EVALUATION OF OXIDATIVE STATUS IN PLASMA OF DMH-EXPOSED RATS ADMINISTERED ETHANOL EXTRACTS OF *VERNONIA AMYGDALINA* LEAVES

BY

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CERTIFICATION

This is to certify that this is a detailed account of the work written by Ed-Osokpro O. Cynthia**,** with matriculation number **LSC2006767** from the department of BIOCHEMISTRY, Faculty of Life Sciences**,** University of Benin.

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DEDICATION

This work is dedicated to God almighty whose love, support, mercy and salvation has guided me through my life and academic pursuit to this point regardless of the puzzling circumstances and the hurdles faced during the period. Also, to my parents whose love and support has strengthened me and kept me going.

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ABSTRACT

Oxidative stress plays a significant role in the pathophysiology of various diseases, including cancer, neurodegenerative disorders, and metabolic syndromes. 1,2-Dimethylhydrazine (DMH) is a known carcinogen that induces oxidative stress via generation of reactive oxygen species (ROS), leading to cellular damage and biochemical alterations. *Vernonia amygdalina* (bitter leaf) is widely recognized for its medicinal properties, particularly its antioxidant potential. This study evaluated oxidative status in the plasma of DMH-exposed Wistar rats administered ethanol extract of *Vernonia amygdalina* leaves. Male Wistar rats (n = 30) were divided into six groups: control, DMH, silymarin, extract, pretreatment and post-treatment groups. After the experimental period, plasma samples were analyzed for oxidative stress biomarkers, including total protein (TP), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), nitric oxide (NO), and vitamins A, C, and E. Results showed that DMH exposure significantly increased lipid peroxidation (MDA) levels, while reducing enzymatic and non-enzymatic antioxidant levels ($p < 0.05$). Treatment with ethanol extract of *V. amygdalina* significantly restored antioxidant enzyme activities, increased GSH and vitamin concentrations, and reduced oxidative damage. Overall, *Vernonia amygdalina* demonstrated potent antioxidant activity, effectively mitigating DMH-induced oxidative stress. These findings provide scientific validation for its traditional use as a medicinal plant and highlight its potential as a natural alternative for managing oxidative stress-related diseases.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In recent years, there has been considerable emphasis on evaluating oxidative status in biological systems, particularly in relation to diseases caused by oxidative stress. Oxidative stress, defined as an imbalance between reactive oxygen species (ROS) generation and antioxidant defenses, is associated with the pathophysiology of multiple illnesses, including cancer, diabetes mellitus, and neurodegenerative disorders (Halliwell, 2015). Studies on oxidative stress and potential treatments has been greatly facilitated by the use of animal models, including rats given 1,2-dimethylhydrazine (DMH) (Bhanot *et al*., 2020).

Vernonia amygdalina, also known as bitter leaf, is a medicinal plant that has been traditionally used across various cultures for its purported health benefits, which are reported to include antioxidant properties (Okwu and Ndu, 2006). The leaves of *V. amygdalina* contain bioactive compounds (flavonoids and phenolic acids), which have demonstrated significant antioxidant properties (Ishola *et al*., 2016). Studies have shown that extracts of the leaves of *V. amygdalina* may help mitigate oxidative damage and promote overall oxidative well-being in several settings (Okwu *et al.*, 2018).

It has been reported that the carcinogenic effect of DMH occurs via free radicals generation. Studies have shown that the incorporation of antioxidant-rich plant extracts can substantially decrease oxidative damage. (Akinmoladun *et al*., 2019).

1.1.1 Studies Hypothesis

The studies hypothesis posits that ethanol extract of *V. amygdalina* leaves can attenuate oxidative stress in DMH-exposed rats.

1.1.2 Aim and Objectives

The aim of this study was to investigate the effect of ethanol extract of *V. amygdalina* leaves on oxidative status in DMH-exposed rats. The objectives were:

1. To extract the leaves of *V. amygdalina* with absolute ethanol
2. To expose Wistar rats to a single intraperitoneal dose of DMH
3. To treat DMH-exposed rats with the plant extract
4. To determine the effect of the plant extract on enzymatic antioxidants in DMH-exposed rats' plasma
5. To determine the effect of the plant extract on non-enzymatic antioxidants in DMH-exposed rats' plasma

1.2 Literature Review

1.2.1 Vernonia amygdalina

Vernonia amygdalina, also referred to as bitter leaf, is a perennial shrub classified under the family Compositae or Asteraceae, native to tropical Africa, and can be found growing both naturally and as a cultivated species throughout sub-Saharan Africa. At full maturity, this plant typically grows to be between one to three meters high, which equates to roughly 23 feet in height. The bark exhibits a rough texture with a grey or brown hue. The foliage is typically medium to dark green in colour, has an oblong-lanceolate shape and usually ranges in size from 10–15 cm in length to 4–5 cm in width, with a distinctive aroma and a bitter flavour. The leaves display visible red veining, a narrow apex and base, a nearly symmetrical base, an entire or finely toothed margin, and a petiole that is commonly short but can be up to 1–2 cm in length and approximately 6mm in diameter. The small, creamy white, thistle-like flower heads of this plant are approximately 10 mm in length, and all of its parts have potential medicinal uses (Degu *et al*. 2024). The plant's roots and leaves are employed in traditional medicine to alleviate conditions such as fever, hiccups, kidney disease and stomach discomfort among other complaints (Ogidi *et al*., 2019). *Vernonia amygdalina* is a versatile plant with a wide distribution, predominantly found in Africa, especially in West Africa, and also present in Asia, with its various parts utilized for multiple purposes. The leaves of this particular plant are notable for containing a varied mixture of nutrients such as proteins, fats, fibers, amino acids, minerals,

vitamins, and carbohydrates (Alara *et al*., 2017). The plant is rich in various bioactive compounds, including flavonoids, alkaloids, saponins, tannins, and sesquiterpene lactones, and it is these compounds that are responsible for its wide range of pharmacological effects (Erasto *et al*., 2006).

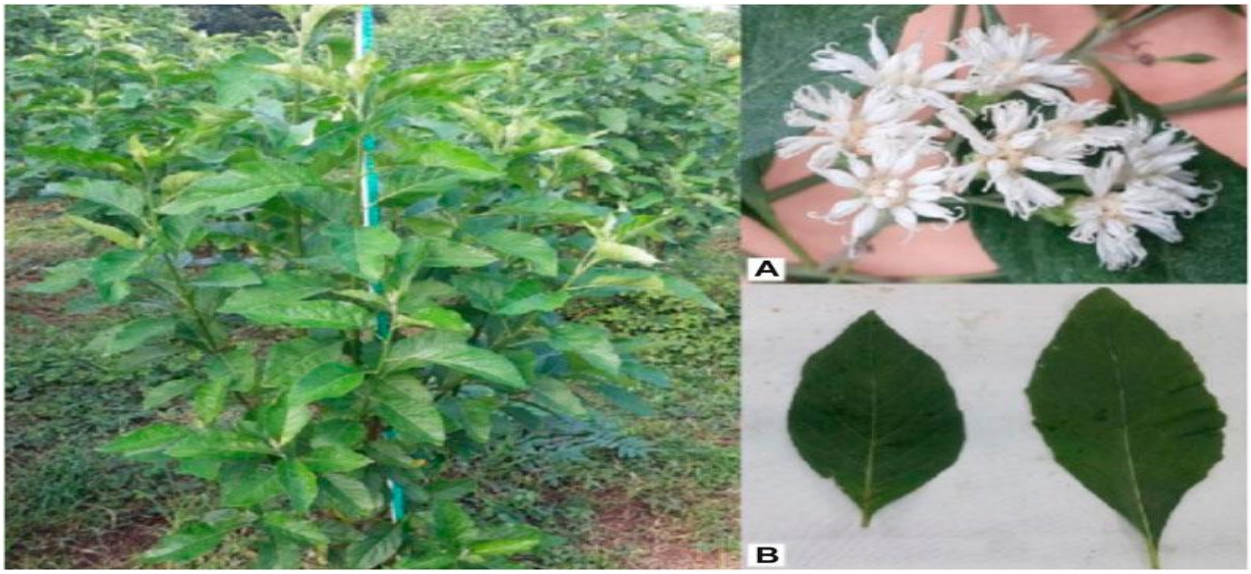

Figure 1.1: *Vernonia amygdalina* plant (left), flowers **(A)**, top right) and leaves **(B)**, bottom right) (Degu *et al.,* 2024).

1.2.2 Traditional Application of *Vernonia amygdalina*

The use of *Vernonia amygdalina* as a medicinal herb began when zoo pharmacologists discovered that sick chimpanzees with empty stomachs would suck the pith and juice from the unpalatable Vernonia plant stalk (which was not a part of their usual diet), in an effort to self-deparasitize, improve their overall body fitness, boost their strength or appetites, and alleviate symptoms of constipation or diarrhea, particularly during the rainy season. The bitter taste of *Vernonia amygdalina* was thought to be a clue for selecting the right plant, the correct plant part, and the suitable amount to consume. In Africa, citizens from lower socioeconomic backgrounds

with limited education also utilized the *Vernonia amygdalina* plant, primarily for cultural and financial reasons. Traditionally, the plant's leaves, consumed as a vegetable, have been used to treat conditions including gastroenteritis, diarrhea, fever, parasite infections, dysentery, diabetes mellitus, and hepatitis, owing to its cytotoxic, antibacterial, antitumor, antivirus, antimalaria, anticancer, anti-inflammatory, antioxidant, antidiabetic, and antiparasitic properties (Yeap *et al*., 2010).

