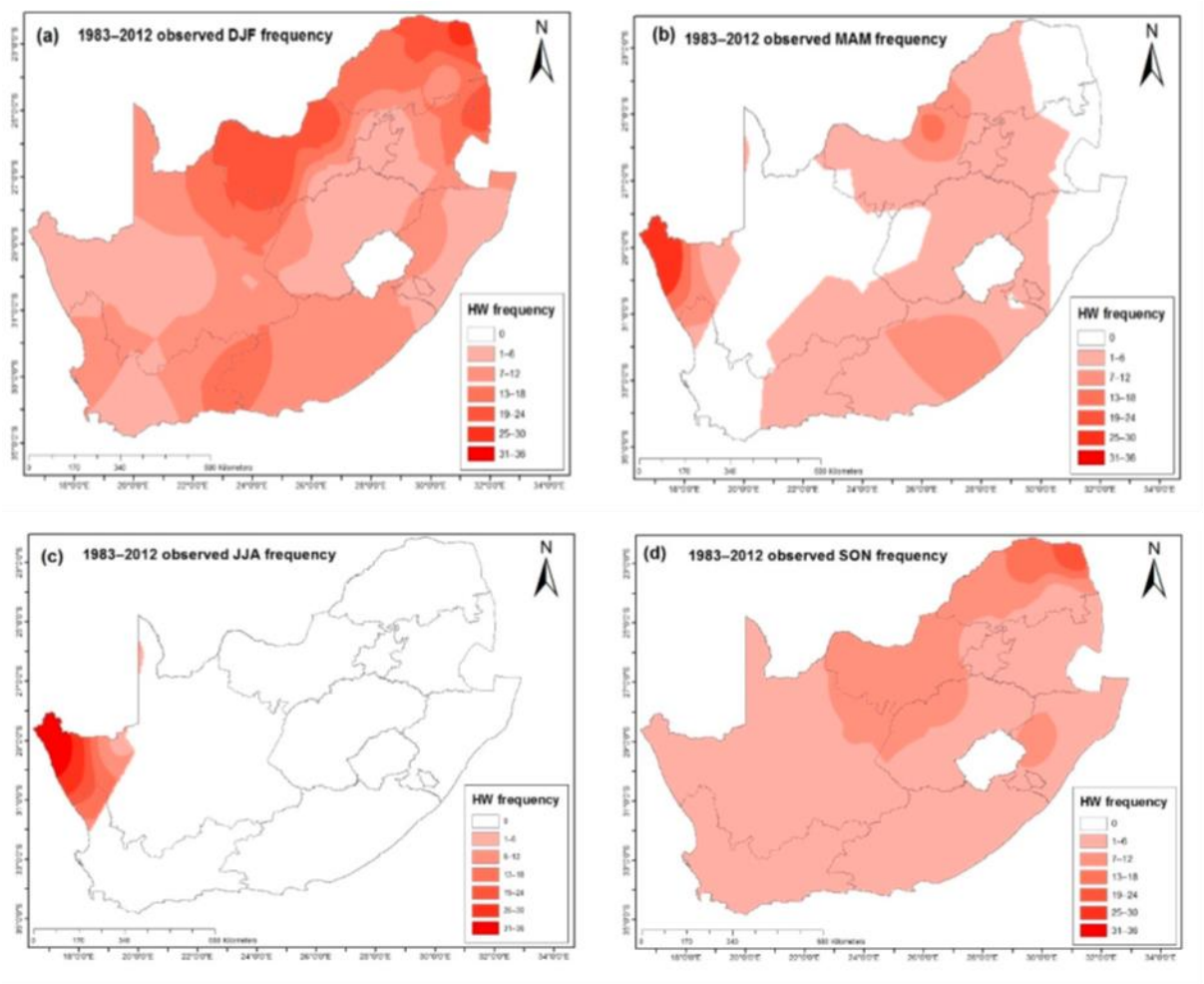

Figure 2. 4 Frequencies of heat waves in South Africa from 1983-2012

Source: Mbokodo *et al*. (2020).

Severe increase in temperatures is significantly associated with the biosybthesis and make-up of secondary metabolites in plants (Verma & Shukla, 2015; Pradhan *et al*., 2017). High

temperature improves secondary metabolites in plant species. According to Zheng *et al.* (2012), stress from high temperatures triggers further production of secondary metabolites, while findings from earlier research showed that plants produced fewer secondary metabolites when exposed to high temperature stress (Christie *et al*., 1994).

Reactive oxygen species (ROS) are liberated in excess when plants are subjected to heat stress, causing oxidative stress in plants (Al-Huqail *et al*., 2020). The activation of adaptation mechanisms in response to adverse environmental conditions, however, enables plants to resist a variety of abiotic stresses. In addition to modulating osmotic pressure, general homeostasis, and antioxidant system regulation, they generate chemicals that gather proteins and cell structures potentially improving the formation of secondary metabolites like phenols (Al-Huqail *et al*., 2020).

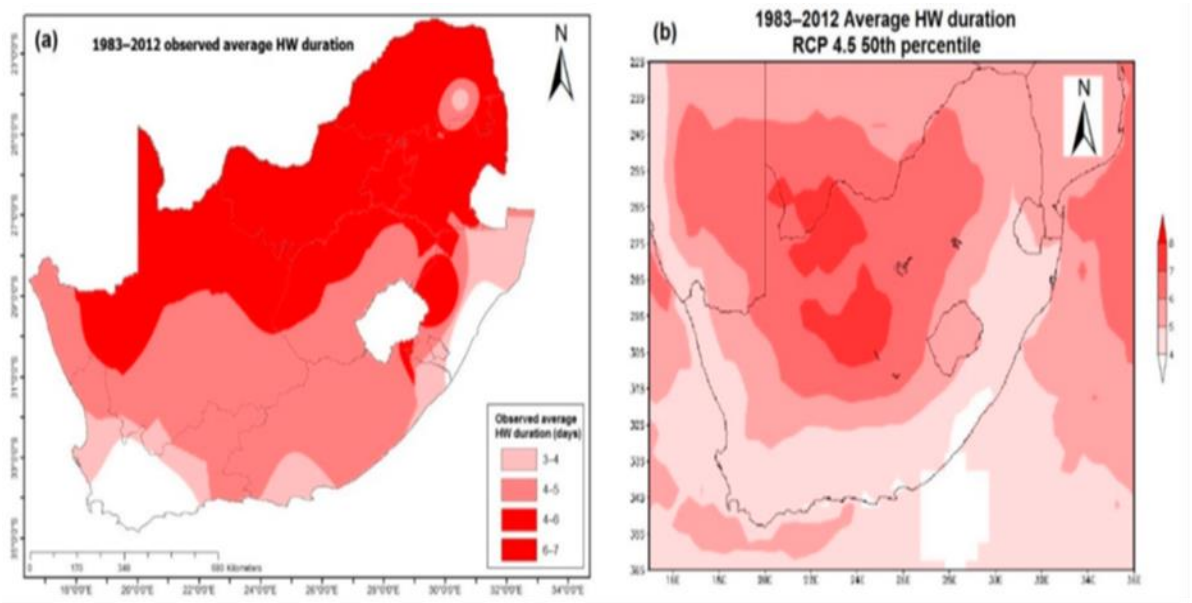

Figure 2. 5 Observed (a) and simulated (b) heat wave average duration in South Africa, respectively, from 1983-2012

Source: Mbokodo *et al*. (2020).

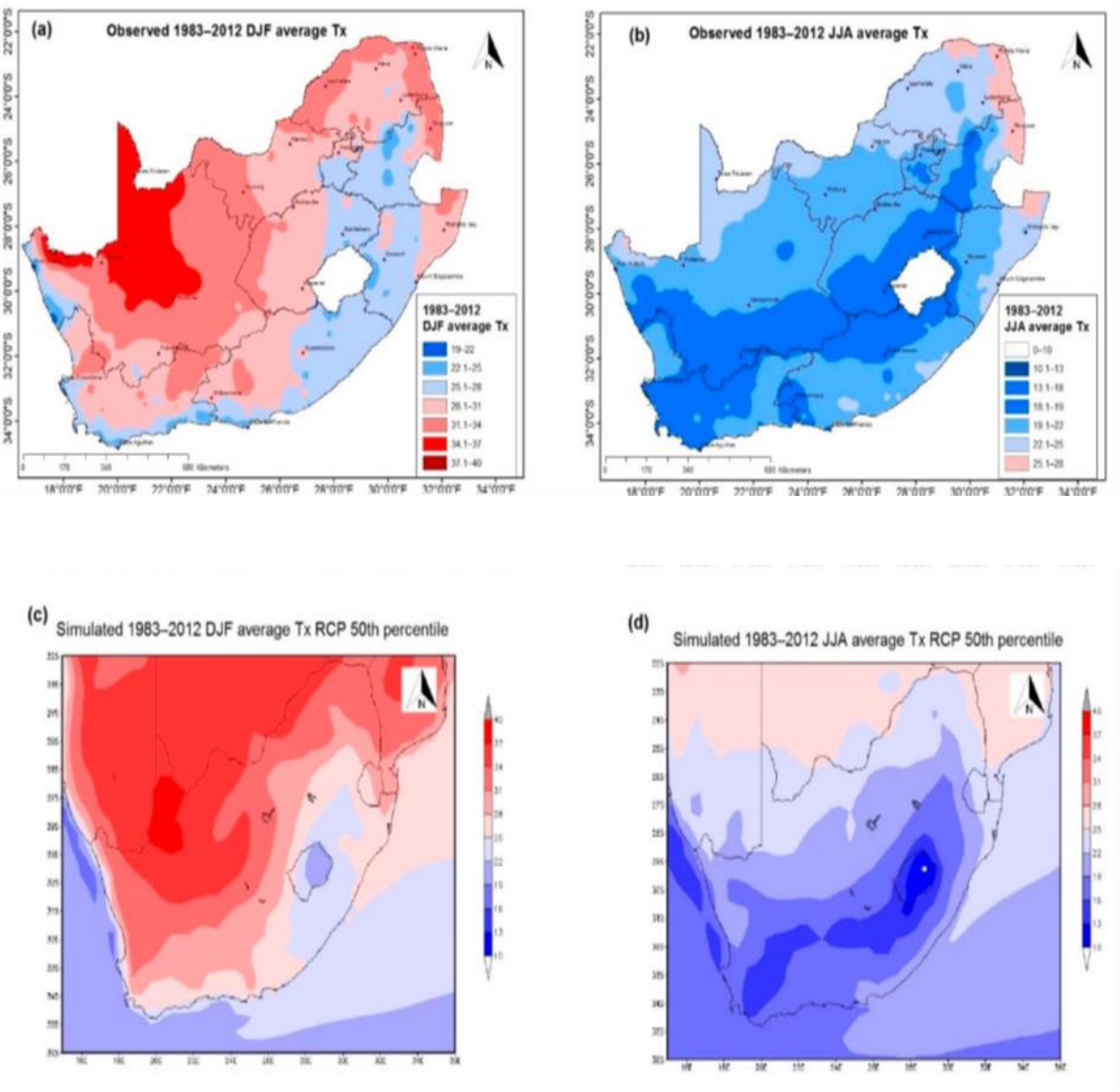

Figure 2. 6Figure 2. 6 (a & b) and simulated (c & d) colder temperature average duration in South Africa from1983-2012

Source: Mbokodo *et al*. (2020).

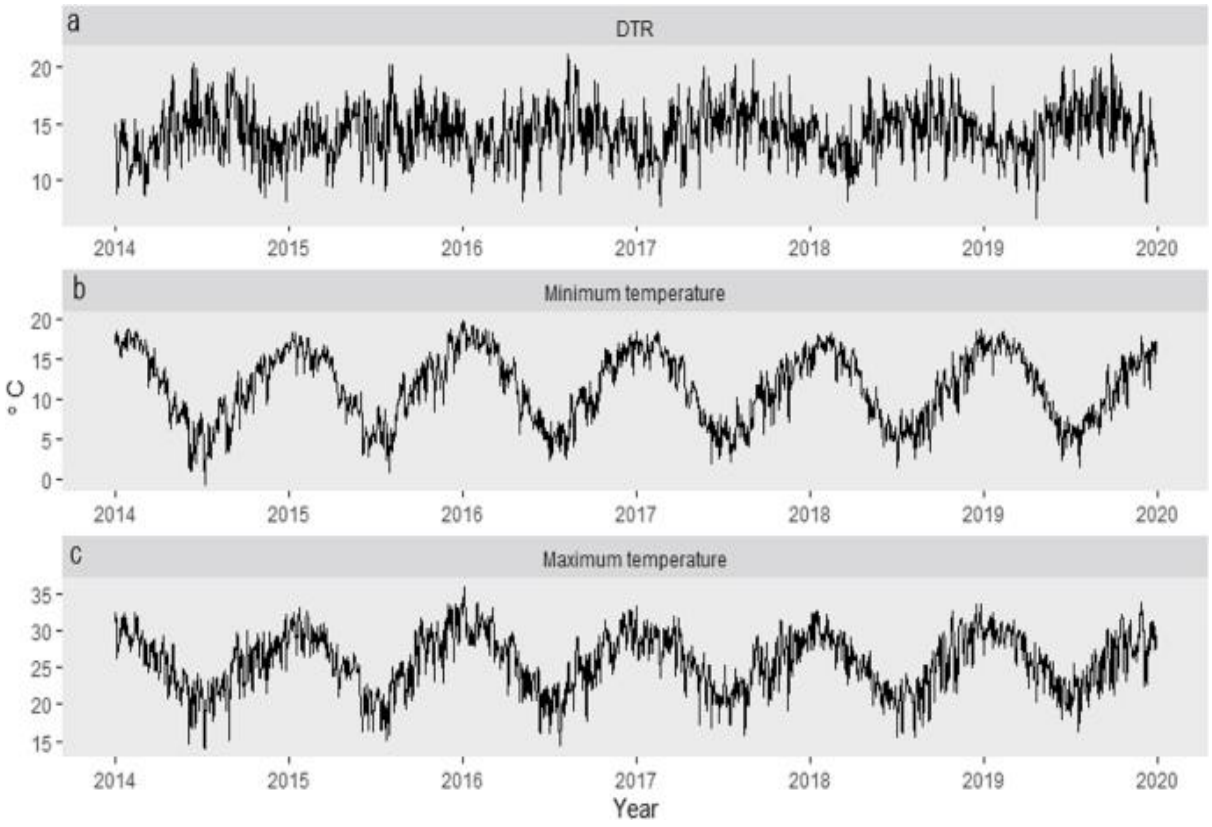

Figure 2. 7 Showing trends in South Africa's diurnal temperature from 2014 – 2019, (a) daily minimum (b) and maximum temperatures (c)

Source: Kapwata *et al*. (2022).

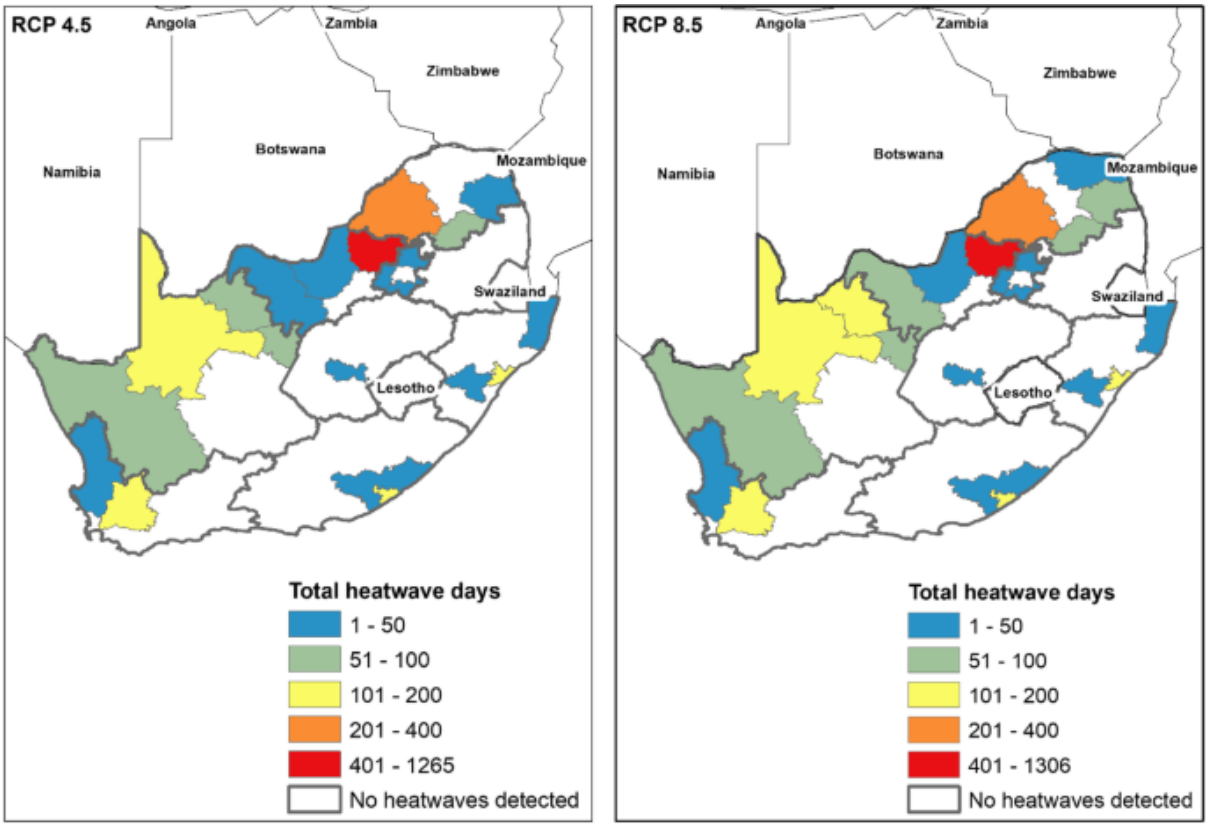

Figure 2. 8 Showing heatwaves simulation in South Africa from the year 2020-2039 through representative concentration pathway scenarios.

Source: Kapwata *et al*. (2022).

Past and future projections into the future show an increase in severely hot temperatures and a receding cold front (Figure 2. 6- 2. 8) (Van der Walt & Fitchett, 2021). By 2039, heatwaves are projected to intensify, largely in the north and east zones of South Africa (Kapwata *et al*., 2022). As indicated by Mbokodo *et al*. (2020), simulations from 2010 to 2039 show a slight increase in overall heat wave frequency, especially in the east, while the lowest heat wave frequency in the west will fall below 10 cycles over 30 years in autumn, winter, and spring (Mbokodo *et al*., 2020).

2.8.2 Drought/water deficit

Due to climate change and rising temperatures, there is an urgency for irrigation of crops and soils. The FAO Statistical Report (2015) reveals the scale of water utilization, with water utilization for irrigation purposes expected to increase by 5.5% from 2008 to 2050.

As stated by Kogan (2018), drought is predominantly an unfavourable abiotic factor on the planet. There are speculations that global warming and shifting climatic conditions will lead to an increase in drought occurrences (IPCC, 2018). There are records of previous drought occurrences, such as the 1997–2009 Millennium Drought in South Australia and the 2011–2017 California Millennium Drought (Freund *et al*., 2017).

In plants, drought stress induces a decline in water uptake and water potential in plants, thus affecting multiple physiological processes and causing irregularities in the biosynthesis of secondary metabolites (Azhar *et al*., 2011). Studies have shown that drought stress has effects on secondary metabolites found in medicinal plants, leading to an increased accumulation of secondary metabolites. A rise in plant flavonoid and phenol levels upon water stress is one example (Azhar *et al*., 2011). In a study on *Pisum sativum* cv. Meteor, Nogués *et al*. (1998) discovered that the flavonoids in this species suffered from dehydration and increased at a rate of 45%. Anthocyanins showed a similar pattern, significantly rising with water deficit as opposed to those that were adequately watered (controls). The improvement seen in phenolic and flavonoid compounds are associated with carbohydrate source and sink stability. Results from previous studies indicate that a reduction in the transference of soluble sugar during water stress affects soluble carbohydrate depository within plant cells (Jaafar *et al*., 2012).

Understanding how much drought stress impacts overall plant function is crucial for improving plant ecology and agricultural management. To investigate soil moisture deficits, various experimental techniques can be used, such as: the controlled water deficit in the greenhouses,

agar-based drought models, hydroponics aqueous culture-based models, and passive pot drying by withholding irrigation. The most used method is to air dry, and constant weighing of each pot, and addition of water to replace water loss with transpiration and to stabilize the desired soil water mark. These techniques are employed during simulation of drought stress in plants (Marchin *et al*., 2020).

2.8.3 Effects of concurrent extreme temperatures and droughts/water deficit on medicinal plants

Heatwaves and drought are considered combined abiotic factors, as explained by Perkins-Kirkpatrick and Gibson (2017). These often happen simultaneously when temperatures are very high and are associated with drought stress and particularly dry air (Perkins-Kirkpatrick & Gibson, 2017).

Severe drought conditions are caused by insufficient rainfall, and elevated air temperatures together with drought stress raise the temperature of plant tissues (Hussain *et al*., 2019). High levels of evaporation result from the cumulative impact of heat and drought stress on plants and soils when temperatures are high (Wahid *et al*., 2007). The findings of Pie *et al*. (1998) demonstrate that water deficit leads to heat stress in plants when sufficient water for replenishment is lacking after evaporation.

Zandalinas *et al*. (2018) and Lamaoui *et al*. (2018) noted the negative effects of these stresses on crops when citrus plants were first subjected to heat stress and then drought. Although these plant species exhibited the same physiological reactions, but when both stressors were present at once, the effect on the plants was greater than when either stress occurred alone. The results from Hussain *et al*. (2019) also revealed the singular and concurrent effect of drought and heat stress conditions on maize morphology, growth, reproduction, and production, while the harshest effects on maize was noted with exposure to drought and heat stress

simultaneously. Insufficient water commonly correlates with nutrient deficiencies within plant's root system rather than a decrease in the amount of deficient nutrients. The main causes of inadequate plant watering or under-irrigation of crops are a lack of precipitation and the depletion of water due to transpiration and evaporative processes. It also affects physiological processes and causes alterations in secondary metabolite biosynthetic pathway and causing a reduction in plant water uptake and water potential (Jaleel *et al*., 2007). In recent findings, it has been demonstrated that plants respond tremendously to stress caused by a combination of antagonistic abiotic factors compared to exposure to individual factors. These results have prompted the need to study combinations of abiotic stresses in plants.

Conducting this study on medicinal plants such as *Portulacaria afra* highlights the need for cultivation and development of drought-tolerant and highly temperature-tolerant medicinal plants, serving as a mitigation strategy against the adverse effects of projected climate change on medicinal plants.

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CHAPTER THREE

Screening of Phytochemical Profile and Biological Activities in the Leaves, Stems and Roots of South African *Portulacaria afra* using Four Extraction Solvents

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This chapter provides insight on the medicinal profile and phytopharmacological activities of *Portulacaria afra's* leaves, stems, and roots using four extraction solvents with different polarities. This chapter was written in scientific paper format according to Biomedical and Pharmacology Journal requirements (Appendix A). It was published as a research paper in the Biomedical and Pharmacology Journal requirements, DOI: https://dx.doi.org/10.13005/bpj/2494 ,

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Screening of Phytochemical Profile and Biological Activities in the Leaves, Stems and Roots of South African Portulacaria Afra using Four Extraction Solvents

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Portulacaria afra, is indigenous to South Africa and has been identified to have several medicinal properties according to traditional knowledge and few studies. The drive around this research is to evaluate the medicinal properties of the leaves, stems and for the first time the roots extracts of Portulacaria afra, using four solvents with different polarities. The aqueous (60°C), methanol, n-hexane and ethyl acetate whole plant extracts of P. afra were investigated for their phytochemical properties, antimicrobial and antioxidant activity. The phytochemical screening revealed that the methanolic and aqueous extracts of the leaves displayed high presence of secondary metabolites compared to n-hexane and ethyl acetate extracts. The methanolic leaves extracts showed strong presence of quinones, phenols, steroid and coumarins while the aqueous leaves extracts contained a moderate presence of saponins, terpenoids, quinones and coumarins. Ethyl acetate leaves extracts revealed a strong presence of tannins, moderate presence of phytosteroids and a low presence of volatile oil. Meanwhile, the leaves extracts with n-hexane showed a considerable amount of saponins with a moderate presence, and a low presence of tannins, volatile oils and terpenoids. The methanolic stems extracts displayed the most significant presence of secondary metabolites, showing a high presence of terpenoids, steroids, phenols and coumarins. The aqueous stems extracts showed a strong presence of glycosides with a moderate presence of saponins. However, ethyl acetate and n-hexane stems extracts displayed a few secondary metabolites with their concentration ranging from medium to low. The ethyl acetate roots extracts displayed a significant elevated amount of quinones with a strong presence. n-hexane roots extracts showed a moderate presence of volatile oil and a low presence of tannins and steroids. Methanolic roots extracts showed a moderate presence of coumarins and glycosides while aqueous roots extracts showed a low presence of glycosides. The overall highest total phenolics contents (TPCs) and total flavonoids contents (TFCs) in all the plant parts, were found to be in the methanol stems extracts and aqueous roots extracts respectively. Next to the methanol leaves and aqueous leaves extracts respectively. However, in the root's extracts, the aqueous extracts showed the highest total phenolics content while the water extracts had the highest total flavonoids contents. The antimicrobial activities of P. afra whole plant extracts with the various four solvents were tested against three microorganisms Escherichia coli, Staphylococcus aureus and Streptomyces griseus using agar-well diffusion method. The Antimicrobial activity of the n-hexane extracts of the leaves, stems and roots of P. afra presented a wide range of inhibition against all the test microorganisms, ethyl acetate leaves extract showed a considerable effect against Staphylococcus aureus while the methanolic extracts were not active. Aqueous roots extracts demonstrated a strong antimicrobial activity against Staphylococcus aureus while the other extracts were not active. The zones of inhibition ranged from 13 to 24 mm for the plant extracts. The antioxidant activity potential of the aqueous, methanol, n-hexane and ethyl acetate extracts of P. afra leaves, stems and roots extracts were observed through a 2, 2 diphenylpicryhydrazyl (DPPH) free radical assay, hydrogen peroxide scavenging (H2O2) and metal chelating activity assay. Ethyl acetate roots extracts exhibited the strongest hydrogen peroxide scavenging activity compared to the other extracts. Meanwhile,

aqueous stems extracts showed the highest antioxidant activity against DPPH radical. Aqueous and n-hexane roots extracts displayed the strongest metal chelating ability. These findings reveal the efficacy of the use of several solvents with different polarities for effective and more accurate extraction of various compounds and indicate that the antimicrobial and antioxidant activity of P. afra parts are dependent on the solvent extracts.

Keywords: Antimicrobial Activity; Antioxidant Activity assays; Extracts; Portulacaria afra; Phytochemical screening; Solvents; Whole plant.

Medicinal plants from prehistoric times have long been employed in practicing traditional medicine. Hundreds of chemical compounds are synthesized by plants to perform various preventive roles like protection from insects, diseases, fungi as well as herbivores[1]. They are also used by humans as a form of medical antidote used for healing purposes and protection from diseases[2-4]. The World Health Organization (WHO) reported the use of traditional plants for therapeutic purposes by 80% of the world's population[5].

In Africa, particularly in certain tribes, plant medicine is not a novel practice, due to its existence over a long time[2,6]. South Africa has about 60 million people and largely, people in the Cape Province, Kwazulu-Natal, Mpumalanga and Limpopo Province continue to rely on those medicinal plants. These plants have proven their efficacy and potency by curing various malignant ailments and diseases like cancer, diabetes, tuberculosis (TB) and benign ones like common cold, arthritis, dysmenorrhoea, and stomach problems[7,8].

Phytochemicals are plant chemicals that are brought about by primary/secondary metabolism. They are involved in biological activities and perform vital functions like plant protection from pathogens, competitors, and predators[9]. They are present in plant foods such as vegetables, fruits, beans, whole grains, nuts, and seeds. It is believed that the consumption of organic plant foods with high phytochemical properties are of great health benefits as they can be used in the avoidance of serious ailments and heart disease conditions[9]. These natural compounds synthesized by plants are categorized into two main groups namely the primary and secondary metabolites[10]. Secondary metabolites serve as mediators of antagonistic ecological interactions between the plants and their habitat but are not vital in their growth, development, and reproduction. They also help to defend plants against disease-causing agents with their medicinal properties (antibiotic, antifungal and antiviral)[11].

Antioxidants are natural or manufactured compounds that slow down the oxidation process; they donate an electron to a free radical without making themselves unstable. This causes the free radical to stabilise and become less reactive[11]. Antioxidant constituents present in plant foods and materials are widely studied by researchers. They have received more attention in the food manufacturing companies and among consumers, due to their health benefits in terms of preventions from and treatment of chronic heart diseases and cancer caused by oxidative stress[12]. Natural antioxidants are the most beneficial for use or consumption because they do not have negative impacts like the synthetic ones and are present in food, fruits, vegetables, and other plant-based, whole foods. Several vitamins, such as vitamins E and C, selenium, and carotenoids, such as beta-carotene, lycopene, lutein, and zeaxanthin are effective antioxidants[11].

According to Pietta[13], phenolic components like flavonoids are responsible for the anti-oxidative effect, while their antioxidant activity is a resultant of the redox characteristics they possess. These redox characteristics absorb and neutralise free radicals, extinguishing singlet and triplet oxygen, or decomposing peroxides. Essentially, the presence of secondary metabolites in medicinal plants influence antioxidant activity by slowing down or preventing oxidative stress or damage[14]. However, to date there are limited scientific records of this.

Portulacaria afra belongs to the Family: *Portulacaceae* or more recently Didiereaceae.

Portulacaria has 7 species in total, the other 6 are: *P. armiana*, *P. carrissoana*, *P. fruticulose*, *P. longipedunculata*, *P. namaquensis*, *P. pygmaea* while *P. afra* and *P. namaquensis* are the only 2 native tree species found in the South Africa. It is a succulent evergreen plant popularly used worldwide. It has various names such as elephant bush (English), spekboom, and porkbush (Afrikaans). The succulent garden plant is indigenous to South Africa, and it is found in several regions in South Africa[15]. It grows in warm weather conditions and can be found growing on rocky hillsides and it is useful for carbon sequestration. In addition, it is useful for reclamation programmes in dry and parched terrain[15].

In *P. afra*, there is very limited scientific documentation on the full phytochemical profile in all the plant parts. Most studies have worked on the physiological aspects such as the leaves and leaf juice used for the treatment of skin conditions and mouth infections[16]. The overarching aim of this study is to establish the phytochemical profile, medicinal properties; phytopharmacological attributes of the leaves, stems and for the first time the roots extracts of *Portulacaria afra,* using four solvents with different polarities.

