THERAPEUTIC PROMISE OF AQUEOUS EXTRACT OF *Portulaca oleracea* L.: ANTIOXIDANT AND ANTI-INFLAMMATORY EFFECTS

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AUGUST, 2025

DECLARATION

I, Wanderi Gladys Wamaitha, duly declare that this thesis is my original work and has not been presented for a degree or other awards in any other university

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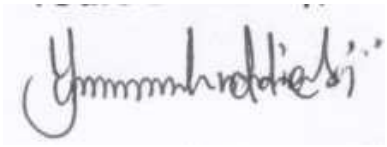
Signature:  Date ……

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DEDICATION

This thesis is dedicated to my family in appreciation of their consistent support, prayers, sacrifices, and encouragement.

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ABBREVATIONS AND ACRONYMS

ANOVA	Analysis of Variance
CAT	Superoxide dismutase
COX	Cyclooxygenase
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ETC	Electron Transport Chain
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
IC_{50}	Half-maximal inhibitory concentration
IL-1β	Interleukin-1beta
LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor kappa B
NSAIDs	non-steroidal anti-inflammatory drugs
PG	Prostaglandin
RBCs	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEM	Standard error of mean
SOD	Superoxide dismutase catalase
TAA	Total antioxidant activity
TFC	Total flavonoid content
TNF-α	Tumor necrosis factor-alpha
TPC	Total phenolic content
TXA2	Thromboxane A2

ABSTRACT

Oxidative stress is an imbalance in which oxidants exceed antioxidants in the body's defense system. Several chronic diseases, including rheumatoid arthritis, cardiovascular disorders and cancer are caused by oxidative stress. Inflammation refers to biological responses caused by oxidative stress, toxic substance, irradiation and pathogens. Synthetic antioxidant and anti-inflammatory drugs already on the market are linked to adverse effects, necessitating the need for an alternative medicinal approach. *Portulaca oleracea* is used by communities living in Embu County to manage inflammation. Nevertheless, the scientific data to confirm this claim was lacking. This study aimed to assess *in vitro* antioxidant and *ex vivo* anti-inflammatory effects, including qualitative phytochemical analysis of *Portulaca oleracea*. Fresh plant sample was obtained from Embu County, Kenya. The sample was air-dried, milled, macerated with water and then freeze dried to obtain a solid extract. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, H_2O_2 radical scavenging, ferric reducing antioxidant power (FRAP), total phenolic content (TPC) and total flavonoid content (TFC) were among the antioxidant assays that were performed according to standard methods. The antioxidant assays used the extract at concentrations of 7, 15, 31, 62, 125, 250, 500 and 1000 µg/ml. In antioxidant assays, ascorbic acid was utilized as the standard. The standards that were used in TFC and TPC assays were rutin and gallic acid, respectively. The *ex vivo* anti- inflammatory assays included hypotonicity-induced hemolysis, heat-induced hemolysis, albumin denaturation and anti-proteinase activity. Indomethacin was utilized as reference drug in the *ex vivo* anti-inflammatory assays. The extract and reference drug used concentrations of 7, 15, 31, 62, 125, 250, 500 and 1000 µg/ml. Standard procedures were used to conduct a qualitative phytochemical analysis. Analyzed data was summarized using mean and the standard error of the mean. Statistical differences between the different concentrations were evaluated using one-way analysis of variance and Tukey's multiple comparisons. Effects of ascorbic acid/Indomethacin and the extract were compared statistically using independent T-test. If p value was <0.05, statistical results were deemed significant. This study found that *Purslane oleracea* extract possesses potent *in vitro* antioxidant effect through FRAP, as well as DPPH radical scavenging and H_2O_2 radical scavenging activities. The extract also revealed considerable amount of TPC and TFC, which are linked with antioxidant effects. The extract also demonstrated *ex vivo* anti-inflammatory effect via inhibitions of: hypotonicity-induced hemolysis, heat-induced hemolysis, albumin denaturation, as well anti-proteinase activity. A concentration- dependent response was seen in the extract's *in vitro* antioxidant and *ex vivo* anti- inflammatory activities. For instance, at the highest concentration of extract(1000 µg/ml), the extract had its highest percentage inhibition on heat-induced hemolysis (72.65%) and its lowest inhibitory percentage (27.14%) at the lowest concentration of(7µg/ml) Phytocompounds including flavonoids, alkaloids, steroids, saponins, terpenoids, cardiac glycosides, tannins, and phenolic acids were detected in the extract according to a qualitative phytochemical analysis. These phytocompounds were linked to the two activities mentioned in this study. The current study concluded that the extract possesses potent *in vitro* antioxidant and *ex vivo* anti-inflammatory effects. The study recommends that the aqueous extract of *Purslane oleracea* can be used to develop alternative anti- inflammatory and antioxidant agents.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Oxidative stress, is a pathological state marked by an imbalance between free radicals and antioxidant defenses. It has been implicated in the progression of numerous chronic diseases. In biological systems, this causes molecular damage and/or disruption of redox signaling and regulation (Sies, 2020). Chronic ailments like lung disease, rheumatoid arthritis, cancer, cardiovascular disease, diabetes, kidney disease, and neurological disorders are linked to oxidative stress (Pisoschi *et al*., 2021). Oxygen metabolism produces free radicals as byproducts, such as hydroxyl radicals, singlet oxygen, peroxynitrite, nitric oxide radicals, hydrogen peroxide and superoxide radicals. These radicals are crucial for cell signaling (Panday *et al*., 2020; Jomova *et al*., 2023). Despite this, environmental stressors like ultraviolet rays, pollutants, ionizing radiation, and heavy metals, as well as xenobiotics such as antiblastic drugs, can greatly increase their production leading to an imbalance that damages cells and tissue (Pizzino *et al*., 2017; Jomova *et al*., 2023).

While reactive oxygen species (ROS) serve physiological roles such as signaling, their excess production that is triggered by the environmental and endogenous stressors can induce tissue damage and oxidative stress

Inflammation is defined as a biological response caused reactive oxygen species, damaged cells, toxic substance, irradiation and pathogens (Chen *et al*., 2018). These

elements may cause acute or chronic inflammatory reactions leading to tissue damage or disease (Pahwa *et al*., 2021). Activating the healing process and eliminating harmful stimuli are two responsibilities of inflammation (Chen *et al*., 2018). Despite their efficacy, synthetic anti-inflammatory drugs often cause adverse effects necessitating safer alternatives. Some of these include glucocorticoids (like betamethasone, budesonide, prednisone, prednisolone, dexamethasone, and fludrocortisone) Nevertheless, these drugs are linked to severe effects including hepatoxicity, nephrotoxicity and gastrointestinal bleeding (Pahwa *et al*., 2021; Chourpiliadis and Aeddula, 2023).

These limitations have driven interest in medicinal plants such as *Portulaca oleracea*, which is traditionally used but lacks sufficient scientific validation. Currently, *Portulaca oleracea* is used by Kenyan populations living in Embu County to manage conditions such as stomach upset, diabetes and inflammation. Its therapeutic properties are attributed to its phytochemical richness as well as the fact it grows annually and usually harvested after maturity (dry seasons) when concentration of phytochemicals is at peak. Nonetheless, the empirical evidence to validate these claims was lacking (Zhou *et al*., 2015). The current research study aimed to determine the *ex vivo* anti-inflammatory and *in vitro* antioxidant activities of *Portulaca oleracea* aqueous extract, including qualitative phytochemical screening.

1.2 Statement of the problem

Despite the increasing prevalence of chronic diseases linked to oxidative stress and inflammation, the conventional drugs used to manage these conditions often produce adverse effects. As such, there is an urgent need for safer, plant-bases alternatives.

Portulaca oleracea is widely used in treating a variety of ailments, including inflammation in Kenyan populations living in Embu County. However, there is insufficient empirical data to validate the acclaimed medicinal use.

1.3 Justification

With rising interest in plant- based therapies, identifying and characterizing the bioactive components in traditional medicinal plants like *Portulaca oleracea* could lead to novel antioxidant and anti-inflammatory agents (Marrelli,2021). The beneficial effects of medicinal plants lie in their phytochemicals such as saponins, phenols, tannins, alkaloids and flavonoids. These phytocompounds have clear physiological effects on the human body. Some of these physiological actions include anti-inflammatory and antioxidants effects, among others (Xu *et al*., 2017; Nunes *et al*., 2022).

These compounds are suspected to contribute to the plant's pharmacological effects, warranting scientific investigation (Kurek *et al*., 2022). Therefore, they may be alternative agents in managing inflammation and oxidative stress. *Portulaca oleracea* is traditionally used in treating several ailments, including inflammation and oxidative stress (Zhou *et al*., 2015). This investigation was designed to screen for *in vitro* antioxidant and *ex vivo* anti-inflammatory effects of *Portulaca oleracea* extract, including qualitative phytochemical screening.

1.4 Hypotheses

i. The *aqueous* extract of *Portulaca oleracea* does not exhibit significant *in vitro* antioxidant activity

ii. The aqueous extract of *Portulaca oleracea* does not exhibit significant *ex vivo* anti-inflammatory activity

iii. The *aqueous* extract of *Portulaca oleracea* does not exhibit phytocompounds linked to antioxidant and anti-inflammatory potential.

1.5 General objective

To determine the *in vitro* antioxidant and *ex vivo* anti-inflammatory effects of aqueous extract of *Portulaca oleracea*.

1.5.1 Specific objectives

i) To determine *in vitro* antioxidant effect of aqueous extract.

ii) To evaluate *ex vivo* anti-inflammatory effect of aqueous extract.

iii) To assess qualitative phytochemical composition of aqueous extract.

1.6 Significance of the study

This study seeks to bridge the knowledge gap regarding the antioxidant and anti-inflammatory activities of aqueous extract of *Portulaca oleracea*. Its findings may contribute to the development of safer, plant-based alternatives to synthetic drugs.

CHAPTER TWO

LITERATURE REVIEW

2.1 Oxidative stress

Oxidative stress arises when reactive oxygen species overwhelm the body's antioxidant defense, leading to cellular damage and diseases. (Sharifi-Rad *et al*., 2020). Numerous causes contribute to increased oxidative stress at the cellular level. They include poor diet, exposure to alcohol, strenuous physical activity, medications, radiation, infections, toxins, cold or trauma. The various antioxidants, which are obtained either directly or indirectly from food, are necessary for protection against oxidative stress (Pizzino *et al*, 2017; Wang and Kang, 2020).