1.2.3 The Dietary Composition and Importance of *Vernonia amygdalina*

Vernonia amygdalina's bitter properties make it suitable for use as a bittering agent in beverages and also as an antimicrobial additive in the brewing of beer. Bitter leaf soup, a well-known Nigerian dish, is typically prepared with leaves and served as both an appetizer and a digestive aid. Goats can be fed with the leaves and shoots as nutritious food. Administering the bitter leaf meal with drinking water was also shown to numerically boost the growth rate of the birds. In Ethiopia, it is utilized to produce honey wine known as 'Tej' and is also used as hops in the brewing of 'tella' beer. The edible portion of *Vernonia amygdalina* makes a significant contribution to human health and food security due to its sufficient content of proximate composition. The leaves of the plant are an excellent source of food due to their high content of protein, dry matter, crude fiber, ash, and key minerals such as sodium, potassium, calcium, magnesium, zinc, and iron. Furthermore, numerous studies have also disclosed varying levels of protein (featuring essential amino acids), moisture, carbohydrates, ash, and fat within the leaves. A study on micronutrients, macronutrients, and minerals revealed a difference in concentration, where magnesium, copper, and lead were found to be elevated in fresh leaves, whereas calcium, ash, fiber, lipid content, and iron were found to be high in dried leaves. The leaf contains oil, starch, and iodine. In addition, the leaf is a good source of various vitamins including vitamin A,

vitamin C (ascorbic acid), vitamin E, vitamin B1, vitamin B2, niacin, and carotenoid (Degu *et al*., 2024).

1.2.4 Chemical Composition of *Vernonia amygdalina*

Vernonia amygdalina has a dry matter content of 21-23%, with 6.5-29.2% crude fiber and 0.137% chlorophyll (75% chlorophyll-a). Hemicellulose levels are higher in dry leaves than fresh ones. The plant contains crude protein (17-33 g/100g DW) and fat (2-15 g/100g DW, with 24.54% saturated and 65.45% polyunsaturated fats, mainly oleic acid). Its high crude protein content indicates its value as a protein source, crucial for animal growth and milk production. *V. amygdalina* leaves, combined with soybean meal, serve as an optimal infant weaning food promoting weight gain. The ash content (10-13 g/100g DW) signifies valuable minerals (e.g., calcium, iron, potassium) and substantial nitrogen, phosphorus, and various exchangeable bases (Calcium, Magnesium, Sodium, and Potassium). High sulfur aids in cyanide detoxification, while low sodium benefits obese individuals. The nutritive values of young and mature leaves show no significant difference. Leaf abrasion reduces nutrient and antinutrient levels, except for carbohydrates. The moisture content of *V. amygdalina* varies between 79.1-82.1%. (Yeap *et al*., 2010).

1.2.5 Ethnomedicinal Uses of *Vernonia amygdalina*

Vernonia amygdalina has a wide range of traditional medicinal uses globally. The plant's leaves, in particular, are used to address a wide range of health issues such as diarrheal disorders, diabetes mellitus management, wound recovery, tonsillitis, headaches, eye infections, intestinal parasites, hepatitis, malaria, gastritis, and gastrointestinal problems. This herb is also utilized in the treatment of evil eye, retained placenta, toothache, anthrax, urine retention, and snake bites (Degu *et al*., 2024). In traditional medicine, practitioners utilize the plant to combat parasites,

fight malaria, and induce bowel movements. The root and twig of the plant are used by the Hausas of Northern Nigeria for treating stomach and gastrointestinal issues, while those in Guinea use a leaf decoction to combat malaria fever and people in Ghana use it to alleviate cough, as well as for the topical treatment of wounds, and it also serves as a digestive tonic, appetizer, and febrifuge. In certain regions of Nigeria, stems are utilized as chew sticks for oral health maintenance and in the management of selected dental issues. In Malawi and Uganda, *V. amygdalina* is utilized by traditional birth attendants to facilitate placenta expulsion after birth, stimulate postpartum uterine contractions, induce lactation, and manage postpartum hemorrhage. Extensive scientific analysis has been conducted on the traditional uses of this plant. Previous studies on *V. amygdalina* , including numerous investigations, has yielded revealing outcomes (Ijeh and Ejike, 2011).

Notably, *V. amygdalina* has applications not only in human medicine but also in ethnoveterinary practices. This species is among the 12 identified Vernonia species used in animal healthcare. In addition, the plant has been noted in zoopharmacognostic applications, where chimpanzees and gorillas employ it for self-treatment purposes (Toyang and Verpoorte, 2013). The important uses of *V. amygdalina* in traditional medicine underscore its importance in healthcare systems. This application extends across human, veterinary, and self-medication practices in animals, highlighting its therapeutic benefits.

Table 1.1: Medicinal uses of different parts of *Vernonia amygdalina* in various countries (Yeap *et al.*, 2010).

Country	**Plant Parts**	**Ailments**
Ethiopia	Leaves (not root)	Stomach disorder, skin wound, diarrhea, scabies,

	Leaves and root	hepatitis, ascariasis, tonsillitis, fever, tapeworm and worm infection. Stomach ache (worm expulsion)
Democratic Republic of Congo	Leaves and root bark	Diarrhea, dysentery, gastroenteritis, malaria, hepatitis, worm infection
Ghana	Leaves decoction	Malaria, fever, constipation, abortifacient, stomach sores, ulcer, pain, upper respiratory tract infections and dermatitis.
Tanzania	Leaves Roots	Snake bite (chew), fever, stomachache, as appetizer Trematode
Nigeria (Hausa of the northern tribe)	Leaves, Root and twig	Stomachache, gastrointestinal troubles, oral hygiene, itches, parasitic infection, ringworm, typhoid fever, headache, diabetes mellitus, constipation, pile (hemorrhoids) and reduces aflatoxin contamination of storage cobs.

1.2.6 Phytochemistry of *Vernonia amygdalina*

Analysis of the plant's phytochemical composition has shown the presence of a range of compounds including flavonoids, alkaloids, saponins, tannins, triterpenoids, sesquiterpene lactones, steroids, cardiac glycosides, oxalates, phytates, cyanogenic glycosides, and phenols. Studies has discovered that these phytochemicals exhibit a diverse range of biological activities, indicating the plant's potential as a medicinal agent (Degu *et al*., 2024).

Alkaloids have been identified as substances that participate in metabolic processes and regulate growth within living organisms. The presence of alkaloids in *V. amygdalina* may be responsible for its effectiveness in antimicrobial applications, as it also disrupts the process of cell division. Alkaloids act as protective chemicals in plants that help deter predators and parasites. This likely confers these group agents with their antimicrobial properties. Flavonoids have been found to have antioxidant properties in both healthy and diseased conditions. Tea flavonoids have been documented to decrease the oxidation of low-density lipoprotein, lower the blood level of cholesterol, and decrease triglycerides. Studies suggests that the presence of flavonoids in plants is also triggered by microbial infection, implying that they possess antimicrobial properties. It's thought that saponins interact with the cholesterol-rich cell membranes of cancer cells, which in turn restricts their growth and survival. Medicinal plants containing saponins are generally responsible for most biological effects associated with human cell growth and division. The presence of saponin in the leaves of *V. amygdalina* lends credibility to its efficacy in treating inflammation. Steroids hold significant value in pharmacy due to their composition of compounds such as sex hormones, and they can be utilized for the production of medication. Studies has shown that terpenoids have potential in the prevention and treatment of various diseases, among them cancer. Terpenoids are also known for possessing antimicrobial, antifungal, anti-parasitic, antiviral, antiallergenic, antispasmodic, anti-inflammatory and immunomodulatory properties. It has been noted that phenolics act as free radical scavengers, thereby preventing oxidative cell damage, and exhibit considerable anticancer properties, potentially influencing cancer cell mechanisms and inhibiting tumour invasion. Consuming these substances can also reduce the likelihood of heart disease and offer anti-inflammatory effects, primarily due to their

capacity to neutralize and eliminate free radicals. Tannin has been found to exhibit antiviral properties and also shows potential in preventing and treating cancer cells (Ali *et al*., 2019).

1.2.7 Pharmacological Activities of *Vernonia amygdalina*

Various studies have found that *Vernonia amygdalina* possesses a range of pharmacological effects, encompassing antimicrobial, antimalarial, antithrombotic, antioxidant, anti-diabetic, laxative, hypoglycemia, antihelmintic, anti-inflammatory, cathartic, anticancer, antifertility, antifungal, and antibacterial properties among many others.

1.2.7.1 Antioxidant activity

Vernonia amygdalina has shown substantial antioxidant properties according to existing reports. Studies have found that plant extracts can neutralize free radicals and reduce oxidative damage. Notably, aqueous extracts from *V. amygdalina* leaves resulted in a substantial decrease in malondialdehyde levels in streptozotocin-induced diabetic rats, as reported in the studies of Alara *et al*. (2017). Earlier studies has demonstrated that the aqueous extract of *V. amygdalina* substantially decreased blood glucose levels following oral administration to alloxan-induced diabetic rabbits (Owolabi *et al*., 2009). The extract obtained from leaves has been investigated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, showing it can scavenge 75-99.3% of DPPH radicals and 96.2-100% of ABTS radicals. Studies involving biochemistry conducted in living organisms found a significant rise in the quantities of antioxidants such as superoxide dismutase, catalase, glutathione, and malondialdehyde in rat subjects exposed to *V. amygdalina* leaf extracts. Studies have shown that these extracts can suppress the bleaching of B-carotene, lipid peroxidation caused by iron ion and ascorbate in a rat liver microsomal system, and also linoleic acid. The antioxidant properties of *V. amygdalina* are mainly due to flavonoids in its extracts (Alara *et al*., 2017).