MATERIALS AND METHODS

Chemicals and solvents used for this study were of analytical grades. Gallic acid, Folin-Ciocalteau reagent, methanol, hexane, ethyl acetate, Mueller-Hinton agar and Baird Parker agar, dimethyl sulfoxide (DMSO), 2, 2 diphenylpicryhydrazyl (DPPH), iron (II) chloride, ferrozine, phosphate buffer, hydrogren peroxide were all purchased from Sigma-Aldrich, USA, order CAS N0. 1162-65-8.

Collection of Plant Material

P. afra plants were collected in July 2021, within the premises of the University of the Witwatersrand, Johannesburg and were propagated from cuttings.

Cutting/growing the plants

Cuttings with green, non-woody stems were selected from a healthy parent *P. afra* plant with absence of diseases and with good features like green leaves, lack of drooping and no dying foliage. Cuttings which were 40-45 cm long and 4cm thick were collected with a pair of sterilised secateurs and transferred into the greenhouse for planting[17].

45 pots of 2 - 2.5 litres were filled to the brim with culterrean potting professional cutting mix soil to hold the stem cutting for rooting. This type of soil was used because it drains better, provides moist conditions and free from pathogens that could be harmful to the cuttings before they become rooted[17].

Cuttings were carefully placed in the half-filled potting mix soil and were gently firmed and then filled to the brim with the potting mix soil[17].

The plants were allowed to grow in the green house for a period of 3 months to acclimatise and develop new roots mass and until new leaves began to appear along the stems. The plants were monitored and watered with 500 ml of water in the greenhouse every two days since it does not require too frequent watering[17].

Sample preparation and extraction

The leaves, stem and roots of *P. afra* were harvested, washed with deionized water and then allowed to dry in a hot air dryer under 40°C for four days. The dried plant material was ground into a fine crude powder using an electric grinder. The powdered plant material was then enclosed in foil and placed in an airtight container, which was stored in the dark under room temperature cupboard until further tests commenced[18].

Preparation of Extracts

The crude plant extract was prepared using four solvents: 80% methanol, hexane, ethyl acetate and 100% distilled water at temperature (60°C). All crude plant extracts were prepared by placing 3g of the powdered plant into separate bottles and adding 30 ml of a solvent. The methanol, hexane, ethyl acetate was placed on a shaker, while the water extracts were prepared with a sonicator and was set at temperature (60°C) for 45 minutes[18-20]. All extracts were shaken for 48 hours, apart from the water extracts. The water extracts were centrifuged to decrease the viscosity of the extracts. All extracts were filtered through a filter paper into vials. The supernatant, which remained, were discarded whilst the vials were enclosed in foil and stored in the refrigerator until the tests were conducted[18-20].

Qualitative phytochemical screening

The preliminary phytochemical analysis was done on all the plant extracts for secondary metabolites discovery and identification by using Harbone[21] (1998), Roghini and Vijayalakshmi[22] methodology with slight modification. The various tests for saponins, flavonoids, glycosides, quinones, phenols, terpenoids, steroids, phytosteroids, volatile oil, and coumarins were observed and the tests results were recorded.

Total Phenolic Contents and Total Flavonoids Contents Evaluation

The total phenolic and flavonoids content of the leaves, stems and roots extracts of *P. afra* with the different solvents were estimated by using the colorimetric Folin-Ciocalteu method. For the analysis, standard gallic acid solutions at different concentrations were used for the calibration curve. The concentrations of the total phenolic contents in the extracts were estimated from the standard curve (Figure 3) and the results were expressed as gallic acid equivalents per gram (mg GAE/g) dry weight of the extract. Total flavonoids contents of the different plant parts extracts were also calculated by making a standard curve of quercentine (Figure 4) and expressed as quercetin per gram (mg RE/g) of dry weight. All determinations were done in triplicates and data was expressed as mean ± standard error. $P < 0.05$ was considered statistically significant.

Antimicrobial activity assay

P. afra antimicrobial activity assay in the leaves, stems and root extracts with the different solvents were determined using the Agar-well diffusion assay described by Jain *et al.*[23] The plant extracts were tested against gram-positive *Staphylococcus aureus* (ATCC 25923), gram-negative *E. coli* (ATCC 25922) and gram-positive *streptomyces griseus*.

Agar-well diffusion method

The Petri dishes were sterilised and put in the autoclave before pouring the nutrient agar medium -Mueller-Hinton agar and Baird Parker agar. Once the agar medium solidified, sterile cotton swabs were used to inoculate the whole surface of the agar medium with the test microorganisms. *S. aureus* was inoculated on the Baird Parker agar plates and *E. coli* was inoculated onto the Mueller-Hinton agar plates as well as the *Streptomyces griseus* plates. Six wells were made in the inoculated media using a 6mm sterile borer. The plant extracts (100il) were impregnated only into five wells while the sixth well was impregnated with the negative control, dimethyl sulfoxide (DMSO). The plates were covered and placed in the fridge for 30 minutes to diffuse. The plates were put in the incubator for 24 hours at 37°C. After the incubation period, the zones of inhibition were measured in millimetres using a ruler. The presence of an inhibitory zone surrounding was indicative of a positive antibacterial activity result. The studies were performed in triplicate and data was expressed as mean ± standard deviation and was calculated by Microsoft excel. $P < 0.05$ was considered statistically significant while Tukey test HSD in RStudio was used to determine where the significance lies.

Evaluation of in vitro Antioxidant Activity

DPPH scavenging activity assay

A 2, 2 diphenylpicryhydrazyl (DPPH) free radical assay by Brand-Williams *et al*[24] was used with a few alterations. 3 g of dried and powdered leaves, stems, and roots materials of *P. afra* was added to 80% methanol solvent and water respectively for the extract's preparation. The scavenging potential of DPPH on the plant extracts were derived by adding 50 mg of DPPH to 100 ml of 80% methanol for preparation of the stock solution. A work solution was then prepared from the stock solution; the dilution mixture was of ratio 1:5 of the stock solution to 80% methanol. Each extract had 5 quantities (10-50 iL). A reaction mixture was made containing 10-50 iL of the extracts and 700 iL of the work solution which was topped with 80% methanol to make up a volume of 1 ml. A blank was made with the use of 80% methanol and the control solution was prepared using the work solution and 80% methanol. The mixtures were kept in the dark for 45 minutes, and then a spectrophotometer was used to measure the absorbance at a wavelength of 517 n. The inhibition (%) was expressed with the following equation:

DPHH Scavenging Effect (%) = [(Absorbance of sample – Absorbance of blank)/Absorbance of control] x 100

Metal chelating activity

To determine the metal chelating activity, the method used by Dinis *et al.*[25] was employed. A volume of 2 ml of extracts was added to 0.25 ml of 250 mM iron (II) chloride, the reaction was

observed when 0.25 ml of ferrozine was added. The mixture was firmly shaken together and allowed to sit at room temperature for 10 minutes. The absorbance of the mixture was measured at a wavelength 562 nm. Methanol served as the blank while the control was without the extracts. The metal chelation (%) was calculated using the following formula:

Metal chelation (%) = {(A0 " A1)/ A0} × 100

A0 is the absorbance of the control and A1is the absorbance with the test samples.

Hydrogen peroxide scavenging (H, O,) assay

The methodology used by Ruch *et al.*[26] was used for this study. 1 ml of extracts was added to 3.4 ml of phosphate buffer (50 mM, pH 7.4). 600 ìL of 40 mM hydrogen peroxide (30%) was added into the initial solution. The mixture was left at room temperature for 40 minutes; the absorbance was measured at a wavelength of 230 nm. The hydrogen peroxide scavenging activity was calculated with the following formula:

Scavenged H, O, (%) = [(Absorbance of control – Absorbance of sample)/Absorbance of control] x 100

Statistical Analysis

The concentration values were then plotted against the % inhibition values using Microsoft excel sheet to generate a trendline equation used to calculate the IC_{50}. A one-way analysis of variance (ANOVA) was used to determine any significant differences. Experiments were all performed in triplicate and data was expressed as mean ± standard error (SE) (when n= 3). $P < 0.05$ was considered statistically significant while Tukey test HSD in RStudio was used to determine where the significance lies.

RESULTS AND DISCUSSION

Table 1 shows the preliminary phytochemical analysis done on the plant parts extracts of *P. afra* for secondary metabolites discovery and identification by using Harbone[21], Roghini and Vijayalakshmi[22] methodology with slight modification. Phytochemicals are plant chemicals that are brought about by primary/ secondary metabolism and are involved in biological activities[9]. It is believed that the consumption of organic plant foods with high phytochemical properties are of great health benefits as they can be used in the avoidance of serious ailments and heart disease conditions[27]. Literature recommends the use of several solvents with different polarities for effective and more accurate extraction of various compounds[28,29]. The solvents used for the extraction were selected according to the degree of polarity of the solute of interest because a solvent of close polarity will be more effective and accurate in dissolution of the solute[30]. The polarity of some often-used solvents in the order of lowest polarities to highest polarities are Hexane < Chloroform < Ethyl acetate < Acetone < Methanol < Water[30]. For this study, the following four solvents were used: (water (60°), *n*-hexane, ethyl acetate, and methanol Table 1).

Fig. 1. Image showing *P. afra* leaves and stems

Fig. 2. Image showing *P. afra* roots

Table 1. Phytochemical screening of *P. afra* samples

No.	Chemical tests	Leaves extracts					Stems extracts					Roots extracts		
		M	H	H_2O	EA		M	H	H_2O	EA		M	H	H_2O EA
1.	Tannins	$FeCl_3$	++	+	-	+++	+	++	++	+	+	+	-	-
2.	Saponins	Froth	+	++	++	+	+	++	++	-	+	-	-	-
3.	Flavonoids	NaOH	-	-	-	-	-	-	-	-	-	-	-	-
4.	Glycosides	Sulphuric acid	-	-	+	-	+	-	+++	-	++	-	++	-
5.	Quinones	Sulphiric acid	+++	-	++	-	-	-	-	+	-	-	+	+++
6.	Phenols	$FeCl_3$	+++	-	++	-	+++	-	-	-	+	-	-	-
7.	Terpenoids	Chloroform	++	+	-	-	+++	-	-	-	+	-	-	-
8.	Steroids	Reaction with Chloroform and sulphuric acid	+++	-	-	-	++	-	-	-	+	+	-	-
9.	Phytosteroids	Reaction with Chloroform and sulphuric acid	-	-	-	++	-	-	-	-	-	-	-	-
10.	Volatile oil	Reaction with NaOH and dilute HCl	-	+	-	+	-	+	-	+	+	++	+	-
11.	Coumarins	Reaction with NaOH	+++	-	++	-	+++	-	-	++	++	-	-	-

M, methanolic extract; H, n-hexane extract; H2O, hot water extract (60°C); EA, ethyl acetate extracts; +++, strongly present; ++, moderately present; +, present; -, not present or not detected.

The phytochemical screening as seen in (Table 1) revealed that the methanolic and aqueous extracts of the leaves displayed high presence of secondary metabolites compared to *n*-hexane and ethyl acetate extracts. Methanol and water commonly extracts chemical compounds like sugars, amino acids and glycosides during phytochemical screening. While ethyl acetate extracts alkaloids, aglycones and glycosides, *n*-hexane is targets waxes, fats, fixed oils and volatile oils for extraction[31]. Several studies have revealed that secondary metabolites like glycosides, phenols, steroids, saponins, alkaloids and terpenoids possess strong free radical scavenging and antioxidant potentials[32]. Glycosides, consisting of terpenoid or steroids and sugar chains, are of great health benefits and biological activities like anti-cancer and anti-inflammatory properties[32]. The methanolic leaves extracts showed strong presence of quinones, phenols, steroid and coumarins. These results were however not in complete accordance with previous work by Tabassum *et al.*[33] on *P. afra* tissue extracts, as findings from their research showed strong presence of tannins, phenols, flavonoids, saponins and glycosides with methanolic extracts and a moderate presence with *n*-hexane fractions which can be linked to the variation in climatic conditions. Consequently, fluctuations in the abiotic factors affect how secondary metabolites are synthesized and gathered. Abiotic factors such as temperature and water deficit are a major hindrance to the reproduction of medicinal plants, which in turn affects the biosynthesis and causes irregularities in the secondary metabolites production and consequent phyto-phamacological activities[34]. The aqueous leaves extracts contained a moderate presence of saponins, terpenoids, quinones and coumarins. Ethyl acetate leaves extracts revealed a strong presence of tannins, moderate presence of phytosteroids and a low presence of volatile oil. Meanwhile, the *n*-hexane leaves extracts showed a considerable amount of saponins with a moderate presence, and a low presence of tannins, volatile oils and terpenoids. The methanolic stems extracts displayed the most significant presence of secondary metabolites, showing a high presence of terpenoids, steroids, phenols and coumarins. The aqueous stems extracts showed a strong presence of glycosides with a moderate presence of saponins. However, ethyl acetate and *n*-hexane stems extracts displayed a few secondary metabolites with their concentration ranging from medium to low. The ethyl acetate roots extracts displayed a significant elevated amount of quinones with a

Table 2. Total phenolic content of *P. afra* samples extracts

	Plant partsTotal phenolic contents (mg/g) Solvents			
	M	H	H_2O (60°C)	EA
Leaves	6073.20±4.10[a]	1237.04±13.47[c]	1038.18±1.17[d]	3008.08±81.65[b]
Stems	9521.01±216.94[b]	1755.55±69.98[a]	1820.67±25.08[a]	1472.73±11.66[a]
Roots	1567.47±29.16[c]	1351.52±11.66[d]	3331.31±58.32[a]	2361.62±11.66[b]

M, methanolic extract; H, *n*-hexane extract; H_2O, hot water extract (60°C); EA, ethyl acetate extracts. All values are expressed as mean ± SE, n=3, and are equivalent to gallic acid (mg/g of GAE). [a]Values with different letters in the same column were significantly ($p < 0.05$) different.

Table 3. Total flavonoid content of *P. afra* samples extracts

Plant parts	Total flavonoid contents (mg/g) Solvents			
	M	H	H_20 (60°C)	EA
Leaves	150.47±1.17[a]	141.34±0[a]	592.70±49.99[b]	108.60±5.13[a]
Stems	107.42±2.04[a]	134.51±1.18[a]	585.63±48.29[b]	114.49±2.04[a]
Roots	359.01±8.16[a]	104.59±8.16[a]	901.30±149.87[b]	154.53±2.36[a]

M, methanolic extract; H, *n*-hexane extract; H_2O, hot water extract (60°C); EA, ethyl acetate extracts. All values are expressed as mean ± SE, n=3, and are equivalent to quercetin (mg/ g of quercetin). [a]Values with different letters in the same column were significantly ($p < 0.05$) different.

strong presence. *n*-hexane roots extracts showed a moderate presence of volatile oil, coumarins and a low presence of tannins and steroids. Methanolic roots extracts showed a moderate presence of coumarins and glycosides while aqueous roots extracts showed a low presence of glycosides. Saponins consist of triterpenoids or steroid, which is responsible for their therapeutic activities[35], while coumarins is known to for its high phytopharmacological properties and particularly anti-cancer, anti-inflammatory and tranquilizer properties[36].

Quantitative analysis of phytochemicals

Quantitative results of phytoconstituents in (Tables 2 and 3) showed the overall highest total phenolics contents (TPCs) and total flavonoids contents (TFCs) in all the plant parts, were found to be in the methanol stems extracts and aqueous roots extracts (9521.01±216.94 and 901.30±149.87 mg/g respectively). Next to the methanol leaves and aqueous leaves extracts (6073.19±4.09 and 150.47±1.17 mg/g respectively). However, in the root extracts, the aqueous extracts had the highest total phenolics and flavonoids contents (3331.31±58.32 and 901.30±149.87 mg/g respectively).

Antimicrobial activity assay

The results of the antimicrobial properties of *P. afra* extracts showed that *n*-hexane extracts of the leaves, stems and roots of *P. afra* presented a wide range of inhibition against all the test microorganisms, ethyl acetate leaves extract showed

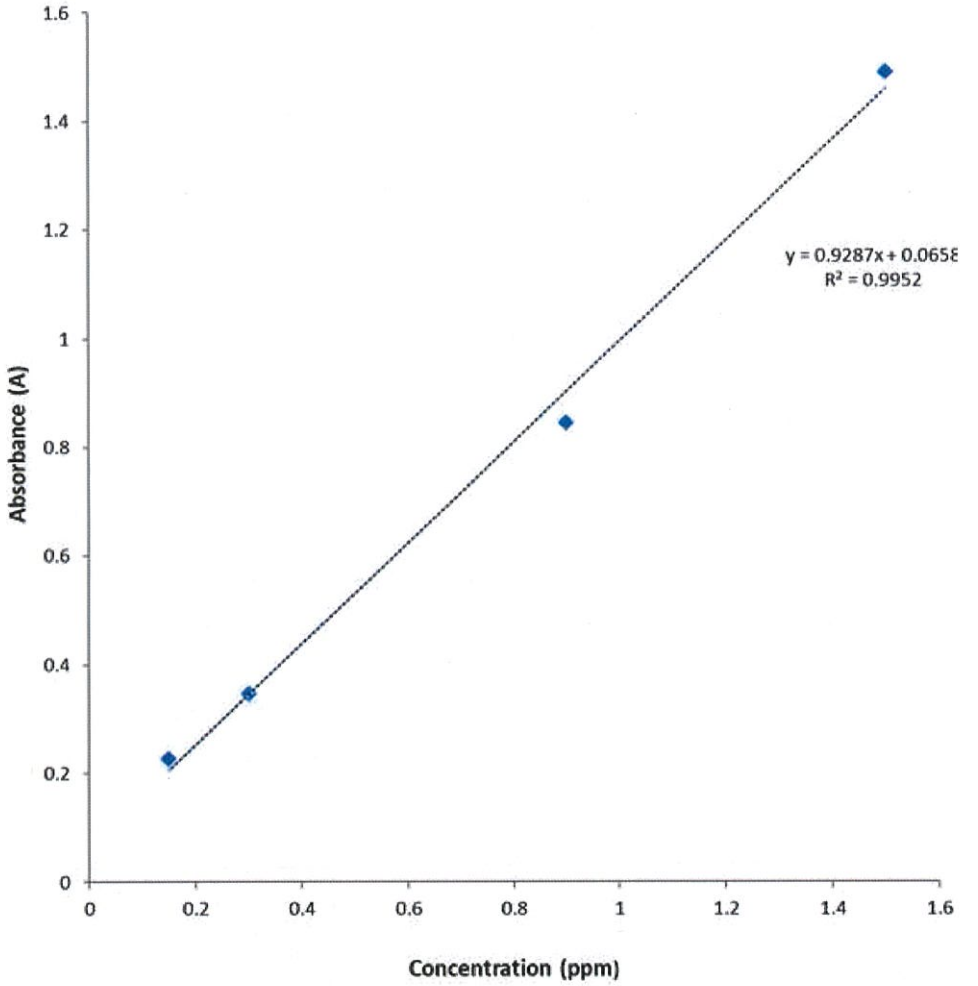

Fig. 3. Gallic acid calibration curve

a considerable effect against *Staphylococcus aureus* while the methanolic extracts were not active (Table 4). Aqueous roots extracts demonstrated a strong antimicrobial activity of 24mm against gram-positive *Staphylococcus aureus* while the other extracts were not active. The zones of inhibition ranged from 13 to 24 mm for the plant extracts. The results of this study are not in agreement with previous studies by Khanyile *et al.*[18] where the leaves extract of *P. afra* was used to determine the inhibitory effect against positive *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The results showed a slight inhibitory effect against the test microorganisms. It is observed that the examined plant extracts showed better activity against gram-positive microorganisms than gram-negative microorganisms. The variation observed in the ability of the extracts to inhibit the growth of these microorganisms could be because of the type of layers possessed by the cell wall of each microorganism. Several factors like chemical constituents in the different solvents, microbial growth and exposure of the test microorganisms may be responsible for the results from this study.

Antioxidant Activity

Antioxidants are natural or synthetic compounds that slow down the oxidation process; they are molecules that donate an electron to a free radical without making themselves unstable. This causes the free radical to stabilize and become

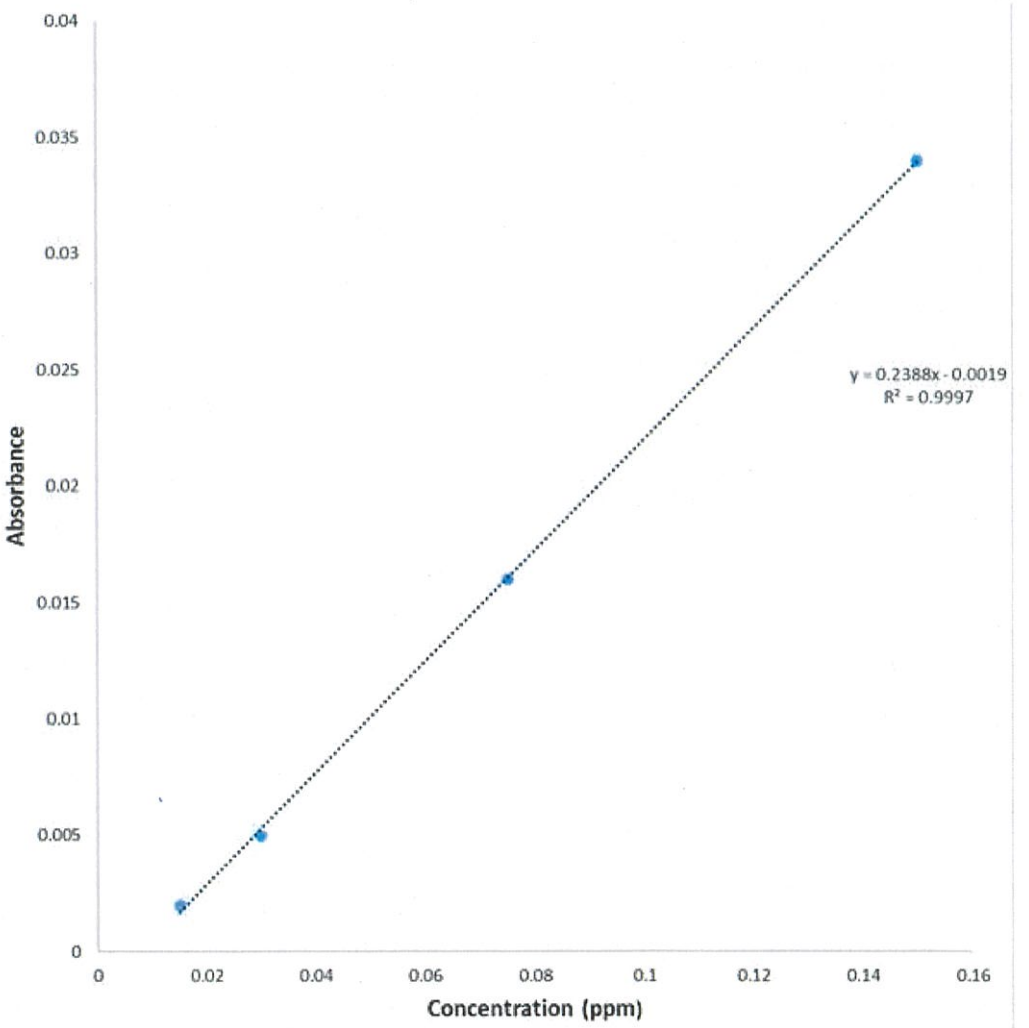

Fig. 4. Quercetin calibration curve

less reactive[11]. They neutralize free radicals and unstable molecules that can harm the cells. In this study, antioxidant activity potential of the aqueous, methanol, *n*-hexane and ethyl acetate extracts of *P. afra* leaves, stems and roots extracts were observed through a 2, 2 diphenylpicryhydrazyl (DPPH) free radical assay, hydrogen peroxide scavenging (H, O,) and metal chelating activity assay. The DPPH free radical is fast and mostly used for antioxidant activity determination against DPPH radical[27]. Meanwhile, hydrogen peroxide is mostly harmful as it transforms into hydroxyl and reactive radical metal complexes[37]. Studies have shown how metal chelating activity is used to determine the antioxidant potential of plant extracts as they interfere with the oxidation process by reacting with free radicals, chelating free catalytic metals and by acting as scavengers of oxygen by-products. Table 5 shows the various antioxidant activity assays used in this study. Aqueous stems extracts showed the overall highest antioxidant activity (0.480mg/ml) against DPPH radical. Ethyl acetate roots extracts exhibited the strongest hydrogen peroxide scavenging activity (0.89 mg/ml) as compared to the other plant part extracts. Aqueous and *n*-hexane roots extracts displayed the strongest metal chelating ability (3.19 and 4.51 mg/ml respectively). Antioxidant constituents present in plant foods and materials are widely studied by researchers. They have received more attention in the food manufacturing companies and among consumers, due to their health benefits in terms of preventions from and treatment of chronic heart diseases and cancer caused by oxidative stress[12].

Table 4. Antimicrobial activity of different solvent extracts of all plant parts of *P. afra*.

Plant parts	Zone of inhibition (mm) of extracts of different solvent				
	Name of microorganism	M	H	H_20 (60°C)	EA
Leaves	*Escherichia coli*	-	16±0.6	-	-
	Staphylococcus aureus	-	18±0.6	-	14±0.6
	Streptomyces griseus	-	18±0.6	-	-
Stems	*Escherichia coli*	-	13±0.6	-	-
	Staphylococcus aureus	-	13±0.6	-	-
	Streptomyces griseus	-	13±0.6	-	-
Roots	*Escherichia coli*	-	15±0.6	-	-
	Staphylococcus aureus	-	17±0.6	24±0.6	-
	Streptomyces griseus	-	18±0.6	-	-

M, methanolic extract; H, *n*-hexane extract; H_2O, hot water extract (60°C); EA, ethyl acetate extracts. All values are expressed as mean ± SD, n=3.

Table 5. Antioxidant activity assay of solvent extracts of *P. afra* plant parts

Plant parts		IC_{50} (±SE, mg/ml) (Extract fraction)			
	Antioxidant assay	M	H	H_20(60°C)	EA
Leaves	DPPH	32.23 ±0.040^a	8.49 ±0.081^b	3.21±0.064^c	1.70±0.162^d
	Hydrogen peroxide	5.44±0.635^b	17.22±0.040^a	17.22±0.040^a	4.13±0.098^b
	Metal chelating	6.66±0.629^a	20.44±0.543^c	35.17±1.415^b	8.43±1.120^a
Stems	DPPH	34.92 ±0.052^b	35.81±0.144^a	0.48±0.064^d	5.74±0.075^c
	Hydrogen peroxide	4.44±0.214^a	2.94±0.017^b	4.70±0.064^a	4.41±0.075^a
	Metal chelating	63.25±0.664^b	6.56±0.600^a	4.23±1.085^a	5.38±1.085^a
Roots	DPPH	29.63±0.267^a	10.23±0.156^b	1.37±0.058^d	3.0±0.092^c
	Hydrogen peroxide	2.12±0.260^c	11.68±0.080^a	3.83±0.110^b	0.89±0.160^d
	Metal chelating	92.34±2.840^b	4.51±0.549^a	3.19±0.554^a	25.28±2.159^c

DPPH, 2, 2 diphenylpicryhydrazyl; M, methanolic extract; H, *n*-hexane extract; H_2O, hot water extract (60°C); EA, ethyl acetate extracts. All values are expressed as mean ± SE, n=3. ^{a} Values with different letters in the same column were significantly ($p < 0.05$) different.

CONCLUSIONS

The results of the present study have shown a unique variation in the estimated phytochemical constituents and biological activities in the leaves, stems and roots extracts of the four different solvents of *P. afra*. It is worthy to note that *P. afra* parts have displayed high therapeutic properties; with a strong presence of quinones in the ethyl acetate roots extracts for the first time. *P. afra* aqueous roots extracts also had the highest TFCs content. Coumarins known for anticancer properties showed a strong presence in the methanolic leaves and stems extracts with a moderate presence in the roots extracts, this could potentially be of good use in the pharmaceutical industries for drug production and future role in public health.

Further research is needed, to determine how abiotic factors would influence therapeutic properties, values and activities of *P. afra.*

ACKNOWLEDGMENTS

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Conflict of interest

The authors declare that there are no conflicts of interest.

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CHAPTER FOUR

Effect of concurrent extreme temperatures and water deficit on the phytochemistry, antibacterial and antioxidant activities of *Portulacaria afra* using four extraction solvents

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This Chapter provides insight on the influence of concurrent extreme temperatures with water-deficit on the medicinal profile, antimicrobial and antioxidant activities of *Portulacaria afra*'s leaf, stem, and root extracts. This Chapter was written in scientific paper format according to Journal of Herbmed and Pharmacology requirements (Appendix B).

It has been revised and resubmitted after a review process (Appendix C) and requests from the journal editor (see below).

Ida Risenga

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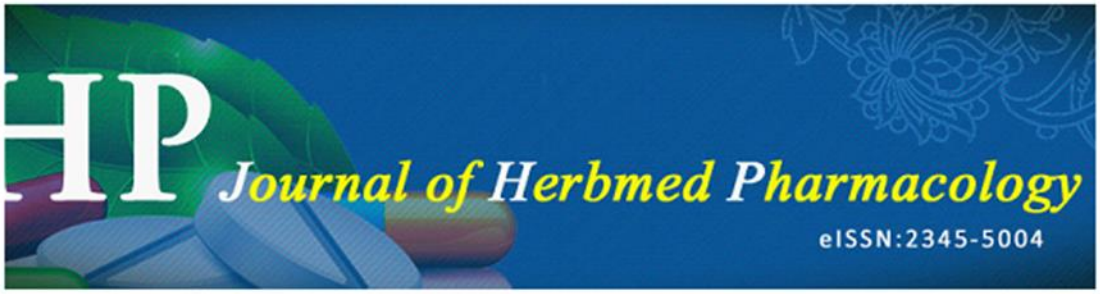

Journal of Herbmed Pharmacology: Revised Manuscript was Received

Dear Ms. Oluwafunbi Christianah Adeleye,

Journal of Herbmed Pharmacology received the revised edition of your manuscript entitled **Effect of concurrent extreme temperatures and water deficit on the phytochemistry, antimicrobial and antioxidant activities of Portulacaria afra using four extraction solvents**.

Thank you for contribution to Journal of Herbmed Pharmacology.