2.1.1 Nitric oxide as an oxidative stress regulator

Nitric oxide is synthesized by nitric oxide synthase (NOS), which exists in three isoforms: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). During inflammation, iNOS is upregulated, leading to sustained and high-output NO production. However, at physiological concentrations, NO helps regulate vascular tone, inhibits leukocyte adhesion, and limits platelet aggregation. This contributes to anti-inflammatory effects and homeostasis (Förstermann & Sessa, 2012). Moreover, excessive NO from iNOS during chronic inflammation can lead to the formation of reactive nitrogen species (RNS), such as peroxynitrite, which can damage proteins, lipids, and DNA, thus exacerbating inflammation (Bogdan, 2015) as well as the onset of some ailments (Sharma *et al*., 2020).

However as an antioxidant, NO can scavenge superoxide radicals and regulate antioxidant enzymes, thus acting as a free radical quencher in some contexts (Radi,

2013).Also as a Pro-oxidant ,when NO reacts with superoxide, it forms peroxynitrite ($ONOO^-$), a potent oxidant that contributes to oxidative damage and cell death (Pacher, Beckman, & Liaudet, 2007). This leads to conditions like neurodegeneration, cardiovascular disease, and sepsis.

2.1.2 Nitrogen and oxygen reactive species

Reactive oxygen and nitrogen species (ROS and RNS) are highly reactive molecules that induce oxidative stress in biological systems. (Jomova *et al*., 2023). The impact of oxidative stress is linked with illness development, aging, and toxicity. Molecular oxygen/nitrogen produces ROS (reactive oxygen species) through the cytochrome P450, Electron Transport Chain (ETC), and other cellular and sub-cellular processes (Jakubczyk *et al*., 2020; Hameister *et al*., 2020).

Majority of living organisms have an efficient defense system that offers them protection against the oxidative damages caused by these ROS and RNS (reactive nitrogen species) (Ahmed and Mohammed, 2020). Some of the ROS include: O_2^- (huperoxide anion), ^-OH (hydroxyl anion), O^- (singlet oxygen), ROO^- (Peroxyl) and RO^- (Alkoxy). Some of the RNS include: $ONOO^-$ (peroxynitrite), NO_3^- (nitrate) and NO_2^- (nitrite). ROS and RNS are generated from non-enzymatic and enzymatic sources (Panday *et al*., 2020; Jomova *et al*., 2023).

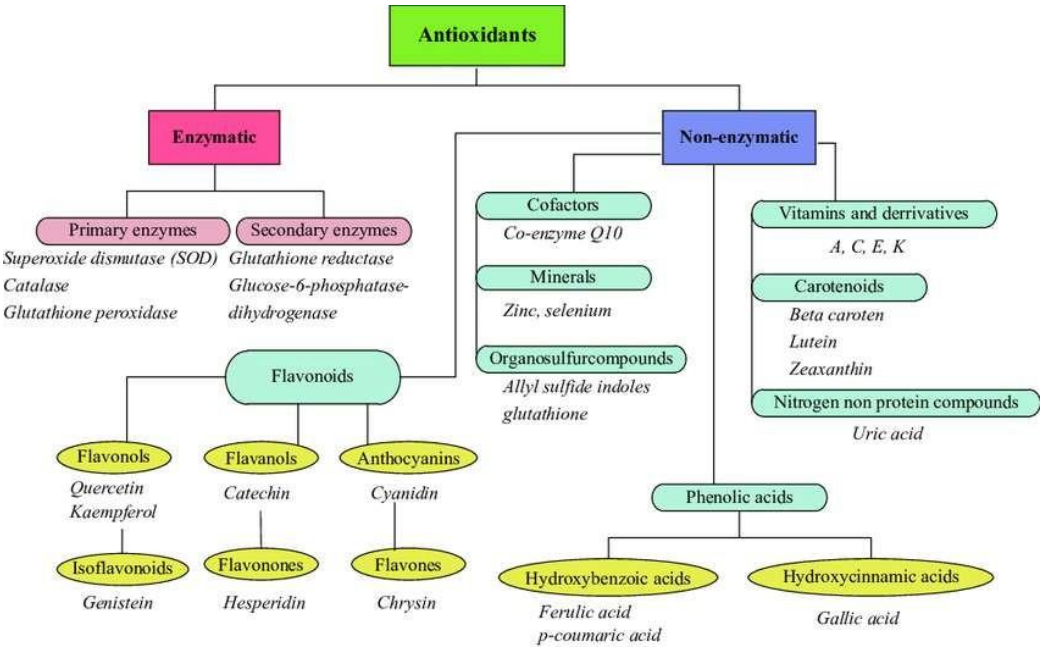
2.1.3 Antioxidants

Any chemical that may prevent or delay the oxidation of other molecules, typically biological substrates like proteins, lipids, or nucleic acids, is considered an antioxidant

(Sharifi-Rad *et al*., 2020). Two different types of reactive species can start the oxidation of these substrates: free radicals and species that are sufficiently reactive even without free radicals to start the oxidation of the aforementioned substrates (Neha *et al*., 2019). As a result, antioxidants can either stabilize or destabilize free radicals before they damage cells. This trait enables the antioxidants to act as our first line of defense. There are several methods that antioxidants use to prevent oxidative stress, such as scavenging of radicals, and chelating unbound catalytic metals (Jomova *et al*., 2023).

2.3.1.Antioxidant function

Biological systems are made up of dynamic equilibrium between oxidation and reduction reactions within a cell or organism. This balance is crucial for maintaining proper cellular function and preventing oxidative damage. It involves a complex interplay of mechanisms that regulate the production and elimination of reactive oxygen species (ROS), reactive nitrogen species (RNS). This balanced redox state plays a key role both physiologically and biochemically as a protective strategy in living organisms. Below is a diagrammatical illustration of the antioxidants classification system.



Together, these mechanisms protect against the damaging effects of oxidants on cells, tissues, and organs (Sharifi-Rad *et al*., 2020). Ideally, antioxidants function well in the aqueous and membrane domains, ROS, chelate redox metals, and positively impact the expression of an organism's genes. Reactive oxygen species are eliminated by antioxidants by the reduction of oxygen, the transformation of radicals into non-radicals, or the suppression of the initial phase of hydroxyl radical formation (Egbujor *et al*., 2021).

Three main defense mechanisms are present in antioxidants. In the first mechanism, antioxidant enzymes are involved. The second mechanism deals with the binding of proteins like albumin and transferrin to elemental ions. These binding proteins impede free radicals' detrimental interactions with biological molecules by suppressing their production. The third mechanism is through free radicals scavenging (Lazzarino *et al*., 2019; Egbujor *et al*., 2021).

2.1.4 Synthetic antioxidants

The need for safer alternatives to the synthetic antioxidants e.g. (Butylated hydroxytoluene, tert-butylhydroxyquinone) motivates the investigation of medicinal plants such as *Portulaca oleracea* (Atta *et al*., 2017; Barbosa *et al*., 2023). These antioxidants are however linked with severe effects such as DNA damage, gastrointestinal toxicities, induce premature senescence, and reduced levels of haemoglobin (Lourenço *et al*., 2019; Barbosa *et al*., 2023).

2.1.5 *In vitro* antioxidant assays

Numerous methods for assessing *in vitro* antioxidant effect of natural substances have been developed. Electron-transfer and hydrogen atom-transfer reactions are two important chemical processes that serve as the foundation for these processes. The following assays are measured by electron transfer reactions in order to ascertain the antioxidant potencies. They form the basis of this study's assessment of *P.oleracea* antioxidant capacity.These assays incude:H_2O_2 radical scavenging, ferric reducing antioxidant power (FRAP), and 2,2-diphenyl-1-picrylhydroxyl (DPPH) radical scavenging assays (Nigussie *et al*., 2023). Total antioxidant activity (TAA), TPC, and TFC are also employed in evaluating the antioxidant benefits of natural products.

2.2 Inflammation

Inflammation refers to the mechanism by which the immune system of the body reacts to an irritant, which can be a foreign object or a noxious chemical (Chen *et al*., 2018). Inflammation is intricately linked with oxidative stress where by increased oxidative stress can also cause inflammation, which can then trigger the emergence of a number of chronic conditions like pancreatitis, rheumatoid arthritis, diabetes, and cancer, among

others. Moreover, a number of transcription factors can be activated by oxidative stress, which in turn results in diverse expression of a number of genes linked to inflammatory pathways (Kiss, 2022).

Inflammation is classified as acute and chronic inflammation depending on the severity and status of symptoms. Acute inflammation is a rapid adaptive reaction that has limited specificity and is triggered on by unpleasant stimuli like tissue injury and infection. The hallmark of chronic inflammation is a persistent, slow-moving inflammation that lasts for months or even years. Chronic inflammation often depends on several factors, such as the cause of injury, the body's capacity to recover, and the extent of damage. It is this type that over the years leads to various conditions such as: rheumatoid arthritis, psoriasis, cardiovascular disease, and allergies. However, acute inflammation is marked by pain, swelling, heat, and redness (Liberale *et al*., 2022; Kiss, 2022).

2.2.1 Physiology of inflammation

The primary function of inflammation is to repair damage or initiate remedial action to ensure the organism's survival and restoring homeostasis. Transferring bloodstream-derived fluid, proteins, and cells to the injured tissues is a crucial first step in this process. The following mechanisms enable this to happen: vasodilation; enhanced vascular permeability; cell infiltration; modifications in biosynthesis and metabolism of cells and organs; activation of the blood plasma enzyme system and immune system (Placha and Jampilek, 2021; Sohrab *et al*., 2023). Some of the key mechanisms that are much relevant to the anti-inflammatory assays in this study included the stabilization of erythrocyte membranes and inhibition of protein denaturation. Whereby during inflammation,

enzymes are released, causing tissue damage. Substances that are known to stabilize erythrocyte membranes e.g. phenolic acids inhibit enzymes release such as the COX-2 and phospholipase A2 which cause the tissue damage and consequently onset of inflammation. This mechanism is tested using red blood cells (RBC) in the ex vivo anti- inflammatory model. Moreover, the process of protein denaturation is much dependent on enzymes such as the lipoxygenases, COX-2 and phospholipase A2 that lead to the release of harmful products (leukotrienes, prostanoids) that induce denaturation process. The presence of some phytocompounds e.g. alkaloids could be linked to the inhibition of protein denaturation

Acute inflammation requires the involvement of early phase mediators. Histamine, serotonin, chemotactic factors (C5a), vascular endothelial products, vasoconstrictors (endothelins, thromboxane A2 (TXA2), prostaglandin G2 (PGG2), vasodilators (nitric oxide, prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), prostaglandin 12 (PGI2), and pro-cytokines (TNF-α (tumor necrosis factor-alpha), and interleukins (1β and IL-6)), are some of these inflammatory mediators. Leukocytes are transported into the tissue by late phase mediators, which are also in charge of controlling vascular reactivity 6-12 hours after the onset of an inflammatory response. Vascular reactivity is regulated by metabolites of arachidonic acid. Vasodilators such as histamine, bradykinin, NO, PGE_2, PGI_2, and C5a are responsible for the production of edema. The chemotactic factors leukotriene B4 (LTB4), N-formylmethionyl peptides from bacteria, and mitochondria of injured host cells are released during this process, directly stimulating the movement of particular phagocytes (Cavaillon and Singer, 2018; Zhong and Shi, 2019; Placha and

Jampilek, 2021).