1.2.7.2 Antibacterial Activity

Vernonia amygdalina has shown substantial antibacterial efficacy against a range of bacterial strains. Studies indicates that various fractions of *V. amygdalina* exhibit efficacy against both gram-positive and gram-negative bacteria. Evidence of the plant's antibacterial properties has been found in extracts obtained from various sections, such as leaves, stems, and roots. The efficacy of extracts differs based on their type and the specific bacterial strain being evaluated. Methanol extracts have been found to possess broad-spectrum antibacterial properties, whereas aqueous extracts have shown effectiveness against specific types of bacteria. The antibacterial properties of *V. amygdalina* suggest its potential as a useful component in a range of applications, such as traditional medicine and possibly the food industry (Yeap *et al*., 2010)

1.2.7.3 Antimalarial Activity

Significant antimalarial properties have been exhibited by *Vernonia amygdalina* in several scientific studies. A significant discovery was made regarding the capability of the ethanolic extract obtained from *Vernonia amygdalina* leaves and root-bark to inhibit parasitemia in mice that had been infected with Plasmodium berghei. *Vernonia amygdalina* shows great potential in fighting malaria parasites efficiently. In addition to its other effects, aqueous extracts from *Vernonia amygdalina* leaves have also been found to be effective in lowering parasite levels in mouse models infected with P. berghei. The water-soluble antimalarial compounds in *Vernonia amygdalina* broaden its potential uses even further. Preliminary studies suggest that the plant has shown potential in augmenting the antimalarial properties of chloroquine, particularly against chloroquine-resistant strains of Plasmodium berghei. This finding has the potential to facilitate combination therapy in treating cases of malaria that are resistant to drugs. Scientific studies

supports the long-standing use of *V. amygdalina* in treating malaria in African communities, linking traditional practices to current scientific findings (Ijeh and Ejike, 2011).

1.2.7.4 Antifungal Activity

Extensive studies have been conducted on *Vernonia amygdalina*, highlighting its antifungal capabilities against numerous fungal pathogens. A study investigated the antifungal properties of *V. amygdalina* leaf extracts against Botrytis cinerea, the causative organism of gray mold disease in tomatoes. Leaves extracted with dichloromethane showed the highest level of antifungal activity, significantly suppressing growth of B. cinerea in laboratory tests and lowering disease occurrence in living organisms. A microscopic examination found that the extract resulted in morphological changes to the fungus, which included withered hyphal tips and shrunken conidia, ultimately resulting in growth inhibition. The dichloromethane extract was found to contain compounds such as squalene, phytol, and triacontane, that could be responsible for the antifungal effect (Yusoff *et al*., 2020). Previous studies have demonstrated the antifungal properties of *V. amygdalina* against a variety of fungal species. It has been documented that the growth of *Colletotrichum lindemuthianum*, a fungus responsible for anthracnose disease in cowpea, can be inhibited (Fadina, 2010). Furthermore, *V. amygdalina* extracts have exhibited antifungal properties against other plant pathogenic fungi (Owoyale *et al*., 2020).

1.2.7.5 Anticancer Activity

Studies indicate that this plant displays significant potential in the treatment of cancer. Studies conducted by Gresham *et al*. (2008) found that extracts from the leaves of *V. amygdalina* exhibit significant anti-proliferative properties against human breast cancer cells that are positive for estrogen receptors (ER+). Studies has continued to investigate its efficacy in treating estrogen receptor-negative breast cancer, a condition that disproportionately impacts African American

women. According to (Gresham *et al*., 2008), *V. amygdalina* extracts were found to suppress the growth of BT-549 cells, a specific ER-positive breast cancer cell line. Several mechanisms proposed by studiesers explain the anticancer properties of *V. amygdalina* . Studies by Utoh-Nedosa (2011) indicates that it blocks the action of sterol 14-α-demethylase, an enzyme essential for the production of ergosterol in cancer cell membranes. Impairing ergosterol synthesis can compromise membrane function and ultimately result in cancer cell mortality. Studies by Izevbigie *et al*. (2004) suggests that *V. amygdalina* may be able to suppress extracellular signal-regulated kinases, which are crucial cell growth and proliferation regulators, thus contributing to its anticancer qualities. This is supported by Yedjou *et al*. (2013), who found that breast cancer cells treated with *V. amygdalina* extracts experienced growth arrest and apoptosis, also known as programmed cell death

1.3 Oxidative Stress

Oxidative stress is a fundamental concept in the realm of cellular and molecular biology, encompassing the delicate balance between the generation of reactive oxygen species and the body's innate antioxidant defense mechanisms. This imbalance can have far-reaching implications, contributing to the pathogenesis of various disease states, including atherosclerosis, diabetes mellitus, cancer, and neurodegenerative disorder (Ray *et al*., 2012). The interaction between pro-oxidants, including reactive oxygen species produced during mitochondrial oxidative metabolism or in reaction to different stimuli, and antioxidants, the body's main defense against these potentially dangerous molecules, is at the core of oxidative stress (Sies, 2015). Oxidative stress has wide-ranging effects on health. ROS-induced cellular damage can result in lipid peroxidation, protein deterioration, and DNA mutations (Halliwell, 2019). Numerous chronic illnesses, such as cancer (Valko *et al*., 2020), neurological conditions

including Parkinson's and Alzheimer's (Butterfield and Kanski, 2020), cardiovascular disease (Heinecke, 2020), and diabetes mellitus (West, 2020), can be exacerbated by this injury.

Moreover, the aging process has been linked to oxidative stress (Sies, 2020). Over time, the buildup of ROS-induced cellular damage may be a factor in the aging-related reduction in cognitive and physical abilities. Vitamins C and E are important antioxidants that help reduce the consequences of oxidative stress (Sies, 2020). By neutralizing ROS, these substances can lessen the harm done to cells. The body's antioxidant defenses can also be strengthened by eating foods high in antioxidants, such as fruits, vegetables, and nuts (Halliwell, 2019). Recent studies have also highlighted the usefulness of other antioxidant techniques, such as exercise (Liu *et al*., 2020) and stress management (Reuter *et al*., 2019), in lowering oxidative stress and boosting general health.

1.3.1 Biomarkers of Oxidative stress

Several biomarkers are used to assess oxidative stress levels in biological systems:

1.3.1.1 Malondialdehyde (MDA)

Malondialdehyde is a metabolic by-product that arises from the peroxidation of lipids, a process marked by the oxidative degradation of lipids within the biological system. (Khan *et al*., 2020). This deterioration transpires when reactive oxygen species (ROS) assault polyunsaturated fatty acids located in cellular membranes, resulting in the synthesis of MDA (Liu *et al*., 2020). Elevated concentrations of MDA function as a pivotal marker of oxidative stress, a state associated with a discord between the generation of free radicals and the organism's capacity to neutralize their deleterious consequences (Halliwell, 2019). Within the domain of oncological studies, the existence of augmented MDA levels has been intimately correlated with diverse

forms of malignancies, such as breast, pulmonary, and colorectal cancer (Mishra *et al*., 2020). By understanding the importance of MDA as an indicator of oxidative damage, studiesers and medical practitioners can obtain essential knowledge regarding the core mechanisms of disease development and evolution (Khan *et al*., 2020).

1.3.1.2 Glutathione (GSH)

Glutathione, is a vital antioxidant that scavenges free radicals causing oxidative damage (Sies, 2020). It helps maintain cellular redox balance by neutralizing harmful molecules (Halliwell, 2019). GSH protects cells from oxidative harm under stress like pollution or UV radiation (Liu *et al*., 2020). Decreased GSH levels signify reduced antioxidant capacity affecting overall health (Singh *et al*., 2020). Low GSH levels can increase susceptibility to diseases linked to oxidative stress (Khan *et al*., 2020). Supporting GSH levels through diet and supplements is crucial (Mishra *et al*., 2020). Maintaining optimal GSH levels is vital for cellular health (Singh *et al*., 2020). Understanding GSH's role in body defense is key to combating oxidative stress (Sies, 2020). Enhancing GSH levels can proactively safeguard health (Halliwell, 2019).

1.3.1.3 Superoxide Dismutase (SOD)

Superoxide Dismutase is an antioxidant that converts superoxide radicals into hydrogen peroxide and oxygen, reflecting the body’s ability to neutralize anions (Mishra *et al*., 2020). Superoxide Dismutase (SOD) is a crucial enzymatic antioxidant in defense against oxidative stress (Johnson *et al*., 2022), reducing harmful effects of free radicals on cells and tissues (Brennan *et al*., 2020). SOD neutralizes unstable superoxide radicals, promoting cellular health (Kumar *et al*., 2022).

SOD activity reveals the body's ability to counteract superoxide anions, which can cause health issues if not managed (Li *et al*., 2022). Monitoring SOD levels helps assess antioxidant status and susceptibility to oxidative damage (Patel *et al*., 2020), linking lower SOD activity to oxidative stress-related conditions (Gupta *et al*., 2022). Understanding SOD's role emphasizes the balance needed for optimal health (Tiwari *et al*., 2022), as inadequate levels can lead to cellular dysfunction and chronic diseases (Bhatia *et al*., 2020). Supporting SOD activity through diet and exercise can enhance natural defense mechanisms (Singh *et al*., 2022).

1.3.1.4 Catalase

Catalase plays a crucial role as an enzymatic antioxidant within cells (Chelikani *et al*., 2022). Its main function is to break down hydrogen peroxide (H2O2) into water (H2O) and oxygen (O2), preventing the accumulation of harmful hydroxyl radicals (Gao *et al*., 2020). When a cell is exposed to high levels of hydrogen peroxide due to oxidative stress, catalase efficiently converts excess H2O2 into less reactive molecules, safeguarding the cell from oxidative damage (Kim *et al*., 2022). Catalase's activity is vital for maintaining cellular homeostasis and protecting biomolecules from oxidative stress-induced damage (Singh *et al*., 2022). In the liver, where catalase is abundant, this enzyme plays a crucial role in detoxifying harmful compounds and ensuring proper organ functioning (Wang *et al*., 2020). Catalase has also been found to be particularly active in peroxisomes, specialized organelles responsible for lipid metabolism and detoxification processes (Lee *et al*., 2022).

1.3.1.5 Glutathione Peroxide

Glutathione peroxide (GPx), is an important enzymatic antioxidant that plays a crucial role in the body's defense against oxidative stress. It catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively, using glutathione as a reducing agent

(Ighodaro & Akinloye, 2017). Interestingly, while GPx is typically considered an enzymatic antioxidant, glutathione itself is a non-enzymatic antioxidant. Glutathione is a tripeptide that serves as a major non-protein thiol involved in cellular antioxidant defense, particularly important in the liver (Wróblewska *et al*., 2023). The intracellular content of glutathione regulates the detoxifying capacity of cells and influences inflammatory and immune responses (Wróblewska *et al*., 2023).