Sincerely Yours,
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1

Effect of concurrent extreme temperatures and water deficit on the phytochemistry, antimicrobial and antioxidant activities of *Portulacaria afra* using four extraction solvents

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Abstract

Introduction: The study focused on *Portulacaria afra*, a plant native to South Africa renowned for its traditional medicinal applications against oral and skin infections. This study aimed to explore, the phytochemical composition, antimicrobial, and antioxidant capabilities of the leaves, stems, and roots under concurrent extreme temperatures (hot and cold) and water deficit conditions to further validate its medicinal significance.

Methods: To achieve this, the study subjected *Portulacaria afra* to various temperatures ranging from 0/5ºC to 35/45ºC combined with water deficit and evaluated its antimicrobial and antioxidant properties. Phytochemical compounds were extracted from different solvent extracts of *P. afra* and analysed. Antioxidant activity was assessed using metal chelating activity, hydrogen peroxide scavenging, and 2,2-diphenylpicrylhydrazyl (DPPH) free radical assays, while antimicrobial efficacy was determined using the agar-well diffusion method against three microbial strains: *Staphylococcus aureus, Escherichia coli, and Streptomyces griseus.*

Results: Results revealed an enhanced presence of 13 phytochemical groups in *Portulacaria afra* extracts compared to controls, with peak accumulation of phenolic and flavonoid

compounds observed under extreme hot temperatures (35/45ºC) with water deficit. Extracts from these conditions exhibited superior inhibitory effects against *Escherichia coli*. Moreover, antioxidant activity peaked at hot temperatures (30/40ºC) for 2,2-diphenylpicrylhydrazyl (DPPH) and metal chelating assays, while the most effective hydrogen peroxide scavenging activity was observed under hot temperatures (35/45ºC) with water deficit.

Antioxidant activity peaked at mid-range hot temperatures (30/40ºC) for DPPH and metal chelating, while the best hydrogen peroxide scavenging activity was observed during concurrent extreme hot temperatures (35/45ºC) with water deficit.

Conclusion: The findings suggest that extreme temperature variations combined with water deficit significantly impact the biological activities of *Portulacaria afra*, highlighting the plant's adaptability and resilience while retaining its therapeutic potential.

Keywords

Portulacaria afra; Extreme temperatures; Water deficit; Whole plant; Phytochemical screening; Antioxidant Activity assays

Implication for health policy/practice/research/medical education: The result of this study contributes new knowledge on how the species responds to the changing climate. Further, it validates that concurrent extreme temperatures and water deficit influences the accumulation of phytochemicals and biological activities of *Portulacaria afra*. The study also recommends the use of the leaf, stem, and root in ethnomedicine as remedy for oral and skin infections, related diseases and as leads for drug formulations in Pharmaceutical industries.

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Running title: Effect of concurrent abiotic factors on *P. afra*

4.1 Introduction

Abiotic factors encompass a spectrum of challenging environmental elements that significantly influence ecological systems. These factors include, but are not limited to, temperature variations, drought occurrences, salinity levels, heavy metal concentrations, water stress, cold temperature exposure, anoxic conditions, high-intensity light exposure, and imbalances in nutrient availability (1).

The impact of abiotic factors, notably temperature fluctuations and water deficits, presents a significant impediment to the reproductive processes of medicinal plants. This interference consequently disrupts biosynthetic pathways, which in turn affects secondary metabolites accumulation and subsequent alterations in phytopharmacological activities (2).

Elevated temperatures induce heat stress, while reduced temperatures result in cold stress. The substantial impact of high temperatures extends to the composition of phytocompounds (3). Elevated temperatures typically correlate with heightened biosynthesis of secondary metabolites in plants, thereby enhancing the production of secondary metabolites across various plant species (4). Conversely, high temperatures inducing stress were observed to result in reduced levels of secondary metabolites in plants (5).

Water deficit results in a decrease of water absorption and potentials within plants, consequently negatively impacting various physiological processes and altering the biosynthesis of secondary metabolites (6). Earlier research has demonstrated the profound impact of water deficit or drought stress on the composition of secondary metabolites in herbal plants, leading to increased accumulation. An instance is the increased proportion of flavonoids and phenolics exhibited in plants as a response to the stress-induced by water deficit (7).

In adverse environmental conditions, plants exhibit resilience against diverse abiotic stresses through the activation of adaptive mechanisms (8). These mechanisms encompass the synthesis

of chemicals facilitating the assembly of proteins and cellular structures, osmotic adjustments, maintenance of general homeostasis, and modulation of the antioxidant system. Additionally, this response potentially leads to heightened production of secondary metabolites such as phenolics (1).

According to recent climate change models, it is more likely than previously thought that plants will experience novel or rigorous concurrent abiotic stresses in the years to come (9).

Earlier research findings have reported that plant responses to stresses caused by combined antagonistic abiotic factors, are phenomenal compared to when exposed to single ones (9). The long-term effects of these abiotic factors may significantly affect the quality and biochemical productivity of medicinal plants such as *P. afra* by disrupting the phytopharmacological activities, metabolic, developmental, and physiological processes (9). These have in turn contributed to identify the need to study abiotic stress combinations within medicinal plant species.

Portulacaria afra is a succulent evergreen plant indigenous to South Africa. The taxonomic classification of *Portulacaria afra* falls within the family *Portulacaceae* or alternatively recently categorized under *Didiereaceae.* According to ethnobotanical knowledge, and a few studies, *P. afra* leaves and leaf juice is renowned for its curative properties against skin diseases and oral infections (10) and phytochemical studies on *P. afra* tissue extracts revealed a strong presence of various phytochemicals (11). Also, it was previously demonstrated that *P. afra* extracts exhibited high levels of therapeutic properties in all plant parts, with a strong presence of coumarins known for anti-cancer properties in the methanolic leaf and stem extract (12). Basson *et al.* (13), also reported how *P. afra* leaves showed significant increase in phytochemicals and biological activities after exposure to elevated CO_2. The lack of comprehensive scientific documentation regarding the impact of combined environmental

factors on *P. afra* is a notable limitation. This prompted an investigation to determine the impact of concurrent extreme temperatures (hot and cold) with water deficit on the phytochemistry, antimicrobial and, antioxidant activities in *P. afra*.

Figure 4. 1 *Portulacaria afra* leaves, stems, and roots

4.2 Materials and Methods

In accordance with standard laboratory protocols, analytical-grade chemicals and solvents were used throughout the experimental procedures. The specific substances, namely gallic acid, Folin-Ciocalteau reagent, methanol, *n*-hexane, ethyl acetate, Mueller-Hinton agar, Baird Parker agar, dimethyl sulfoxide (DMSO), 2,2-diphenylpicrylhydrazyl (DPPH), and iron (II) chloride, were procured from Sigma-Aldrich, USA, bearing the corresponding CAS numbers1162-658.

In this study, the Conviron specifications were meticulously detailed: the model N0- PGW 40, bearing the serial N0- 170028, manufactured in China (280033R01). It operates at 230/400 Volts, 50 Hz frequency, and a three-phase system. The total input amperage is 29.2, with the compressor drawing 65.5 Amps, while designed to function under high pressure at 400 PSI and low pressure at 200 PSI. Control, lighting, heater, fan, and drier systems require 2.0, 9.4, 4.3,

2.6 HP, and 10.9 Amps respectively. Additionally, specific details regarding receptacle, glycol pump, motor, and auxiliary amperages were included. The system's minimum circuit ampacity (MCA) is 31.2 Amps, with a maximum overcurrent protection (MOP) set at 32 Amps, and a robust short circuit withstand capacity of 5000 Amps RMS.

Plant Material collection

In July 2021, cuttings of *Portulacaria afra* were cultivated at the University of the Witwatersrand in Johannesburg, South Africa (26.1899° S, 28.0319° E) for propagation. Green cuttings with non-lignified stems were carefully chosen from a pathogen-free parent plant of *P. afra*. To hold the stem cuttings for roots, 225 pots measuring 2 to 2.5 litres each were filled with professional cutting mix soil from Culterrean Potting.

The plants underwent a three-month acclimatization period and root system development in the greenhouse facilities within the premises of University of the Witwatersrand in Johannesburg, South Africa (26.1899° S, 28.0319° E). The plant growth was observed and sustained by administering 500 ml of water per potted plant every 48 hours due to their low water requirement.

Table 4. 1 Experimental Design

	Control	Treatment A	Treatment B	Treatment C	Treatment D
Water deficit	**500ml of water every 2nd day.**	**No water**	**No water**	**No water**	**No water**
Temperatures (˚C night/day)	**25/27**	**0/5**	**10/15**	**30/40**	**35/45**
Episodic harvesting frequency (hrs)	**Once off**	**48, 96, 144**	**48, 96, 144**	**48, 96, 144**	**48, 96, 144**

Sample size per harvest: n= 15/harvest

The conviron simulations settings used for the treatments were in accordance with the South African Department of Environmental Affairs predictions (14), IPCC (15), and Mbokodo *et al.* (16) records on the variations in the current and anticipated climatic conditions. The treatments were selected in terms of temperature ranges and the span of heat and cold waves. Control temperature ranges were based on records from the South African Weather Service. The different temperature settings used were control (25/27ºC), (0/5ºC), (10/15ºC), (30/40ºC), and (35/45ºC). Each plant was placed in the controlled climate simulation chamber (Conviron chambers, PGW40) with the high and low temperatures set concurrently with water deficit while other parameters such as CO_2 (400 ppm), humidity (60%), light (160 nits/level 1) pH, salinity, were kept at ambient. The lightning was programmed to operate for a duration of 12 hours daily, spanning from 6 am to 6 pm (17).

Five plants were harvested episodically 3 times for up to 6 days (144hrs), 48hrs (48, 96, 144) (Table 4. 1). Samples were subjected to oven drying at 40°C for a period ranging between 2 to 3 days post-harvest. This procedure was repeated three times for each of the treatments. Where,

n=45 control (25°), n=45 for (0/5°C), n=45 (10/15°C), n=45 (30/40°C), n=45 (35/45°C) with a total sample size of 225.

Sample preparation and extraction

The leaves, stems and roots were systematically gathered and subjected to a sequential process: initial harvesting, followed by thorough washing using deionized water, and subsequent drying in a hot air dryer maintained at 40°C for a duration of four days. Post-drying, the plant materials were finely ground into a crude powder using an electric grinder. This powdered form was then enclosed in foil and secured in an airtight container. Subsequently, it was stored in a dark cupboard at room temperature (32ºC) until required for subsequent testing procedures (18).

Preparation of extracts

The plant extract of the leaves, stems and roots were produced through sequential extraction using: 80% methanol, *n*-hexane, ethyl acetate, and distilled water, boiled at 60°C. Each extraction involved placing 3g of powdered plant material into individual glass bottles with cover, with the addition of 30 ml of the respective solvents (19). The methanol, *n*-hexane, ethyl acetate extracts were placed on a shaker, the water extract underwent preparation using a sonicator at a specific temperature (60°C) for a duration of 45 min. The shaking of all extracts lasted 48 hrs, except for the water extracts. The water extracts underwent centrifugation aimed at reducing their viscosity, followed by filtration through filter paper into vials. The residual supernatant was removed, and subsequently, the vials were enveloped in foil and refrigerated at 4ºC until the commencement of the tests.

Preliminary Phytochemical Screening Tests

The chemical constituents within the leaves, stems, and roots extracts underwent evaluation through visual observation for alterations in colour or precipitation with the adoption of the procedure described by Roghini and Vijayalakshmi (20). A comprehensive screening was

conducted to test for the presence of quinones, coumarins, phenols, carbohydrates, terpenoids, flavonoids, glycosides, steroids, saponins, amino acids, phytosteroids, and volatile oils, and the observation was documented.

Evaluation of Total Phenolics and Total Flavonoids Contents

Folin-Ciocalteu reagent assay was used for the approximation of the total phenolics and flavonoids content in *Portulacaria afra* extracts using the procedure outlined by Teffo *et al.* (21). Standard solutions of gallic acid at varying concentrations were used to construct a calibration curve. The concentrations of total phenolic content within the extracts were extrapolated from this curve, using the equation: $y=0.9287x+0.0658$, $r^2=0.9952$. Denoting the findings as gallic acid equivalents per gram (mg GAE/g) of the extract's dry weight.

Similarly, the total flavonoid contents of the plant parts were assessed using a quercetin-based standard curve, using the equation: $y=0.2388x-0.0019$, $r^2=0.9997$ with the results expressed as quercetin per gram (mg RE/g) of dry weight.

Antibacterial activity assay

The antibacterial inhibitory activity of *Portulacaria afra* extracts was assessed by following the agar-well diffusion procedure described by Teffo *et al*. (22) with a few modifications. The test microorganisms and agar were procured from Thermo-Fisher Laboratory Specialties (Pty) Ltd. Briefly, Petri dishes were first sterilized and autoclaved prior to the addition of Mueller-Hinton agar and Baird Parker agar. Once the agar solidified, sterile cotton swabs were used to uniformly inoculate the surfaces of the agar with specific test microorganisms. Baird Parker agar plates were inoculated with Gram+ *Staphylococcus aureus* (ATCC 25923), whereas Mueller-Hinton agar plates were inoculated with Gram- *Escherichia coli* (ATCC 25922), and Gram+ *Streptomyces griseus* and were incubated at 37ºC for 24hours.

Six wells were created in the inoculated media with successful growth of pathogens, using a 6 mm sterile borer. Subsequently, 100 µl of plant extracts were introduced into five of these wells, while the sixth well served as the negative control (dimethyl sulfoxide (DMSO). The plates were then covered and refrigerated for 30 minutes to allow for diffusion before being transferred to an incubator set at 37°C for 24 hours. Following the incubation period, the diameters of the zones of inhibition were measured in millimetres using a ruler. The presence of an inhibitory zone indicated positive antibacterial activity. The experiments were conducted in triplicate, and the data were analysed using Microsoft Excel to express the results as mean ± standard deviation. $P < 0.05$ was used to determine the statistical significance of the findings.

In vitro antioxidant activity assessment

The assessment of antioxidant potential was conducted using the metal chelating activity assay, hydrogen peroxide scavenging (H_2O_2), and the 2,2-diphenylpicrylhydrazyl (DPPH) free radical assay for the respective plant parts as described below:

DPPH scavenging activity assay

A 2, 2 diphenyl picryhydrazyl (DPPH) free radical assay described by Basson *et al*. (23) was used with some modifications. In the determination of the scavenging potential of DPPH, 50 mg of DPPH was dissolved in 100 ml of 80% methanol to create a stock solution. From this stock solution, a working solution was derived at a ratio of 1:5 with 80% methanol. Each extract had 5 concentrations, ranging from 10 to 50 µL, and was mixed with 700 µL of the working solution which was then adjusted to a final volume of 1 ml with 80% methanol to create a reaction mixture. A control solution was prepared using the working solution and 80% methanol, while a blank was generated using solely 80% methanol. These mixtures were then incubated in darkness for 45 minutes, after which their absorbance was measured at a

wavelength of 517 nm using a spectrophotometer. The inhibition (%) was expressed with the following equation:

DPPH Scavenging Effect (%) = [(Absorbance of sample – Absorbance of blank)/Absorbance of control] x 100

Metal chelating activity

The metal chelating antioxidant assay was carried out by following the procedure of Adusei *et al*. (24) with few modifications. 2 ml of extracts was combined with 0.25 ml of a 250 mM iron (II) chloride solution with an addition of 0.25 ml of ferrozine. The resulting mixture was vigorously shaken and left at room temperature (32ºC) for a duration of 10 minutes. Following this, the absorbance was quantified at a wavelength of 562 nm. Methanol was used as the blank, while a control group (without extracts) was used. The percentage of metal chelation was determined with the prescribed formula:

Metal chelation (%) = {(A0 – A1)/ A0} × 100

A0 represents the absorbance of the control while A1 stands for the absorbance of the test samples.

Hydrogen peroxide scavenging (H_2O_2) assay

The hydrogen peroxide scavenging activity was determined by adapting the methods of Bouabid *et al*. (25). 1 ml of extracts was combined with 3.4 ml of phosphate buffer (50 mM, pH 7.4). Following this, 600 µL of 40 mM hydrogen peroxide (30%) was introduced into the solution. The resulting mixture was left at room temperature (32ºC) for a duration of 40 minutes. Subsequently, the absorbance of the solution was determined using a spectrophotometer at a wavelength of 230 nm. The hydrogen peroxide scavenging activity was quantified using the provided formula:

Scavenged H_2O_2 (%) = [(Absorbance of control – Absorbance of sample)/Absorbance of control] x 1

Statistical Analysis

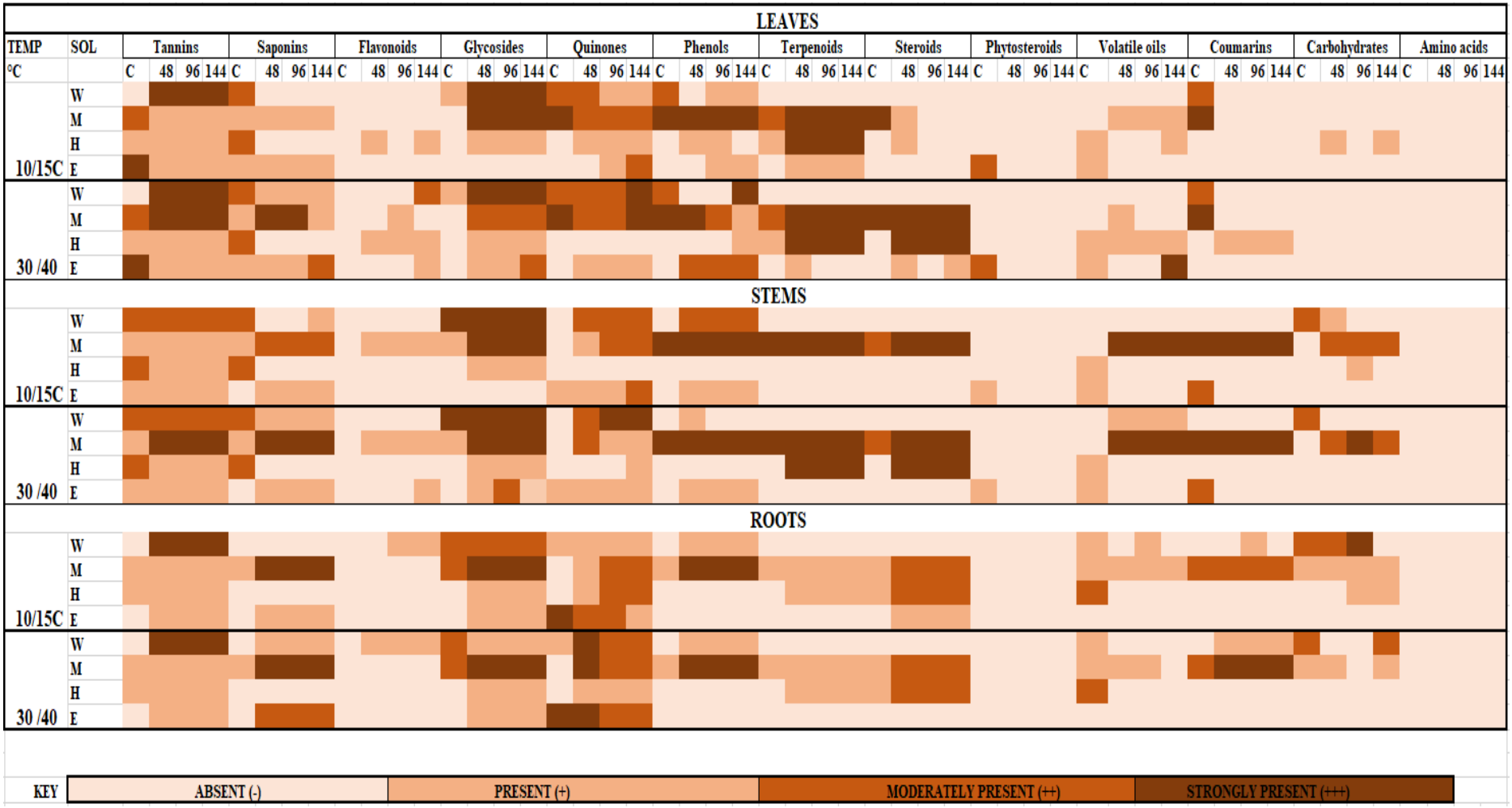
The concentration values were plotted against the % inhibition values in Microsoft Excel to derive a trend line equation facilitating the calculation of the IC_{50}. A one-way analysis of variance (ANOVA) was used to ascertain significant differences. All experiments were conducted thrice, and data were presented as mean ± standard error (SE) for n = 3. $P < 0.05$ was used to determine the statistical significance of the findings and Tukey HSD post hoc tests was used to ascertain where the significance lies.

4.3 Results

Qualitative screening of phytochemicals

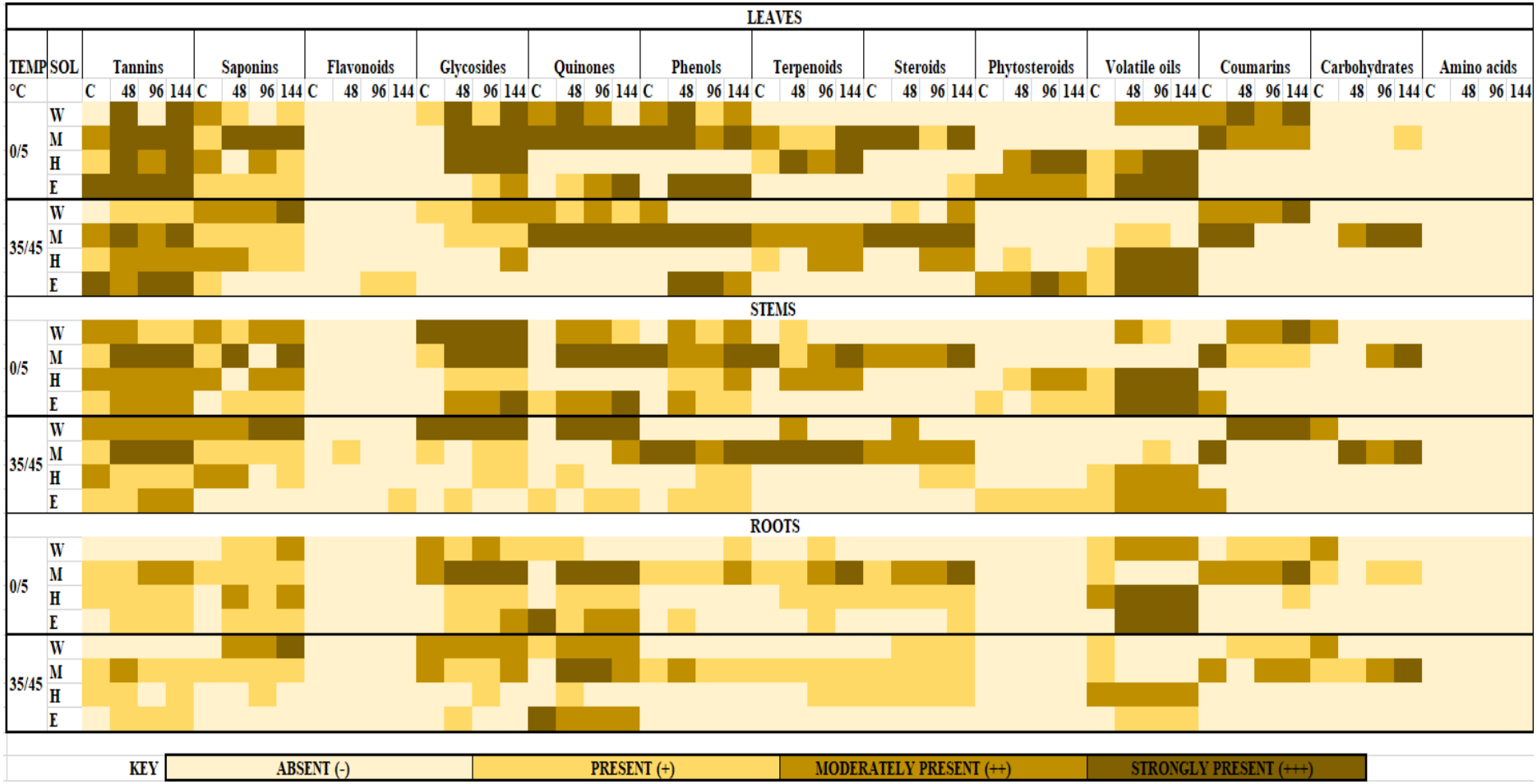
The aqueous (60°C), methanol, *n-hexane*, and ethyl acetate extracts of *P. afra* subjected to concurrent extreme hot and cold temperatures plus water deficit were screened for their phytochemical constituents. For the qualitative phytochemical screening, the leaf, stem, and root extracts were evaluated for the presence of saponins, flavonoids, glycosides, quinones, phenols, terpenoids, steroids, phytosteroids, volatile oil, carbohydrate, amino acids and coumarins. In general, phytochemicals in the plant parts varied according to the different solvents mentioned above. Increasing trends were noticed in the phytochemical presence under concurrent cold & hot temperatures with water deficit condition as shown on the heat map (Tables 4.2 and 4.3). Table 4.2 shows the fluctuations in the presence of phytochemical contents of the leaf, stem, and root of *P. afra*, when plant parts were subjected to concurrent mid-range cold & hot temperatures (10/15°C and 30/40°C) with water deficit conditions. Table 4.3 summarizes the results of the preliminary phytochemical screening under concurrent extreme cold & hot temperatures (0/5°C and 35/45°C) with water deficit conditions.

Table 4. 2 Heat map for the phytochemical tests of *Portulacaria afra* extracts exposed to temperatures (10/15°C and 30/40°C) and water deficit



C, control samples; W, hot water extract (60ºC) extract; M, methanolic extract; H, *n*-hexane extract; E, ethyl acetate extract

Table 4. 3 Heat map for the phytochemical tests of *Portulacaria afra* extracts exposed to temperatures (0/5°C and 35/45°C) and water deficit



C, control samples; W, hot water extract (60ºC) extract; M, methanolic extract; H, *n*-hexane extract; E, ethyl acetate extract

Quantitative analysis of phytochemicals

Quantitative results for the total phenolics (TPC) and total flavonoid content (TFC) in Figure 4. 2 – 4. 9, show the variations in the phytoconstituents across all the plant extracts, under concurrent cold and hot temperatures [(10/15ºC), (30/40ºC)] and [(0/5ºC), (35/45ºC)] with water deficit condition. The highest TPC and TFC in the plant extracts in comparison with the control samples were found in cold temperatures (10/15ºC) with water deficit settings (Figures 4. 2 and 4. 3 respectively). The above were recorded in the aqueous stem extracts after a 144-hour treatment period and aqueous leaf extracts after a 48-hour treatment period (9921.55±8.91mg GAE/g and 7529.09±3.12 mg QE/g; Figures 4. 2 and 4. 3 respectively) with control samples values for TPC (1820.67±25.08 mg GAE/g) and TFC values (592.70±49.99 mg QE/g) in the aqueous stem and leaf extracts respectively. ANOVA results showed a significant difference ($P < 0.05$) in *P. afra,* Tukey test in RStudio revealed where the significance lies among plant parts.

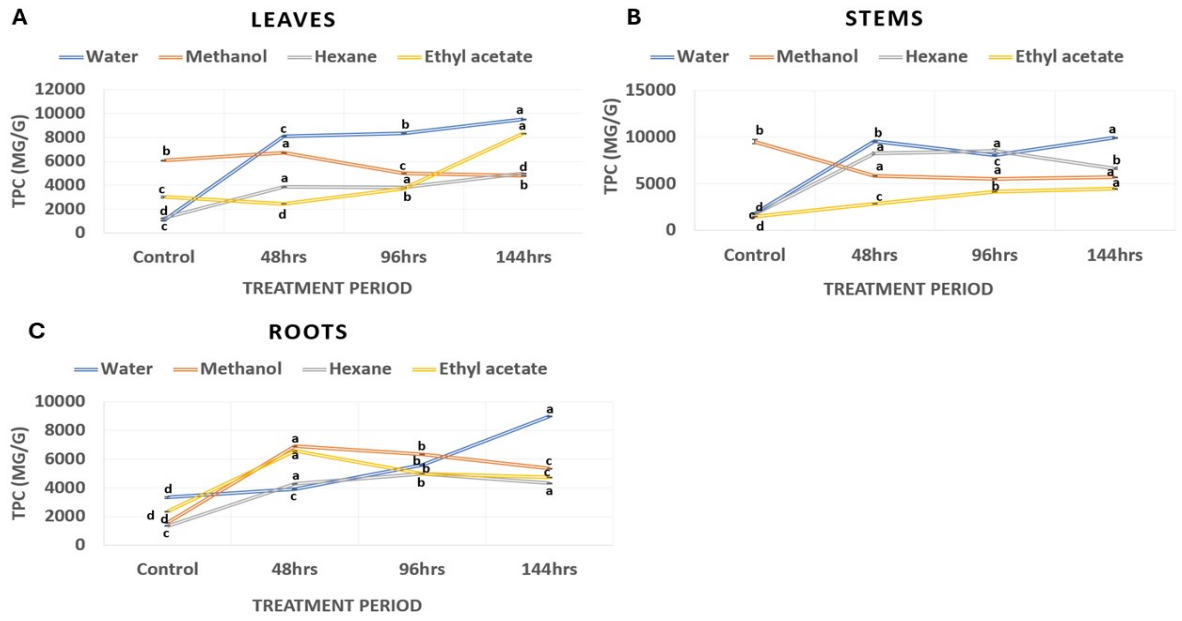


Figure 4. 2 Total Phenolic content in *Portulacaria afra* leaves (A), stems (B) and roots (C) extracts using four solvents exposed to 10/15ºC and water deficit. All values were presented as mean ± SE, n=3, and were quantified in terms of gallic acid (mg/g of GAE). Significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same group.