2.2.2 Conventional treatment of inflammation

Inflammation is treated using NSAIDs (like celecoxib, naproxen, mefenamic acid, diclofenac, ibuprofen, etoricoxib and indomethacin) and glucocorticoids such as hydrocortisone, methylprednisolone and prednisolone. However, the limitations of these treatments have spurred interest in plant- based therapies (Ghlichloo and Gerriets, 2022; Yasir *et al.*, 2022).

NSAIDs primarily function by inhibiting the cyclooxygenase (COX) enzyme. Thrombins, prostaglandins, and prostacyclins cannot be synthesized from arachidonic acid without cyclooxygenase. Cyclooxygenase is composed of two isoenzymes, cyclooxygenase-1 and cycloxygenase-2. The body establishes a constitutive expression of COX-1 to sustain platelet aggregation, renal function, and the mucosa lining the gastrointestinal system. Instead of constitutively producing COX-2, the body produce COX-2 through inducible expression during an inflammatory reaction. Since most NSAIDs inhibit the two cyclooxygenase enzymes, they are generally not selective. NSAIDs that exclusively target COX-2, like celecoxib, have a distinct adverse effect profile because they only impact COX-2. The COX-2 selective NSAIDs are important because they are believed to reduce inflammation without affecting the gastric mucosa, especially since the primary mediator for preserving the integrity of the stomach mucosa is COX-1, whereas COX-2 plays crucial function in inflammation (Jahnavi *et al*., 2019; Ghlichloo and Gerriets, 2023).

2.2.3 *Ex vivo* anti-inflammatory assays

Several *ex vivo* anti-edema assays are highly utilized in assessing the anti-inflammatory effect of medicinal extracts and other potential anti-inflammatory agents. These *ex-vivo* anti-edema assays include albumin denaturation, anti-proteinase, heat and hypotonicity induced hemolysis and membrane stabilization assays (Das *et al*., 2022; Fayez *et al*., 2023).

2.3 Alternative agents and Phytochemicals

This study focuses on evaluating the phytochemical composition of *P.oleracea* which contains the the bioactive compounds.However they are classified into : primary and secondary phytochemicals. Primary phytochemicals include amino acids, common sugars, purines, and pyrimidines, whereas secondary phytocompounds (secondary metabolites) includes alkaloids, flavonoids, terpenes, phenolic acids, curcumines, saponins, and glycosides (Shin *et al*., 2020; Nwozo *et al*., 2023).

A class of secondary phytochemicals known as phenolic compounds has a strong ability to scavenge oxidants, and inhibition of hydrolytic and oxidative enzymes. Flavonoids and phenolic acids have a variety of antioxidant properties. These properties include the capacity to scavenge a broad range of oxidants, including reactive oxygen, hypochlorous acid, hydroxyl ions, peroxynitrous acid, and superoxide. Additionally, flavonoids can chelate ions by reducing the pro-oxidant capacity of metal ions (Hassanpour and Doroudi, 2023; Nwozo *et al*., 2023).

Phytochemicals manage inflammation through suppression of cell signaling pathways,

including 5-lipoxygenase (5-LOX), signal transducer and activator of transcription 3, NF-κB, phosphatidylinositol 3-kinases, mitogen-activated protein kinase, and COX-2 (Jang and Lee, 2023). Additionally, they promote the synthesis of pro-inflammatory cytokines (like interleukins-(1β and 6), and TNF-α) and inhibit anti-inflammatory cytokines (such as interleukins-(4 and 10)) (Zhang *et al*., 2019; Shin *et al*., 2020).

2.4 *Portulaca oleracea* L.

2.4.1 Description of *Portulaca oleracea*

Purslane, or *Portulaca oleracea*, belong to Portulacacea family. It is an annual and succulent plant that may reach a height of 40cm (Zhou *et al*., 2015). The stem appears smooth and reddish, thick, fleshy, succulent, and hairless and up to 20 inches long. The leaves are thick, fleshy, succulent, spatula-shaped, hairless and approximately 1 inch long or less. The leaves may be opposite or alternate at stem joints. Flowers are yellow in colour with five regular parts about 6 mm wide. Rainfall greatly determines the time of the year that the flowers appear. Fruit are egg-shaped capsules that split in the middle, releasing a large number of tiny, glossy black seeds (Heydari *et al*., 2019).

Figure 2. 1: A photo of *Portulaca oleracea* L.

2.4.2 Distribution of *Portulaca oleracea*

Purslane is well distributed in North Africa to East Africa, Middle east, Indian and Southern Europe, (Zhou *et al*., 2015).

2.4.3 Medicinal uses *Portulaca oleracea*

The plant is rich in medicinal uses. It has been used to treat headache, stomachache, dysentery, intestinal worms, fever, diarrhea, carbuncle, eczema, diabetes, atherosclerosis, oxidative stress, inflammation, vascular endothelial dysfunction, gastrointestinal diseases, osteoporosis, psoriasis, and urolithiasis (Zhou *et al*., 2015). It is an immunomodulatory agent and eliminates both the free radicals and carcinogens from the body. Purslane aids in breakdown of cholesterol thus prevention against cardiovascular disease. Moreover, it has also anti-depressant properties thus can be used in treating disorders such as depression (Malekinejad et al.,2017).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Medicinal Plant Sample Harvesting and preparation

A traditional medical practitioner associated with Kenyatta University(Biochemistry department)assisted in the collection of fresh *Purslane oleracea* whole plant from Mbeere North Sub-County, Embu County, Kenya. Information about the plant's local name, its therapeutic applications, and the method for the preparation of various concoctions were also acquired. *Purslane oleracea* is an annual weed and it is usually harvested when mature for various medicinal uses. A purposive sampling strategy was used across 15 locations in January 2023. Collection adhered to the local biodiversity guidelines. Consent was obtained from the traditional healer involved in the collection. Ethical approval was granted by Ethic Review Committee, (Approval No. PKU/2773/11898). After thoroughly cleaning the plant sample with distilled water and chopping it into tiny pieces, the sample was then let to air dry at room temperature. Following the drying process, the material was ground into an evenly fine powder and placed in airtight container.

3.2 Extraction

Two liters of distilled water were used to dissolve 500 grams of the medicine powder. The mixture was left for 48 hours followed by decantation, using a muslin cloth and then filtered using Whatman® GF/C glass microfiber filter paper. The resulting filtrate was freeze-dried to obtain a solid extract which was packed into an airtight container and refrigerated at 4 ℃ waiting for subsequent experiments. The percentage yield was computed based on a formula used by Moriasi *et al*. (2020);

$$\% \text{ Yield} = \frac{\text{Weight of extract}}{\text{Weight of the powered sample}} \times 100$$

3.3 Study design

A completely randomized experimental design was employed to minimize selection bias and ensure comparability across treatment groups. The locale (Mbeere North, Embu County) was chosen based on the documented traditional use of *P.oleracea* and ease of access to fresh plant material.

3.3.1 Variables

These are normally classified into: Independent Variables and Dependent Variables. Independent variables are referred to as the set of variables manipulated or changed in an experiment to observe their effect on the dependent variables (the outcome being measured).In this study, the Independent variables included; aqueous extract concentrations that ranged between 7 -1000 µg/ml as well as the varying concentrations of the reference drug(Indomethacin) that were compared against the extract at similar ranges. The Dependent Variables refer to the outcomes that are affected by the independent variables in an experiment. However, in this study it involved the % inhibition various absorbance values as well as the antioxidant anti-inflammatory markers e.g. Superoxide dismutase, Glutathione reductase, IL-10, IL-1.

3.4 Evaluation of *in vitro* antioxidant activity

3.4.1 DPPH radical scavenging activity assay

This was achieved using a protocol used by Guchu *et al*. (2020) with few adjustments. The *P. oleracea* extract was serially diluted at 1000, 500, 250, 125, 62, 31, 15, and 7

µg/ml. Afterwards, 300 µl of the resulting solution was added to DPPH (3 ml) in separate test tubes. A similar setup was used for ascorbic acid (standard). The resulting mixture were then incubated for thirty minutes after which the absorbances were read using a spectrophotometer (UV-6100 UV/VIS Spectrophotometer 190-1100 nm/1.8 nm, China) at 517 nm. The control consisted of the DPPH and distilled water. This assay was executed in triplicates. The formula used by Skaperda *et al*. (2021) was applied to compute for DPPH radical scavenging activity (%);

$$\text{Radical scavenging activity (\%)} = \frac{\text{Ab control} - \text{Ab sample}}{\text{Ab control}} \times 100$$

Where; Ab = absorbance

3.4.2. FRAP assay

This assay was executed following a procedure used by Sasikumar *et al*. (2020) with few adjustments. Sequential dilutions of the extract and ascorbic acid (standard) were prepared at 1000, 500, 250, 125, 62, 31, 15, and 7 µg/ml. 1 ml of each serial dilution was blended with potassium ferricyanide (2.5 ml, 0.2 M) and phosphate buffer with a pH of 6.6. Following the addition of 2.5 milliliters of 10 % TCA (tricarboxylic acid), the mixture was heated at 50 °C for twenty minutes in a water bath. Subsequently, centrifugation was carried out at 3000 rpm for ten minutes. This led to the formation of a supernatant from which 2.5 ml was carefully obtained and added to 0.05 ml of 0.1 % $FeCl_3$ and distilled water (2.5 ml). The absorbances (at 700 nm) were measured using a spectrophotometer. The blank consisted of phosphate buffer, TCA (2.5 ml), potassium ferricyanide (2.5 ml), and distilled water (1.5 ml). The assay was also carried out in triplicates.