1.3.2 Non-Enzymatic Antioxidants

Antioxidants play a crucial role in maintaining the delicate balance of the body's oxidative state, protecting cells from the detrimental effects of free radicals and reactive oxygen species. Among the various classes of antioxidants, non-enzymatic antioxidants have garnered significant attention due to their diverse mechanisms of action and potential therapeutic applications.

1.3.2.1 Glutathione reductase

Glutathione reductase (GR) is a vital enzymatic element of the antioxidant defense system, essential for preserving the reduced form of glutathione (GSH), a non-enzymatic antioxidant. GR participates in the glutathione redox cycle, reducing oxidized glutathione (GSSG) to GSH using NADPH as a cofactor (Anjum, 2010). This cycle sustains cellular redox homeostasis and mitigates oxidative stress. GR's significance is highlighted in various studies across biological systems and pathological conditions. While most organisms rely on both glutathione and thioredoxin systems for antioxidant protection, insects lack glutathione reductase, making the thioredoxin system essential for their defense (Gřešková & Petřivalský, 2024). This evolutionary

modification underscores the importance of redox systems in different organisms. In summary, glutathione reductase is a fundamental component of the antioxidant defense system, working with other enzymes and non-enzymatic antioxidants like GSH to maintain cellular redox equilibrium. Its role has been studied in diverse contexts, from plant stress responses to human ailments, illustrating its broad relevance in biological systems (Zińczuk *et al*., 2019).

1.3.2.2 Nitric oxide

Nitric oxide is a unique non-enzymatic antioxidant that has gained significant attention recently. This free radical gas is synthesized by various cell types, including endothelial cells, neurons, and immune cells, playing a crucial role in many physiological processes (Laitonjam, 2012). As an antioxidant, nitric oxide can directly scavenge superoxide radicals, preventing the formation of the highly reactive peroxynitrite anion. It also indirectly enhances antioxidant defenses by promoting antioxidant enzyme expression and inhibiting their oxidative inactivation. (Fukai & Ushio-Fukai, 2011) Beyond its antioxidant properties, nitric oxide is a versatile signaling molecule regulating important physiological functions, such as vasodilation, neurotransmission, and immune response. Disruptions in nitric oxide production, bioavailability, or signaling mechanisms have been linked to the development and progression of various pathological conditions, including cardiovascular disorders, neurological diseases, and chronic inflammatory states (Mukherjee & Gogoi, 2011).

1.3.2.3 Vitamin C

Vitamin C (ascorbic acid) is a potent non-enzymatic antioxidant protecting cells against oxidative stress. It inhibits lipid and protein oxidation in human plasma exposed to various oxidative stresses (Frei, 2004). As a water-soluble antioxidant, vitamin C scavenges reactive oxygen species (ROS) such as singlet oxygen, superoxide, hydroxyl, and water-soluble peroxyl

radicals (Adikwu & Deo, 2013). Vitamin C exhibits a dual nature, acting as an antioxidant under physiological conditions and a prooxidant under pathological conditions (Macan *et al*., 2019). Some studies have shown that vitamin C can induce the decomposition of lipid hydroperoxides independent of metal interactions, suggesting potential DNA damage (Lutsenko *et al*., 2002. It functions by donating electrons to free radicals, regenerating other antioxidants like vitamin E, and reducing lipid peroxidation (Adikwu & Deo, 2013). Studies have demonstrated its hepatoprotective effects in animals and humans, particularly in non-alcoholic steatohepatitis and fatty liver disease (Adikwu & Deo, 2013). Vitamin C has been shown to decrease oxidation-induced mutations in human cells, supporting its role in maintaining genomic integrity (Lutsenko *et al*., 2002).

1.3.2.4 Vitamin E

Vitamin E, specifically α-tocopherol, is a crucial non-enzymatic antioxidant protecting tissues from uncontrolled lipid peroxidation (Galli *et al*., 2022). As a potent peroxyl radical scavenger, it acts as a chain-breaking antioxidant that prevents free radical damage propagation in biological membranes (Traber & Packer, 1995). Vitamin E's antioxidant function is important in conditions of oxidative stress, such as chronic kidney disease (CKD) and diabetes (Shirpoor, 2007). While vitamin E supplementation has shown promising results in animal experiments and observational studies, randomized clinical trials in humans have failed to demonstrate consistent clinical benefits (Meulmeester *et al*., 2022). Vitamin E's effects may depend on the nature of both oxidants and substrates being oxidized, suggesting a more nuanced role (Niki, 2021). Vitamin E's antioxidant properties extend beyond its direct radical-scavenging ability. It can act as a gene regulator, influencing the expression of endogenous antioxidant enzymes (Elgendey *et al*., 2022). Vitamin E works with other antioxidants, such as glutathione and vitamin C, to maintain cellular

redox balance and protect against oxidative damage (Acker *et al*., 2000; Niki, 2021). Understanding the interplay between vitamin E's antioxidant activity, capacity, and catabolism may provide crucial insights into its preventive and therapeutic potential in oxidative stress-related diseases (Meulmeester *et al*., 2022).

1.3.2.5 Vitamin A

Vitamin A (retinol) is a crucial non-enzymatic antioxidant protecting the body against oxidative stress. It acts as a precursor for bioactive compounds, including retinaldehyde and retinoic acid isomers (Jakaria *et al*., 2023). Retinol and its metabolites inhibit ferroptosis, a form of programmed cell death caused by iron-dependent phospholipid peroxidation, with potency superior to α-tocopherol (Jakaria *et al*., 2023). While vitamin A is generally beneficial, its effects can be dose-dependent. Lower doses of retinol palmitate have been associated with cancer risk prevention against oxidative damage. However, at high doses, it can generate reactive oxygen species, cytotoxicity, and apoptosis in test systems (Melo-Cavalcante *et al*., 2018). This highlights the importance of maintaining appropriate vitamin A levels. Vitamin A serves as a vital non-enzymatic antioxidant in the body's defense against oxidative stress. Its ability to penetrate the blood-brain barrier and exhibit neuroprotective properties in animal models emphasizes its importance (Jakaria *et al*., 2023). However, the potential risks associated with high doses underscore the need for careful consideration when using vitamin A supplements, particularly in cancer treatment (Melo-Cavalcante *et al*., 2018).

1.3.3 Mechanisms of Oxidative Damage

The mechanisms of oxidative damage can be categorized into several key processes:

1.3.3.1 Lipid Peroxidation

ROS can trigger the peroxidation of lipids, especially polyunsaturated fatty acids within cell membranes. The process produces lipid peroxides that can break down into aldehydes and other reactive substances, ultimately causing damage to cell membranes and compromising cellular structure and function (Halliwell and Gutteridge, 2015).

1.3.3.2 Protein Oxidation

The structure and function of proteins can be altered through oxidative modifications, specifically carbonylation. Consequences can include a loss of enzymatic function, the formation of protein clusters, and ultimately, cellular malfunction (Davies, 2016).

1.3.3.3 DNA Damage

ROS can cause strand breaks and base alterations, including 8-oxoguanine, among other types of DNA damage. These kinds of changes can lead to genomic instability and mutations, which can accelerate aging and the onset of cancer (Lindahl, 1993).

1.3.3.4 Mitochondrial Dysfunction

Mitochondria are major producers of reactive oxygen species (ROS) due to their function in aerobic respiration. Mitochondrial dysfunction can be caused by oxidative stress, resulting in reduced ATP synthesis and elevated release of factors that promote apoptosis, ultimately leading to cell death (Zorov *et al*., 2014).

1.3.3.5 Inflammatory Responses

Inflammation can also be triggered by oxidative stress initiating several signaling pathways. The activation of the nuclear factor kappa B (NF-κB) pathway can lead to the production of pro-inflammatory cytokines, thus creating a cycle of oxidative damage and inflammation (Schafer and Bäumer, 2015).

1.3.4 Importance of Evaluating Oxidative Status

Assessing an organism's oxidative state is essential for grasping and controlling numerous health issues. An imbalance between reactive oxygen species (ROS) production and antioxidant defenses leads to oxidative stress, contributing to chronic diseases and aging (Luo *et al*., 2019). Tracked oxidative stress biomarkers offer useful information about health status, disease likelihood, and treatment success. Assessing oxidative status is crucial for several reasons. It enables early identification of oxidative stress-linked diseases (Zhao *et al*., 2023), aids in evaluating antioxidant treatments and lifestyle modifications (Husain *et al*., 2023), and facilitates understanding of pathological conditions and ageing processes (Luo *et al*., 2019). Measuring oxidative stress accurately poses challenges. Traditional biomarkers lack reliability and specificity, leading to conflicting study outcomes (Marrocco *et al*., 2017). Researchers stress the importance of using various biomarkers and developing more reliable techniques (Husain *et al*., 2023). Combined oxidative damage and antioxidant defense profiles offer a more complete view of oxidative stress levels (Cutler, 2005). Non-invasive testing methods, like saliva-based tests, can simplify oxidative status monitoring in diverse environments (Rubio *et al*., 2018). In summary, assessing oxidative status remains vital for health evaluation and disease control, with ongoing efforts to enhance measurement precision and reliability.