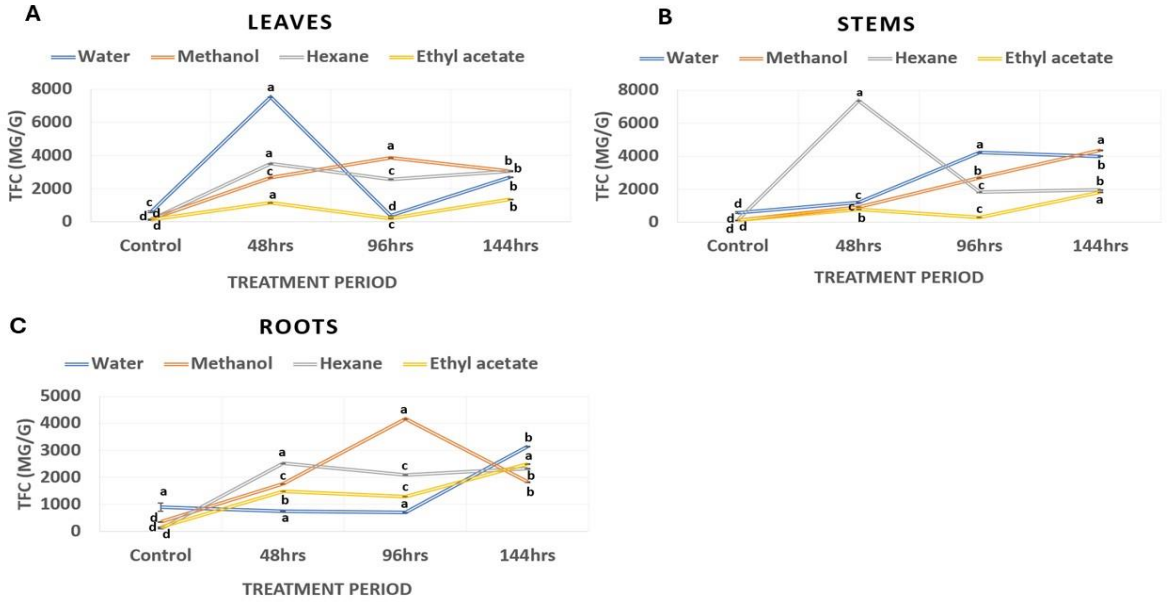


Figure 4. 3 Total Flavonoid content in *Portulacaria afra* leaves (A), stems (B) and roots (C) extracts using four solvents exposed to 10/15ºC and water deficit. All values were presented as mean ± SE, n=3, and were quantified in terms of quercetin concentration (mg/g of quercetin). Significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same

group.

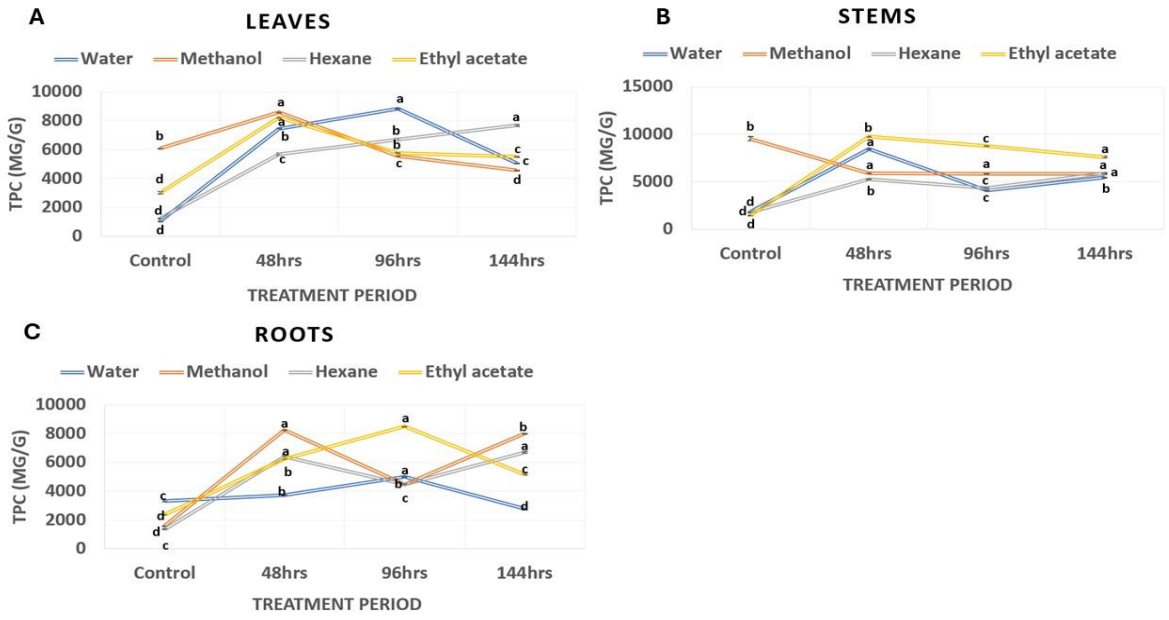


Figure 4. 4 Total Phenolic content in *Portulacaria afra* leaves (A), stems (B) and roots (C) extracts using four solvents exposed to 30/40ºC and water deficit. All values were presented as mean ± SE, n=3, and were quantified in terms of gallic acid (mg/g of GAE). Significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same group.

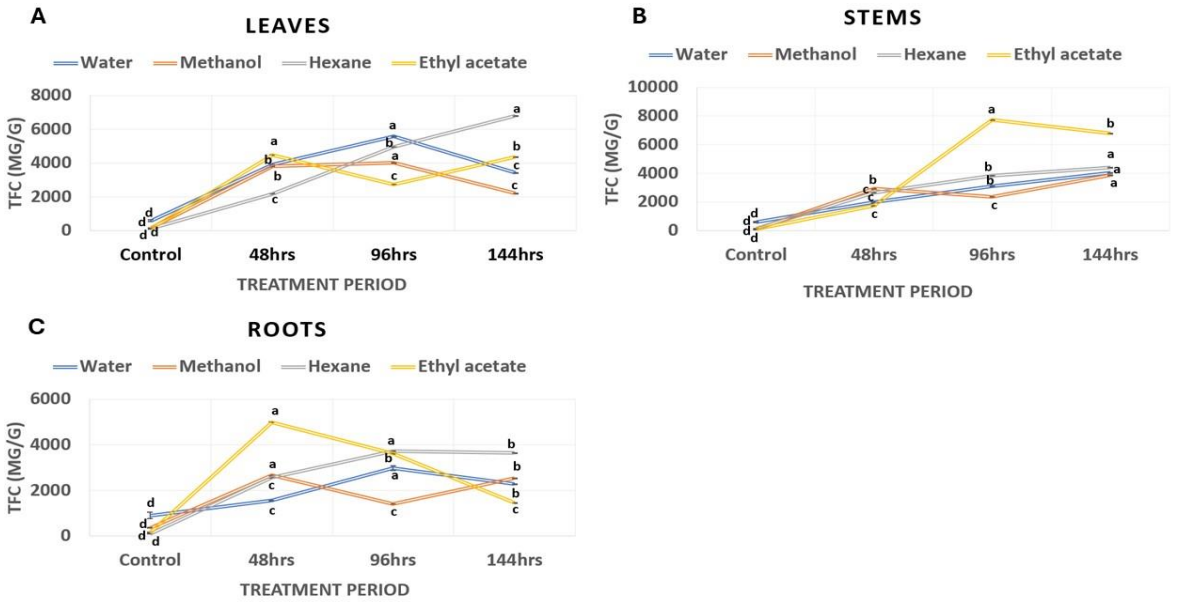


Figure 4. 5 Total Flavonoid content in *Portulacaria afra* leaves (A), stems (B) and roots (C) extracts using four solvents exposed to 30/40ºC and water deficit. All values were presented as mean ± SE, n=3, and were quantified in terms of quercetin concentration (mg/g of quercetin).

Significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same group.

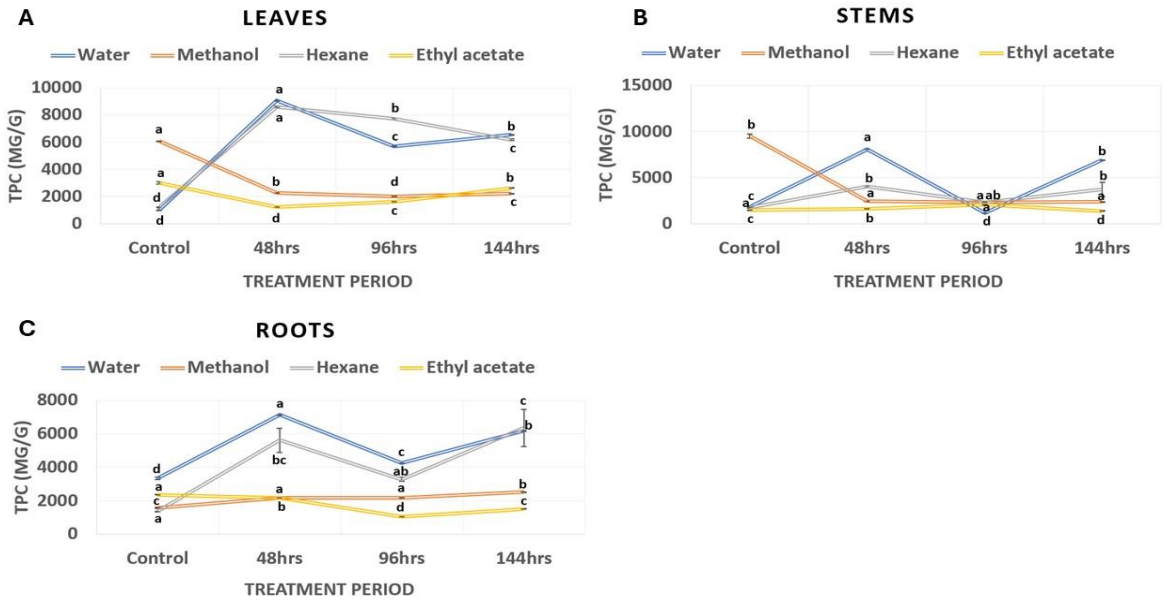


Figure 4. 6 Total Phenolic content in *Portulacaria afra* leaves (A), stems (B) and roots (C) extracts using four solvents exposed to 0/5ºC and water deficit. All values were presented as mean ± SE, n=3, and were quantified in terms of gallic acid (mg/g of GAE). Significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same group.

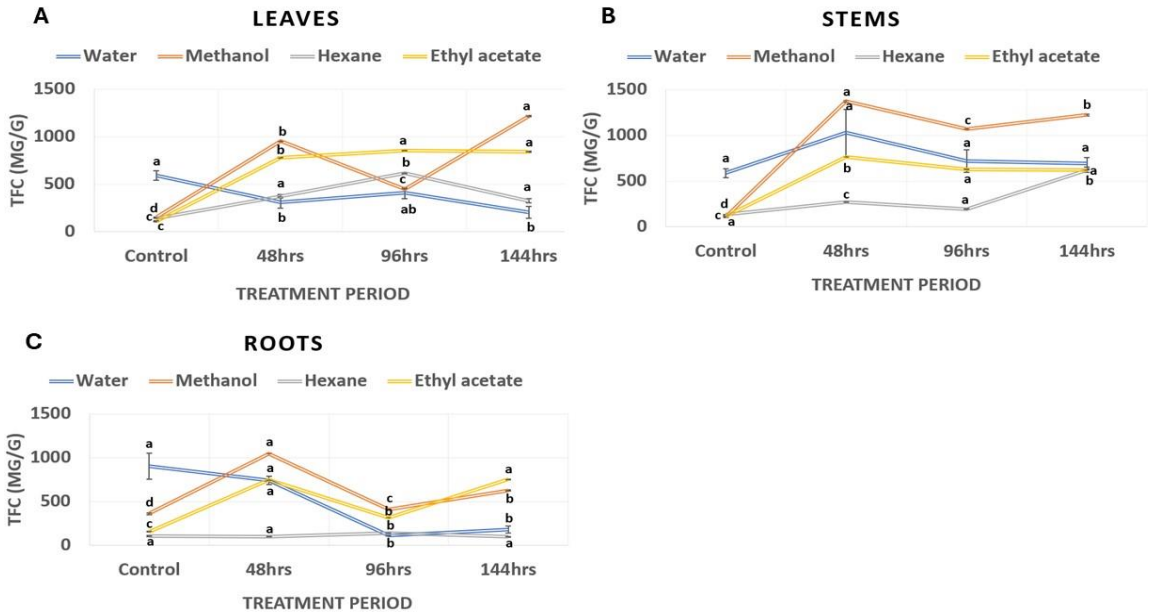


Figure 4. 7 Total Flavonoid content in *Portulacaria afra* leaves (A), stems (B) and roots (C) extracts using four solvents exposed to 0/5ºC and water deficit. All values were presented as

mean ± SE, n=3, and were quantified in terms of quercetin concentration (mg/g of quercetin). Significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same group.

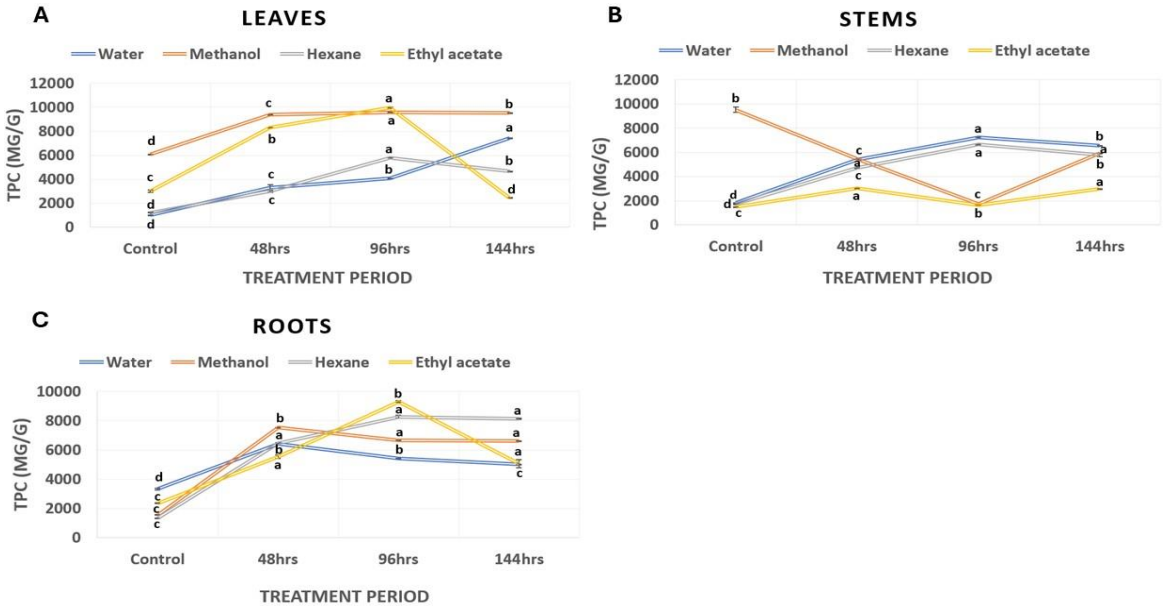


Figure 4. 8 Total Phenolic content in *Portulacaria afra* leaves (A), stems (B) and roots (C) extracts using four solvents exposed to 35/45ºC and water deficit. All values were presented as mean ± SE, n=3, and were quantified in terms of gallic acid (mg/g of GAE). Significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same group.

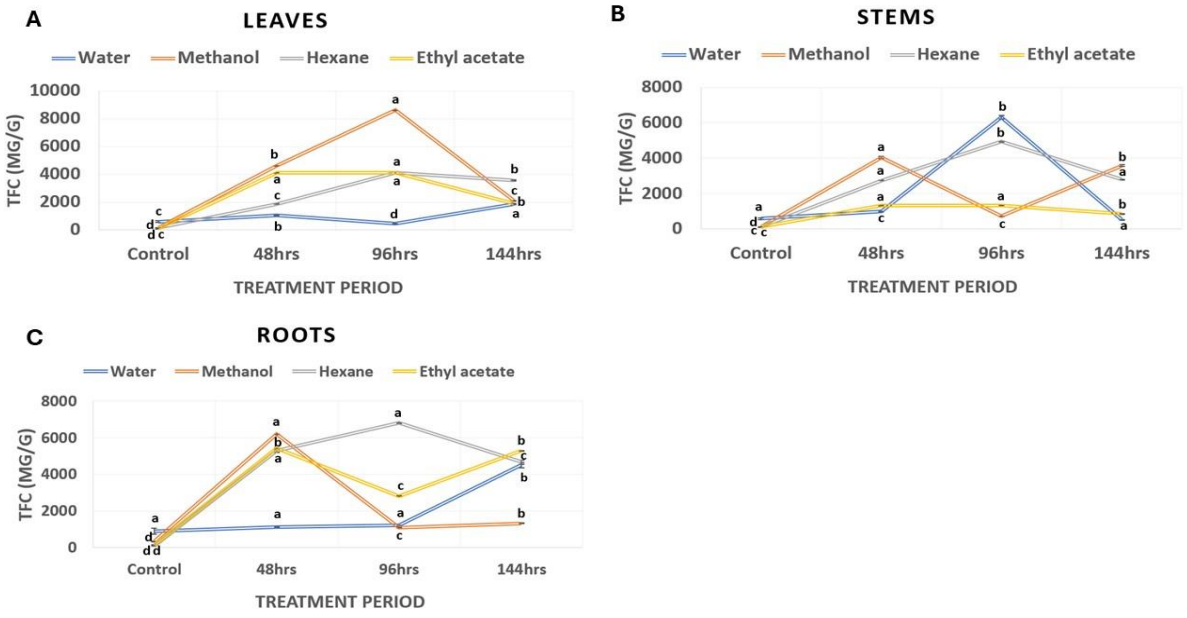


Figure 4. 9 Total Flavonoid content in *Portulacaria afra* leaves (A), stems (B) and roots (C)

extracts using four solvents exposed to 35/45ºC and water deficit. All values were presented as mean ± SE, n=3, and were quantified in terms of quercetin concentration (mg/g of quercetin). Significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same group.

Antibacterial activity assay

Observed variation in the inhibitory effect of *Portulacaria afra* extracts against the tested microorganisms are presented in Table 4. 4 – 4. 7. The highest level of inhibitory effect of 21mm against gram-negative *Escherichia coli* in comparison with the control samples was exhibited by the methanolic root extracts after a 48hr-treatment period, under combined extreme hot temperatures (35/45ºC) and water deficit, (Table 4. 7). This was also followed by a considerable effect of 14mm against *Escherichia coli* in the methanolic stem extracts (Table 4. 7). Furthermore, the inhibitory activities observed in the ethyl acetate extracts were intermediate, ranging from 9-12mm, with an increasing trend when compared with the control samples (Table 4. 7). No inhibitory activity was observed against *Escherichia coli* across all the plant extracts exposed to hot temperatures (30/40ºC), however, intermediate inhibitory activities ranged from 11-13mm against gram-positive *Streptomyces griseus* and *Staphylococcus aureus* (Table 4. 6). Under the cold temperatures (10/15ºC) with water deficit condition (Table 4. 4), a slight inhibitory effect of 11mm was observed only in the *n*-hexane and ethyl acetate root extracts against gram-positive *Streptomyces griseus* and *Staphylococcus aureus* after a 48hr and 96-hour treatment period respectively, with a decline in activity when compared with the control samples. However, there were no inhibitory activities observed across all the plant extracts exposed to cold temperatures (0/5ºC) with water deficit condition (Table 4. 5). From these findings, the examined plant extracts exposed to temperatures [(30/40ºC) and (35/45ºC)] with water deficit condition showed better antimicrobial activity against all the tested microorganisms. ANOVA results showed a significant difference ($P <$

0.05) in *P. afra* extracts. The findings indicate that the antimicrobial activity of *P. afra* extracts is subject to the plant part and temperature with water deficit conditions.

Table 4. 4 Antibacterial activity of *Portulacaria afra* extracts exposed to 10/15°C and water deficit

	Zone of inhibition (millimetres) of extracts															
		Treatment period (hours)														
	Microorganisms	**Methanol**				**Hexane**				**Water (60°C)**				**Ethyl Acetate**			
		C	**48**	**96**	**144**	**C**	**48**	**96**	**144**	**C**	**48**	**96**	**144**	**C**	**48**	**96**	**144**
Leaves	*Escherichia coli*	0	0	0	0	16±0.6	0	0	0	0	0	0	0	0	0	0	0
	Staphylococcus aureus	0	0	0	0	18±0.6	0	0	0	0	0	0	0	14±0.6	0	0	0
	Streptomyces griseus	0	0	0	0	18±0.6	0	0	0	0	0	0	0	0	0	0	0
Stems	*Escherichia coli*	0	0	0	0	13±0.6	0	0	0	0	0	0	0	0	0	0	0
	Staphylococcus aureus	0	0	0	0	13±0.6	0	0	0	0	0	0	0	0	0	0	0
	Streptomyces griseus	0	0	0	0	13±0.6	0	0	0	0	0	0	0	0	0	0	0
Roots	*Escherichia coli*	0	0	0	0	15±0.6	0	0	0	0	0	0	0	0	0	0	0
	Staphylococcus aureus	0	0	0	0	17±0.6	0	0	0	24±0.6	0	0	0	0	0	11±0.3	0
	Streptomyces griseus	0	0	0	0	18±0.6	11±0.1	0	0	0	0	0	0	0	0	0	0

C, Control sample extract; 48, 48hr-treatment extract; 96, 96hr-treatment extract; 144, 144hr-treatment extracts. Values are presented as mean ± Standard Deviation, n=3.

Table 4. 5 Antibacterial activity of *Portulacaria afra* extracts exposed to 0/5°C and water deficit

	Zone of inhibition ((millimetres) of extracts **Treatment period (hours)**															
	Microorganisms	**Methanol**				**Hexane**				**Water (60ºC)**				**Ethyl Acetate**			
		C	**48**	**96**	**144**	**C**	**48**	**96**	**144**	**C**	**48**	**96**	**144**	**C**	**48**	**96**	**144**
Leaves	*Escherichia coli*	0	0	0	0	16±0.6	0	0	0	0	0	0	0	0	0	0	0
	Staphylococcus aureus	0	0	0	0	18±0.6	0	0	0	0	0	0	0	14±0.6	0	0	0
	Streptomyces griseus	0	0	0	0	18±0.6	0	0	0	0	0	0	0	0	0	0	0
Stems	*Escherichia coli*	0	0	0	0	13±0.6	0	0	0	0	0	0	0	0	0	0	0
	Staphylococcus aureus	0	0	0	0	13±0.6	0	0	0	0	0	0	0	0	0	0	0
	Streptomyces griseus	0	0	0	0	13±0.6	0	0	0	0	0	0	0	0	0	0	0
Roots	*Escherichia coli*	0	0	0	0	15±0.6	0	0	0	0	0	0	0	0	0	0	0
	Staphylococcus aureus	0	0	0	0	17±0.6	0	0	0	24±0.6	0	0	0	0	0	0	0
	Streptomyces griseus	0	0	0	0	18±0.6	0	0	0	0	0	0	0	0	0	0	0

C, Control sample extract; 48, 48hr-treatment extract; 96, 96hr-treatment extract; 144, 144hr-treatment extracts. Values are presented as mean ± Standard Deviation, n=3

Table 4. 6 Antibacterial activity of *Portulacaria afra* extracts exposed to 30/40°C and water deficit

	Zone of inhibition ((millimetres) of extracts **Treatment period (hours)**															
	Microorganisms	**Methanol**				**Hexane**				**Water (60ºC)**				**Ethyl Acetate**			
		C	**48**	**96**	**144**	**C**	**48**	**96**	**144**	**C**	**48**	**96**	**144**	**C**	**48**	**96**	**144**
Leaves	*Escherichia coli*	0	0	0	0	16±0.6	0	0	0	0	0	0	0	0	0	0	0
	Staphylococcus aureus	0	0	0	0	18±0.6	0	0	0	0	0	0	0	14±0.6	0	0	0
	Streptomyces griseus	0	0	0	0	18±0.6	0	0	0	0	0	0	0	0	0	0	0
Stems	*Escherichia coli*	0	0	0	0	13±0.6	0	0	0	0	0	0	0	0	0	0	0
	Staphylococcus aureus	0	0	0	11±0.1	13±0.6	0	12±0.1	0	0	0	0	0	0	0	0	0
	Streptomyces griseus	0	0	0	0	13±0.6	0	0	0	0	0	0	0	0	0	11±0.1	0
Roots	*Escherichia coli*	0	0	0	0	15±0.6	0	0	0	0	0	0	0	0	0	0	0
	Staphylococcus aureus	0	0	0	0	17±0.6	0	0	0	24±0.6	13±0.1	0	0	0	0	0	0
	Streptomyces griseus	0	0	0	0	18±0.6	0	0	0	0	0	0	0	0	0	0	0

C, Control sample extract; 48, 48hr-treatment extract; 96, 96hr-treatment extract; 144, 144hr-treatment extracts. Values are presented as mean ± Standard Deviation, n=3.

Table 4. 7 Antibacterial activity of *Portulacaria afra* extracts exposed to 35/45°C and water deficit

	Zone of inhibition ((millimetres) of extracts **Treatment period (hours)**															
	Microorganisms	**Methanol**				**Hexane**				**Water (60ºC)**				**Ethyl Acetate**			
		C	**48**	**96**	**144**	**C**	**48**	**96**	**144**	**C**	**48**	**96**	**144**	**C**	**48**	**96**	**144**
Leaves	*Escherichia coli*	0	0	0	10±0.1	16±0.6	0	0	0	0	0	0		0	0	10±0.2	11±0.3
	Staphylococcus aureus	0	0	0	0	18±0.6	0	0	0	0	0	0	0	14±0.6	0	0	0
	Streptomyces griseus	0	0	0	0	18±0.6	0	0	0	0	0	0	0	0	0	0	0
Stems	*Escherichia coli*	0	14±0.1	0	0	13±0.6	0	0	0	0	0	0	0	0	0	12±0.1	12±0.3
	Staphylococcus aureus	0	0	0	0	13±0.6	0	0	0	0	0	0	0	0	0	0	0
	Streptomyces griseus	0	0	0	0	13±0.6	0	0	0	0	0	0	0	0	0	0	0
Roots	*Escherichia coli*	0	21±0.1	0	0	15±0.6	0	0	0	0	0	0	0	0	9±0.1	11±0.2	10±0.1
	Staphylococcus aureus	0	0	0	0	17±0.6	0	0	0	24±0.6	0	0	0	0	0	0	0
	Streptomyces griseus	0		0	0	18±0.6	0	0	0	0	0	0	0	0	0	0	0

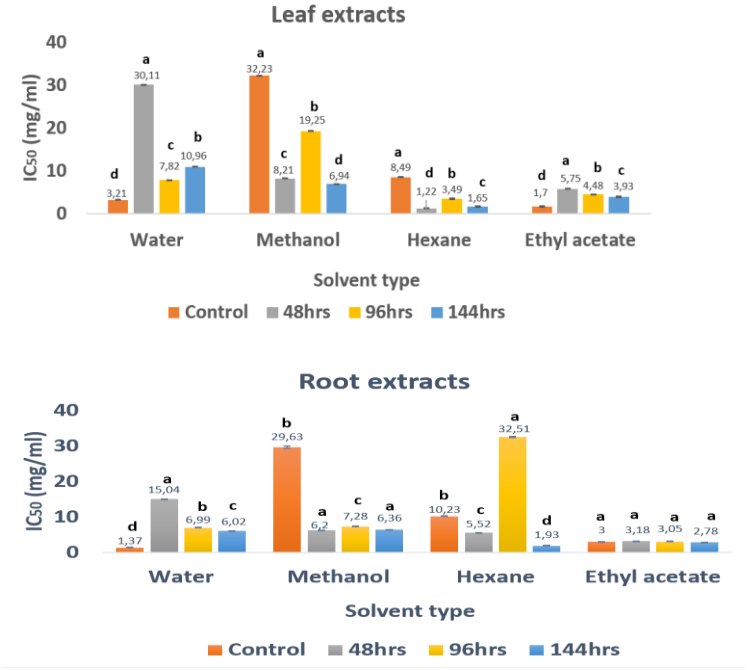
C, Control sample extract; 48, 48hr-treatment extract; 96, 96hr-treatment extract; 144, 144hr-treatment extracts. Values are presented as mean ± Standard Deviation, n=3

Antioxidant Activity

The *in vitro* antioxidant potential of all the extracts was determined by a 2, 2 diphenylpicryhydrazyl (DPPH) free radical assay, hydrogen peroxide scavenging (H_2O_2), and metal chelating activity assay, where IC_{50} value is the amount of extract required to decrease the absorbance of antioxidant radicals by 50%. Lower IC_{50} values indicate stronger antioxidant scavenging ability of the extracts. Figure 4. 10 – 4. 21 graphically show the various changes observed in the antioxidant activities of *Portulacaria afra* extracts under hot and cold temperatures concurrently and water deficit, when compared with the control samples. Under the cold temperatures [(10/15ºC) and (0/5ºC)] plus water deficit condition (Figure 4. 10 – 4. 15), a significant increasing trend of antioxidant capacity was observed across all plant parts when compared to the control samples. Similarly, under hot temperatures [(30/40ºC) and (35/45ºC)] with water deficit conditions (Figure 4. 16 – 4. 21), an increase in antioxidant activity was observed in some of the extracts in comparison with the control samples. The highest DPPH and metal-chelating antioxidant activity was observed under hot temperatures (30/40ºC) in the ethyl acetate root extracts after a 96-hour treatment period and the methanolic leaf extracts after a 144-hour treatment period (0.26±0.065 mg/ml; Figure 4. 16) and (0.40±0.078 mg/ml respectively; Figure 4. 17). However, *n*-hexane stem extracts under concurrent extreme hot temperatures (35/45ºC) with water deficit conditions showed the strongest hydrogen peroxide scavenging activity (0.14±0.048 mg/ml; Figure 4. 21) after a144-hour treatment period.