3.4.3 H_2O_2 scavenging assay

A protocol used by Arika *et al*. (2019) was employed in H_2O_2 scavenging assay. The H_2O_2 solution (40 mM) was prepared in phosphate buffer (2.5 ml) at a pH of 7.4. The extract and ascorbic acid serial dilutions (1000, 500, 250, 125, 62, 31, 15 and 7 µg/ml) were prepared and H_2O_2 (0.6 ml) added. The mixture was incubated (10 minutes) and then absorbance read using a spectrophotometer (230 nm). The control was made of phosphate buffer. The assay was executed in triplicates. A formula used by Hussen and Endalew, (2023) was applied to compute for percentage H_2O_2 radical scavenging activity as follows;

$$\text{Radical scavenging activity (\%)} = \frac{\text{Ab Control} - \text{Ab sample}}{\text{Ab control}} \times 100$$

Where; Ab = absorbance

3.4.4 TFC assay

The extract TFC assay was executed following a method used by Idamokoro and Afolayan, (2020), with few adjustments. First, serial dilutions of the extract and rutin (standard) were prepared at 1000, 500, 250, 125, 62, 31, 15, and 7 µg/ml. The extract/rutin (0.3 ml), $NaNO_2$ (0.15 ml, 0.5 M), 30 % methanol (3.4 ml), and $AlCl_3 \cdot 6H_2O$ (0.15 ml, 0.3 M) were blended, left standing for five minutes, and then NaOH (1 ml, 1 M) was added and thoroughly mixed. The absorbances were then read utilizing a spectrophotometer (510 nm). The assay was executed in triplicate. A rutin standard curve was generated and then used to quantify the extract rutin TFC equivalent.

3.4.5 TPC assay

The Folin-Ciocalteu reagent test was utilized to assess the extract's TPC, utilizing a methodology that was followed by Chaves *et al*. (2020) with some minor adjustments.

First, serial dilutions of the extract were made at 1000, 500, 250, 125, 62, 31 and 15 µg/ml. A milliliter of Folin-Ciocalteu reagent (made by diluting it with distilled water at a ratio of 1:10 v/v) was mixed with one milliliter of the extract and then one milliliter of 20 % sodium carbonate was added. After agitating the mixture for five minutes, the mixture was incubated for thirty minutes (at 40 °C). The absorbances were read using a spectrophotometer (760 nm). The TPC assay was executed in triplicates. A standard curve for TPC was generated using gallic acid (1000, 500, 250, 125, 62, 31, 15 and 7 µg/ml). The extract's TPC was calculated using its gallic acid equivalent.

3.5 Determination of *ex vivo* anti-inflammatory assay

3.5.1 Preparation of extracts concentration

Eight experimental concentrations were used in this study; (1000, 500, 250, 125, 62, 31, 15 and 7 µg/ml). To obtain a 1000 µg/ml extract concentration, 0.01 g of *P. oleracea* extract was put in normal saline (10 ml). The rest of 500, 250, 125, 62, 31, 15, and 7 µg/ml extract concentrations were obtained by 10-fold serial dilutions of the solutions with 1000 µg/ml extract concentration. The same protocol was applied when preparing standard drug (Indomethacin) concentrations.

3.5.2 Red blood cells preparation

Blood samples were obtained from healthy laboratory mice under institutionally approved ethical protocols (Ethical Clearance No: PKU/2773/11898). This packed red blood cells drawn from mice was preserved in Alseiver's solution. Determination of *ex vivo* anti-inflammatory effects was carried out by first conducting membrane stabilization activity. This involved combining the packed red blood cells with Alseivers solution (anticoagulant) in a ratio of 1:1. After centrifugation for ten minutes at 3000 rpm, the

resulting red blood cells (RBCs) were triple-washed with isosaline solution. Isosaline solution was then used to prepare the RBC suspension (10% v/v). Use of the animal samples was carried out under ethical approval of the Ethics Review Committee following ARRIVE guidelines.

3.5.3 Hypotonicity-induced hemolysis

This assay was executed following a protocol used by Chirumamilla and Taduri, (2023) with minor adjustments. Briefly, serial dilutions of the extract and reference drug indomethacin (1000, 500, 250, 125, 62, 31, 15 and 7 µg/ml) were prepared. One milliliter of the serial dilutions was prepared using normal saline and then distilled water (1 ml) added to form a hypotonic saline solution. The RBCs suspension (0.5 ml) in 1 ml of 150 Mm phosphate buffer (pH of 7.4) was added to each serial dilution of the extract and Indomethacin. The mixtures were incubated (1 hour) at 37 °C and later, centrifuged for five minutes at 3000 rpm. The resulting supernatants were aspirated and then absorbances were estimated using a spectrophotometer (560 nm). The RBCs suspension and distilled water were used as the control. The assay was executed in triplicates. The hemolysis generated by distilled water was assumed to be 100%. The percent protection of hemolysis by the extract was computed in accordance to a formula used by Rastogi *et al*. (2018) as follows;

$$\text{Percentage protection} = 100 - \frac{\text{Ab sample}}{\text{Ab control}} \times 100$$

Where; Abs = Absorbance

3.5.4 Heat-induced hemolysis

This assay was executed following a method used by Gunathilake *et al*. (2018) with minor adjustments. Serial dilutions of *P. oleracea* extract (1000, 500, 250, 125, 62, 31, 15

and 7 µg/ml) were prepared with phosphate buffer (pH of 7.4) and then 1 ml of the serial dilutions mixed with 10% RBCs suspension (1 ml). The reference drug indomethacin was prepared following the same protocol. The mixture was then centrifuged once more for five minutes at 2500 rpm after being incubated for thirty minutes at 56 °C and cooled using running water. The mixture's turbidity was determined with the use of a spectrophotometer (560 nm). The RBCs (10%) suspension and isosaline solution were utilized as the control. The assay was executed in triplicates. The hemolysis inhibition (%) was computed based on a formula used by Rastogi *et al*. (2018) as follows;

$$\text{Percentage hemolysis inhibition} = \frac{\text{Ab control} - \text{Abs control}}{\text{Ab control}} \times 100$$

Where Ab = absorbance

Moreover, this particular assay was also based on the mechanism whereby the breakdown of red blood cells due to heat, contributes to inflammation by releasing hemoglobin and heme, which are known damage-associated molecular patterns (DAMPs). These DAMPs trigger inflammatory responses through various receptors and signaling pathways, promoting a state of hyperinflammation and hypercoagulability.

Here's a more detailed breakdown:

3.5.5 Inhibition of albumin denaturation

This assay was executed following a procedure used by Das *et al*. (2022) with minor adjustments. The mixture consisted 1% bovine albumin aqueous solution (1.5 ml) and the extract (1 ml) at serial dilutions of 1000, 500, 250, 125, 62, 31, 15, and 7 µg/ml. The reference drug, indomethacin was prepared using a similar procedure. The blank used a mixture of normal saline (1 ml) and albumin solution (1.5 ml). Once the mixes were ready, they were put in a water bath for twenty minutes (at 37 °C). The temperatures were

further increased to 51 ºC for twenty minutes and then let to cool at room temperature. The absorbances were measured at an absorbance of 660 nm. The assay was conducted in triplicates. The protein denaturation inhibition (%) was obtained according to a formula used by Priyadharshini *et al*. (2023) as follows;

$$\text{Protein denaturation inhibition (\%)} = \frac{\text{Ab control} - \text{Abs control}}{\text{Ab control}} \times 100$$

Where; Ab = absorbance

3.5.6 Determination of anti-proteinases activity

This assay was performed following a protocol by Gunathilake *et al*. (2018) with minor adjustments. Briefly, the extract (1 ml) at serial dilutions of 1000, 500, 250, 125, 62, 31, 15 and 7 µg/ml was mixed with Tris-HCl buffer (1ml, 20 mM, pH = 7.4) and 0.06 ml of trypsin (10 µg/ml). A similar setup was used for the reference drug, indomethacin. The resulting mixtures were incubated for 5 minutes (at 37 °C) and then casein (0.8 % w/v, 1 ml) was added. After twenty minutes of incubation at 37 °C, the mixture was stopped by adding two milliliters of 70% perchloric acid. The control had normal saline, Tris-HCl buffer, trypsin, casein and Per chloric acid. The mixture was centrifuged and the supernatant absorbances were determined utilizing a spectrophotometer at 210 nm. The assay was done in triplicates. The anti-proteinase activity (%) was computed based on a formula used by Basyal *et al*. (2021);

$$\text{Antiproteinase activity (\%)} = \frac{\text{Ab control} - \text{Abs control}}{\text{Ab control}} \times 100$$

Where; Ab = absorbance

3.5.7 Validity and Reliability of Experimental Procedures

All procedures were conducted in triplicate to ensure reproducibility. Standard methods were adapted from peer-reviewed sources to ensure procedural validity. Positive controls (ascorbic acid, indomethacin) were used for internal consistency.

3.6 Qualitative phytochemical analysis

The following qualitative phytochemical analysis was conducted in accordance to standard laboratory procedures:

3.6.1 Tannins test

The extract (0.5 g) was put in normal saline (5 ml) in a test tube, heated in the water bath and then filtered. Thereafter, 150 µl of $FeCl_3$ (0.1%) was added to the filtrate. Tannins were recognized by a blue-green color (Snehlata *et al*., 2018).

3.6.2 Alkaloids test

The extract (0.5 g) was put in 1% HCL (5 ml), warmed gently for 3 minutes in the water bath, filtered and subjected to Dragendorff's test and Mayer's test. In the first test, 100 µl of Dragendorff's reagent was put in 2 ml of the filtrate. Alkaloids were identified as a reddish-brown/orange solution. In the second test, 100 µl of Mayer's reagent was put in 2 ml of the filtrate. Alkaloids were shown by the development of cream precipitate. (Jared *et al*., 2018).

3.6.3 Saponins test

The extract (0.5 g) was mixed with normal saline (5 ml) in a test tube. The resultant solution was heated in the water bath, cooled and shaken vigorously. After two minutes, a steady, continuous foaming showed the presence of saponins (Snehlata *et al.,* 2018).

3.6.4 Cardiac glycosides test (Keller-Killiani test)

In this assay, the extract (0.2 g) was blended with chloroform (5 ml) in a test tube and evaporated to dryness. Approximately, 0.4 ml of glacial acetic acid (with traces of $FeCl_3$) and concentrated sulphuric acid (0.5 ml) were added respectively. A blue acetic layer indicated presence of cardiac glycosides (Snehlata *et al*., 2018).

3.6.5 Steroids test

This assay involved the use of 3 drops of the Liebermann-Burchard reagent that was added to the extract (1 ml). Steroid presence was indicated by a reddish-purple color (Usman *et al*., 2009).

3.7 Statistical data analysis

Descriptive and inferential statistics were conducted using GraphPad Prism v10.1.2. ANOVA with Turkey's post hoc was chosen for its suitability in comparing means across multiple groups. Qualitative phytochemical tests were interpreted based on the standard colorimetric reactions and compared with literature references.