1.3.5 Oxidative Stress and Cancer Development

Oxidative stress is a crucial factor in cancer progression, influencing multiple pathways. It can result in DNA damage, mutations to tumor suppressor genes, and disruption of cellular functions, key early occurrences in carcinogenesis (Nourazarian *et al*., 2014). Despite being often linked to cancer initiation and progression, oxidative stress can have opposing effects. Studies indicate that higher levels of ROS can help prevent tumor growth by causing cell death and cellular ageing (Canli *et al*., 2017). Certain chemotherapy medications achieve therapeutic effects by inducing oxidative stress in damaged cells (Udensi and Tchounwou, 2014). Oxidative stress in cancer is characterized by a complex and often paradoxical set of circumstances. It can impact several cellular processes and signaling pathways, causing uncontrolled cell growth, formation of new blood vessels, and cancer spread (Nourazarian *et al*., 2014). Understanding the complex connection between oxidative stress and cancer is essential to create successful prevention and treatment methods. Antioxidant-based treatments or redox-modulating agents may offer potential in managing cancer by addressing oxidative stress (Iqbal *et al*., 2024; Saleh *et al*., 2023). Further studies are necessary to fully understand the intricate mechanisms and create targeted treatments that specifically target cancer cells without harming healthy cells.

1.4 1, 2-Dimethylhydrazine (DMH)

1,2-Dimethylhydrazine (DMH), also known as unsymmetrical dimethylhydrazine, is a potent carcinogen which has been extensively studied for its ability to induce colon cancer in laboratory animals. Frequently, it is also used as rocket fuel due to its stability and high energy content. It is classified as a potent DNA alkylating agent within the hydrazine class, with natural occurrence in cycads (Venkatachalam *et al*., 2020). DMH has been widely used as a model carcinogen to

investigate the mechanisms of colon cancer development in rodents, providing valuable information about the biochemical, molecular, and histological aspects of various stages of colon cancer formation (Venkatachalam *et al*., 2020). Worth noting is the fact that while 1,2-dimethylhydrazine is a potent carcinogen for the colon in rats and mice, 1-methylhydrazine, a related compound, does not have this effect (Hawks and Magee, 1974). The variation in carcinogenic potential has prompted studiesers to study the metabolic and biochemical effects of these substances. Studies shows that DMH undergoes several metabolic processes before reaching the colon, where it produces reactive oxygen species (ROS) and the final carcinogen that causes DNA alkylation and triggers colon cancer development (Venkatachalam *et al*., 2020). The studies history of 1,2-dimethylhydrazine has shown its significance in uncovering the mechanisms of colon cancer development and carcinogenesis. Studies using animal models exposed to DMH have proven to be a vital approach for evaluating the effects of natural and pharmacological substances in colon cancer studies, providing valuable insights into the condition in both humans and animals. (Venkatachalam *et al*., 2020).

This synthetic organic compound, 1,2-Dimethylhydrazine, began its history in the early 1900s, where it was initially discovered and produced synthetically by studiers. Since then, 1,2-Dimethylhydrazine has received considerable attention in the scientific community because of its various uses and potential health effects. The initial studies conducted in the early 20th century centered on examining the chemical properties, reactivity, and synthetic applications of 1,2-Dimethylhydrazine, thereby setting the stage for further studies in multiple disciplines. With growing usage, especially in the manufacture of rocket propellants and agricultural chemicals, the environmental and biological effects of 1,2-Dimethylhydrazine were subject to increasing examination. Studies on this compound has been ongoing since the latter half of the 20th century

and continues into the present era, covering its synthesis and characterization as well as its reactivity, transformations, and potential uses in the field of biomedicine.

1.4.1 Synthesis and Characterization of 1,2-Dimethylhydrazine

The synthesis of 1,2-Dimethylhydrazine is a widely accepted process, typically achieved by the reaction of methylamine hydrochloride and urea, then the addition of sodium nitrite at low temperatures to yield the required compound (Wu *et al*., 2023). The 1,2-Dimethylhydrazine obtained can be further purified and characterized through a range of analytical methods, including infrared spectroscopy, Raman spectroscopy, and X-ray diffraction analysis (Parvarinezhad and Salehi, 2020). 1,2-Dimethylhydrazine's chemical properties have been thoroughly examined, and its distinctive characteristics have made it a highly sought-after starting material in organic synthesis. Scientists have synthesized new ligands based on hydrazine compounds, such as 1,2-Dimethylhydrazine, and have identified the structures of these ligands by employing different spectroscopic methods (Parvarinezhad and Salehi, 2020)

1.4.2 Health Implications of DMH Exposure

1,2-dimethylhydrazine (DMH) is a highly potent cancer-causing agent commonly used in animal studies to induce colon cancer. This compound functions as a potent agent that alters DNA, generating reactive oxygen species, and subsequently leads to the alkylation of DNA in the colon, thereby triggering the process of carcinogenesis (Venkatachalam *et al*., 2020). Exposure to DMH results in the formation of abnormal crypt formations, increased production of inflammatory cytokines, and the development of colon tumors in rodents (Yamashita *et al*., 2017). Notably, although DMH is predominantly linked to colon cancer, it can also cause other types of malignancies. Heterozygous mice lacking the msh2 gene developed additional cancers outside the colon following DMH exposure, including trichofolliculoma, angiosarcoma, and lymphoma

(Colussi *et al*., 2001). It implies that genetic factors can affect the likelihood of developing cancer caused by DMH beyond the colon. The health effects of DMH exposure go beyond the induction of cancer. Oxidative stress is triggered, accompanied by heightened lipid peroxidation and alterations in the activity of antioxidant enzymes within erythrocytes (Harzallah *et al*., 2012). These results emphasize the far-reaching effects of DMH exposure and stress the need for examining its influence on various organ systems, rather than focusing solely on the colon, in order to grasp its full health consequences.

1.4.3 Environmental Implications and Biological Relevance

1,2-Dimethylhydrazine is widely utilized in several industrial processes, such as the manufacture of agricultural chemicals and rocket propellants; however, owing to its extensive use, it has become a potential environmental pollutant (Parvarinezhad and Salehi, 2020). Studies have revealed that exposure to 1,2-Dimethylhydrazine can lead to a variety of health problems, encompassing respiratory disorders, skin irritation, and potential carcinogenic consequences. Extensive studies has been conducted into 1,2-Dimethylhydrazine's biological significance, examining not only its environmental impact but also its potential uses and effects in biomedicine, such as its function as a cancer-causing substance and its possible therapeutic uses.

1.4.4 Structural Features of 1,2-Dimethylhydrazine

The chemical compound 1,2-Dimethylhydrazine is a transparent, colourless liquid, consisting of two methyl groups bonded to a central hydrazine molecule, resulting in distinctive chemical properties that influence both its effects on living organisms and its resistance to environmental degradation (Venkatachalam *et al*., 2020). The structural setup enables the molecule to bind with cellular elements in a specific manner, possibly resulting in both carcinogenic and therapeutic outcomes (Punvittayagul *et al*., 2021). The addition of two methyl groups to the hydrazine

backbone raises the lipophilicity of 1,2-Dimethylhydrazine, thereby increasing its capacity to traverse cell membranes and bind with intracellular receptors. The structural feature impacts metabolic processes, with methyl groups susceptible to oxidation, resulting in the creation of reactive intermediates (Aachary *et al*., 2015). The hydrazine part of the compound can engage in several chemical reactions, such as nucleophilic substitutions and redox processes, which could impact its toxicity and its possible uses in medicine (Flesher and Lehner, 2016).

1.4.5 Mechanisms of DMH-Induced Oxidative Stress

DMH (1,2-dimethylhydrazine) triggers oxidative stress in colorectal cancer models through lowered antioxidant enzyme activity and heightened levels of lipid peroxidation byproducts in diverse tissues. A study involving male Wistar rats found that DMH treatment led to decreased activities of antioxidant enzymes and lower levels of reduced glutathione (GSH) in the colon, liver, kidney, and heart (López-Mejía *et al*., 2021). Cellular damage occurred concurrently with elevated levels of lipid peroxidation products, which signify an oxidative imbalance. The study notably discovered that Callistemon citrinus, which contains a high amount of phenolics and terpenoids, was able to reverse DMH-induced oxidative stress. Supplementation with C. citrinus helped to preserve or even enhance the activity of antioxidant enzymes and decrease lipid peroxidation products to levels comparable to those in the control group (López-Mejía *et al*., 2021). The findings imply that natural antioxidants can offer a protective benefit against oxidative damage caused by DMH. Ultimately, DMH triggers oxidative stress primarily by weakening the body's antioxidant defense mechanisms and accelerating lipid peroxidation. The potential for developing preventive strategies against colorectal cancer and other oxidative stress-related diseases is underscored by the ability of antioxidant-rich compounds to reduce these effects.

1.5 Silymarin

Silymarin, a naturally occurring flavonoid complex derived from the milk thistle (Silybum marianum) plant, has been extensively studied for its remarkable therapeutic potential across various medical conditions. This versatile compound has been studied for its potent antioxidant, anti-inflammatory, and cytoprotective activities (Kanitkar *et al*., 2008). This complex mixture, primarily composed of silibinin, silydianin, and silychristine, exhibits antioxidant, anti-inflammatory, and antiviral activities (Vargas-Mendoza, 2014). Silymarin has garnered attention as a potential natural treatment for liver conditions. An analysis of nine high-quality clinical trials found that silymarin yielded a beneficial impact in managing chronic liver conditions (Stickel and Schuppan, 2007). Scientific studies have shown silymarin possesses liver-protective properties in multiple animal studies, demonstrating its ability to neutralize the damaging effects of carbon tetrachloride, a well-documented liver toxin. Silymarin's liver-protecting properties stem from a complex mechanism of action. Studies show that silymarin's liver-protecting properties are primarily due to its ability to regulate key antioxidant enzymes, which eliminate harmful free radicals and reactive oxygen species, maintaining the liver's structural and functional integrity (Jia *et al*., 2013). Studies have also found that silymarin has benefits beyond liver health, including mitigating the negative effects of chemotherapy and radiotherapy, making it a promising complementary treatment for cancer patients (Stickel and Schuppan, 2007). Silymarin's antioxidant capabilities are particularly important in countering health problems caused by oxidative stress (Fraschini *et al*., 2002). This compound's anti-inflammatory properties could provide extra benefits in inflammatory conditions.