Data available here reveal excellent antioxidant activity of all plant parts. The methanolic leaf extracts showed the strongest antioxidant activity against ferric ion radical, ethyl acetate root extracts also exhibited a very strong scavenging ability against DPPH radical, while *n*-hexane stems extract scavenged hydrogen peroxide radical much better, indicating the strongest

antioxidant activity of *Portulacaria afra* across the three different assays. All IC_{50} values were less than 1 mg/ml with extracts showing significant differences ($P < 0.05$)



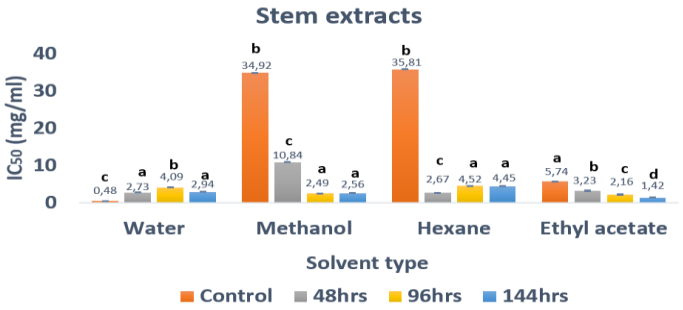
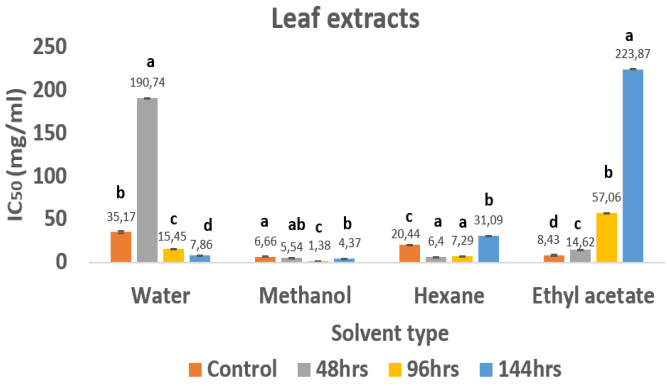
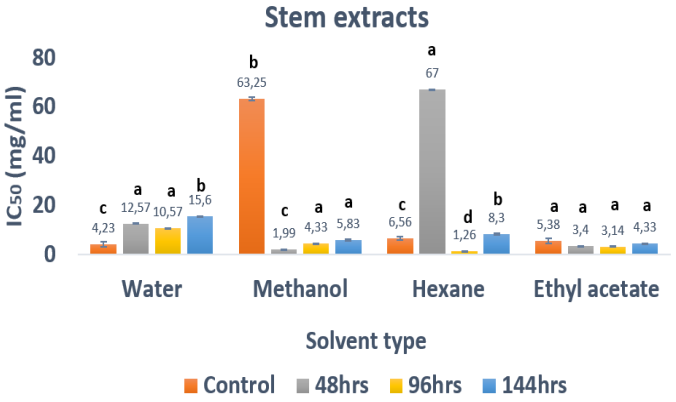


Figure 4. 10 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity in *Portulacaria afra* plant parts exposed to 10/15°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test).





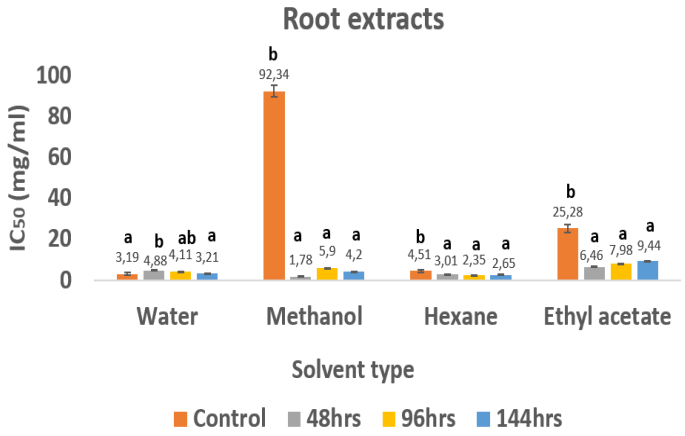


Figure 4. 11 Metal chelating antioxidant activity in *Portulacaria afra* plant parts exposed to 10/15°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test).

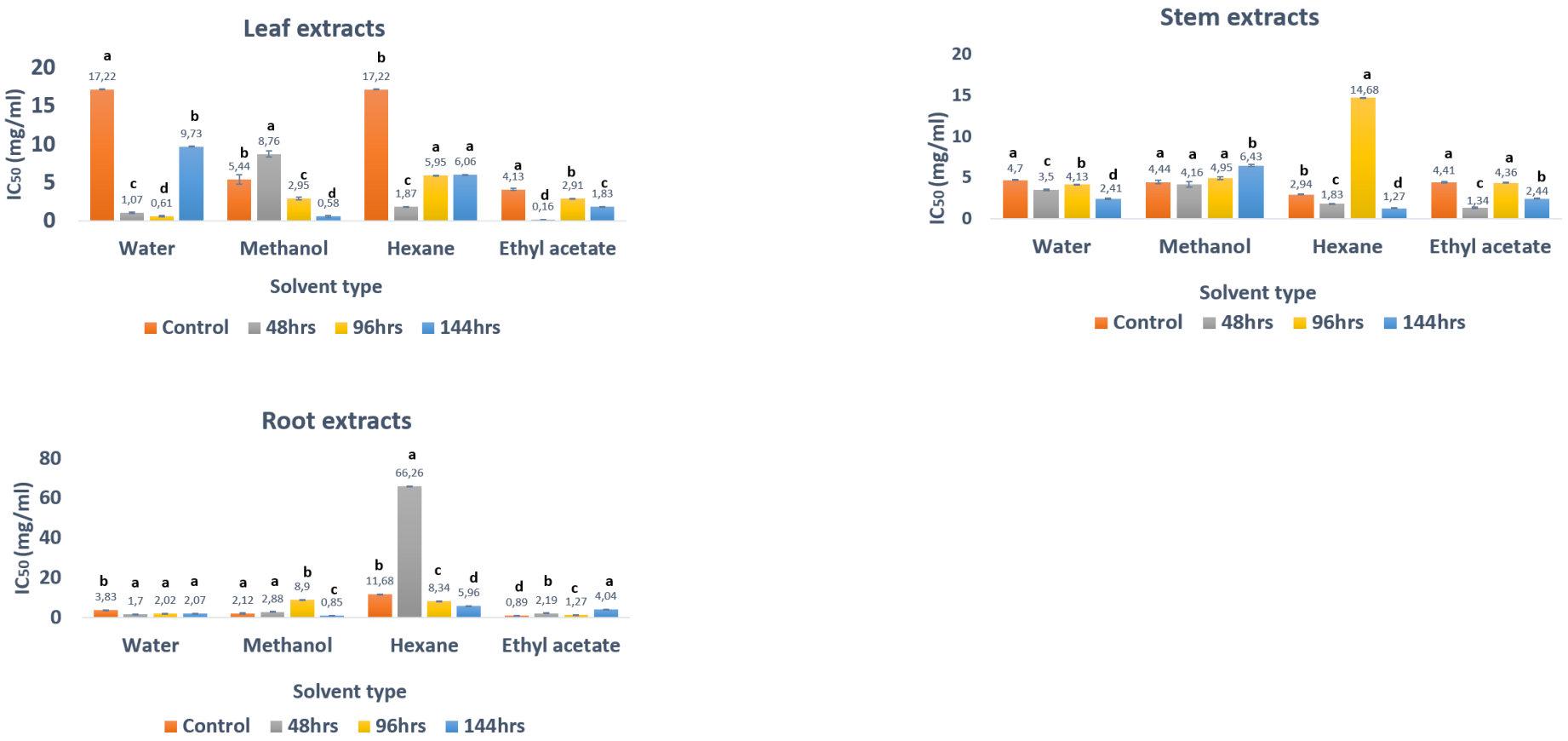
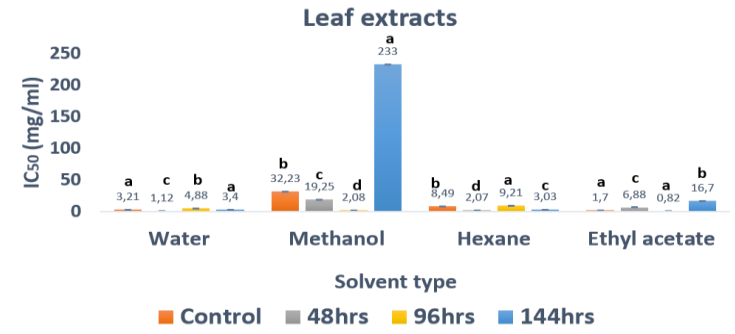
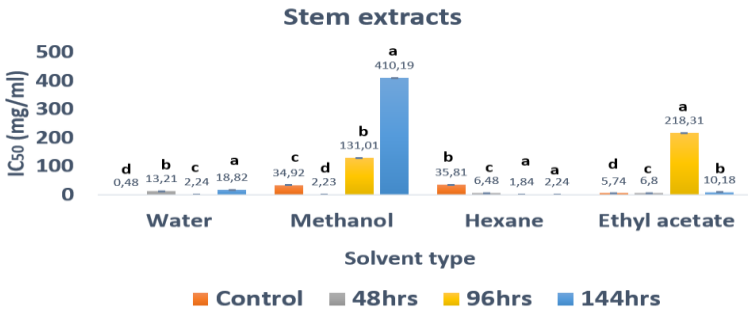


Figure 4. 12 Hydrogen peroxide antioxidant activity in *Portulacaria afra* plant parts exposed to 10/15°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test).





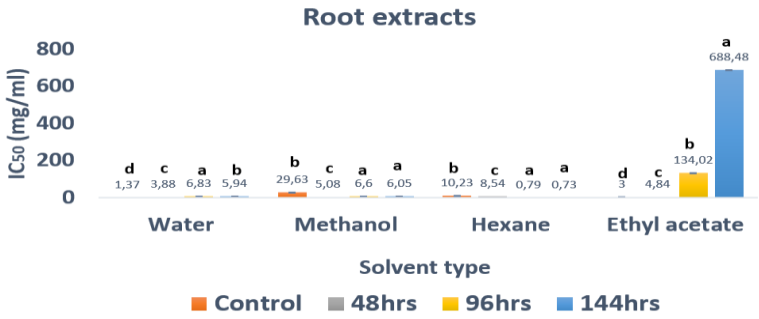
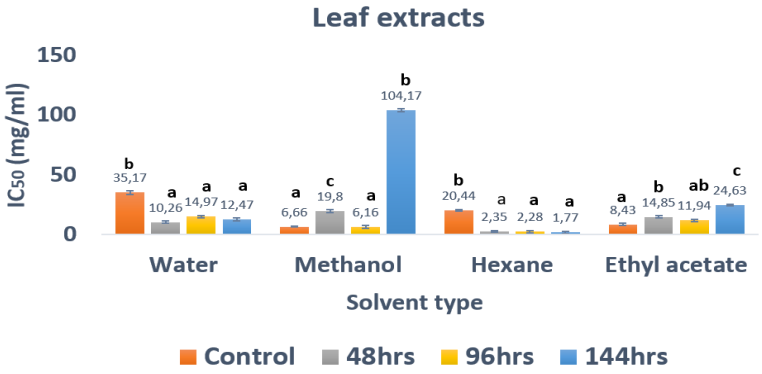
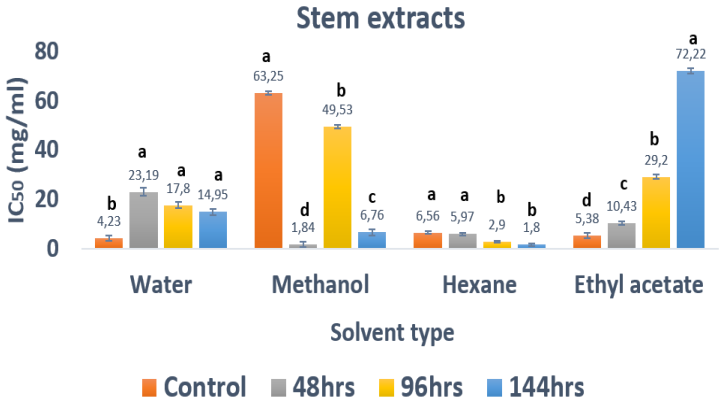


Figure 4. 13 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity in *Portulacaria afra* plant parts exposed to 0/5°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test).





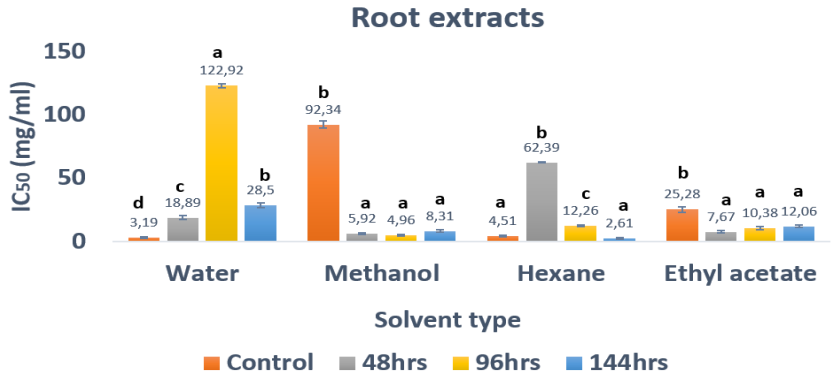


Figure 4. 14 Metal chelating antioxidant activity in *Portulacaria afra* plant parts exposed to 0/5°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test)

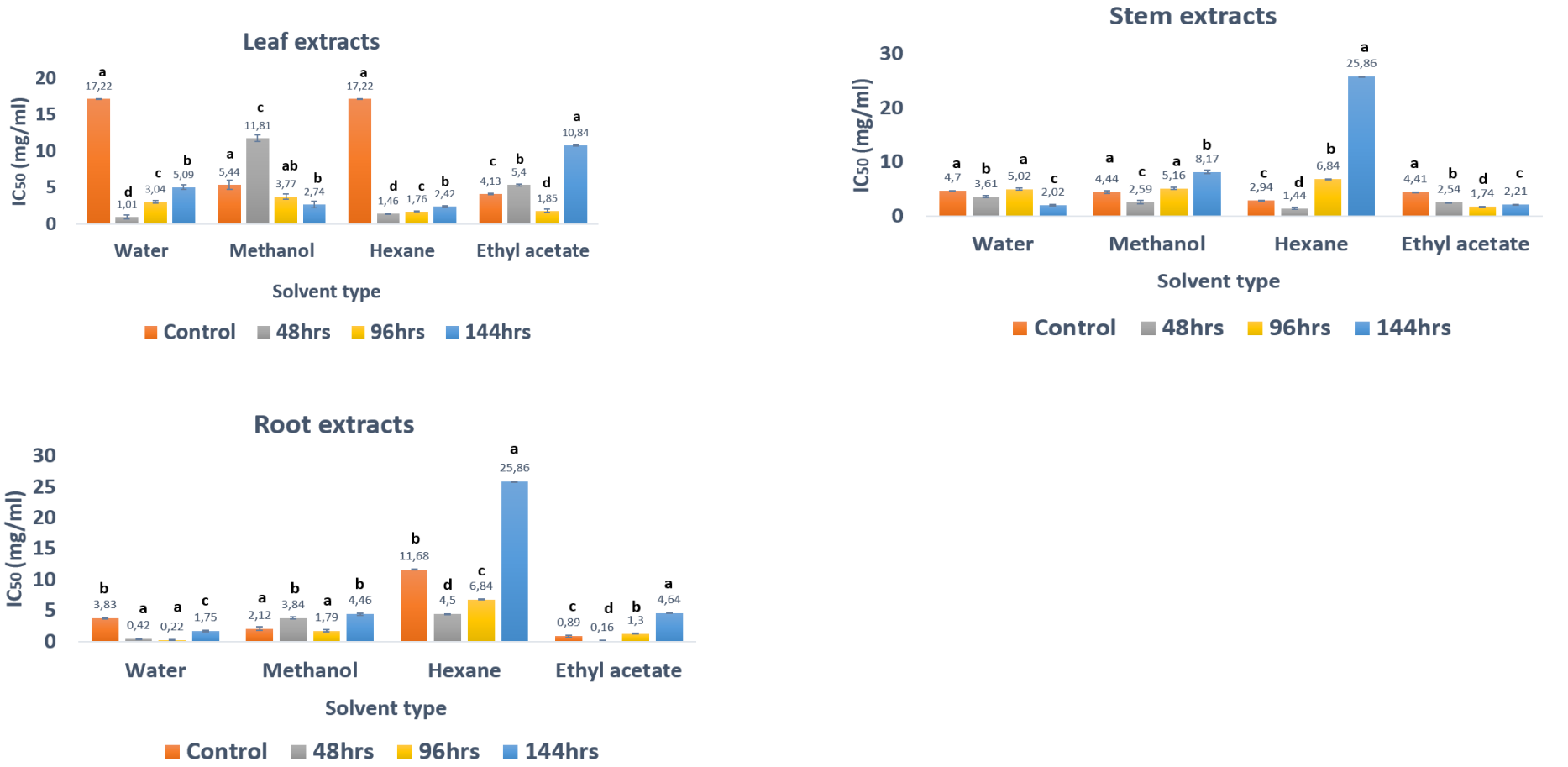
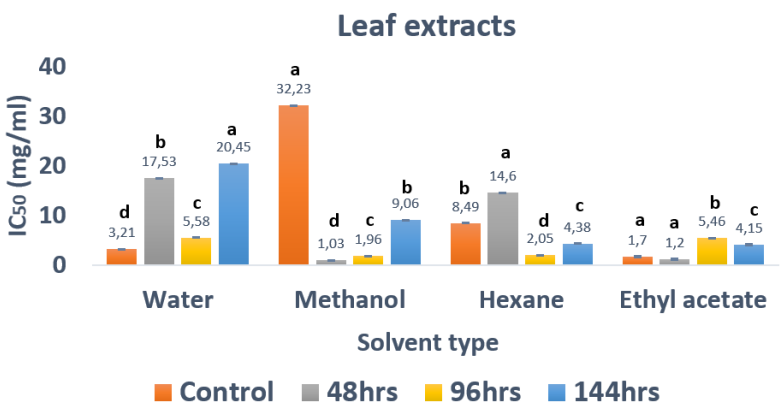
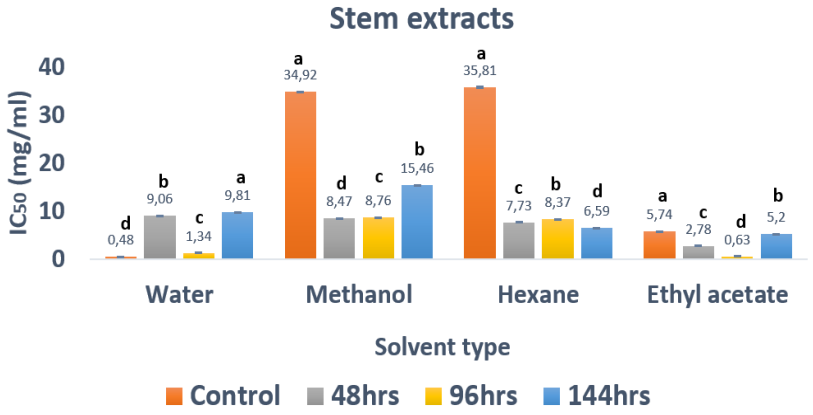


Figure 4. 15 Hydrogen peroxide antioxidant activity in *Portulacaria afra* plant parts exposed to 0/5°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test).





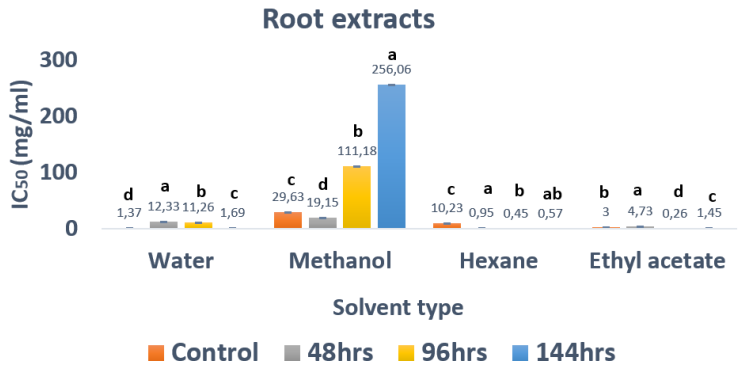
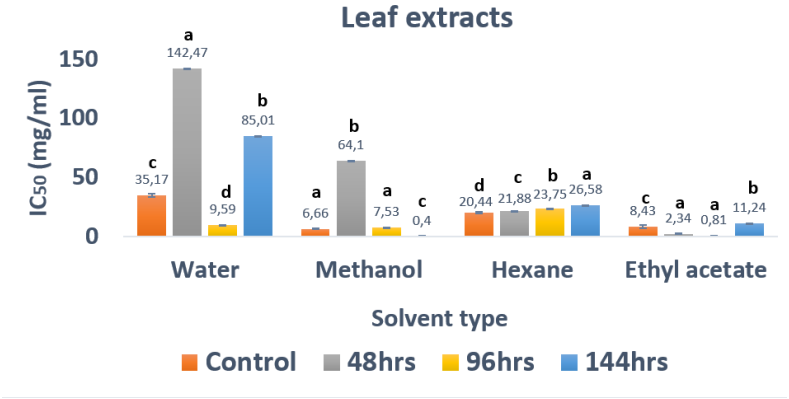
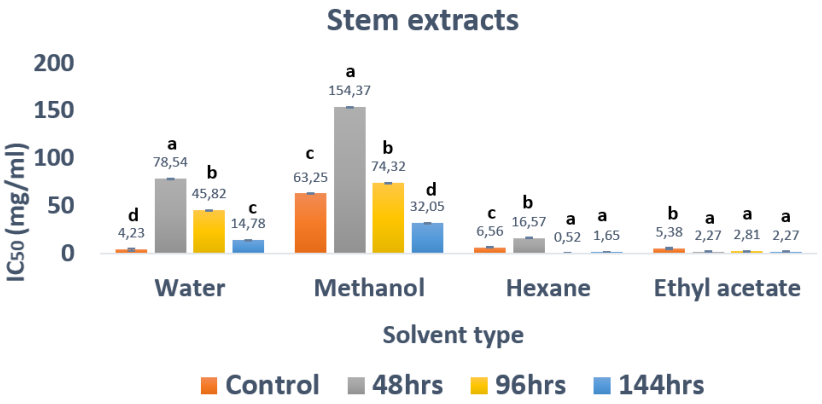


Figure 4. 16 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity in *Portulacaria afra* plant parts exposed to 30/40°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a,b,c, d) within the same column (Tukey's post-hoc test).





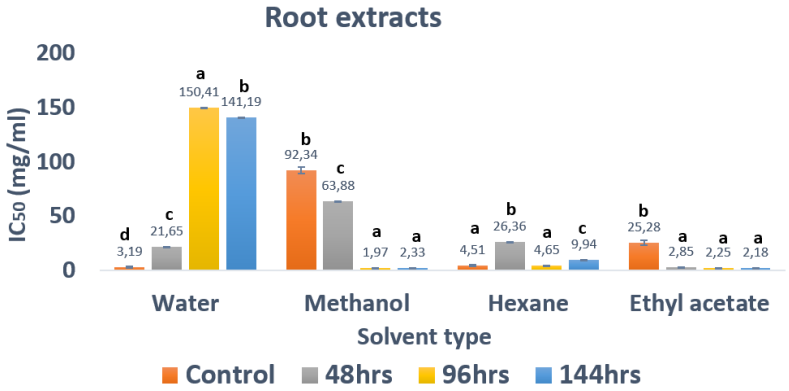


Figure 4. 17 Metal chelating antioxidant activity in *Portulacaria afra* plant parts exposed to 30/40°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test).

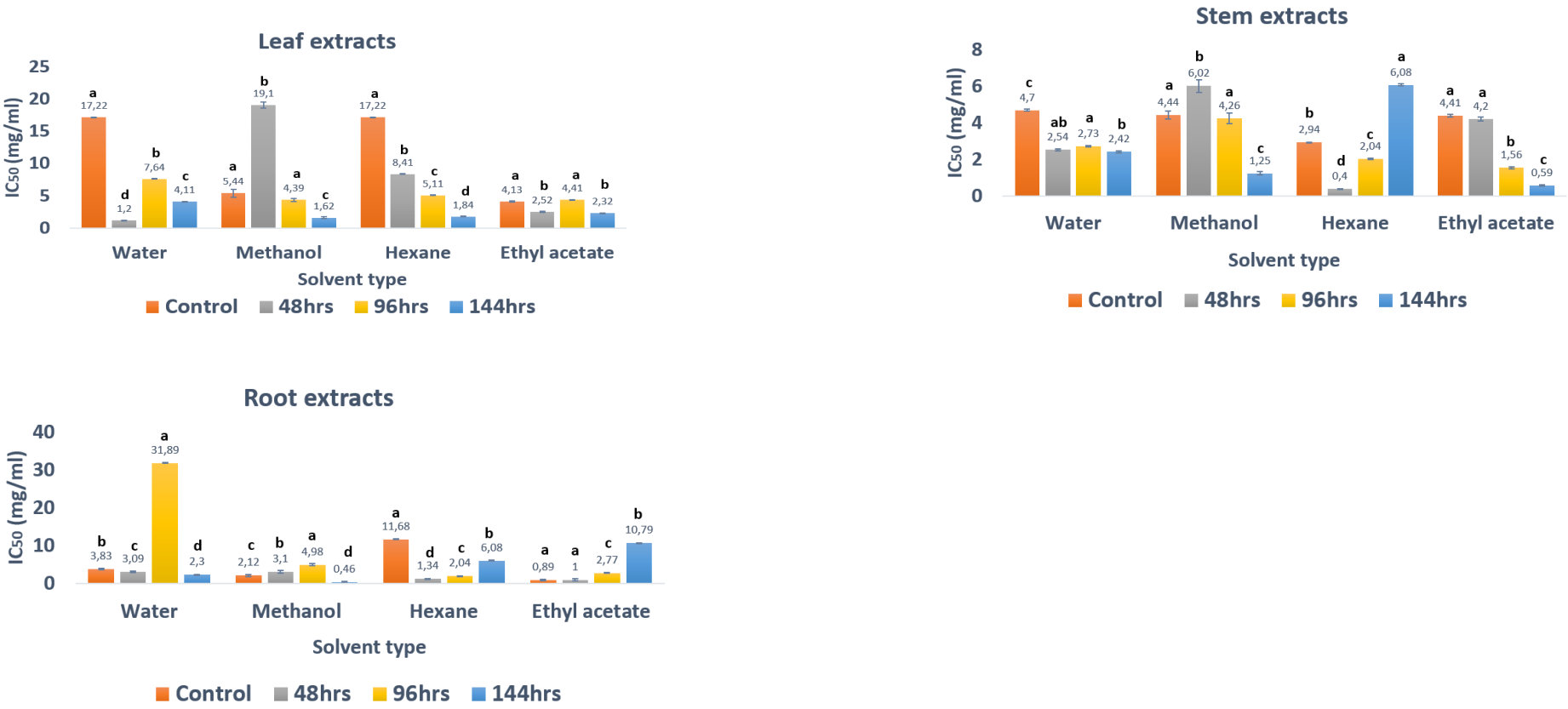


Figure 4. 18 Hydrogen peroxide antioxidant activity in *Portulacaria afra* plant parts exposed to 30/40°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test).

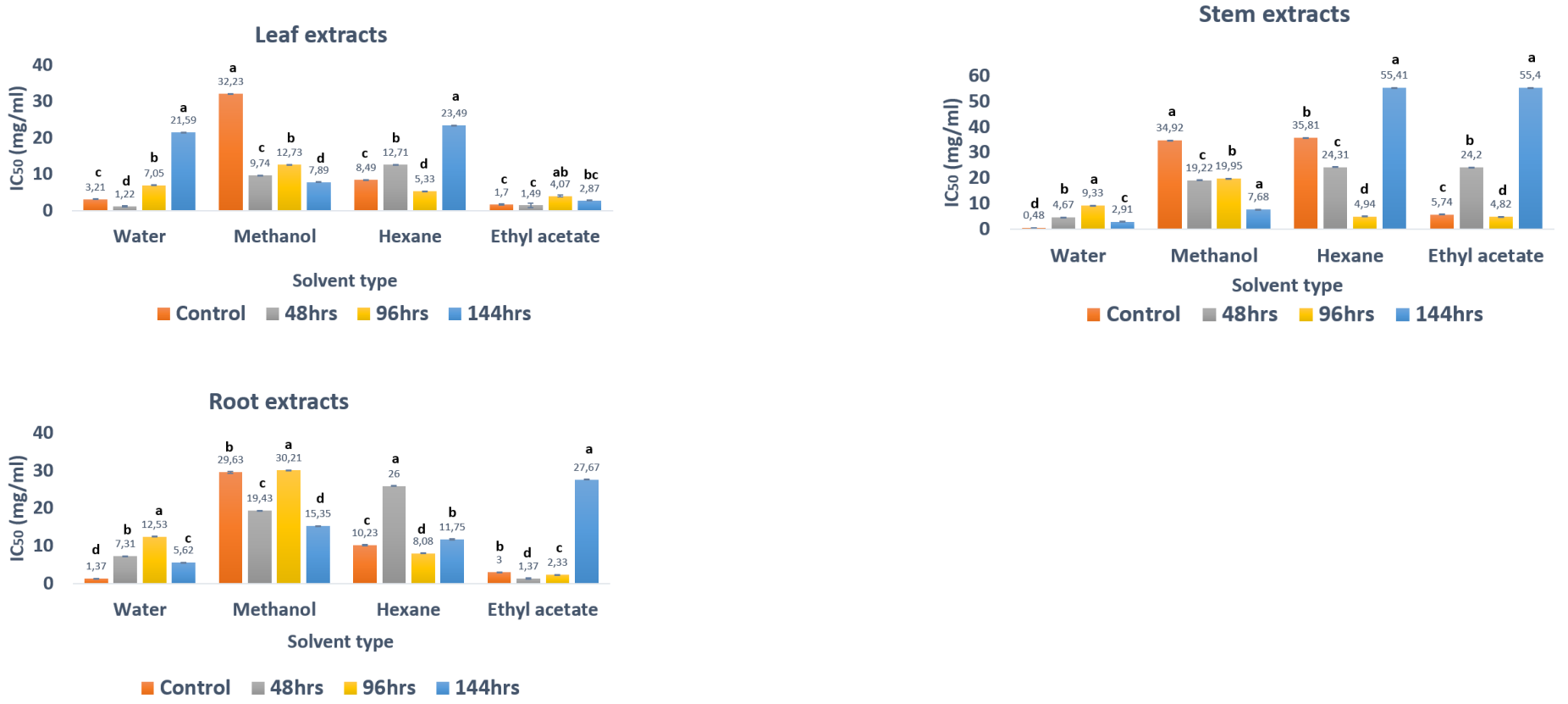


Figure 4. 19 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity in *Portulacaria afra* plant parts exposed to 35/45°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test).