CHAPTER FOUR

RESULTS

4.1 *In vitro* antioxidant effect of aqueous extract of *Purslane oleracea*

4.1.1 DPPH radical scavenging effect of aqueous extract of *Purslane oleracea*

The DPPH radical scavenging activity of *P. oleracea* aqueous extract increased significantly in a concentration- dependent manner($p<0.05$), with maximal activity at 1000µg/ml.Despite showing antioxidant potential, the extract's IC50 (58.66 µg/ml) was significantly higher than that of ascorbic acid (27.16 µg/ml), suggesting a comparative lower potency.

Table 4.1: DPPH scavenging effect of *Purslane oleracea* aqueous extract

	DPPH scavenging activity (%)	
Concentrations (µg/ml)	**Ascorbic acid**	**Extract**
1000	86.09 ± 1.01^{a}	74.45 ± 1.61^{b}
500	82.11 ± 0.95^{a}	66.67 ± 0.92^{b}
250	72.81 ± 1.11^{a}	59.59 ± 1.08^{b}
125	66.82 ± 1.02^{a}	53.05 ± 1.24^{b}
62	58.03 ± 0.93^{a}	44.81 ± 0.91^{b}
31	47.45 ± 1.27^{a}	37.92 ± 1.25^{b}
15	33.49 ± 1.35^{a}	23.73 ± 0.74^{b}
7	26.10 ± 1.40^{a}	13.19 ± 1.21^{b}
IC_{50}	27.16 ± 0.60^{B}	58.66 ± 0.64^{A}

Descriptive statistics (mean±SEM) with distinct superscript lower-case letters along the row differed significantly (($p<0.05$), using independent t-test).

As illustrated in figure 4.1, the extract of *Purslane oleracea* revealed a significant increase in DPPH radical scavenging effect from concentration ($p<0.05$) of 7 to 1000 µg/ml. The extract had concentration-dependent DPPH radical scavenging effect.

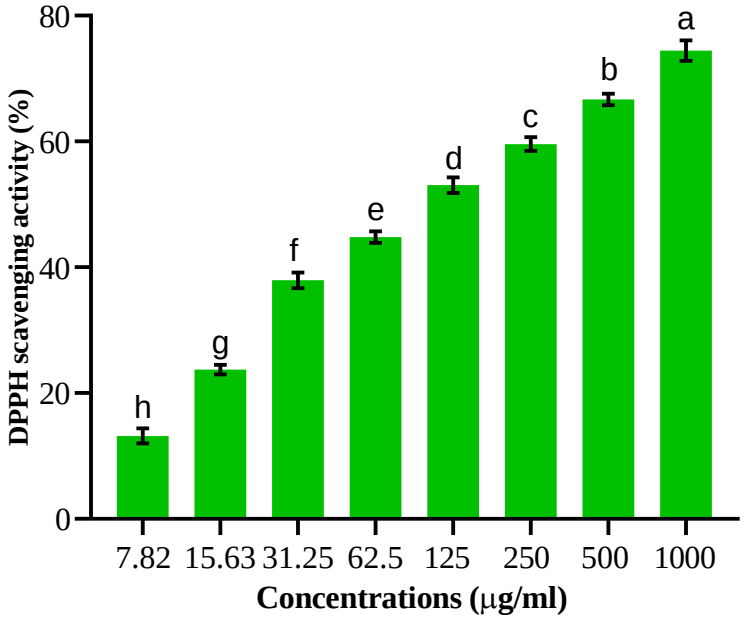


Figure 4.1: DPPH radical scavenging effect of *Purslane oleracea* aqueous extract. Bars with distinct lowercase letters show significant variations ($p<0.05$) using One-Way Analysis of Variance (ANOVA) and Tukey's multiple comparisons.

4.1.2 *In vitro* H_2O_2 radical scavenging effect of aqueous extract of *Purslane oleracea*

Although the extract exhibited hydrogen peroxide scavenging activity, the higher IC50 compared to ascorbic acid implies it may require higher concentrations to achieve similar oxidative stress reduction.

Table 4.2: H_2O_2 radical scavenging effect of *Purslane oleracea* aqueous extract

	H_2O_2 scavenging activity (%)	
Concentrations (µg/ml)	**Ascorbic acid**	**Extract**
1000	94.95±0.83[a]	86.70±1.26[b]
500	91.51±0.90[a]	81.25±1.25[b]
250	86.21±0.97[a]	72.84±1.11[b]
125	78.98±1.04[a]	65.38±1.23[b]
62	64.90±1.27[a]	56.57±1.46[b]
31	53.85±1.46[a]	36.62±1.54[b]
15	43.99±1.37[a]	33.17±1.32[b]
7	38.06±1.16[a]	17.23±1.04[b]
IC_{50}	49.36±0.23[B]	89.91±3.30[A]

Descriptive statistics (mean±SEM) with distinct superscript lower-case letters along the row differed significantly ((p<0.05), using independent t-test).

As presented in figure 4.2, the H_2O_2 radical scavenging effect of *Purslane oleracea* aqueous extract increased significantly from concentration (p<0.05) of 7 to 500 µg/ml. A concentration-dependent response was noted in the extract's DPPH radical scavenging effect.

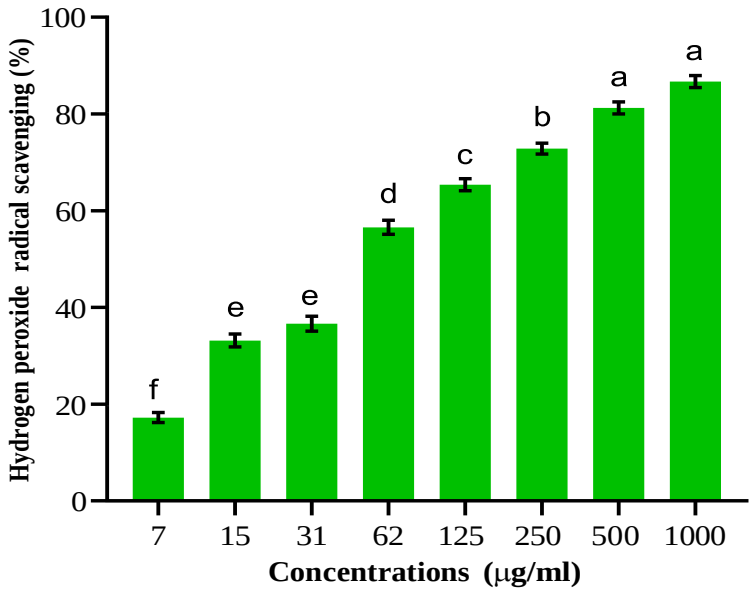


Figure 4.2: H_2O_2 radical scavenging effect of *Purslane oleracea* aqueous extract.
Bars with distinct lowercase letters show significant variations (p<0.05) using One-Way Analysis of Variance (ANOVA) and Tukey's multiple comparisons.

4.1.3 FRAP of *Purslane oleracea* aqueous extract

The extract demonstrated ferric reducing antioxidant power (FRAP) that increased with concentration, although its potency remained significantly lower than the ascorbic acid standard at all levels ($p<0.05$). This my indicate fewer electron-donating bioactives in the aqueous extract.

Table 4.3: FRAP of *Purslane oleracea* aqueous extract

	Ferric reducing antioxidant power (nm)	
Concentrations (µg/ml)	**Ascorbic acid**	**Extract**
1000	0.871 ± 0.03^{a}	0.587 ± 0.01^{b}
500	0.659 ± 0.02^{a}	0.396 ± 0.01^{b}
250	0.505 ± 0.02^{a}	0.277 ± 0.01^{b}
125	0.362 ± 0.02^{a}	0.185 ± 0.01^{b}
62	0.288 ± 0.02^{a}	0.138 ± 0.01^{b}
31	0.209 ± 0.01^{a}	0.087 ± 0.01^{b}
15	0.170 ± 0.01^{a}	0.066 ± 0.01^{b}
7	0.127 ± 0.01^{a}	0.021 ± 0.01^{b}

Descriptive statistics (mean±SEM) with distinct superscript lower-case letters along the row differed significantly (($p<0.05$), using independent t-test).

As illustrated in figure 4.3, the FRAP of the extract increased significantly from concentration ($p<0.05$) of 31 to 1000 µg/ml. The extract's FRAP responded in a concentration-dependent response.

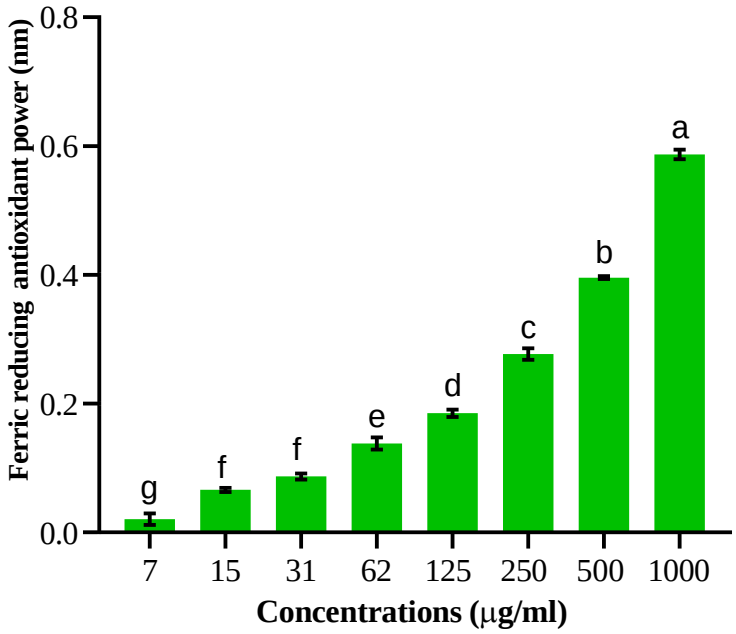


Figure 4.3: FRAP of *Purslane oleracea* aqueous extract.
Bars with distinct lowercase letters show significant variations ($p<0.05$) using One-Way Analysis of Variance (ANOVA) and Tukey's multiple comparisons.

4.1.4 TPC of *Purslane oleracea* aqueous extract

As depicted in figure 4.4, the *Purslane oleracea* aqueous extract exhibited a significant rise in TPC (gallic acid equivalent) across concentrations ranging from ($p<0.05$) 15 to 1000 µg/ml.