1.6 Plasma

Plasma is a vital part of blood, acting as the liquid carrier in which cells and numerous biomolecules are dispersed. This component accounts for roughly 55% of the total blood volume and is crucial in disease diagnosis and biomedical studies due to its abundance of biomarkers (Wang *et al*., 2021). Plasma, a crucial element of blood, is often isolated from whole blood for both medical treatment and scientific study. Notably, though centrifugation has long been used to separate plasma, there is increasing interest in creating microfluidic devices for plasma separation that offer more compact and efficient alternatives. Technologies for passive self-separation, founded on microchannel geometry and hydrodynamic forces, are particularly promising due to the ease of their fabrication and user-friendly attributes (Wang *et al*., 2021). The separation and storage of blood components, particularly plasma, pose distinct difficulties. For each component, there are particular storage conditions and temperature specifications that must be met in order to preserve therapeutic efficacy (Basu and Kulkarni, 2014). This blood component plays a vital role with substantial implications for both clinical and studies purposes. Investigations into its separation and analysis remain ongoing, with advancements in technologies such as microfluidics presenting new opportunities for more efficient data processing. Despite advancements, challenges persist in maintaining the quality and effectiveness of plasma and other blood components throughout their shelf life, especially given the introduction of new processing and storage techniques (Acker *et al*., 2016).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

The materials used for this study included plain tubes, nose mask, spatula, micropipette (Labline stock centre), pasteur pipette (Avon Healthcare), centrifuge (80.2 Techmel and Techmel USA), spectrophotometer (Search Tech.721G), refrigerator, analytical weighing balance, test tube racks, test tubes (Pyrex, England), beaker (Pyrex, England), porcelain crucible (Pyrex, England), large bowls for extracts, wooden cages, gavage, measuring cylinder (Pyrex), gloves, scissors, dissecting kit (Atico Medical Pvt. Ltd), Cotton wool, Syringes (2ml and 5ml, Atico Medical Pvt. Ltd), lithium heparin tubes, drug sachet, chloroform, ice, aluminum Foil.

2.2 Reagents

The reagents used for this study were distilled water, pyrogallol, phosphate-buffered H_2O_2, hydrogen tetraoxosulphate VI acid (H_2SO_4), phosphate buffer, trichloroacetic acid (TCA), thiobarbituric acid (TBA), hydrochloric acid (HCl), stock TCA-TBA-HCl, carbonate buffer, greiss reagent, potassium permanganate solution (KMnO4), normal saline, adrenaline

2.3 Plant Sample Collection and Identification

Fresh *V. amygdalina* leaves were collected from Oluku, Benin City, Nigeria. Specimen of the leaves was identified by a Botanist in the Department of Plant Biology and Biotechnology.

2.4 Experimental Animals

Male adult Wistar rats weighing between 130-150g were obtained from the Biochemistry Department, University of Benin, Benin City. They were handled with national and institutional guidelines for care and use of laboratory animals. The rats were handled according to international, national and institutional guidelines for laboratory animal care as set by the Canadian Council of Animal Care (1984).

They were housed in cages and allowed to acclimatize for 14 days During acclimatization, they had ad libitum access to rat feed and water some rats were later transferred to metabolic cages They were kept in well-ventilated rooms at 28±2°C under a 12-hour light/dark cycle.

2.5 Measurement of Body Weights

The weight of each rat was measured weekly after acclimatization. This was done to ascertain the effect of the various feed constituents on their body weights.

2.6 Experimental Design

The 30 Albino Wistar rats were randomly divided into groups of six, with each group comprising five rats. These rats were then marked at different parts of their bodies using picric acid to facilitate identification during administration. Upon the start of the administration, each group of rats served different purposes. Group 1 served as normal control group with rats that were not induced with DMH, Group 2 was induced with DMH, Group 3 was treated with standard drug silymarin, Group 4 was administered ethanol extract of *Vernonia amygdalina*, Groups 5 which served as post-treatment group was administered the ethanol extract of *Vernonia amygdalina* after being induced DMH at 200mg/body weight, and group 6 which served as pre-treatment

group was administered the ethanol extract of *Vernonia amygdalina* before being induced DMH at 200mg/body weight. After a period of 14 days post-administration, the rats were euthanized. Cotton wool, which had been saturated with chloroform, was positioned within a transparent container equipped with a lid. The chloroform served the purpose of a mild anesthetic agent. The rats that were housed within the container were subsequently extracted, and an incision was made utilizing dissecting instruments. Blood samples were procured from both the abdominal aorta and the heart utilizing a 5 ml syringe. The collected blood was then transferred into a properly labeled sample container. Following centrifugation for a duration of 10 minutes, the bottles containing anticoagulants were retrieved from the whole blood. The resultant serum and plasma were subsequently preserved in a laboratory refrigerator maintained at a temperature of 4°C.

2.7 Biochemical Assays

2.7.1 Determination of Concentration of Plasma Total Protein

Principle

Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex.

Procedure

Biuret reagent (2.5 mL) was added to 0.05 mL of plasma and 0.05 mL of standard. The blank contained 2.5 mL of Biuret and 0.05 mL of distilled water. The solution in each tube was incubated for 10 min at 37°C, and the absorbance was read at 546 nm against the reagent blank.

Calculations

When measurements are taken at 546 nm, total protein concentration may be calculated as follows:

- When measurements are taken at 540nm, total protein concentration may be calculated as follows:

Total protein (g/L) = 190 x A_{sample}

Total protein (g/dL) = 19 x A_{sample}

- When using a standard

Total Protein Concentration = $\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}$ x Standard Conc.

2.7.2 Estimation of Malondialdehyde (MDA) Level

This is based on the method of Guttridge and Wilkins (1982), a modification of the procedure used by Hunter, *et al*., (1963). The principle that underlies this assay is that MDA – a product of lipid peroxidation when heated with thiobarbituric acid (TBA), in the presence of an acid, forms a pink or reddish complex that is measured spectrophotometrically at 532nm.

Procedure

An aliquot of the liver homogenate was added to 3.0 mL of TCA–TBA–HCl reagent and mixed thoroughly by swirling. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed via centrifugation at 1000 g for 10 min. The absorbance of the clear supernatant was measured against a reference blank at 535 nm.

Calculation:

The MDA concentration of each sample is calculated as shown below:

$$\frac{O.D \times V_t \times 1000}{a \times V \times L \times Y}$$

Where, O.D = Absorbance of sample test at 535nm

V_t = Total volume of the reaction mixture = 3.6ml

a = Molar extinction coefficient of product = $1.56 \times 10^5\ M^{-1}cm^{-1}$

L = Light path = 1.0cm

V = Volume of sample homogenate used = 0. 6ml

Y = mg of tissue in the sample used

The unit of MDA is moles/mg wet tissue.

2.7.3 Estimation of Superoxide Dismutase (SOD) Activity

This is based on the method of Misra and Fridovich (1972). The principle upon which this assay is based is as follows: Adrenaline auto-oxidizes rapidly in aqueous solution to adrenochrome whose concentration can be determined spectrophotometrically at 420nm. The auto-oxidation depends on the presence of superoxide anions (O^{2-}). Superoxide dismutase (SOD) inhibits this auto-oxidation by catalyzing the breakdown of superoxide anions. The degree of inhibition is

thus an indication of the SOD activity. The amount of enzyme producing 50% inhibition is defined as one unit of the enzyme activity.

Procedure

Liver homogenate (0.2 mL) was added to 2.5 mL of 0.05 M carbonate buffer (pH 10.2) and allowed to equilibrate. The reaction was initiated by the addition of 0.3 mL of freshly prepared 0.03 mM adrenaline as a substrate. The solution was mixed by inversion. The reference tube contained 2.7 mL of carbonate buffer and 0.3 mL of adrenaline, while the blank contained 2.5 mL of carbonate buffer, 0.2 mL of distilled water, and 0.3 mL of 0.03 mM adrenaline. The increase in absorbance at 420 nm due to the formation of adrenochrome was monitored every 30 sec for 120 sec. One unit of SOD activity was taken as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome within 120 s.

Calculation:

$$\% \text{ inhibition} = \frac{(O.D_{test} - O.D_{reference})(0.022)}{O.D_{test}} \times \frac{100}{1}$$

$$\text{Enzyme activity (units/mg protein)} = \frac{\% \text{ inhibition}}{50 \times Y}$$

Where, Y = g of protein in the volume of sample.

N.B.: 1 unit of SOD activity is taken as the amount of SOD required to cause 50% inhibition of the auto-oxidation of adrenaline to adrenochrome per minute.

2.7.3 Estimation of Catalase Activity

This is based on the method of Cohen, *et al.,* (1970). This estimation is based on the measurement of the rate of decomposition of hydrogen peroxide (H_2O_2), after the addition of the material containing the enzyme.

Catalase catalyzes the reaction: $2H_2O_2 \longrightarrow 2H_2O + O_2$

The quantity of hydrogen peroxide decomposed is directly proportional to the concentration of the enzyme in the sample. The hydrogen peroxide produced in tissues is measured by reacting it with excess potassium permanganate ($KMNO_4$) and then measuring the residual $KMNO_4$ spectrophotometrically at 480nm. The detailed protocol is illustrated below:

Procedure

Liver homogenate (0.5 mL) was placed in ice-cold test tubes, and the blank contained 0.5 mL of distilled water. Cold phosphate-buffered H_2O_2 (30 mM, 5 mL) was added to both blank and sample tubes at fixed intervals and was mixed by inversion. After 3 min, the reaction was stopped by the rapid addition of 1 mL of 6 M H_2SO_4. The tubes were mixed thoroughly by inversion, after which 7 mL of 0.01 M $KMnO_4$ was added. Absorbance was read at 480 nm within 3 min.