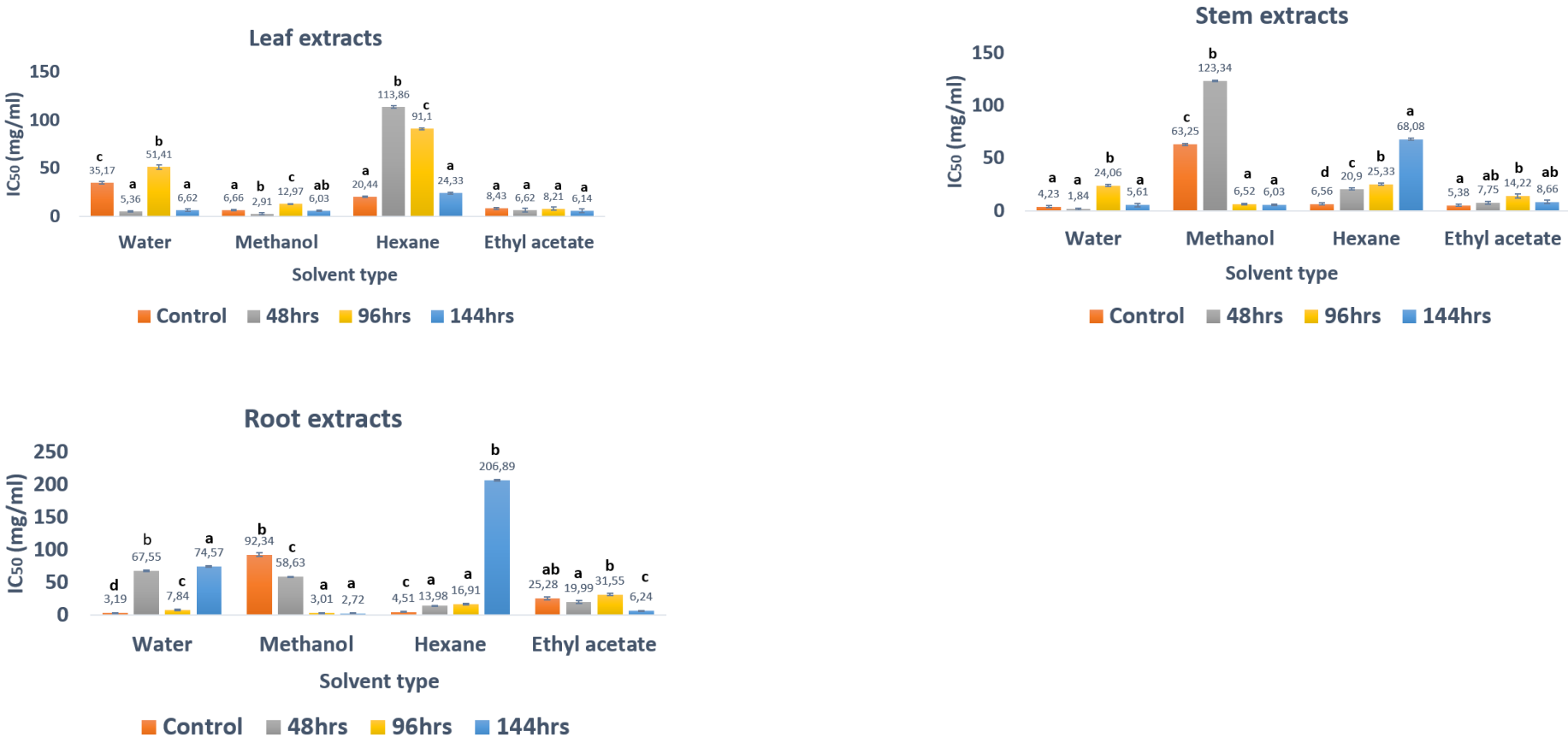
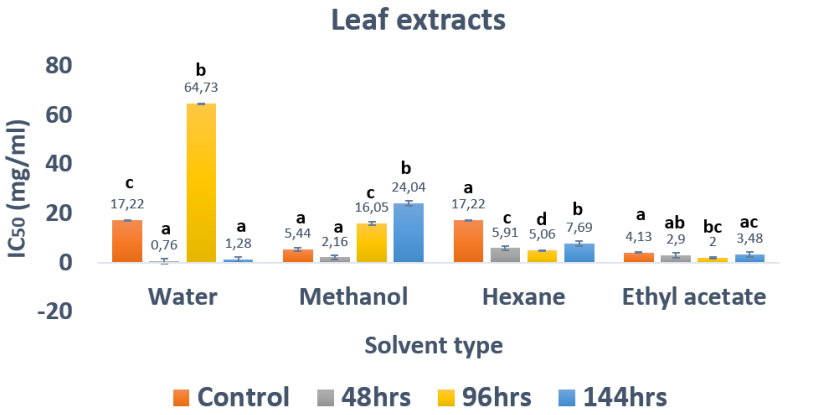
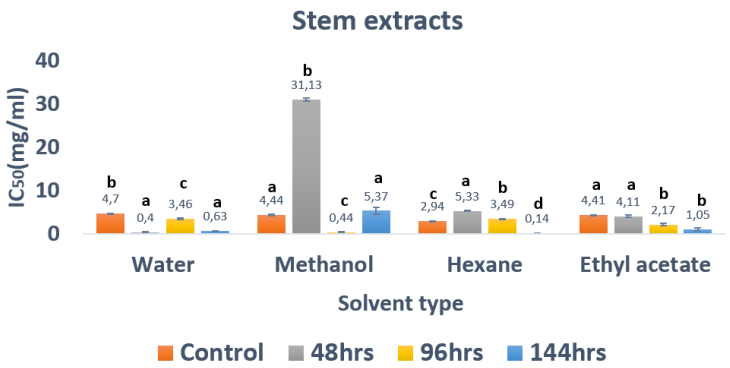


Figure 4. 20 Metal chelating antioxidant activity in *Portulacaria afra* plant parts exposed to 35/45°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test).





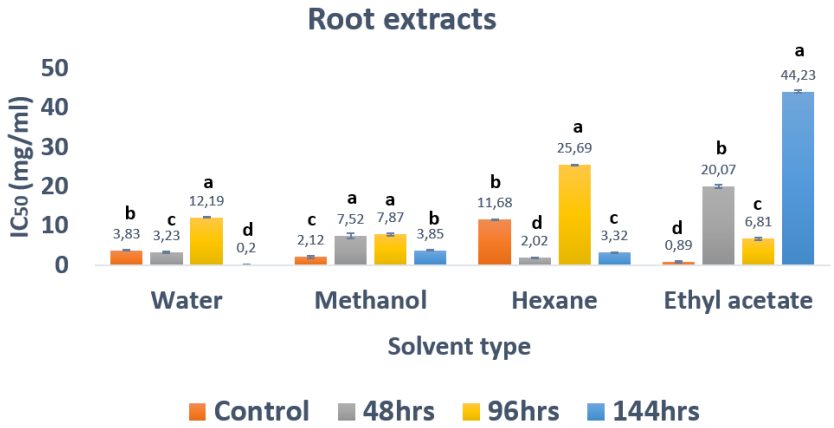


Figure 4. 21 Hydrogen peroxide antioxidant activity in *Portulacaria afra* plant parts exposed to 35/45°C and water deficit. IC_{50} values were denoted as (Mean±SE, mg/ml), n=3, Error bars=standard error values (SE). Statistically significant differences ($p < 0.05$) were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test).

4.4 Discussion

Components such as root, stem, leaf, flower, fruits, and seed are categorised according to the synthesis, accumulation, and distribution patterns of phytochemicals (26). In specific organs, tissues, and cells, distinct regulatory pathways and transport routes facilitate the synthesis of diverse secondary metabolites across various parts of medicinal plants. This phenomenon arises due to the complexity and diversity inherent in these secondary metabolites (26).

Extraction solvents are important in the extraction of potential antioxidant compounds, and are determined by variations in chemical characteristics, polarities, and solubility of these compounds (27). Hence, the solvents options for extraction of phytochemicals in this study were chosen based on the polarity of the solute, for better accuracy in dissolving the solute (27). The presence of phytochemicals in the leaves, stems, and roots extracts of *P. afra* exposed to various treatments, when compared with the control, showed that the methanol extracts, across all the parts, in both hot & cold conditions showed the most presence of phytochemicals, followed by the aqueous extracts. This is in accordance with earlier and current research as they are mostly recommended for phytochemical extractions (28), due to their high polarity index of 5.1 and 10.2 respectively (27), which also validates the use of hot water extraction in traditional medicine.

Previous findings have documented that methanol and water target sugars, amino acids, and glycosides during phytochemical extraction. On the other hand, alkaloids, aglycones and glycosides are commonly extracted with ethyl acetate while *n*-hexane extracts waxes, fats, fixed oils and volatile (29).

Tannins are complex polyphenolic biomolecules with abundant hydroxyls and carboxyl groups, they form strong complexes with macromolecules. They are known for anti-inflammatory and curative properties, used in treating piles, burns, and venereal diseases, and

serve as insecticides and regulators of plant growth (30). The strong presence of tannins observed across all solvents in the leaf extracts, this could be responsible for anti-inflammatory and curative properties of *P. afra*, thus validating its suitability and potency for the treatment of piles, burns and some venereal diseases.

In the pharmaceutical industry, volatile oils are commonly utilized flavouring agents and are known particularly for their antibacterial and carminative attributes (31). Interestingly, in this study, *n*-hexane, and ethyl acetate extracts across all plant parts exposed to hot (35/45ºC) & cold (0/5ºC) (Table 4. 3) temperatures with water deficit showed the highest presence of volatile oil concentration, when compared to the other solvents. The concentrations ranged from low to high, the observed outcome is not completely in accordance with earlier research, indicating that warmer seasons or hotter temperatures present better concentrations of volatile oil and essential oils (32). In contrast to the above reports, studies have shown how low temperature enhances the production of several groups of volatile terpenoids in essential oil glands on the leaf surface (33). Similar observation was reported for the increase in monoterpenes in sage leaves (*Salvia officinalis L.*) and peppermint essential oil (*Mentha piperita L.*) mostly under low temperatures (33). The increase observed in the volatile oil concentration across the different temperature settings in this study, could suggest the response of this plant species to the physical and chemical stress impacted simultaneously by the combined effect of both extreme temperatures and water deficit than with individual stress. Thus, resulting to the production of terpenoids, which are bioactive compounds that confer several biological activities (32). Conversely, volatile oils were produced in low to high quantity in all plant parts under the temperatures [hot (30/40ºC); & cold (10/15ºC)] (Table 4.2), while methanolic stem extracts detected a strong presence of volatile oils in both treatments.

Phenols are the most prevalent class of phytoconstituents that perform protective roles by shielding plants from pathogens and antagonistic abiotic factors (34). Methanolic extracts showed a high presence of phenols in all plant parts under the temperatures [hot (30/40ºC); & cold (10/15ºC)] (Table 4.2), with water deficit when compared with the control samples. Meanwhile, the concentrations of terpenoids and steroids, also showed an increase in comparison with the control sample extracts across all plant parts under both temperatures with water deficit.

Coumarins present in all plant parts of *Portulacaria afra*, which is commonly used in cancer treatment, renal cell carcinoma and blood diseases has caught the attention of many researchers for its photochemotherapy and therapeutic application in cancer (35). Results as shown in the heat map (Table 4. 3) suggest that the production of coumarins is best under hot (35/45ºC) & cold (0/5ºC) temperatures with water deficit. The methanolic and aqueous extracts of all plant parts, showed a significant increase in comparison to the control in both hot (35/45ºC) and cold (0/5ºC) temperatures with water deficit (Table 4. 3). Plant carbohydrates are important biochemical indicators of temperature acclimation and cold tolerance development (33). Therefore, the carbohydrate concentration across all plant extracts, compared to the control samples showed an increasing trend only in the methanolic extracts. This observation shifted from absent to considerable amounts in both hot (35/45ºC) & cold (0/5ºC) temperatures with water deficit (Table 4. 3). While in the methanolic stem extracts exposed to temperatures [hot (30/40ºC); & cold (10/15ºC)] temperatures with water deficit (Table 4. 2), an increasing pattern was also noted.

Studies have shown that water stress may amplify the accumulation of phytochemicals (36), contrary to the belief of the negative impact of severe water deficit. This has been extensively researched to establish its effects on the secondary metabolite profile, frequently leading to

increasing output in most cases, which indicates higher quality (37). The qualitative phytochemical data from this study, however, is attributed to the simultaneous impact of both extreme hot and cold temperatures with water deficit stress.

A broad class of polyphenols with the chemical structure benzoyl-pyrone are called flavonoids. They are prevalent everywhere in plants, produced by the phenylpropanoid pathway and perform a wide range of pharmacological actions (38). It is believed that the organoleptic properties of fresh fruits, fruit juices, and wine are also significantly influenced by phenolic chemicals, including tannins and flavonoids. Therefore, their potential impact on human health will primarily depend on the environmental variables that regulate their amounts and quality of accumulation in plants.

It was observed that hot temperatures (35/45ºC) with water deficit increase the accumulation of total phenolics and total flavonoids in *Portulacaria afra* (Figures 4. 8 and 4. 9), in comparison with cold temperatures (0/5ºC) and water deficit. Al-Huqail *et al*. (1) reported similar higher accumulation of total phenolics content (TPC) in plants under stress, especially in basil plants (*Ocimum basilicum L.*) treated with high temperature, as well as an increase in total phenolics content (TPC) and total flavonoids content (TFC) concentrations in response to water stress and a highly significant increase in flavonoids under high climate temperature condition. The increase in the contents of the chemical compounds analysed, agrees with data in the literatures indicating that this is a response to the generation of reactive oxygen species. Reactive oxygen species (ROS) are produced by plants in response to environmental stress factors such as shade, excessive salt levels, extreme temperatures, drought, or water deficit, which when present in excess can lead to oxidative stress. Moreover, a vast variety of substances, such as arylpyrones and styrylpyrones, stibenes, tannins, coumarins, flavonoids, lignins, and lignans, are included in the category of phenolics and they are produced in response

to abiotic stress (7). Consequently, they protect the plants and increase their tolerance against various abiotic and biotic stresses (7). Phenolic compounds, due to their redox attributes, act as antioxidants, effectively neutralizing singlet oxygen, displaying notable pharmacological significance. They have demonstrated antioxidative, anticarcinogenic, antibacterial, and anti-inflammatory effects in previous findings (1), which supports the traditional therapeutic use of *P. afra*. Additionally, exogenous factors are responsible for the biosynthesis and accumulation of phenolic compounds while endogenous factors, developmental stage, and tissue differentiation (39) are responsible for the site of synthesis in plants. As a result, variation in the climatic, biotic, and environmental factors experienced during seasons can be used to explain the observed differences across seasons. Hence, the content of phenolic compounds and their temperature-dependent fluctuations as seen in this study, suggest that there is a link between their distinct functions in each unique plant parts, and the stimuli causing their synthesis.

Lately, there has been a significant rise in antibiotic resistance, creating a growing therapeutic challenge. Using medicinal plant materials is one way to lessen antibiotic resistance (40). Plants create a wide range of chemicals to defend themselves against different diseases. Plant extracts with targets locations different from those used by antibiotics are anticipated to be effective against microorganisms with drug resistance (41).

The antimicrobial activity results of *P. afra* extracts showed that the antibacterial activity of the extracts is subject to temperature with water deficit (Table 4. 4 – 4. 6). The inhibitory activity observed in the methanolic root and stem extracts (Table 4. 7) against *Escherichia coli*, are similar to the report of previous research demonstrating maximum antibacterial activity against *Escherichia coli* by the leaves of *Amaranthus spinosus L.* during the summer month May-June (42). The antibacterial activity observed could be correlated with the maximum

building up of bioactive compounds in the methanolic stem and root of *P. afra* as a response to the concurrent effect hot temperatures (35/45ºC) with water deficit. Conversely, the other extracts across the plant parts were not active against *Staphylococcus aureus* and *Streptomyces griseus.* A similar trend was observed in *Harpephyllum caffrum* bark extracts where the highest inhibitory activity was detected with plant material collected during summer (December) with increasing antibacterial activity against gram-negative bacteria whereas it declined against gram-positive bacteria (43). Meanwhile in hot temperatures (30/40ºC) with water deficit (Table 4. 6), no positive antimicrobial activity was observed against *Escherichia coli* across all the plant extracts. However, intermediate inhibitory activities, ranging from 11-13mm were observed against gram-positive *Streptomyces griseus* and *Staphylococcus aureus.* Here, the different fractions of the extracts appeared to be more effective against the gram-positive microorganisms. These results can be explained by studies that have proven that gram-negative bacteria have an outer membrane permeability barrier, which makes them more resistant to antimicrobial treatments than gram-positive bacteria (44). This might also explain the inhibitory effect seen against *S. aureus* and *S. griseus* to the extracts and non-effectiveness of the tested fractions against *E. coli*.

The slight inhibitory effect of 11mm observed only in the *n*-hexane and ethyl acetate root extracts against gram-positive *Streptomyces griseus* and *Staphylococcus aureus* under cold temperatures (10/15ºC) with water deficit (Table 4), is partly in line with findings from Ncube *et al*. (45), where the best antibacterial activity was observed in winter season from dichloromethane bulb extracts against *S. aureus.* The observed variation in efficacy to inhibit microorganism growth may be as a function of the distinct cell wall layers characteristic of each microorganism (46). The outcomes of this study might be linked to various factors, including the chemical composition of distinct solvents, temperature variations, specific plant parts used, microbial proliferation, and the extent of exposure of the test microorganisms (46).

Antioxidants inhibit and delay oxidation process, by transferring electrons to free radicals to limit fluctuations and to prevent further reaction (47). The antioxidant activity and medicinal power of plants are significantly dependent on the secondary metabolites present in them, which in turn results to a reduction of oxidative stress by absorbing and neutralising free radicals (48). Depending on the amount of antioxidant molecules present in plants, which are greatly influenced by their growth environment, plants have various antioxidant properties (49).

Natural antioxidants attract attention in research, and food production, for their therapeutic properties and defence against oxidative stress-induced diseases (50). In fact, they are most beneficial for use since they are mostly free of negative side effects in comparison to the synthetic ones (49).

Antioxidant potential of *Portulacaria afra* leaves, stems, and roots extracts were investigated through a 2, 2 diphenylpicryhydrazyl (DPPH) free radical assay, hydrogen peroxide scavenging (H_2O_2) and metal chelating activity assay.

The variations observed in Figure 4. 10 – 4. 21 in this study could potentially be because of the dissimilarities in the concentration or potency of active compounds in each part of *P. afra* under these stress conditions (51). In addition, different antioxidant enzymes have temperature sensitivity, and they can only function at specific temperatures (52). Their actions also vary according to the crop varieties, their growth phases, and the growing season's tolerance or susceptibility to temperature (53). Moreover, the availability of various precursors that the plant needs to produce the active components can vary depending on the temperature and time of the year (54).

Thus, the results from this study are similar to Basson *et al*. (13), where accumulation of phytochemicals and biological activities in *P. afra* were found to either remain constant or

increased after exposure to elevated CO_2. Consequently, these results suggest that the responses in the biological activities in the plant parts under concurrent hot and cold temperatures with water deficit condition serve as an adaptive and protective mechanism of *P. afra* against the concurrent stressors exposed to in this study.

4.5 Conclusions

In conclusion, the phytochemical content, and biological activities in *Portulacaria afra* were significantly influenced by extreme temperature simulations and water deficit. The observed variability in plant responses provided significant insights into the diverse reactions of various plant parts to distinct environmental stimuli and extraction solvents. These include the modulation of secondary metabolites, alterations in antimicrobial properties, and adjustments in antioxidant activities, highlighting fluctuations in response to the combined abiotic factors. It is evident that abiotic factors in combination may influence the biological activities of *P. afra*, which shows the adaptation and resilience of the plant reflected in its ability to still provide therapeutic treatment against targeted diseases. Further studies are still recommended for the characterization of the bioactive compounds in these extracts for *in vivo* studies.

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Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical considerations

Ethical issues regarding (plagiarism, misconduct, data fabrication, falsification, double publication or submission, and redundancy) have been carefully observed by authors

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CHAPTER FIVE

The *In Vitro* Assessment of Antidiabetic Activity of the Plant Extracts obtained from *Portulacaria afra* Jack. grown under Concurrent Extreme Temperatures and Water-deficit conditions

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This chapter provides information on the effect of concurrent extreme temperatures with water deficit on the antidiabetic activity of *Portulacaria afra* leaf, stem, and root extracts. This Chapter was written in scientific paper format according to Biomedical and Pharmacology Journal requirements (Appendix A). It was published as a research paper in the Biomedical and Pharmacology Journal requirements, DOI: https://dx.doi.org/10.13005/bpj/2859

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The *In Vitro* Assessment of Antidiabetic Activity of the Plant Extracts Obtained from *Portulacaria afra* Jack. Grown under Concurrent Extreme Temperatures and Water-deficit Conditions

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The escalating global prevalence of diabetes mellitus presents a significant health concern, prompting exploration into alternative treatments. Recent research highlights the efficacy of newly developed bioactive medications sourced from plants in managing diabetes, surpassing currently used oral hypoglycemic drugs. Medicinal plants' therapeutic characteristics are from secondary metabolites and are greatly influenced by environmental factors. This study investigated the antidiabetic properties of *Portulacaria afra*, using various extraction solvents under different temperature settings with water deficit conditions, using an in vitro model. Aqueous, methanol, ethyl acetate, and n-hexane extracts from leaf, stem, and root were evaluated for antidiabetic potential under different treatments. Overall, extracts substantially increased in antidiabetic capacity compared to control samples. Aqueous leaf extracts at mid-range cold temperatures (10/15ºC) demonstrated the strongest antidiabetic activity, with an IC50 value of 2.33±0.832mg/ml after a 96-hour treatment. Under extreme cold temperatures (0/5ºC) with water deficit, ethyl acetate stem extracts showed the highest inhibitory action (IC50 2.85±0.111mg/ml). Aqueous stem extracts under hot temperatures showed the strongest inhibitory activity (IC50 1.70±0.666mg/ml) after a 48-hour treatment. Notably, the study provides the first data on the antidiabetic potential of P. afra's leaf, stem, and root extracts, particularly under temperature and water deficit conditions. This could be useful as leads worthy for further drug development against diabetes and related symptoms. The observed a-amylase inhibitory activity in aqueous and ethyl acetate stem extracts is most likely due to the polar compounds, establishing a foundation for future investigations.

Keywords: Antidiabetic activity; Extreme temperatures; *Portulacaria afra;* Solvents; Water-deficit; Whole plant.

The significant role of herbal plants in the field of medicine is due to their inherent therapeutic efficacy. Despite the advancements in modern medicine, numerous diseases, including diabetes mellitus and its associated complications caused by the production of advanced glycation end products, are being treated with medicines of plant origin[1]. In developing nations, where most of the population has low resources and no access to contemporary treatments, plants remain important in the management of diabetes[1]. Due to the negative side effects of using insulin and oral hypoglycemic medications, the desire for alternate methods of treating diabetes, such as plant-based medications, has increased in industrialized nations[2].

According to WHO recommendations, the use of plant-derived hypoglycemic treatments within traditional medicinal practices holds significant importance[3]. The antihyperglycemic effects of these treatments are due to their capacity to enhance pancreatic tissue functionality through increased insulin secretion or decreased intestinal glucose absorption. Therefore, treatment with herbal medicines protects β-cells and minimizes glucose fluctuation.

Diabetes mellitus (DM) is a lethal health condition marked by impaired insulin synthesis, which is either hereditary or acquired, and by diminished organ response to secreted insulin. Increased blood glucose levels brought on by such a shortage can harm various bodily systems, including blood vessels and nerves[4].

The World Health Organization (W.H.O.) predictions show that the number of diabetic patients globally would surge from 171 million in 2000 to exceeding 366 million by 2030[5]. While According to data from Statistics South Africa[6], non-communicable diseases accounted for 58% of the country's increase in mortality rates between 1997 and 2018. Of these, 12% of adult patients had diabetes, making the condition the second leading cause of death in South Africa. These predicted increases in global diabetes prevalence represent a significant burden on the health system.

The primary objective of treating diabetes mellitus is to successfully lower the increased blood sugar levels. To reduce post-prandial hyperglycemia, the predominant therapeutic strategies for managing diabetes centre on impeding the breakdown of dietary starch via carbohydrate hydrolysing enzymes such as α-amylase and α-glucosidase, alongside stimulating or enhancing insulin activity within target tissues through the utilization of oral hypoglycemic agents[7].

Furthermore, the reduction of post-prandial hyperglycemia is achieved by delaying glucose absorption by blocking α-amylase or α-glucosidase in the gastrointestinal system. This procedure slows down the breakdown of carbohydrates, reducing the pace of glucose uptake which in turn dampens the postprandially induced rise in plasma glucose[8]. Acarbose, miliglitol, and voglibose, are some of the inhibitors currently in clinical use, these inhibitors' primary negative effects are gastrointestinal, including bloating, stomach pain, diarrheal, and flatulence[9].

Secondary metabolites with high therapeutic values like phenolics, flavonoids, glycosides, coumarins, saponins, terpenoids, alkaloids, and others are typically found in plants and contribute to their pharmacological qualities[10]. Also, plants contain antioxidants which are known to have therapeutic potentials for various diseases. The main function of natural antioxidants is to activate endogenous antioxidants to combat oxidative damage[11].

Different factors such as environmental conditions, seasonal variations, plant parts, treatment/harvest period and extraction solvents affect both the growth of plants and the biosynthesis of phytochemicals production, chemical composition, antioxidant, and antidiabetic activities of plants[12]. Additionally, recent studies have shown that plant responses to stresses caused by combined antagonistic abiotic factors, are phenomenal compared to when exposed to single ones.

In this study we have selected *Portulacaria afra,* a succulent medicinal plant and a member of the *Portulacaceae* or *Didiereaceae* family. It is commonly known as elephant bush (English), spekboom, and porkbush (Afrikaans). *P. afra* is native to South Africa, Kenya, and Mozambique, it thrives mostly in subtropical biomes. [13]. *P. afra* has recorded high phytochemical contents and pharmacological evidence have shown its antioxidant and antimicrobial properties Tabassum *et al.*[14] and Adeleye & Risenga [15]. The *in-vitro* antioxidant activity observed in *P. afra* whole plant extracts gives a preliminary prediction for the antidiabetic activity. To the best of our knowledge, there is no scientific documentation on the antidiabetic potential of *Portulacaria afra* leaf, stem, and root extracts.

Hence, the aim of our study is to assess the α-amylase inhibitory potential of the leaf, stem, and root extracts of *P. afra* using four distinct extraction solvents characterized by different polarities, and to investigate the effect of concurrent extreme temperatures (hot & cold) and water deficit on the antidiabetic activity of the different parts. This study is essential for determining exposure duration and temperature with water deficit-dependent

effects on the plant extracts for the optimal antidiabetic activity, which could potentially be useful in drug production as a remedy for diabetes.

MATERIALS AND METHODS

The chemicals and solvents utilized in this investigation were of analytical variety. The following items were procured from Sigma-Aldrich, USA, under CAS Number 1162-65-8: methanol, *n*-hexane, ethyl acetate, dimethyl sulfoxide (DMSO), potato starch, 3,5-dinitro salicylic acid (DNSA), sodium potassium tartrate, and sodium hydroxide.

The Conviron system specifications for Model No. PGW 40, Serial No. 170028, manufactured in China (280033R01) are as follows: Voltage - 230/400V, Frequency - 50Hz, Phase - 3, Total Input Amperage - 29.2A, Compressor Rated Load Amperage (R.L.A) - 65.5A, High Design Pressure - 400 PSI, Low Design Pressure - 200 PSI, Control Amperage - 2.0A, Lighting Amperage - 9.4A, Heater Amperage - 4.3A, Fan Horsepower - 2.6 HP, Drier Amperage - 10.9A, along with additional specifications for Receptacle Amperage, Glycol Pump Amperage, Motor Amperage, and Auxiliary Amperage. The Minimum Circuit Ampacity (MCA) required is 31.2A, with a Maximum Overcurrent Protection (MOP) of 32A, and a Short Circuit With-Stand Capacity of 5000 Amps RMS.

Plant Material collection

Portulacaria afra specimens were propagated via cuttings in July 2021 at the University of the Witwatersrand, Johannesburg, South Africa.

Cutting and growing of plants

Healthy cuttings displaying characteristics such as vibrant green leaves, absence of drooping, and no signs of fading foliage were selectively collected from a disease-free parent plant. Cuttings measuring 40–45 cm in length and 4 cm in thickness were harvested using sterile secateurs and subsequently transplanted into the greenhouse environment for cultivation[16].

A total of 225 pots, each with a capacity ranging from 2 to 2.5 litres, were used to contain stem cuttings for root development. These pots were filled with Culterrean Potting's professional cutting mix soil, chosen for its superior drainage, moist environment maintenance, and absence of potentially harmful pathogens detrimental to the initial rooting phase of the cuttings.

The cuttings were delicately inserted and secured within the partially filled potting mix soil before being surrounded by additional potting mix soil[17].

The plants were further kept in the greenhouse at the University of Witwatersrand, Johannesburg, South Africa, for three months to allow them to adapt, develop new root systems, and produce new leaves along the stems[18]. In the greenhouse setting, plants monitoring was conducted while receiving a consistent watering regimen of 500 ml administered every two days due to their low-frequency watering requirements[16]. Sample size per harvest: n= 15/harvest

The settings for the conviron simulations used for the treatments were derived from forecasts regarding changes in the present and future climate conditions by the South African Department of Environmental Affairs [19], IPCC [20], and Mbokodo *et al.* [21] records. The temperature settings used in this study were chosen based on the length of heat and cold waves from earlier predictions, [(Control (25/27ºC); mid-range high (30/40ºC); and mid-range low (10/15ºC); extreme high (35/45ºC); and extreme low (0/5ºC)].

According to Janda *et al*.[22], each plant was placed in a controlled climate simulation chamber (Conviron chambers, PGW40) with water deficit and high and low temperatures set simultaneously. Ambient conditions for CO_2 (400 ppm), humidity (60%) and light (160 nits/level 1), pH, and salinity were maintained. It was arranged for the lights to be on for 12 hours per day, from 6 am to 6 pm while the South African Weather Service's records[23] were used to determine the control temperature ranges.

The plants were exposed to treatments and not watered for up to 144 hours (6 days), and the control samples were placed under 25°C (ambient) and watered every second day with 500ml of water[24]. The controls were maintained at a maximum nighttime temperature of 25°C (7pm to 5am) and a maximum daytime temperature of 27°C (5am to 7pm). Plants (A, B, C, and D) while receiving treatment did not receive any water. Samples for treatments A and B were subjected to cold temperatures of 0 and 10°C at night and 5 and 15°C during the day. For the C and D treatments,

samples were exposed to hot temperatures of 30 and 40°C maximum at night and 35 and 45°C maximum during the day.

Five plants were harvested for all plant parts episodically 3 times for up to 6 days (144hrs), where plants were harvested every 48hrs (48, 96, 144) (Table 1). Harvested samples were oven airdried under 40°C for 2 to 3 days (Loveys *et al*., 2002). This procedure was repeated three times for each of the treatments. Where, n=45 control (25°), n=45 for (0/5°C), n=45 (10/15°C), n=45 (30/40°C), n=45 (35/45°C) with a total sample size of 225.