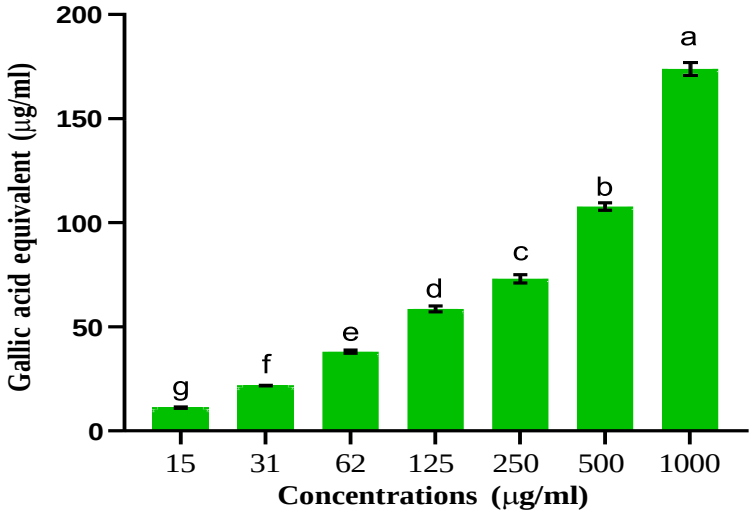


Figure 4.4: TPC of *Purslane oleracea* aqueous extract.
Bars with distinct lowercase letters show significant variations ($p<0.05$) using One-Way Analysis of Variance (ANOVA) and Tukey's multiple comparisons.

4.1.5 TFC of *Purslane oleracea* aqueous extract

As shown in figure 4.5, the TFC (rutin equivalent) of the extract increased significantly from concentration ($p<0.05$) of 15 to 1000 µg/ml.

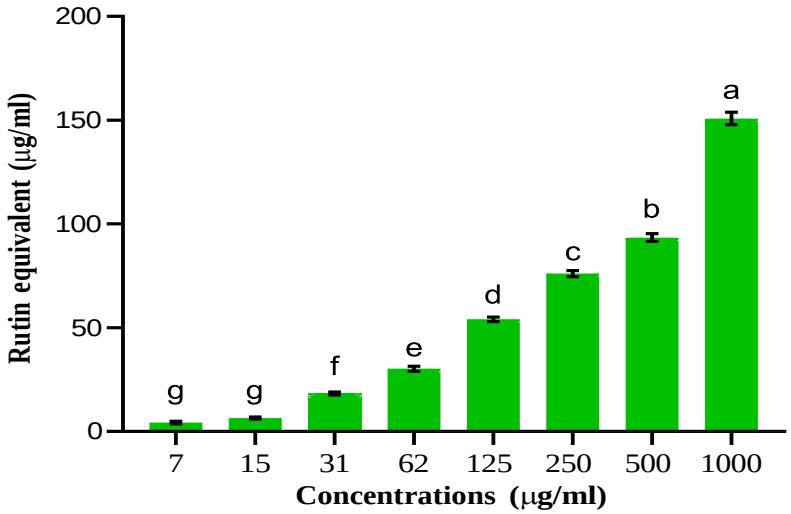


Figure 4.5: TFC of *Purslane oleracea* aqueous extract.
Bars with distinct lowercase letters show significant variations ($p<0.05$) using One-Way Analysis of Variance (ANOVA) and Tukey's multiple comparisons.

4.2 *Ex vivo* anti-inflammatory effect of aqueous extract of *Purslane oleracea*

4.2.1 Effect of aqueous extract of *Purslane oleracea* on heat-induced hemolysis

As depicted in table 4.4, the effect of the extract demonstrated changes in inhibition of heat-induced hemolysis. The effect of the aqueous extract had a significantly lower inhibition of heat-induced hemolysis relative to reference drug, Indomethacin ($p<0.05$) at the tested concentrations. Although less effective than Indomethacin, the aqueous extract demonstrated notable membrane stabilizing activity, indicative of anti-inflammatory potential through the inhibition of lysosomal release

Table 4.4: Effect of *Purslane oleracea* aqueous extract on heat-induced hemolysis

	Heat-induced hemolysis inhibition (%)	
Concentration (µg/ml)	**Indomethacin**	**Extract**
1000	81.41±0.98[a]	72.65±0.93[b]
500	75.43±0.93[a]	65.17±0.93[b]
250	70.30±0.57[a]	57.05±0.74[b]
125	64.10±0.98[a]	51.07±0.93[b]
62	59.19±0.77[a]	44.23±0.98[b]
31	54.49±0.74[a]	38.03±0.77[b]
15	48.93±0.77[a]	32.48±0.77[b]
7	40.39±0.98[a]	27.14±1.40[b]

Descriptive statistics (mean±SEM) with distinct superscript lower-case letters along the row differed significantly (($p<0.05$), using independent t-test).

As illustrated in figure 4.6, the *Purslane oleracea* aqueous extract noted a significant increase in inhibition of heat-induced hemolysis from concentration ($p<0.05$) of 7 to 1000 µg/ml. The heat-induced hemolysis was inhibited by the extract in a concentration-dependent response.

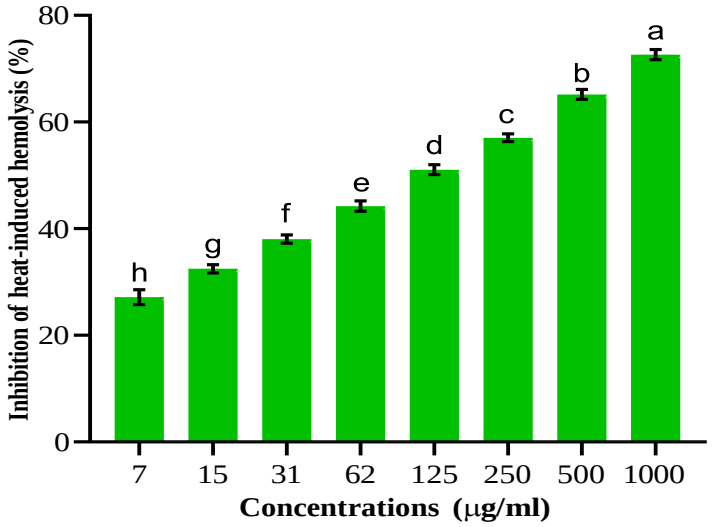


Figure 4.6: Effect of *Purslane oleracea* aqueous extract on heat-induced hemolysis. Bars with distinct lowercase letters show significant variations ($p<0.05$) using One-Way Analysis of Variance (ANOVA) and Tukey's multiple comparisons.

4.2.2 Effect of *Purslane oleracea* aqueous extract on hypotonicity-induced hemolysis

As illustrated in table 4.5, the effect of *Purslane oleracea* extract caused changes in inhibition of hypotonicity-induced hemolysis at concentrations of 7, 15, 31, 62, 125, 250, 500 and 1000 µg/ml. The effect of the extract had a significantly lower hypotonicity-induced hemolysis inhibition relative to ($p<0.05$) the effect of indomethacin.

Table 4.5: Effect of *Purslane oleracea* aqueous extract on hypotonicity-induced hemolysis

	Hypotonicity-induction hemolysis inhibition (%)	
Concentrations (µg/ml)	**Indomethacin**	**Extract**
1000	84.44 ± 1.47^{a}	70.89 ± 1.24^{b}
500	76.11 ± 1.47^{a}	66.00 ± 0.88^{b}
250	68.89 ± 1.11^{a}	50.44 ± 1.74^{b}
125	54.44 ± 1.11^{a}	38.56 ± 1.83^{b}
62	41.11 ± 1.11^{a}	28.89 ± 2.02^{b}
31	34.44 ± 1.11^{a}	18.67 ± 1.17^{b}
15	25.78 ± 1.13^{a}	12.33 ± 0.84^{b}
7	17.89 ± 0.80^{a}	6.89 ± 0.87^{b}

Descriptive statistics (mean±SEM) with distinct superscript lower-case letters along the row differed significantly (($p<0.05$), using independent t-test).

As shown in figure 4.7 below, the effect of *Purslane oleracea* aqueous extract caused a significant rise in the inhibition of hypotonicity-induced hemolysis from concentration of 15 to 1000 µg/ml ($p<0.05$). The extract inhibited hypotonicity-induced hemolysis in a concentration-dependent manner.

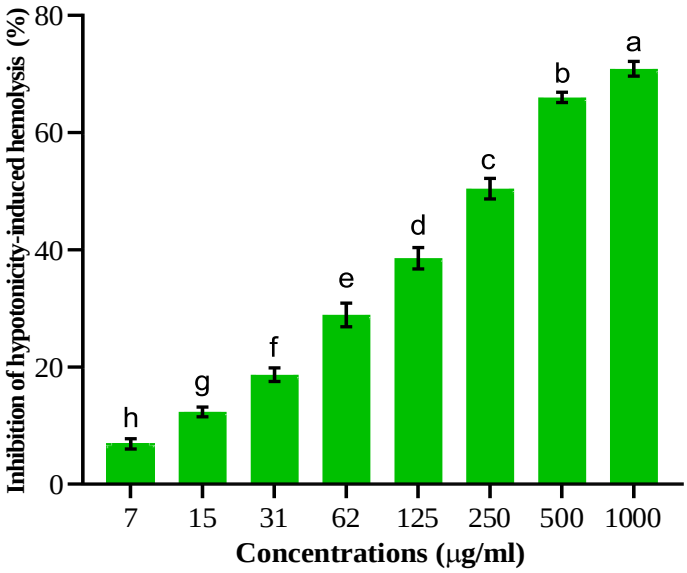


Figure 4.7: Effect of *Purslane oleracea* aqueous extract on hypotonicity-induced hemolysis.
Bars with distinct lowercase letters show significant variations ($p<0.05$) using One-Way Analysis of Variance (ANOVA) and Tukey's multiple comparisons.

4.2.3 Effect of *Purslane oleracea* aqueous extract on albumin denaturation

Since protein denaturation is a hallmark of inflammation, the extract's ability to inhibit this process, though weaker than Indomethacin, indicates a therapeutic potential in attenuating inflammatory protein misfolding or degradation

Table 4.6: Effect of *Purslane oleracea* aqueous extract on albumin denaturation

	Inhibition of albumin denaturation (%)	
Concentrations (µg/ml)	**Standard**	**Extract**
1000	86.11±0.96[a]	70.37±1.85[b]
500	81.85±0.81[a]	68.52±1.85[b]
250	77.78±1.60[a]	59.26±1.85[b]
125	70.74±0.81[a]	51.85±1.85[b]
62	59.07±1.03[a]	42.59±1.85[b]
31	47.59±1.30[a]	31.48±1.85[b]
15	42.41±0.98[a]	20.00±1.16[b]
7	30.93±1.30[a]	15.37±0.81[b]

Descriptive statistics (mean±SEM) with distinct superscript lower-case letters along the row differed significantly ((p<0.05), using independent t-test).

As depicted in figure 4.8, the effect of *Purslane oleracea* extract noted a significant increase in albumin denaturation inhibition from concentration (p<0.05) of 15 to 500 µg/ml. A concentration-dependent response was noted in the extract's inhibition of albumin denaturation.