Calculation:

The activity of catalase in each sample is calculated thus:

$$\frac{O.D/min \times Vt \times 1000}{M \times V \times L \times Y}$$

Where, O.D = Absorbance of sample test at 480nm

Vt = Total volume of the reaction mixture = 13.5ml

M = Molar extinction coefficient of H_2O_2 = $43.6M^{-1}$ cm^{-1}

L = Light path = 1.0cm

V = Volume of sample homogenate used = 0.5ml

Y = mg of protein in tissue used

2.7.4 Estimation of Plasma Vitamin E (Sauberhch, *et al*., 1974)

This is based on the reduction of ferric ions to ferrous ions by tocopherols (i.e vitamin E) after xylene extraction of the blood sample. The ferrous ion reacts with the α-α-dipyridly to give a red colour which is measured at 520nm.

Reagent preparation:

Linoleic Acid Solution (0.02M): Prepare a 0.02M linoleic acid solution in ethanol.

Procedure:

Mix the sample containing vitamin E with linoleic acid solution and a free radical initiator (e.g., azo compounds) and incubate the mixture at a specified temperature for a defined period. Then measure the degree of linoleic acid oxidation by monitoring the formation of conjugated dienes at a specific wavelength (e.g., 234nm).

Calculation:

$$C_X = \frac{A_X \times C_S}{A_S}$$

Where A_X = Absorbance of the test sample

A_S = Standard sample = 0.852

C_S = Concentration of known standard solution = 50mg/dl

2.7.5 Estimation of Plasma Vitamin A

Standard vitamin A calibration curve was determined by method of Neeld and Pearson, (1963).

Procedure:

1ml of the plasma, 1ml of 95% ethanol and 1ml of hexane was transferred to a centrifuge tube and stoppered. This was vigorously vortexed for 2 minutes and centrifuged at 2000g for 5 minutes. After centrifuging, 1ml of the upper hexane layer was taken and the absorbance of the extract read at OD_{450nm} against a hexane blank. The content of the cuvette when measured was poured back into the test tube and then evaporated to dryness in a water bath at 35-40^{O}C. 0.2ml chloroform was then added to the test-tubes, 0.7ml of antimony trichloride reagent (225g $SbCl_3$ per liter of washed chloroform) were added and the optical density at 620nm read immediately after the addition of antimony trichloride reagent.

Vitamin A standard solution

Vitamin A acetate standard (100 µg/mL) was used. 0.1g of this standard was dissolved in chloroform and diluted to 100ml in a volumetric flask. This is the stock standard solution and was also made prior to use. Vitamin A standards were prepared from the stock standard to give solutions containing 0.2, 0.4, 0.6, 0.8, 1.0 µg/ml in the concentration of 20, 40, 60, 80, and 100 µg/ml respectively. For preparation of the standard curve, 0.2ml aliquots of these standards were pipette for reaction with the $SbCl_3$ reagent.

$$Cx = \frac{\text{Abs sample}}{\text{Abs Std}} \times 100$$

Absorbance Standard = 0.385

Standard concentration = 100 µg/ml

2.7.6 Estimation of Plasma Vitamin C (Ascorbic acid)

Vitamin C was estimated by the method described by Omaye *et al*., (1979).

Reagents

TCA (6%), 2,4-dinitrophenyl hydrazine reagent (2%) in 9N H_2SO_4, Thiourea (4%), Sulphuric acid (85%). Standard ascorbic acid solution: 100µg / ml in 4% TCA

Procedure

To 0.5ml of plasma, 1.5ml of 6% trichloroacetic acid (TCA) was added and centrifuged at 500g for 20 min. To the supernatant, 0.5ml of 2,4 -dinitrophenyl hydrazine (DNPH) reagent (2% DNPH and 4% thiourea in 9N sulphuric acid) was added and incubated for 3 hours at room temperature. After incubation, 2.5ml of 85% sulphuric acid was added. The standard sample will be prepared as the same process carried out on the test sample without serum while a known concentration of ascorbic acid will be used as the standard solution. The absorbance of the test sample (A_X) and of the standard sample (A_S) was read at 530nm.

Calculations

$$C_X = \frac{A_X \times C_S}{A_S}$$

Where A_X = Absorbance of the test sample

A_S = Standard sample = 0.416

C_S = Concentration of known standard solution = 10mg/ml

2.7.7 Estimation of Plasma Concentration of Reduced Glutathione (GSH)

Plasma concentration of reduced glutathione was estimated by the method of Ellman (1959)

Reagents

5, 5^{1}-dithiobis-2-nitrobenzoic acid (DTNB), Sodium citrate, Trichloroacetic acid (TCA)

Procedures

To 1.0ml of plasma, 2.5ml 10% of trichloroacetic acid (TCA) was added and centrifuged at 3000rmp for 10mins. 1.0ml of the supernatant was treated with 0.5ml of Ellman's reagent (0.0189% DTNB and 1% sodium citrate) and 3.0ml of 0.3M phosphate buffer (pH 8.0). The yellow colour of the mixture developed was read immediately 412nm.

$$\text{GSH concentration} = \frac{A_{test} \times Cstd}{A_{std}}$$

$$\text{\% Reduced Glutathione} = \frac{\text{GSH concentration}}{\text{Total glutathione concentration}} \times 100$$

Where A_{test} = Absorbance of sample

A_{std} = Absorbance of standard = 0.245

Cstd = Concentration of standard = 100

2.7.8 Estimation of Glutathione Peroxidase Activity (GPx)

This was determined by the method of Nyman (1959).

Principle:

This is based on the oxidation of pyrogallol to purpuragallin by peroxidase, resulting to a deep brown colouration, which is read at 430nm.

Reagent Preparation

Pyrogallol (20mM): 0.2552g of pyrogallol was dissolved in 100ml of distilled water.

Procedure:

To an aliquot of plasma (0.2ml), 2.5ml of phosphate buffer, 2.5ml of H_20_2, 1.5ml of pyrogallol was added. The reaction was allowed to stand for 30mins at room temperature. A deep colour was formed, which was read at 430nm.

Calculation:

Enzyme activity = $\frac{OD/min \times vt \times Df}{E \times Vs \times Y}$

Where OD = Absorbance of test

Vt = Total volume of reaction mixture = 6.7ml

Df = Dilution factor = 33.5

E = Molar extinction coefficient (12/M/cm)

Vs = Volume of sample = 0.2ml

Y = mg of protein used

2.7.9 Estimation of Plasma Concentration of Nitric Oxide

Nitric oxide was assayed by the method of Marcocci *et al*., (1994).

Principle:

When sodium nitroprusside is dissolved in aqueous solution, nitric oxide is spontaneously generated from it at physiological pH (7.2) which interacts with oxygen to produce nitrite ions that can be estimated by the use of Greiss reagent. The absorbance of the pink colour is read at 540nm

Procedure:

To 0.5ml of serum, 0.5ml sodium nitroprusside prepared in 10mM of potassium phosphate buffer (pH 7.4) was added and incubated at 25°C for 15min. At the end of incubation, the absorbance was taken and the samples were allowed to react with 1.0ml of Greiss reagent containing equal volume of solution A (2% sulfanilamide and 4% H_3P0_4) and B (0.2ml naphthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was read at 540nm.

Calculation:

$$\frac{A_0 - A_1}{A_0} \times 100$$

A_0 = Absorbance before reaction with greiss reagent.

A_1 = Absorbance after reaction with greiss reagent.

2.7.10 Estimation of Plasma Concentration of Glutathione Reductase (GR)

Glutathione Reductase was determined using the method of Carlberg and Mannervik (1985).

Principle:

GR catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as a cofactor. The rate of NADPH oxidation, monitored by the decrease in absorbance at 340 nm, indicates GR activity.

Procedure:

Tissue samples were homogenized in 0.1 M phosphate buffer with a pH of 7.4. The assay mixture consisted of 0.1 mL of tissue homogenate, 0.2 mL of 0.1 M GSSG, and 0.2 mL of 1 mM NADPH in 2.5 mL of phosphate buffer. The decrease in absorbance at 340 nm was monitored for 5 minutes. The activity of glutathione reductase (GR) was quantified as nmol of NADPH oxidized per minute per mg of protein.

CHAPTER THREE

RESULTS

3.1 Effect of Ethanol Extract of *V. amygdalina* Leaves on Rats Plasma Oxidative Status

Exposure of adult male Wistar albino rats to DMH led to significant reductions in the concentrations of antioxidant enzymes and molecules, while increasing index of lipid peroxidation (MDA) and total protein (TP) ($p < 0.05$). However, treatment of the rats with ethanol extract of *V. amygdalina* leaves markedly increased the levels/activities of the antioxidant enzymes/molecules, but it reduced MDA and total protein concentrations ($p < 0.05$). These results are presented in Tables 3.1 – 3.3.

Table 3.1: Activities of Antioxidant Enzymes

Group	Catalase (U/min) x 10^{-2}	SOD (U/min) x 10^{-2}	GPx (U/min) x 10^{-3}	GR (U/min) x 10^{-3}
Control	80.00 ± 3.10	76.00 ± 9.00	52.55 ± 1.75	35.50 ± 0.50
DMH	18.77 ± 0.43	11.00 ± 0.58	21.87 ± 4.44	9.00 ± 0.00
Siymarin	60.80 ± 0.80	61.50 ± 0.50	48.10 ± 2.40	30.50 ± 5.50
Extract Only	53.90 ± 5.60	51.50 ± 10.50	39.80 ± 0.50	30.00 ± 1.00
Post-treatment 1	47.15 ± 3.95	48.20 ± 16.80	38.50 ± 1.00	29.50 ± 0.50
Pre-treatment 1	41.85 ± 3.65	53.00 ± 4.00	39.15 ± 0.15	29.50 ± 1.50

N = 5

Table 3.2: Levels of Glutathione, Total Protein and Malondialdehyde

Group	GSH (mg/mL)	% GSH	MDA (mole/mg tissue) x 10^{-3}	TP (g/dL)
Control	37.03 ± 0.08	78.07 ± 0.44	4.00 ± 1.00	5.19 ± 0.10
DMH	9.10 ± 0.19	11.50 ± 0.82	15.67 ± 1.20	10.11 ± 2.79
Siymarin	34.20 ± 1.81	70.00 ± 0.75	6.50 ± 2.50	5.39 ± 0.03
Extract Only	27.75 ± 1.89	70.38 ± 1.00	6.50 ± 2.50	5.46 ± 0.23
Post-treatment 1	31.46 ± 1.04	72.01 ± 2.38	5.50 ± 0.50	5.23 ± 0.06
Pre-treatment 1	30.83 ± 0.26	72.50 ± 3.75	5.50 ± 1.50	5.30 ± 0.09