Sample preparation and extraction

The leaf, stem, and root of *P. afra* were gathered and cleansed using deionized water. Subsequently, these specimens were subjected to a four-day drying process at 40°C in a hot air dryer. Following this, an electric grinder was employed to finely powder the dried plant material. The powdered samples were then sealed in foil and stored in an airtight container within a dark cabinet at room temperature (32ºC) until further analysis commenced[25].

Plant extract preparation

The crude plant extract was obtained by employing a series of sequential solvent extractions. A mixture of 80% methanol, *n*-hexane, ethyl acetate, and 100% distilled water (60°C), were chosen based on their respective polarities, was utilized[26]. Each extraction involved placing 3g of powdered plant material into individual containers and adding 30 ml of the designated solvent[27]. The methanol, *n*-hexane, and ethyl acetate extractions were put on a shaker, while the aqueous extracts underwent sonication at a temperature of 60°C for 45 minutes. The shaking process of all solvents lasted for 48 hours, except for the aqueous extracts[28]. Aqueous extracts underwent centrifugation to reduce viscosity, after which all extracts were filtered using filter paper into vials[29]. The resulting supernatant was discarded, and the vials were sealed with foil, and then refrigerated at 4ºC before subsequent analyses.

Preparation of buffer and reagent

The buffer and reagents were prepared according to the methodology outlined by Kamtekar *et al*.[30] and Geethalakshmi *et al*.[3] with slight modification.

The phosphate buffer solution was prepared by initially adding 800 ml of distilled water to a suitable container. Subsequently, 20.214 g of Sodium Phosphate Dibasic Heptahydrate powder and 3.394 g of Sodium Phosphate Monobasic Monohydrate were successively introduced into the 800 ml distilled water. The pH of the resulting solution was adjusted to the desired level of 6.9 using either HCl or NaOH. Distilled water was then incrementally added until the total volume reached 1 L.

For the preparation of the DNSA reagent, 1 g of 3,5-dinitrosalicylic acid was dissolved in 50 ml of distilled water while shaking with a magnetic stirrer on a hot plate set at 90-95°C. Following this, 30 g of Potassium sodium tartrate tetrahydrate was dissolved in 20 ml of distilled water and gradually combined with the previous solution, resulting in the appearance of a milky yellow colour. Subsequently, 20 ml of 2(N) sodium hydroxide solution was added, leading to the development of a transparent orange colour. The final volume was adjusted to 100 ml using distilled water. After complete dissolution, the solution underwent filtration through filter paper, followed by transfer into a dark glass bottle for storage at room temperature (32ºC).

Evaluation of *in vitro* Antidiabetic Activity

***In vitro* inhibitory α-amylase assay**

The determination of α-amylase inhibitory activity was conducted following the methodology outlined by Kumar *et al*.[31] with few alterations. The leaf, stem, and root extracts of *P. afra* underwent drying, whereas aqueous extracts were subjected to lyophilization at -85º C.

The solvent-extracted residues were dissolved in a solution of 10% dimethyl sulfoxide (DMSO). Subsequently, all resulting extracts were standardized to a concentration of 100 mg/ml. Triplicates of five serial dilutions were meticulously prepared for each extract, encompassing a concentration range of 20 to 100 mg/ml.

In this study, 500 µl of plant extract was subjected to incubation alongside 500 µl of α-amylase solution for a duration of 10 minutes at a room temperature of 32 °C. The α-amylase solution, comprising 2 units/ml, was prepared by dissolving 0.001 g of α-amylase in a solution containing 100 ml of 0.02 M sodium phosphate buffer (pH 6.9) and 6.7 mM sodium chloride. Following this initial incubation period, 500 µl of a 1% starch solution, derived from the mixture of

1 g of potato starch with 100 ml of distilled water, heated and stirred for 15 minutes, was introduced into the mixture. Subsequently, the combined solution underwent a further 10-minute incubation period at room temperature (32°C).

To cease the reaction, 1 ml of DNSA reagent was introduced to the mixture, followed by a 5-minute incubation in a hot water bath (85°C). The termination of the reaction was indicated by a visible change in colour of the reaction mixture to orange red after the stipulated 5-minute incubation, upon which the mixture was removed from the water bath and allowed to cool to room temperature. The volume was then adjusted to 5 ml with distilled water. Blank experiments were conducted by substituting the enzyme with buffer, while the plant extract served as the positive control. Subsequently, the absorbance of the resultant solution was measured at 540 nm using a spectrometer. The absorbance was measured with a spectrometer at 540 nm. The inhibition (%) which is the sample concentration (mg/ml) required to decrease the absorbance by 50 % of alpha amylase was expressed with the following equation:

$$(Ac^{+}) - (Ac^{-}) - (As - Ab) \div (Ac^{+}) - (Ac^{-}) \times 100$$

Where, Ac^{+} is the absorbance of 100% enzyme activity (only solvent with enzyme),

Ac^{-} is the absorbance of 0% enzyme activity (only solvent without enzyme),

As is the absorbance of test sample with enzyme,

Ab that absorbance of test sample without enzyme.

Statistical Analysis

The concentration values were graphed against the % inhibition values employing Microsoft Excel to derive a trend line equation which facilitating the calculation of IC_{50}. Statistical analysis was done with a one-way analysis of variance (ANOVA) to ascertain significant differences. All experiments were conducted in triplicate, and data were presented as mean ± standard error (SE) when n = 3. Statistical significance was determined at $P < 0.05$, with Tukey test HSD in RStudio used to pinpoint specific significant differences.

RESULTS AND DISCUSSION

Plants are exposed to a variety of biotic and abiotic elements as they grow and develop, and in response, they activate their defence mechanism. These fluctuating and sometimes harmful external environmental factors interact with plants[32]. Being non-motile organisms, plants have complex alternate defence mechanisms that use a wide range of chemical compounds as coping mechanisms for stressful situations. Secondary metabolites have a significant impact on how plants adjust to their environment[32]. The capacity of plants to synthesize these metabolites is nearly infinite.

Environmental factors, seasonal variations, plant part, and harvest/treatment period,

Fig. 1. *Portulacaria afra* leaf and stem

Fig. 2. *Portulacaria afra* root (arrow)

affect the chemical composition, antioxidant, and antidiabetic activities of medicinal plants[33].

Maintaining close to normal levels of glycemic control in both the fasting and post-prandial phases is the key therapeutic objective for diabetic patients. Numerous natural resources have been studied in relation to inhibiting the generation of glucose from carbohydrates in the gut or absorbing glucose from the intestine[7]. The α-amylase (á-1, 4-glucan-4- glucanohydrolases) is one of the main secretory products of the pancreas and salivary glands and is present in bacteria, plants, and higher animals. It aids in the digestion of starch and glycogen. Inhibiting their activity in the human digestive system is thought to be useful in managing diabetes by reducing the absorption of glucose produced when these enzymes break down starch[7].

In this study, four extraction solvents were used to prepare *P. afra* leaf, stem, and root extracts under concurrent extreme hot and cold temperatures, mid-range high (30/40ºC); and mid-range low (10/15ºC), extreme high (35/45ºC); and extreme low (0/5ºC) with water deficit conditions. The extracts were evaluated for *in vitro* antidiabetic activity using the *in vitro* inhibitory α-amylase assay, where IC_{50} value is the amount of extract required to inhibit α-amylase activity by 50%. Lower IC_{50} values indicate stronger antidiabetic ability of the extracts, which was used to detect the potential antidiabetic effect of *P. afra* extracts. *P. afra* parts showed significant differences, with extracts showing significant differences ($P < 0.05$). The antidiabetic activity of each part of *P. afra* extract at different treatment periods is shown in Figures 3-6. A notable increasing trend of antidiabetic capacity was seen across all parts when compared to the control samples under simultaneous cold temperatures [mid-range low (10/15ºC); and extreme low (0/5ºC)] with water-deficit conditions. As shown in Figure 3, the aqueous leaf extracts under mid-range cold temperatures (10/15ºC) showed the strongest antidiabetic activity with IC_{50} value of (2.33±0.832mg/ml) amongst the plant's parts after a 48-hour treatment period while the IC_{50} value for the control sample was (208.818±0.064mg/ml). Figure 4 shows the antidiabetic activity under concurrent extreme cold temperatures (0/5ºC) and water deficit, where ethyl acetate stem extracts showed the highest inhibitory action with IC_{50} value of (2.85±0.111mg/ml) against α-amylase after a 96-hour treatment period, while the IC_{50} value for the control sample was (324.16±0.111mg/ml). The variation observed in the activity among the plant organs at the different treatment periods suggests a possible corresponding shift in the accumulation of some compounds responsible for the activity[12]. Since most of these phytochemicals are produced in response to external stimuli such as light intensity, moisture/water deficit stress, and temperature amongst others[34,35].

Similarly, Figures 5 and 6 show observed variations in the α-amylase inhibitory activity of the plant extracts under concurrent hot temperatures [mid-range high (30/40ºC); and extreme high (35/45ºC)] with water deficit conditions. A shift in antidiabetic activity was observed in the extracts in comparison with the control samples. The antidiabetic activity of the aqueous stem extract under the mid-range hot temperatures (30/40ºC) showed the strongest inhibitory activity with IC_{50} (1.70±0.666mg/ml) after a 48-hour treatment period while a similar trend was observed also in the aqueous stem extracts under the extreme hot temperatures (35/45ºC) with the highest inhibitory

Table 1. Experimental Design

	Control	Treatment A	Treatment B	Treatment C	Treatment D
Water deficit	500ml of water every 2nd day.	No water	No water	No water	No water
Temperatures (°C night/day)	25/27	0/5	10/15	30/40	35/45
Episodic Harvest frequency(hrs)	Once off	48, 96, 144	48, 96, 144	48, 96, 144	48, 96, 144

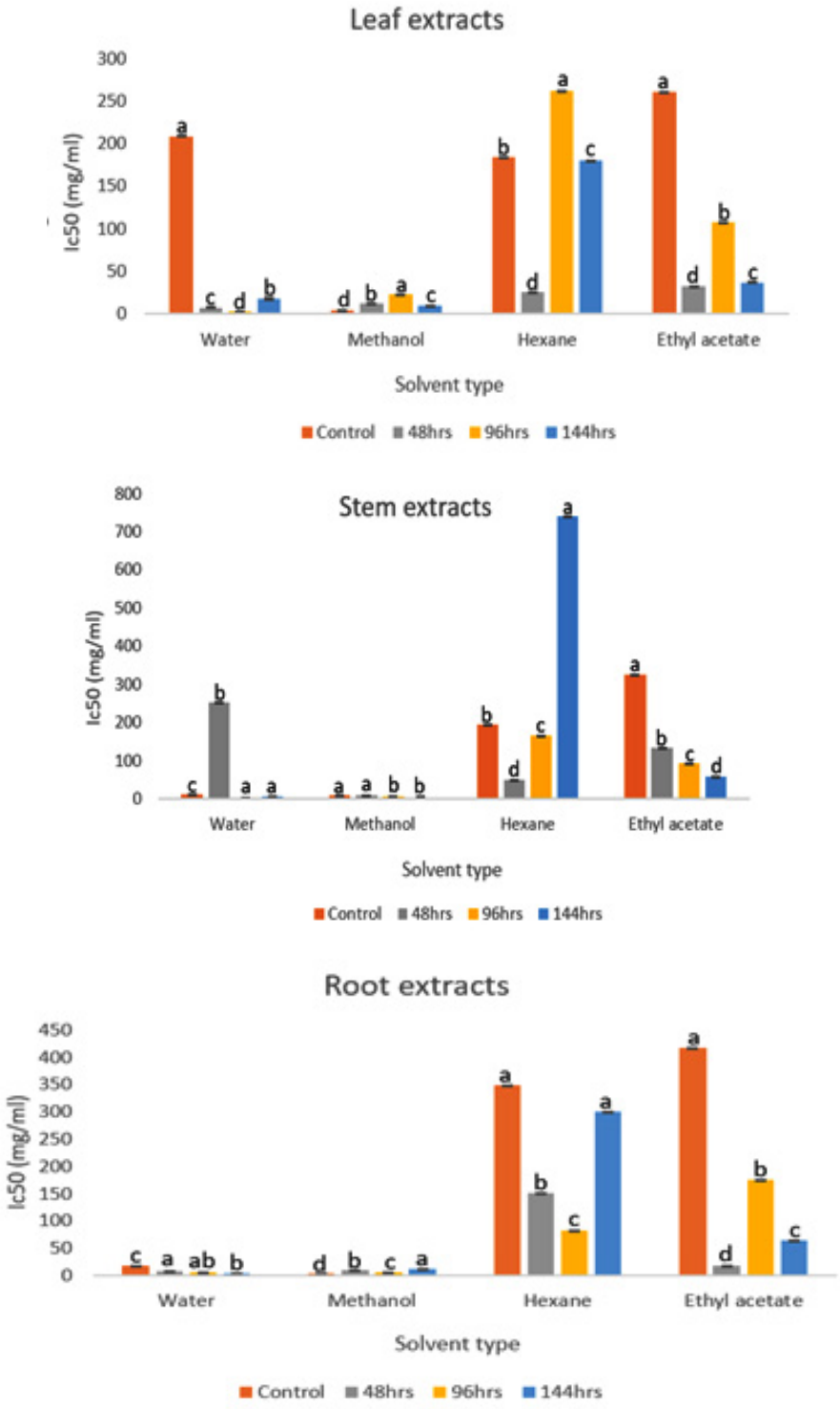

Fig. 3. Graphical representation of antidiabetic activity in *P. afra* plant parts under 10/15°C with water deficit conditions. All IC_{50} values were presented as (±SE, mg/ml), n=3, and [a]Values with different alphabets were significantly ($p < 0.05$) different, error bars=standard error values (SE)

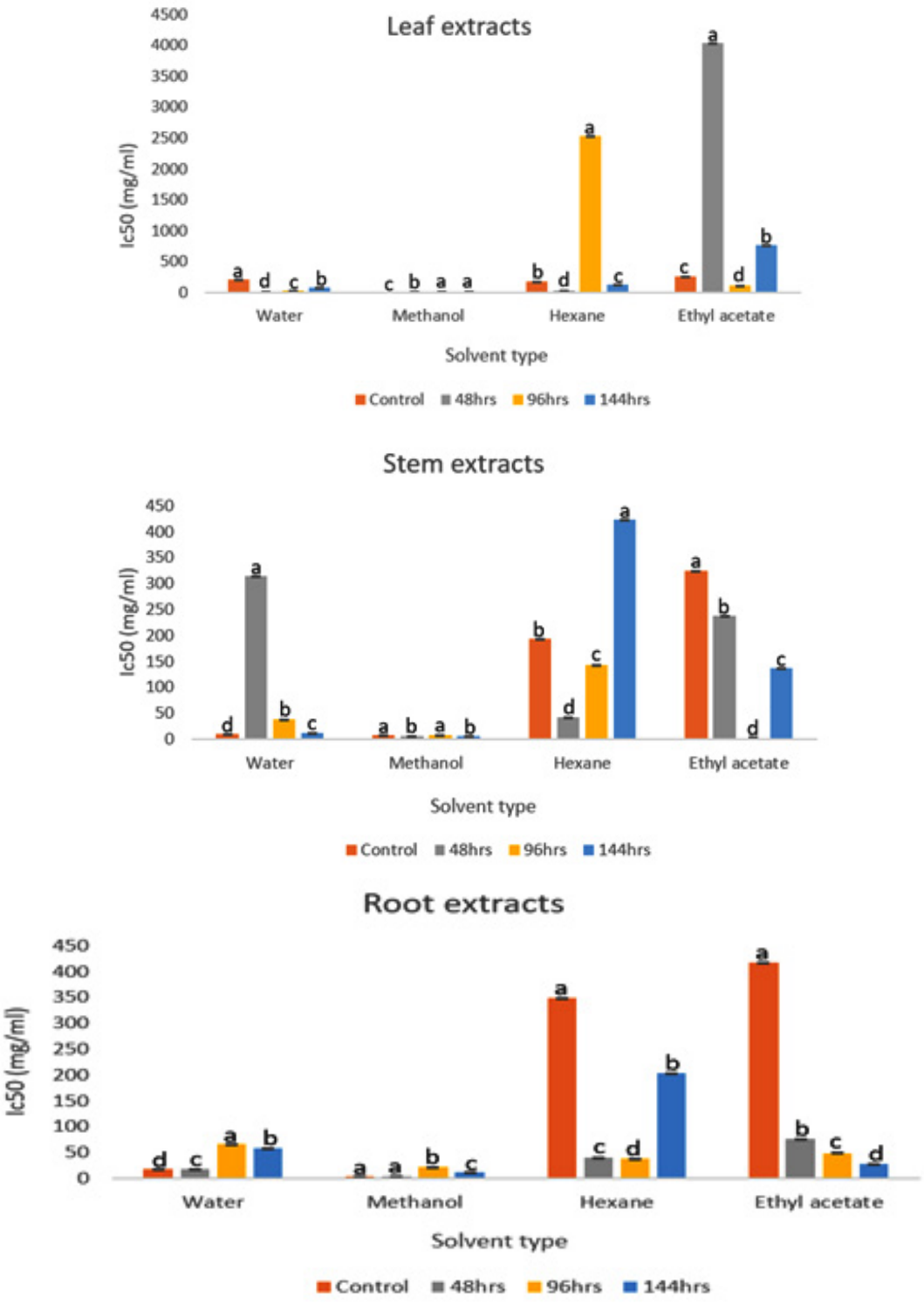

Fig. 4. Graphical representation of antidiabetic activity in *P. afra* plant parts under 0/5°C with water deficit conditions. All IC_{50} values were presented as (±SE, mg/ml), n=3, and [a]Values with different alphabets were significantly ($p < 0.05$) different, error bars=standard error values (SE)

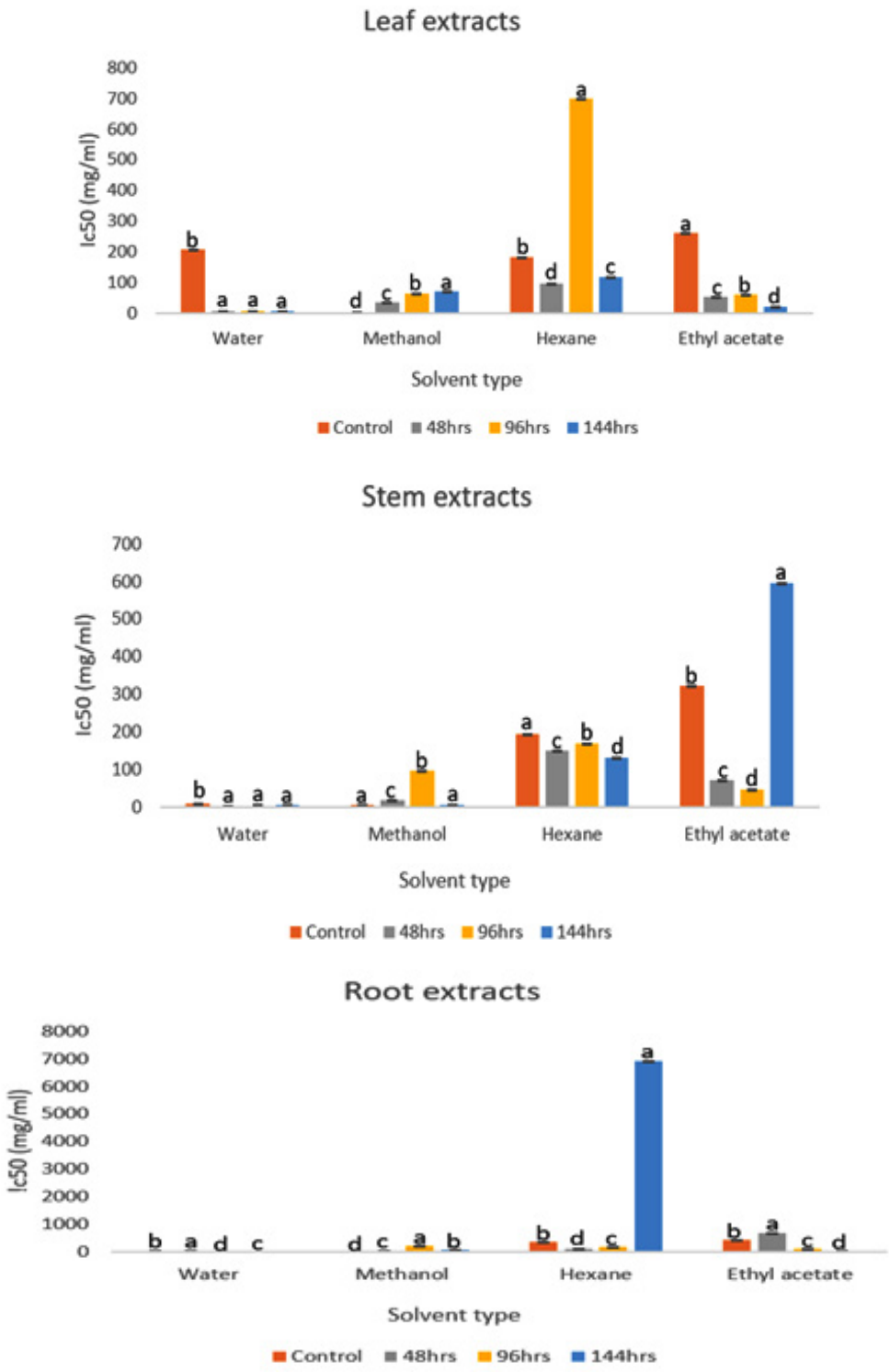

Fig. 5. Graphical representation of antidiabetic activity in *P. afra* plant parts under 30/40°C with water deficit conditions. All IC_{50} values were presented as (±SE, mg/ml), n=3, and [a]Values with different alphabets were significantly ($p < 0.05$) different, error bars=standard error values (SE)

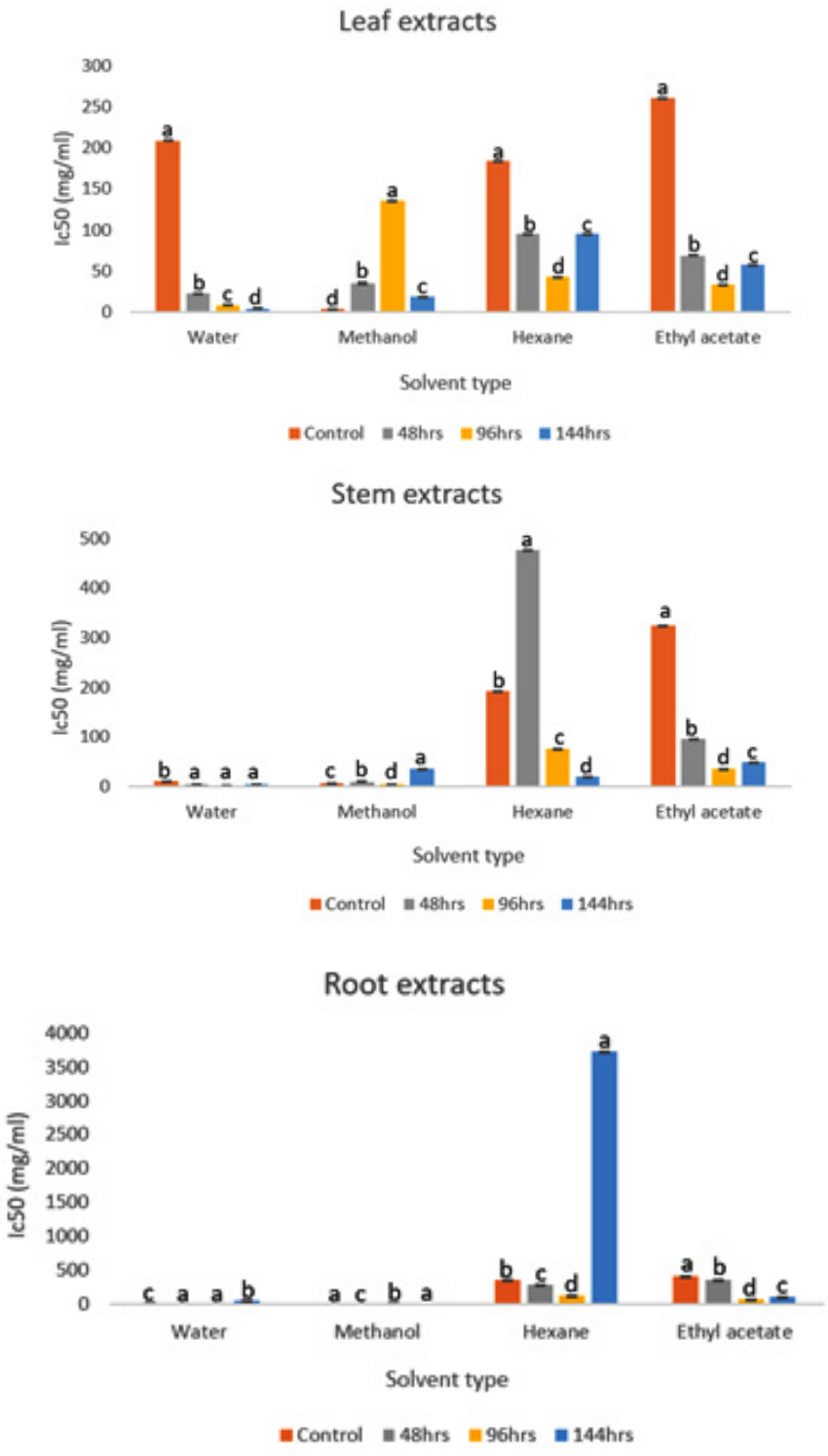

Fig. 6. Graphical representation of antidiabetic activity in *P. afra* plant parts under 35/45°C with water deficit conditions. All IC_{50} values were presented as (±SE, mg/ml), n=3, and [a]Values with different alphabets were significantly ($p < 0.05$) different, error bars=standard error values (SE)

activity with IC_{50} (1.99±0.626mg/ml) after a 96-hour treatment period, with IC_{50} (10.61±0.127mg/ml) for the control sample.

Considering all these, the overall strongest inhibitory activity against α-amylase enzyme activity under both cold and hot temperatures with water deficit condition was found in the aqueous stem extracts under the mid-range hot temperatures (30/40ºC); with IC_{50} value (1.70±0.666mg/ml) after a 48-hour treatment period. Similar result was observed in Usman *et al*. [33] where the oil from (hot-dry) season harvested plants showed stronger antidiabetic activity than the essential oil from the harvested plants during the (wet-cold) season. Research by Chelladurai and Chinnachamy[10] also reported the antidiabetic activity of aqueous stem extract of *Salacia oblonga* to be effective against α-amylase.

The marked variation in antidiabetic activity in this study may also be attributed to the polar and non-polar compounds present in the plant parts based on the extraction solvent. From a wide array of experiments, it has been established that methanol and water targets sugars, amino acids, and glycosides during phytochemical screening. Conversely, alkaloids, aglycones and glycosides are extracted with ethyl acetate while *n*-hexane extracts waxes, fats, fixed oils and volatile[36,37].

The high presence of various phytochemicals like tannins, terpenoids, phenolics and flavonoids previously detected in *P. afra* parts from earlier findings [14, 15], have potential inhibitory effects on α-amylase enzyme due to their ability to bind with proteins, which in turn contributes to the lowering of postprandial hyperglycemia[38].

Additionally, an extensive range of compounds, including arylpyrones and styrylpyrones, stibenes, tannins, coumarins, flavonoids, lignins, and lignans, fall within the phenolics category, being synthesized in reaction to abiotic stress[39].

This correlates to this study's observations and might explain the observed inhibitory effects across the different extracts of *P. afra*. (More information advised to be written for Results)

The *in vitro* antidiabetic potential of all the extracts was determined by *in vitro* inhibitory α-amylase assay, where IC_{50} value is the amount of extract required to inhibit α-amylase activity by 50%. Lower IC_{50} values indicate stronger antidiabetic ability of the extracts, which was used to detect the potential antidiabetic effect of *P. afra* extracts. Figures 3-6 experimentally reveal the various changes observed in the antidiabetic activities of *P. afra* plant parts' extracts with different solvents and treatment periods, under hot and cold temperatures [mid-range high (30/40ºC); and mid-range low (10/15ºC), extreme high (35/45ºC); and extreme low (0/5ºC)] concurrently with water deficit conditions. *P. afra* plant parts showed significant differences, with extracts showing statistically significant differences ($p < 0.05$), and were indicated by different letters (a, b, c, d) within the same column (Tukey's post-hoc test).

CONCLUSIONS

The present study explores the potential antidiabetic activity of *P. afra* leaf, stem, and root with four extraction solvents and with focus on the inhibitory effects on α-amylase under extreme temperatures with water deficit condition. The results of this study also highlighted the effect of concurrent extreme temperatures with water deficit condition, treatment period, plant part and extraction solvent on the antidiabetic potential of *P. afra.*

The antidiabetic potential of *P. afra* leaf, stem and root extracts with the control samples were characterized by their inhibitory effect on α-amylase activity. *P. afra* leaf, stem and root have significant inhibitory effects against α-amylase enzyme. A marked variation in the antidiabetic activity were observed among the plant parts based on the extraction solvent and treatment period for all temperature ranges with water deficit condition. Of the extracts tested from *P. afra*, aqueous stem extracts under mid-range hot temperatures after a 48-hour treatment period showed the strongest inhibition on the enzyme (α-amylase) across all treatments, which may be attributed to the polar compounds present based on the solvent extraction and the temperature exposure duration. This study is the first to report the antidiabetic potential of the leaf, stem, and root of *P. afra* with the effect of concurrent extreme temperatures and water deficit.

Conclusively, the results of the study indicate that *P. afra* leaf, stem and root possess great antidiabetic potential from the *in vitro* assay

performed. Therefore, the antidiabetic property can be credited to the rich phytochemicals and antioxidant activity possessed by the plant, according to earlier research. For future production of this species, this work has shown how environmental factors may influence the antidiabetic potential of specific plant sections. Subsequent research is necessary to isolate antidiabetic compounds and conduct *in vivo* studies.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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CHAPTER SIX

GENERAL DISCUSSION

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

6.1 General discussion

In numerous developing nations, medicinal plants are extensively used by billions of individuals owing to the limited access to modern medical resources, their cost-effectiveness, efficacy, and alignment with cultural beliefs and preferences (Sen & Batra, 2012). The therapeutic properties of these organisms are as a result of the production of secondary metabolites, whose presence and subsequent activities are influenced by various environmental factors, including geographical location, cultivar fertility, utilized plant part, temperature, seasonal variations, and the timing of collection (Kamatou *et al*., 2008). The simultaneous occurrence of temperature and water deficit factors may influence the abundance of secondary metabolites (also referred to as phytochemicals in this study) within plant species, consequently impacting the anticipated phytopharmacological properties associated with medicinal plants (Punetha *et al.*, 2022).