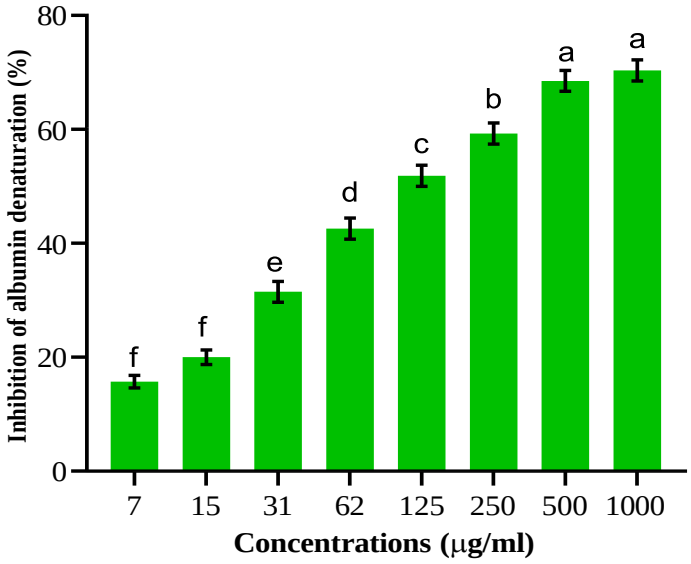


Figure 4.8: Effect of *Purslane oleracea* aqueous extract on albumin denaturation.
Bars with distinct lowercase letters show significant variations (p<0.05) using One-Way Analysis of Variance (ANOVA) and Tukey's multiple comparisons.

4.2.4 Effect of *Purslane oleracea* aqueous extract on anti-proteinase activity

The effect of the extract revealed alterations in anti-proteinase activity at concentrations of 7, 15, 31, 62, 125, 250, 500 and 1000 µg/ml (Table 4.7). The effect of the extract had a significantly lower anti-proteinase activity in comparison to the effect of ($p<0.05$) indomethacin at the studied concentrations (Table 4.7).

Table 4.7: Effect of *Purslane oleracea* aqueous extract on anti-proteinase activity

	Anti-proteinase activity (%)	
Concentrations (µg/ml)	**Indomethacin**	**Extract**
1000	83.15±1.29[a]	73.48±1.56[b]
500	77.78±1.29[a]	65.23±1.29[b]
250	65.59±1.64[a]	53.05±1.29[b]
125	57.35±0.95[a]	43.73±1.29[b]
62	46.24±1.24[a]	31.54±1.29[b]
31	37.63±0.62[a]	22.22±1.29[b]
15	28.67±1.29[a]	11.47±1.56[b]

Descriptive statistics (mean±SEM) with distinct superscript lower-case letters along the row differed significantly (($p<0.05$), using independent t-test).

The effect of the extract noted a substantial increase in the anti-proteinase activity from concentration ($p<0.05$) of 15 to 1000 µg/ml (Figure 4.9). The extract exhibited concentration-dependent anti-proteinase action (Figure 4.9).

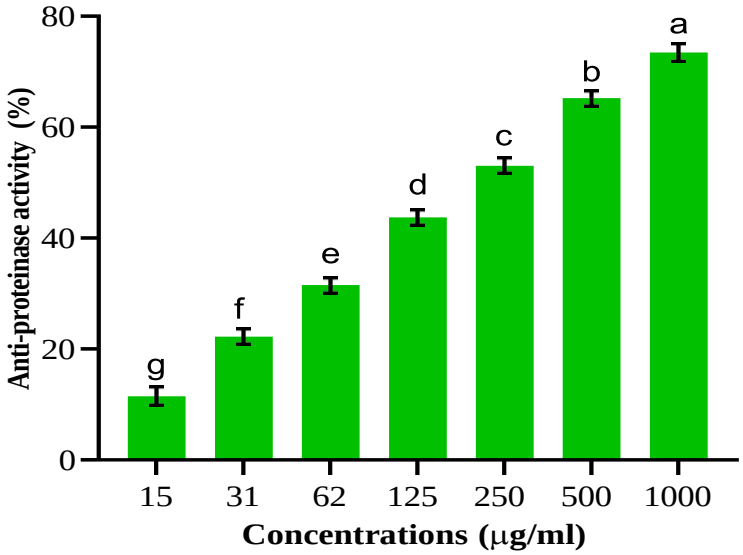


Figure 4.9: Effect of *Purslane oleracea* aqueous extract on anti-proteinase activity. Bars with distinct lowercase letters show significant variations ($p<0.05$) using One-Way Analysis of Variance (ANOVA) and Tukey's multiple comparisons.

4.3 Qualitative phytochemical composition of *Purslane oleracea* aqueous extract

The analysis identified tannins, alkaloids, saponins, cardiac glycosides, steroids, flavonoids, terpenoids and phenolics (Table 4.8).

Table 4.8: Qualitative phytochemical composition of *Purslane oleracea* aqueous extract

Secondary metabolite	Status
Tannins	+
Alkaloids	+
Saponins	+
Cardiac glycosides	+
Steroids	+
Flavonoids	+
Terpenoids	+
Phenolic acids	+

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Plant-based antioxidants (phytochemicals) can scavenge oxidants and mitigate oxidative stress related inflammatory ailments such as rheumatoid arthritis (Guan *et al*., 2021). Flavonoids and phenolic acids are responsible for the antioxidant activities of medicinal plants and also have anti-inflammatory activities (Herrera-Rocha *et al*., 2022; Al-Khayri *et al*., 2022). Inflammation can be attenuated by suppressing generation of pro- inflammatory cytokines (interleukins-(1β and 6), and TNF-α), nitric oxide, COX-2, and PGE2, among other inflammatory mediators. Free radicals are synthesized by inflammatory biomolecules and have the ability to irreversibly damage cell membranes (Rastogi *et al*., 2018; Yan *et al*., 2023).This background supports the rationale for evaluating the antioxidant and anti-inflammatory effects of *Portulaca oleracea* as conducted in the present investigation

This study found that *Purslane oleracea* aqueous extract had *in vitro* antioxidant activities. This was indicated by H_2O_2 radical scavenging and DPPH radical scavenging activities, including FRAP of the extract. The findings also indicated adequate amounts of TPC and TFC which are associated with antioxidant effects (Di Sotto and Di Giacomo, 2023). The study also identified saponins, tannins, alkaloids, terpenoids and steroids that are linked with antioxidant activities (Kedir *et al*., 2023).

The extract demonstrated concentration-dependent DPPH radical scavenging, suggesting

an increase proton donating ability at higher concentrations. However, its IC_{50} value was significantly higher than that of ascorbic acid, indicating lower potency. This trend aligns with the findings from *Solanum khasianum*, *Aloe vera* and *Irvingia gabonesis (*Chirumamilla and Taduri, 2023*;* Manye et al., 2023*),* which also exhibit variable potency depending on phytochemical richness.

The extract's DPPH radical scavenging activity demonstrated an IC_{50} (50% of radicals are scavenged at this concentration.) that was substantially higher than the standard, ascorbic acid. This suggested that the standard had a better DPPH radical scavenging activity in comparison to *Purslane oleracea* aqueous extract. The extract exhibited DPPH radical-scavenging action that was concentration-dependent. This suggested that the extract's ability to scavenge DPPH radicals rose as the concentration increased. These findings were consistent with those from other medicinal plants, including *Solanum khasianum* Clarke (Chirumamilla and Taduri, 2023), *Irvingia gabonensis* (Otitolaiye *et al*., 2023), and *Aloe vera* (Manye *et al*., 2023).

This study also revealed that *Purslane oleracea* extract had H_2O_2 radical scavenging activity. Hydrogen peroxide is naturally produced by humans at low concentrations during normal metabolic processes (Sadiq, 2023). In humans, hydrogen peroxide can rapidly pass through cell membranes and react with both Fe^{2+} and Cu^{2+} to produce hydroxyl radicals, which are responsible for DNA damage and lipid peroxidation. Hydrogen peroxide can also decompose to hypochlorous acid, which may produce hydroxyl radical (Kador and Salvi, 2022; Kettle *et al*., 2023). The principle behind the *in*

vitro H_2O_2 free radical scavenging technique is that the absorbance of H_2O_2 reduces when H_2O_2 radicals are converted into water and by an antioxidant (Salemcity *et al*., 2020; Alqub *et al*., 2023). The extract's ability to scavenge H_2O_2 radicals may be ascribed to flavonoids and phenolic acids, which could have donated electrons to H_2O_2 radical, thereby neutralizing it to water and oxygen (Kedir *et al*., 2023).

The IC_{50} of H_2O_2 radical scavenging effect of ascorbic acid (standard) was considerably lower relative to that of the extract. This indicated that the extract had lower activity in comparison to that of the standard. The extract's ability to scavenge H_2O_2 radicals was concentration-dependent. This could be ascribed to the increase in the concentration of phytocompounds that possess hydrogen peroxide radical scavenging activity. These findings corroborate with those of other medicinal plants such as *Luisia tenuifolia* Blume (Sakthipriyadarsini and Kumar, 2022), *Cocos nucifera* L. (Ambe *et al*., 2023) and *Xerophyta spekei* Baker (Nyalo *et al*., 2023).

This study also found that the extract had potent FRAP. The assay for FRAP involves a change in color from yellow to green based on the test sample reducing power. The antioxidants usually reduce Fe^{3+} (ferricyanide) complex to the ferrous form (Fe^{2+}). One method of estimating Fe^{2+} is to measure absorbance at 700 nm. It has been demonstrated that FRAP occurs through the donation of an electron to neutralize free radicals. A rise in absorbance signifies a higher reducing power (Chirumamilla and Taduri, 2023; Nwozo *et al*., 2023). The FRAP of the extract in this study could therefore be ascribed to phytochemicals like flavonoids and phenolic acids, which could have donated an

electron, hence reducing Fe^{3+} to Fe^{2+}, an indication of antioxidant activity.

Although the extract's reducing power was significantly lower than that of ascorbic acid, the dose-response curve indicated increasing antioxidant strength with higher concentrations, likely due to flavonoid and phenolic content (Di Sotto and Di Giacomo, 2023).

It is conceptualized that polyphenols' antioxidant effect is attributed to their redox properties, which allow them to act as singlet oxygen quenchers, hydrogen donors, reducing agents, and potentially metal chelators (Basyal *et al*., 2021; Das *et al*., 2022).

Flavonoids are polyphenols that are composed of a C6-C3-C6 carbon skeleton, where carbon atoms are arranged in three phenolic rings, A, B, and C. The C ring typically contains oxygen. Flavones, flavan-3-ols, isoflavones, flavanones, flavonols, dihydroflavonols, proanthocyanidins, and anthocyanidins are among the sub-classes of the flavonoids (Doloking *et al*., 2022). The absence of glycosylated groups and the presence of hydroxy groups increase the flavonoids' antioxidant potential. Flavonoids exhibit their antioxidant activities through scavenging free radicals (such as lipid peroxides, superoxides, hydroxylated compounds and single oxygen molecules), chelating metals, suppressing the activity of lipoxygenases and activating the production of antioxidant enzymes characterized by radical scavenging capacity. The capacity of flavonoids to transfer a hydrogen atom from a hydroxyl group to a free radical and eventually stabilize it accounts for their strong inhibitory effect on free radicals

(Hassanpour and Doroudi, 2023; Olszowy-Tomczyk and Wianowska, 2023).

Polyphenols with a functional group of carboxylic acid are called phenolic acids. Phenolic acids are categorized into two: hydroxycinnamic and hydroxybenzoic acids. The carboxylic acid functional groups of hydroxybenzoic acids are directly linked to the phenol ring, while the carboxylic acid functional groups and the phenol rings of hydroxycinnamic acids are separated by two double-bonded carbons (Saqib and Rahman, 2022). The ability of phenolic acids to scavenge free radicals is due to the presence of carboxylic groups, hydroxyl groups and conjugated ring structures (Sehrawat *et al*., 2022). The phenol moieties donate hydrogen atoms, which scavenges free radicals and confers antioxidant properties. Phenolic acids have additional known modes of action that include electron donation-mediated radical quenching and singlet oxygen quenching (Kumar and Goel, 2019).

The total phenolic and total phenolic concentrations in this study were correlated with the extract's ability to scavenge H_2O_2 and DPPH radicals, as well as its ferric reducing power. These findings implied that the polyphenolic components of the extract could be responsible for neutralizing free radicals. These findings corroborated with those of other medicinal plants like *Aloe vera* (Bista *et al*., 2020), and *Bauhinia purpurea* (Htay *et al*., 2023).

This investigation also demonstrated that the extract had *ex vivo* anti-inflammatory effect. It was found that the extract suppressed albumin denaturation, hypotonicity-induced

hemolysis, heat-induced hemolysis, and proteinases activity. Lysosomal hydrolytic enzymes are typically released into wounded tissues during inflammation, leading to diseases like arthritis. Drugs and herbal preparations with anti-inflammatory potentials are screened and studied using a variety of techniques *in vitro*. These methods include lysosomal membrane stability, erythrocyte membrane stabilization, and decrease of protein denaturation (Yadav *et al*., 2020).

The present study utilized a simple and reproducible method to stabilize erythrocyte membranes subjected to heat-induced and hypotonic-induced hemolysis (Yadav *et al*., 2020). The aqueous extract of *Purslane oleracea* exhibited erythrocyte membrane stabilization effect by suppressing heat-induced and hypotonicity-induced hemolysis. Since the erythrocyte membrane and lysosomal membrane are analogous, this implies that the extract may also stabilize lysosomal membranes. The control of the inflammatory response is largely dependent on the stabilization of the lysosomal membrane, which limits the release of activated neutrophil lysosomal contents such as proteases, which exacerbate tissue damage and inflammation when released extracellularly (Uwaya *et al*., 2020; Yadav *et al*., 2020).

The reference drug, indomethacin had better heat and hypotonicity-induced hemolysis inhibition relative to the extract. The reference drug, indomethacin exerts its anti-edema effect via COX-2 inhibition necessary for the synthesis of prostaglandin E2 (Ghlichloo and Gerriets, 2022), thereby stabilizing the erythrocyte membrane. The erythrocyte membrane stabilizing effect of the extract could be explained by phytocompounds such

as flavonoids, phenolic acids, terpenoids and alkaloids. According to reports, these phytocompounds inhibit lipoxygenases, COX-2, and phospholipase A2, which exerts anti-inflammatory properties (Zhang and Virgous, 2019), thereby stabilizing the erythrocyte membrane. The erythrocyte membrane was stabilized by the extract in a concentration-dependent manner, indicating that the effect increased as the concentration increased. Similar studies have documented medicinal plants with membrane-stabilizing effects such as *Neurada procumbens* (Aslam *et al*., 2023) and *Solanum khasianum* Clarke (Chirumamilla and Taduri, 2023). This membrane-stabilizing activity may be attributed to the extract's alkaloids and flavonoids, which are known to inhibit lipoxygenase and COX-2, pathways crucial in inflammation (Zhang and Virgous , 2019)

The current investigation also found that *Purslane oleracea* aqueous extract prevented albumin denaturation. When proteins are exposed to external stressors or substances such as heat, organic solvents, concentrated inorganic salts, strong acids or bases, they lose their secondary and tertiary structures, a process known as denaturation. When denatured, most biological proteins (such as albumin) cease to possess their biological functions. One extensively studied mechanism of inflammation is protein denaturation (Basyal *et al*., 2021). The effect the extract to inhibit albumin denaturation was investigated to establish *ex vivo* anti-inflammation effects. The extract was found to inhibit albumin denaturation, suggesting an anti-inflammatory effect. The presence of phytocompounds such phenolic acids, alkaloids, flavonoids, and terpenoids may account for the extract's anti-inflammatory action. There are reports of these phytocompounds having anti- inflammatory properties (Jang and Lee, 2023), thereby suppressing albumin denaturation.

The extract's effect on albumin denaturation was dose-dependent, indicating that its potency rose with increasing concentration. The effect of indomethacin was better than that of the extract. Indomethacin inhibits the COX-2 pathway (Ghlichloo and Gerriets, 2022), thereby lowering the amounts of prostanoids and leukotrienes, necessary for albumin denaturation. One of the mechanisms through which the extract could have inhibited albumin denaturation is the suppression of enzymes that generate eicosanoid, such as phospholipase A2, COX-2, and lipoxygenases. This leads to a decrease in the amounts of prostanoids and leukotrienes necessary for albumin denaturation (Nguemnang *et al*., 2019; Basyal *et al*., 2021). These results agree with studies carried out on other medicinal plants such as *Phlomis crinita* (Boutennoun *et al*., 2023) and *Alchornea cordifolia* (Oruka and Achuba, (2023). The inhibition of albumin denaturation increased significantly with dose, though lower than Indomethacin. This again underscores the moderate efficacy *of P. oleracea* extract, possibly due to lower concentrations of active anti-inflammatory constituents.

The present investigation also demonstrated the anti-proteinase activity of *Purslane oleracea* aqueous extract *ex vivo.* Proteinases are components of lysosomes that induce tissue damage and inflammation when released extracellular. The development of tissue damage during inflammation is greatly influenced by leukocyte proteinases, and cells are generally protected against inflammation by proteinase inhibitors (Gunathilake *et al*. 2018; Oruka and Achuba, 2023). The anti-proteinases effect of the extract could be attributed to phytocompounds such as alkaloids, terpenoids, flavonoids, saponins, and

phenolic acids. These secondary metabolites are documented to exert anti-inflammatory activities (Jang and Lee, 2023). Although the extract was less potent than Indomethacin, its dose-dependent inhibition of proteinases aligns with trends observed in *Neurada procumbens and Phlomis crinita (*Aslam et al., 2023; Boutennoun et al., 2023).

The phytocompounds that were identified in *Purslane oleracea* extract, including phenolic acids, alkaloids, terpenoids, flavonoids, and saponins, may be link to the anti- inflammatory and antioxidant activities reported in this study. Furthermore, a correlation between the antioxidant and anti-inflammatory properties was demonstrated by the study's findings. These findings imply that the antioxidant activity of *Purslane oleracea* extract may account for the anti-inflammatory effect that was reported in the present study.

5.2 Conclusions

This study concludes that:

i. The aqueous extract of *P. oleracea* demonstrated significant DPPH, FRAP and H_2O_2 scavenging activity.

ii. The extract showed dose-dependent *ex-vivo* anti-inflammatory effects through hemolysis inhibition, proteinase suppression and protection against albumin denaturation.

iii. The identified phytochemicals – phenolics, flavonoids, alkaloids – are likely contributors to the observed therapeutic activities.

5.3. Recommendations

5.3.1 Recommendations from the study

The aqueous extract of *P.oleracea* holds promise for development of phytopharmaceuticals targeting oxidative stress and inflammation. Specific compounds within the extract should be further evaluated for efficacy and safety in preclinical models.

5.3.2 Recommendations for further studies

i. In vivo efficacy testing using animal models of oxidative stress and inflammation.
ii. Toxicity profiling including acute, sub-acute and chronic exposure.
iii. Bioassay-guided fractionation, structural elucidation (e.g., via LC-MS, NMR) and pharmacological screening of isolated compounds.
iv. Mechanistic studies on molecular targets (e.g., COX-2, NF-kB, Nrf2 pathways).

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APPENDICES

Appendix I: Ethical approval

KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

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Our Ref: KU/ERC/ EXEMPTION/VOL.I (001) Date 10th August 2023

Wanderi Gladys Wamaitha
P.0 Box 43844-00100
Nairobi

Dear Ms. Wamaitha,

APPLICATION NUMBER: PKU/2773/11898- PHYTOCHEMICAL PROFILES, IN VITRO ANTIOXIDANT ANTI-INFLAMMATORY POTENTIAL OF PORTULACA OLEVACEA AQUEOUS EXTRACT"

1. IDENTIFICATION OF PROTOCOL

The application before the committee is with a research topic " **Phytochemical profiles, in vitro antioxidant anti-inflammatory potential of Portulaca olevacea Aqueous extract**"

2. APPLICANT

Wanderi Gladys Wamaitha

3. SITE

4. DECISION

The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines and **EXEMPTED from having an Informed Consent for research participants.**

5. **ADVICE/CONDITIONS**

i. Progress reports are submitted to the KU-ERC every six months and a full report is submitted at the end of the study.
ii. Serious and unexpected adverse events related to the conduct of the study are reported to this committee immediately they occur.
iii. Notify the Kenyatta University Ethics Committee of any amendments to the protocol.
iv. Submit an electronic copy of the protocol to KUERC.

When replying, kindly quote the application number above.
If you accept the decision reached and advice and conditions given please sign in the space provided below and return to KU-ERC a copy of the letter.

PROF. JUDITH KIMIYWE
CHAIRMAN ETHICS REVIEW COMMITTEE

I Hondeni Gladys Namatha accept the advice given and will fulfill the conditions therein.

Signature..... Dated this day of 11th August 2023

cc.
DVC-Research Innovation and Outreach

Appendix II: Research permit