The botanical history of South Africa encompasses a diverse array of flora, intertwined with a rich tapestry of cultural diversity that incorporates traditional healing practices within distinct ethnic groups (Ncube *et al*., 2011). Despite comprehensive ethnobotanical documentation, scientific data regarding the medicinal attributes of indigenous plants remain limited. Among these, *Portulacaria afra*, native to South Africa, has been recognized within folklore and a sparse number of studies for its purported medicinal properties. This research study was initiated to further document scientifically the phytochemical profile, therapeutic properties, phytopharmacological attributes of *Portulacaria afra* which also includes the assessment of the effects of concurrent extreme temperatures and water deficit on its biological activities. The starting point was the assessment of the therapeutic properties of leaf, stem, and, notably, root extracts, with the use of multiple solvents.

This paved a way for conducting phytochemical tests which were achieved both qualitatively and quantitatively.

> Considering the link between the presence of these phytochemicals and their biological activities, how will concurrent extreme temperatures and water deficit influence the accumulation of phytochemicals and phytopharmacological attributes in the plant parts?

To answer this over-arching question, a subset of questions was developed, as presented below:

1. How will concurrent extreme temperatures and water deficit impact the phytochemical content of *Portulacaria afra* ?
2. Will concurrent extreme temperatures and water deficit improve the antibacterial activity in *Portulacaria afra* ?
3. Will concurrent extreme temperatures and water deficit improve the antioxidant activity in *Portulacaria afra* ?
4. What impact will concurrent extreme temperatures and water deficit have on the antidiabetic potential of *Portulacaria afra* ?
5. In relation to the expectations as noted in points 1-4 above, can any conclusions be drawn from the impact of these simulated abiotic factors and the phytochemical accumulation and performance of the biological activities in the plant part samples?

The answers to these questions have been addressed in the chapters 3-5 presented in the thesis.

6.2 Overall Findings from The Research Presented in Chapters 3-5

Exposure to extreme temperatures and water deficit stress in combination increased the secondary metabolites production in *Portulacaria afra* plant parts. The results of this study showed a temperature and solvent dependent variation in the types of phytochemicals extracted

from the plant parts with the various treatment periods. The increase of detected secondary metabolites under concurrent cold temperatures and water deficit, especially after a 144-hour treatment period across all plant parts can be explained as the possible response of the phytochemicals to the external stimuli, such as the extreme cold temperatures and water deficit stress in combination (Ncube *et al*., 2011). Studies have shown that variations in plant parts' activity throughout the different times of the year may suggest a corresponding variation in the accumulation of certain chemicals responsible for the activity (Azizi *et al*., 2016). One important physical aspect that has a significant impact on how plants grow and develop is temperature. Extreme temperature stress, which can take the form of extreme heat or cold, has an impact on the fundamental physiological, biochemical, and molecular functions of medicinal plants. Some studies have shown both increasing and decreasing trend in the production of secondary metabolites because of temperature and water deficit stress in combination (Punetha *et al*., 2022). This correlates to the observations in this study and might explain the irregularities observed in the secondary metabolites' accumulation across the different extracts of *Portulacaria afra*.

Due to the simultaneous effects of severe temperature and drought/water deficit stress, numerous publications have noted sharp increases in total flavonoids and total phenolic content (Azhar *et al*., 2011). This is in accordance with the findings in this study and can be used to justify the significant high total phenolic and total flavonoid content observed in plant parts when compared to the control. In addition, the production of ROS is hindered with increasing secondary metabolites, which in turn shields the plants from the effect of antagonistic ecological factors (Radácsi *et al*., 2010). These secondary metabolites are biosynthesised to counteract the over production of ROS by the peroxisomes, chloroplast, and mitochondria. ROS are produced in excess by the matrix and membrane of peroxisomes, the electron transport chain (ETC) of mitochondria (Complex I, Ubiquinone, and Complex III), and both chloroplast

photosystems (PSI and PSII) (Irato & Santovito, 2021). The enzymatic and non-enzymatic antioxidant defence mechanisms aided in protecting the plant from excessive stress, ameliorated the tolerance to stress and mitigated the effects of concurrent extreme temperatures and water deficit stress on *Portulacaria afra.*

The strong *in vitro* antioxidant activities observed, in all experimental studies (chapter 3 and 4) for both under extreme hot and cold temperatures in combination with water deficit stress can be attributed to the presence of numerous non-enzymatic antioxidants which include ascorbate (vitamin c), carotenoids, vitamin E, phenolic, flavonoids and many more. Non-enzymatic antioxidants are important for managing and regulating ROS generated during abiotic stress exposure (Concato *et al*., 2022). The presence of phenols, tannins, flavonoids, terpenoids, saponins, volatile oils, were stronger under extreme hot and cold temperatures in combination with water deficit stress, this might explain the antioxidant, antidiabetic and antimicrobial activities observed in this study.

6.3 The interrelationship between the objectives investigated

The preliminary phytochemical analyses (chapters 3) show the extracted phytocompounds believed to have contributed to the biological activities (also referred as phytopharmacological activities in this thesis). There is a clear interconnection in the outcomes of the objectives identified in this study based on the following factors: the extraction solvents; treatment period; plant parts; and phytochemical content. The findings herein support the antioxidant potential of the extracts, with their probable association with alpha amylase inhibitory efficacy via oxygen-free radical scavenging. Numerous bioactive phytoconstituents found in plants, including polyphenols, flavonoids, terpenoids, anthocyanins, and vitamins, have been documented to exhibit a wide array of activities, such as antioxidant, antidiabetic, anticancer, antiaging, antiallergic, anti-inflammatory, antimicrobial, and other pharmacological effects

(Walczak *et al*., 2022). This correlates to the observations recorded in this study and might explain the link between concentrations of these phytochemicals within the species components with the performance of the antioxidant, antimicrobial and antidiabetic activities observed.

6.4 Conclusion

In conclusion, the findings in the study indicate that abiotic factors, particularly in combination significantly influence the presence and content of pharmacologically active secondary metabolites in *Portulacaria afra* Jacq. It can be can also be inferred that in addition to extraction of samples using solvent with different polarities, plant part, procedure, the state of sample and treatment period also matter in phytochemical extraction and accumulation. One of the key coping mechanisms used by plants under stress is the synthesis of secondary metabolites. This idea implies that secondary metabolites might protect the plant from harm brought on by various abiotic stimuli. This illustrates the diverse responses of plant parts to simultaneous environmental stresses, demonstrating alterations in secondary metabolite levels, antioxidant efficacy, as well as antimicrobial and antidiabetic activities. It is evident that abiotic factors in combination may influence the production of secondary metabolites, which could increase the output of phytomedicine and enhance the production of phytochemicals.

The results derived from this investigation may significantly influence the methodologies employed by ethnobotanists and pharmaceutical companies in determining optimal environmental parameters for the cultivation and harvesting of *P. afra*. This optimization aims to augment the production of secondary metabolites or specific chemical compounds, thereby enhancing antioxidant, antimicrobial, and antidiabetic properties within the respective plant components traditionally utilized in medicinal applications, while concurrently mitigating the risk of overexploitation of specific plant parts.

Despite the impact of climate change on all life forms and predictions, the development of the phytopharmaceutical and aromatic Industries can be aided by the cultivation of medicinal and aromatic crops because of the higher accumulation of phytochemicals in response to stress and climate change. This can also help to safeguard the natural resources that are under threat from overuse. For future production of this species, this work has demonstrated how environmental factors can have an impact on the antioxidant, antimicrobial and antidiabetic properties, of specific plant parts, and the isolation of secondary metabolites.

Additional investigations are warranted to precisely characterize the bioactive compounds within these extracts and flower derivatives for *in vivo* studies and elucidating further chemical constituents for the utilization of this plant across diverse pharmaceutical domains and facilitation of specific biological functions. Also, chromatographic, toxicological, and nutritional investigations are recommended in future research.

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Appendices

Appendix A: Author guidelines and sample manuscript for the Biomedical and Pharmacology Journal (see next page).

Biomedical and Pharmacology Journal

Healthcare Monitoring System Based on Sensor Network for Cardiac Patients

Principal Author[1*], First Author[2] and Second Author[1,2]

[1*]Department, University Name, City, Country, Pincode, orcid id

[2]Department, University Name, City, Country, Pincode, orcid id

Corresponding Author E-mail: Corresponding author@gmail.com

Orcid ID: 0000-000X-XXXX-XXXX

Abstract

The abstract should be concise and elicit a clear indication of the objective, scope, and results of the paper in order for readers to determine if the full text will be of particular interest to them. All the abbreviations must be spelled out fully when first mentioned in the abstract of the report.

Keywords

The authors must provide up to 6 keywords for indexing purposes. Only established abbreviations may be proposed as keywords. All the keywords must be arranged in alphabetical order.

Introduction

In this part, there should be a concise review of the problem, the relevant methodologies and a clear establishment of the goal(s) of the study.

Material and methods

This section consists of elaboration of the objective of the study, and step-by-step details of the experiment. The following are to be specified:

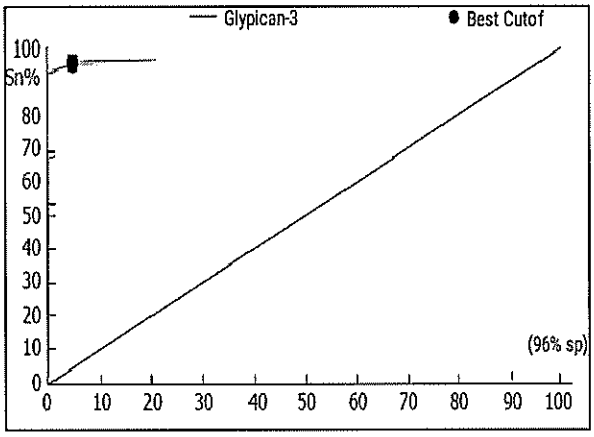
- The original names of instruments and reagents.
- Manufacturer's name (company, country).
- Source from which the particular strain has been obtained.

Table 1: Legend for table 1

Title	Value 1	Value 2	Value 3
Description 1	100	100	100
Description 2	1m	100m	500m
Description 3	240	32767	250
Description 4	250 Kbps	2.1 Mbps	450 Mbps
Description 5	5MHz	902MHz	160MHz

Results and Discussion

The results and discussion section should have a brief account of the so-obtained experimental data, along with tables and figures. The section should be completed with a fulfilling conclusion, addressing the questions and problems raised in the Introduction section.



Graph 1: Legend for graph 1

Figures and Tables

The tables should be marked with a number and a title. The figures must contain the captions. Figure/Photos submitted must be of high resolution. Proper reference must be given for the figures and photos.

Figure 1: Legend for figure 1

Conclusion

The conclusion should summarize the major findings of the investigation. The purpose or hypothesis of the study stated earlier should be addressed.

Acknowledgement

This section must appear before References. The Acknowledgments should contain the names of individuals, organizations and funding agencies that aided in underwriting and reporting the particular work.

Conflict of Interest

All authors are requested to disclose any conflict of interest including honorarium, grants, membership, employment, ownership of stock or any other interest or non-financial interest such as personal or professional relation, affiliation and knowledge of the research topic.

Funding Source

Please provide the source of financial support if any.

Reference Format

1. Burwen D. R, Banerjee S. N and Gaynes R. P. Ceftazidime resistance among selected nosocomial gram-negative bacilli in the United States. J. Infect. Dis., 1994; 170: 1622-1625.

Note: It is to be ensured that every reference cited in the text is present in the reference list, and vice versa.

Appendices

Appendix B: Author guidelines for Journal of Herbmed and Pharmacology (see next page).

JHP Journal of Herbmed Pharmacology
eISSN:2345-5004

eISSN: 2345-5004 (https://portal.issn.org/resource/ISSN/2345-5004)

For Author

Author Guidelines

The benefits of publishing in the Journal of Herbmed Pharmacology:

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- Easy and quick online submission
- Rapid publication
- Indexing in high quality databases

Terms of submission

Unsolicited manuscripts will be reviewed for publication with the following understanding:

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2. The paper neither has been published already nor is being under review elsewhere.

3. Upon acceptance, the paper may not be published elsewhere without the permission of Journal of Herbmed Pharmacology.

4. The published paper is the sole property of Journal of Herbmed Pharmacology and may be edited before publication.

Manuscripts should be only submitted by the online (http://www.herbmedpharmacol.com/Login) system (http://www.herbmedpharmacol.com/Login). The manuscripts that be sent by other methods such as email will not been evaluated. The submitting author is responsible for ensuring that the article's publication has been approved by all the other coauthors. It is also the authors' responsibility to ensure that the articles emanating from a particular institution are submitted with the approval of the necessary institution. The submitting author takes responsibility for the paper during submission and peer review.

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All manuscripts are subject to peer review and are expected to meet standards of academic excellence. Submissions will be considered by the technical editor and section editor and "if not rejected right away" by peer-reviewers,

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Journal of Herbmed Pharmacology is an open-access journal. Open access publication is not without costs. To cover a section of the costs, papers that are accepted for publication following peer review incur a publishing fee of 200 USD for Original and Reviews, and 100 USD for Case reports and letters.

Withdrawal Policy for authors

Authors are free to withdraw an article at no charge – as long as it is withdrawn within 15 days of its initial submission.

If you have concerns or questions about it, please contact us for further discussion. We welcome your input.

Structures

Units of measurement should be presented simply and concisely using System International (SI) units.

Types of Articles

Manuscripts should be presented as one of the following formats.

Full Original Researches

A full-length original research article (up to ~5000 words, including tables, figures, and references) presents novel findings relevant to the aims and scope of the journal.

Reviews

A full-length critical review (up to ~8000 words, including tables, figures and references) provides an abstract and discussion of the relevant literature about any topic covered within the aims and scope of the journal. Review articles should be sharply focused, well-focused, well-documented examinations of timely related issues.

Mini-Reviews

Mini-Reviews are sharply focused, well-focused, well-documented examinations of timely related issues (up to ~4000 words, including tables, figures and references). The issues may be of a controversial nature, or may address a more narrowly focused area than those typically covered in a review.

Short Communications

Short Communications are preliminary reports (up to ~2000 words, including tables, figures and references).

Commentaries

Commentaries present the author's considered opinion (up to ~1000 words limited to one figure/table and limited references) on an original article to be published in the journal and usually submitted by the reviewers.

Case reports

These reports should include an introduction, case description, discussion, and conclusions. Patient confidentiality must be maintained. Any identifying information must not be published, and any specific details or description that may compromise patient anonymity, should be omitted. The patient's consent should be obtained when possible.

Letters to Editor

Letter to Editor presents the author's opinion (up to ~1000 words limited to one figure/table with limited references). If such a letter criticizes an article already published in the journal, then the authors of the original article will be given a chance to respond in the same issue in which the letter is published.

Preparation of Manuscripts

Text

Submitted manuscripts to JHP must be in DOC format as follows. **Authors are strongly recommended to consult a recently published article in this journal** and consider the following instructions.

Title page

Title: Lengthy systematic names and complicated/numerous chemical formulae should be avoided where possible.

Authors' names: Full names (First, Middle and Last) for all the authors of an article should be given and specified with superscript number(s) for the affiliation(s) [e.g., Mark Junior Smiths[1]]. The name of the corresponding author(s) should be specified with an asterisk after name (e.g., Mark Junior Smiths*). Where the family name may be ambiguous (e.g., a double name), please indicate this clearly.

Affiliation: Affiliation of all the authors should be given and specified with superscripted number before address (e.g., [1]Faculty of .....).

Running title: A very short running title should be given.

Corresponding author: Full address, telephone, and fax numbers (with country and area code) and email of the corresponding author(s) should be given.

Abstract page

Abstract: A factual concise abstract (up to 250 words) is required for every manuscript. The abstract should briefly state the ***Introduction***, *Methods*, ***Results*** and ***Conclusion***. An abstract is often presented separately from the article; hence it must be able to stand alone. Referencing should be avoided. Also, non-standard or uncommon abbreviations should be avoided, however, if essential they must be defined at their first mention in the abstract itself.

Key words: Immediately after the abstract, three to six relevant keywords should be included. (Notice: Readers are increasingly used search engines to find literature using keywords; thus, recognizable and searchable keywords should be given to maximize the visibility of the article.)

Notice: Original research papers can also be published in a brief format. Submitted papers that are of interest but are not acceptable as a full-length original contribution are offered by the editor to be published in this section. Also, the authors can primarily submit their papers for consideration of publication in this section. An unstructured abstract not longer than 200 words is required for this section. The body of the manuscript should not exceed 2000 words, and no heading or subheading should be used. Tables and/or Figures should be limited to 2 ones and references to 15 in maximum.

Introduction

This section should clearly and briefly (up to 600 words) provide an adequate background with relevant references, avoiding a detailed literature survey or a summary of the results. The last paragraph should address the main objectives of the work.

Materials and Methods (Patients and Methods for clinical investigations)

This section should provide sufficient details to allow the work to be reproduced, with details of the supplier (i.e., company's name, city, country) and catalogue number when appropriate. Methods already published, should be indicated by a reference: only relevant modifications should be described. The company's name, city and country of manufacturer of the major equipment should be given. Unexpected hazards encountered during the experimental work should be noted. Any unusual hazards inherent in the use of chemicals, procedures or equipments in the investigation should be clearly identified. In cases where a study involves the use of live animals or human subjects, the author should include a statement that all experiments were performed in compliance with the relevant laws and institutional guidelines, and also state the institutional committee(s) that has approved the experiments. They should also include a statement that informed consent was obtained for any experimentation with human subjects.

Results

Results should be clear, descriptive and concise. Attention should be paid to the matter of significant figures and tables. The same data should not be presented in more than one figure or in both a figure and a table. Basically, as a rule, interpretation of the results should be reserved for the discussion section of a full original research article.

Discussion

The discussion should explore the significance of the results of the work (without repeating them) in comparison with others similar reports. Extensive citations and discussion of published literature should be avoided.

Conclusion

The main question of the work should be very concisely stated and the final conclusions of the study may be presented in a short "Conclusions" section, which may stand alone or form a subsection of a Discussion or Results and Discussion section(s).

References

The authors are responsible for the accuracy of all references. These should be numbered sequentially as superscripts in order of their appearance in the text and listed in a separate section following the text, double-spaced. All authors and inclusive page numbers should be

limited to published works; unpublished data or personal communications should be indicated parenthetically in the text.

Numbered references should appear at the end of the article and should consist of surnames and initials of all authors when six or less (when seven or more list the first six and add et al). Also title of article, name of the journal, year, volume, first and last page numbers should be given.

Authors. Title. Journal. Year; Volume(Issue): Pages.

For books, names and initials of all authors, the full title, place of publication, publisher, year of publication, and page numbers should be given.

Authors. Title. Place Published: Publisher; Year. Number of Pages.

Tables

Tables should be created with a word processor and saved in either DOC or RTF format.

Notice: Please embed tables in your text.

Figures

The figures must be minimum 300 dpi resolution at full size (size when printed in journal).

Notice: Please embed figures in your text.

Ethical Issues

Please see the ethical issue provided in the below link

Every experimental or clinical study may raise controversial ethical issues (e.g., Institutional Ethical Approval for working with animal or human subjects). Thus, the Journal of Herbmed Pharmacology expects all authors, reviewers, and editors to consider COPE (http://www.publicationethics.org/), ICMJE (http://www.icmje.org/) and Equator Network (http://www.equator-network.org/)'s reporting guidelines in medical ethics plus scientific writing. If any, authors should state related declaration(s), otherwise the following sentence should be given "None to be declared". Please take a look at the review process in Journal of Herbmed Pharmacology.

The cover letter must include a statement declaring that the study complies with current ethical considerations.

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or redundancy) must completely considered by the authors.

Authors reporting experimental studies on human subjects must include a statement of assurance in the Materials and Methods section of the manuscript reading that: (1) informed consent was obtained from each patient enrolled in the study and (2) the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (http://en.wikipedia.org/wiki/Declaration_of_Helsinki)as reflected in a priori approval by the institution's human research committee. In studies involving animal experimentation, provide assurance that all animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/olaw.htm)" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Every experimental or clinical study may raise controversial ethical issues (e.g., Institutional Ethical approval for working with animal or human subjects).

http://en.wikipedia.org/wiki/Declaration_of_Helsinki (http://en.wikipedia.org/wiki/Declaration_of_Helsinki)

http://grants.nih.gov/grants/olaw/olaw.htm (http://grants.nih.gov/grants/olaw/olaw.htm)

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Ethical considerations

Human subjects

Authors reporting experimental studies on human subjects must include a statement of assurance in the Materials and Methods section of the manuscript reading that, the project was done with consideration of ethical issues and obtaining license from the ethics committee of local university/research center and obtaining the written consent of participants. Also, it

was done according to ethical standards of human experimentation in accordance to the Helsinki Declaration (www.cirp.org/library/ethics/helsinki (http://www.cirp.org/library/ethics/helsinki)).

Animal subjects

Authors reporting experimental studies on animal subjects must include a statement of assurance in the Materials and Methods section of the manuscript reading that, the project was done with consideration of ethical issues and obtaining license from the ethics committee of local institute. Also, the general care of the experimental animals used for this study was done in compliance with the Animal Welfare Act (http://www.nap.edu/openbook.php?record_id=5140&page=114).

Informed Consent

In the case of research on human subjects, informed consent and other ethical considerations should be mentioned in the "methods" section of the manuscript. The author should include a statement that informed consent was obtained for any experimentation with human subjects. As *JHP* follows ICMJE (http://www.icmje.org/journals.html#B), please consider their guidelines (http://www.icmje.org/urm_main.html) for more information. In cases where a study involves the use of live animals or human subjects, the author should also include a statement that all experiments were performed in compliance with the relevant laws and institutional guidelines, and also state the institutional committee(s) that has approved the experiments. Moreover, the templates can be seen from WHO.

http://www.icmje.org/ (http://www.icmje.org/)

http://www.icmje.org/urm_main.html (http://www.icmje.org/urm_main.html)

http://www.who.int/rpc/research_ethics/informed_consent/en/

Conflict of Interests

The authors must declare any conflict of interest of contributed authors very briefly in a separate paragraph at the end of the paper. All sources of funding should be declared; unless otherwise the following sentence should be given "Authors declare no conflict of interests".

To prevent the information on potential conflict of interest for authors from being overlooked or misplaced, mention this information in the cover letter. The authors must identify any potential financial conflicts of interest before the review process begins. Declared conflict of interest will not automatically result in rejection of paper but the editors reserve the right to publish any declared conflict of interest alongside accepted. The following would generally be regarded as potential conflicts of interest:

1. Direct financial payment to an author for the research or manuscript production by the sponsor of a product or service evaluated in an article.

2. Ownership of shares by an author in the company sponsoring a product service evaluated in an article (or in a company sponsoring a competing product).

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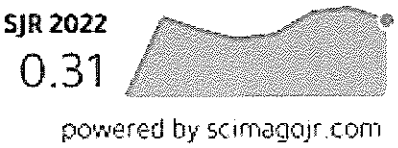

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Appendices

Appendix C: Confirmation email with reviewer comments and request to resubmit the manuscript to Journal of Herbmed and Pharmacology (see next page).

Ida Risenga

From: Oluwafunbi Adeleye <2415662@students.wits.ac.za>
Sent: 13 March 2024 04:00 PM
To: Ida Risenga
Subject: Fwd: Decision on your manuscript submitted to Journal of Herbmed Pharmacology (JHP)

---------- Forwarded message ---------
From: <master@herbmedpharmacol.com>
Date: Sun, 4 Feb 2024, 20:45
Subject: Decision on your manuscript submitted to Journal of Herbmed Pharmacology (JHP)
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Decision on your manuscript submitted to Journal of Herbmed Pharmacology (JHP)

Dear Oluwafunbi Christianah Adeleye,

Thank you for submitting your manuscript "Effect of concurrent extreme temperatures and water deficit on the phytochemistry, antimicrobial and antioxidant activities of Portulacaria afra using four extraction solvents" to Journal of Herbmed Pharmacology (JHP). Our editorial team and reviewers completed the evaluation of your manuscript. They have concluded that your manuscript has potential for publication but cannot be accepted in its present form. The referees have recommended a number of modifications.

You may resubmit a revised version but it will be re-evaluated and there is no guarantee that even with revision it will necessarily be accepted. It is essential that these comments are addressed before a revised manuscript can be re-considered.

Failure to fully address or refute these will result in rejection of the manuscripts. May I ask you to submit your revised manuscript with the attachment of a separate "word" file, stating item by item the revisions made, at your earliest convenience. Please highlight new changes in resubmitted document with red font and be informed that: 1. The revised manuscript should be returned to us within 28 days. Papers received after this period of time will

be handled as a new submission. 2. If you disagree with a reviewer's comment/suggestion, please provide me with an explanation in your cover letter. 3. You can't change, remove or add any author upon resubmission. Thank you so much for your interest in our journal.

Best Regards,

Editor in Chief

Reviewer id-38563 Comments

- The introduction is too long and must be summarized. Use an introduction paragraph, a justification paragraph, and a third objective paragraph.
- Figures 1 and 2 can be combined.
- No great graphic treatment is displayed. Many tables, from tables 4 to 10, can be joined into graphs and be more attractive.
- Tables 12 to 15 can be joined since many null data spaces exist.
- Figures 3 to 14 must be redone, improving the graphical treatment and using R studio.
- The manuscript requires a lot of information processing.

Limitations:

- Limited information on the specific abiotic stress conditions or combinations studied, hindering a comprehensive understanding of the broader impact of multiple stressors on medicinal plants.
- Focus on the effects of abiotic stress on secondary metabolite production in P. afra without exploring the underlying molecular mechanisms or pathways involved.
- Lack of information on the long-term effects of abiotic stress on the growth, development, and overall productivity of medicinal plants.

Reviewer id-44091 Comments :

1. Please explain how plants live in extreme temperatures. So, it is crucial to investigate the various effects of temperature, water deficit and solvents, which may play a role in phytochemical, antimicrobial and antioxidant activity.

2. Please explain what effect this plant has on the antimicrobial activity of the extract depending on the part of the plant and the temperature of the water. What about normal conditions? Is there a significant difference? And do solvents have an essential role in showing results?

3. Please use concise and easy-to-understand explanations if the explanations are complicated.

Reviewer id-4102 Comments:

General comments:

1. The lack of novelty, 2. The presentation of data lacks systematic organization and challenging to interpret, 3. The antimicrobial data

testing results exhibit deficiencies, characterized by a notable frequency of missing or incomplete data entries, which detract from their overall coherence and reliability

Introduction

It is advisable to focus on the background of the research –

-Explanation regarding the influence of abiotic and biotic factors on plant conditions is too broad, please provide a concise overview

- Please elaborate on why P. afra plants were chosen for this study. Is it because these plants are commonly used as a primary medical ingredient?

- Why were parameters such as phytochemistry, antimicrobial, and antioxidant properties chosen for testing? Please connect these choices with the primary objectives of the research.

- It would be appreciated to provide an addition clarification regarding P. afra, supplemented with an explanation as to why this particular plant has been chosen for the research.

- Please ensure all measured parameters are included. Also, explain that the plants are harvested from South Africa

Method –

Please provide additional information regarding the source of P. afra plants, and which part of the plant is utilized for testing purposes

- The method should be written succinctly.

- Please provide the details of all storage temperatures

- Please consolidate both points (Antimicrobial activity assay and Agar well diffusion method) into a more concise form.

- A statistical analysis in method can be written as a separate point

Result and Discussion

- The presentation of tables 4 through 15 appears to be rather challenging to understand. It would be great if the presentation of these tables could be enhanced to be more comprehensible. - Please also state that significant differences are indicated by differences in alphabetical notation (a, b, c, d) for all tables and figures

- Please also list several studies discussing the active ingredients contained in P. afra and compare them with the research findings obtained.

- What is the significance of a high increase in antioxidant values? It was previously mentioned that antioxidant activity could be due to elevated ROS levels. It would be better to explain the pathway between ROS, plant growth conditions, and the resulting antioxidant levels in this experiment

Conclusion

- Conclusion should be written concisely, encompassing all findings and addressing the research title

Reference

- References should be current, with a maximum publication date of no more than 10 years ago - Ensure consistency in citation style

Editor comments:

The manuscript has various technical problems.

1. Professional English checking and writing are recommended. Please note that this journal ONLY accepts articles written in good English for publication. Authors who are not native English speakers, should please ensure to have their article corrected by a native English speaker or by a professional language editor for any grammatical, semantical/stylistic and typographical errors. Authors who are native English speakers should ensure that their article has been revised for language, grammar, and style (where appropriate). A section was edited as an example.

2. Reduce the manuscript to the than 9000 words.

3. Tables and figures should be understandable without referring to the text. Hence, the title should be complete, the abbreviations should be defined, and the comparisons should be clear (Just indicating the p-values is not accepted), ….

4. Scientific names should be in *italic* form.

5. All abbreviations should be presented in full and the abbreviation in parenthesis when appearing for the first time or in each table and figure.

6. The similarity index of your manuscript should be less than 20% with no more than 3% similarity from a single source. Please check for plagiarism.

7. Please consult a recently published paper in JHP and add the followings:

ORCID IDs of all the authors.

Running title.

Implication for health policy/practice/research/medical education: ???

Please cite this paper as: Family name first and then the initials of the first and middle names. Title. J Herbmed Pharmacol. x;x(x):x-x]A1[

Authors' contributions

Due to recent changes in PubMed Policies, our journal has to publish its papers with the new "Authors' contributions" style, onwards. On this basis, "Authors' contributions" should be clarified based on the following items: Please do not change item names and do not add any other items.

Authors' Contribution:

Conceptualization:

Data curation:

Formal analysis:

Funding acquisition:

Investigation:

Methodology:

Project administration:

Resources:

Software:

Supervision:

Validation:

Visualization:

Writing–original draft:

Writing–review & editing:

Please add author names (**Full names**) in front of each above-mentioned contributor role if applicable.

Conflict of interest

Ethical considerations

??????? (Which committee confirmed the protocol??? Ethical code???)

Funding/Support

??????? (Grant number???)

8. References:

References should be edited and reformatted. They are not formatted correctly. Some of them are not complete.

Et al., after six authors.

The journal names (Abbreviations) should be adapted from NLM Catalogue (PubMed).

The names of a journal start with a capital letter Example: J Ethnopharmacol.

- Please also be informed that the publication fee is 200 USD for normal and 250 USD for fast-track publication.

Appendix D: Symposium Attendance certificate