N = 5

Table 3.3: Concentrations of Nitric Oxide and Antioxidant Vitamins

Group	NO (µmole/L)	Vitamin A (mg/mL)	Vitamin C (mg/mL)	Vitamin E (mg/mL)
Control	28.55 ± 0.95	41.83 ± 0.63	17.77 ± 0.11	49.24 ± 0.51
DMH	66.30 ± 2.20	7.28 ± 0.25	2.32 ± 0.18	21.04 ± 0.48
Siymarin	44.80 ± 9.90	22.67 ± 0.42	17.25 ± 0.24	42.97 ± 3.43
Extract Only	46.70 ± 4.10	21.35 ± 0.15	17.25 ± 0.06	41.14 ± 0.74
Post-treatment 1	49.30 ± 6.70	21.67 ± 0.04	17.20 ± 0.11	39.44 ± 5.99
Pre-treatment 1	49.75 ± 8.35	19.79 ± 2.69	17.37 ± 0.12	41.91 ± 2.52

N = 5

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 Discussion

Oxidative stress plays a crucial role in various pathological conditions, including cancer, neurodegenerative diseases, and metabolic disorders. The current study evaluated the effect of *Vernonia amygdalina* on DMH-induced oxidative stress in Wistar rats, comparing its efficacy with silymarin, a known hepatoprotective agent. The findings indicated that DMH exposure significantly increased oxidative stress markers, such as lipid peroxidation index (MDA), while reducing enzymatic and non-enzymatic antioxidants. However, treatment with *V. amygdalina* significantly ameliorated the condition by restoring antioxidant enzyme activities, increasing glutathione (GSH) levels, and enhancing plasma vitamin concentrations. These results are in agreement with previous studies, such as Akinmoladun *et a*l. (2019), which demonstrated that DMH induces oxidative stress by ROS, leading to cellular damage.

The significant increase in malondialdehyde (MDA) levels following DMH exposure in this study aligns with the work of Ali *et al*. (2019), which also observed increased lipid peroxidation in DMH-treated models. Malondialdehyde (MDA), a well-known biomarker of oxidative stress and lipid peroxidation, is commonly elevated in oxidative stress-induced diseases, including cancer and cardiovascular disorders (Zhao *et al*., 2023). The administration of *V. amygdalina* significantly reduced MDA levels, supporting its antioxidant potential. This is in agreement with Alara *et al.* (2017), who reported that *V. amygdalina* leaf extracts possess strong free radical-scavenging properties due to their high flavonoid and polyphenol content.

Furthermore, the depletion of enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), in DMH-exposed rats was reversed upon administration of *V. amygdalina* . This agrees with findings by Bhanot et al. (2020), who reported that antioxidant-rich plant extracts can counteract DMH-induced oxidative damage by restoring antioxidant enzyme activity . Similarly, Patel *et al*. (2020) highlighted that the presence of bioactive compounds in medicinal plants, such as flavonoids and alkaloids, contributes to the upregulation of endogenous antioxidant enzymes, thereby reducing oxidative stress . These findings suggest that *V. amygdalina* could serve as an effective natural therapy for managing oxidative stress-related diseases.

In comparison with silymarin, the study revealed that both *V. amygdalina* and silymarin significantly improved antioxidant enzyme activities. However, silymarin exhibited slightly superior effects in restoring glutathione peroxidase (GPx) activity, indicating its strong hepatoprotective and cytoprotective properties. These results align with studies by Jia et al. (2013) and Stickel & Schuppan (2007), who confirmed that silymarin is effective in reducing oxidative stress by modulating key antioxidant pathways . Despite this, *V. amygdalina* demonstrated comparable efficacy to silymarin in restoring overall oxidative balance, suggesting that it could be a potential alternative therapy for oxidative stress-related conditions.

Additionally, non-enzymatic antioxidants, including glutathione (GSH), vitamin A, vitamin C, and vitamin E, were significantly reduced in DMH-treated rats, consistent with findings by Iqbal et al. (2024), who highlighted that chronic oxidative stress depletes essential antioxidants, impairing cellular defense mechanisms . However, treatment with *V. amygdalina* significantly restored these antioxidant levels, further validating its protective role against oxidative damage.

These findings align with the results of Frei (2004), who demonstrated that vitamin C and E supplementation effectively reduces oxidative stress in plasma.

Conclusion

This study demonstrated that ethanol extracts of *Vernonia amygdalina* significantly improved oxidative stress markers in DMH-exposed Wistar rats. DMH administration resulted in increased lipid peroxidation, depletion of antioxidant enzymes, and reduction in non-enzymatic antioxidant levels. However, treatment with *V. amygdalina* extract effectively reversed these effects, enhancing the antioxidant defense system and reducing oxidative damage.

The findings suggest that *Vernonia amygdalina* possesses potent antioxidant, hepatoprotective, and cytoprotective properties, comparable to the standard antioxidant drug, silymarin. Given its effectiveness in mitigating DMH-induced oxidative stress, *V. amygdalina* may serve as a natural therapeutic agent for managing oxidative stress-related conditions, including cancer, metabolic disorders, and neurodegenerative diseases.

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APPENDICES

Total Protein

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	2	5.1850	.13435	.09500	3.9779	6.3921	5.09	5.28
2	2	10.1100	3.94566	2.79000	-25.3403	45.5603	7.32	12.90
3	2	5.3900	.04243	.03000	5.0088	5.7712	5.36	5.42
4	2	5.4550	.31820	.22500	2.5961	8.3139	5.23	5.68
5	2	5.2250	.07778	.05500	4.5262	5.9238	5.17	5.28
6	2	5.5350	.12021	.08500	4.4550	6.6150	5.45	5.62
7	2	5.2950	.12021	.08500	4.2150	6.3750	5.21	5.38
8	2	5.5300	.21213	.15000	3.6241	7.4359	5.38	5.68
Total	16	5.9656	1.91956	.47989	4.9428	6.9885	5.09	12.90

Multiple Comparisons

Dependent Variable:VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1	2	-4.92500*	1.40398	.008	-8.1626	-1.6874
		3	-.20500	1.40398	.888	-3.4426	3.0326
		4	-.27000	1.40398	.852	-3.5076	2.9676
		5	-.04000	1.40398	.978	-3.2776	3.1976
		6	-.35000	1.40398	.809	-3.5876	2.8876
		7	-.11000	1.40398	.939	-3.3476	3.1276
		8	-.34500	1.40398	.812	-3.5826	2.8926
	2	1	4.92500*	1.40398	.008	1.6874	8.1626
		3	4.72000*	1.40398	.010	1.4824	7.9576

	4	4.65500*	1.40398	.011	1.4174	7.8926
	5	4.88500*	1.40398	.008	1.6474	8.1226
	6	4.57500*	1.40398	.012	1.3374	7.8126
	7	4.81500*	1.40398	.009	1.5774	8.0526
	8	4.58000*	1.40398	.011	1.3424	7.8176
3	1	.20500	1.40398	.888	-3.0326	3.4426
	2	-4.72000*	1.40398	.010	-7.9576	-1.4824
	4	-.06500	1.40398	.964	-3.3026	3.1726
	5	.16500	1.40398	.909	-3.0726	3.4026
	6	-.14500	1.40398	.920	-3.3826	3.0926
	7	.09500	1.40398	.948	-3.1426	3.3326
	8	-.14000	1.40398	.923	-3.3776	3.0976
4	1	.27000	1.40398	.852	-2.9676	3.5076
	2	-4.65500*	1.40398	.011	-7.8926	-1.4174
	3	.06500	1.40398	.964	-3.1726	3.3026
	5	.23000	1.40398	.874	-3.0076	3.4676
	6	-.08000	1.40398	.956	-3.3176	3.1576
	7	.16000	1.40398	.912	-3.0776	3.3976
	8	-.07500	1.40398	.959	-3.3126	3.1626
5	1	.04000	1.40398	.978	-3.1976	3.2776
	2	-4.88500*	1.40398	.008	-8.1226	-1.6474
	3	-.16500	1.40398	.909	-3.4026	3.0726
	4	-.23000	1.40398	.874	-3.4676	3.0076
	6	-.31000	1.40398	.831	-3.5476	2.9276
	7	-.07000	1.40398	.961	-3.3076	3.1676
	8	-.30500	1.40398	.833	-3.5426	2.9326
6	1	.35000	1.40398	.809	-2.8876	3.5876
	2	-4.57500*	1.40398	.012	-7.8126	-1.3374
	3	.14500	1.40398	.920	-3.0926	3.3826
	4	.08000	1.40398	.956	-3.1576	3.3176

		5	.31000	1.40398	.831	-2.9276	3.5476
		7	.24000	1.40398	.869	-2.9976	3.4776
		8	.00500	1.40398	.997	-3.2326	3.2426
	7	1	.11000	1.40398	.939	-3.1276	3.3476
		2	-4.81500*	1.40398	.009	-8.0526	-1.5774
		3	-.09500	1.40398	.948	-3.3326	3.1426
		4	-.16000	1.40398	.912	-3.3976	3.0776
		5	.07000	1.40398	.961	-3.1676	3.3076
		6	-.24000	1.40398	.869	-3.4776	2.9976
		8	-.23500	1.40398	.871	-3.4726	3.0026
	8	1	.34500	1.40398	.812	-2.8926	3.5826
		2	-4.58000*	1.40398	.011	-7.8176	-1.3424
		3	.14000	1.40398	.923	-3.0976	3.3776
		4	.07500	1.40398	.959	-3.1626	3.3126
		5	.30500	1.40398	.833	-2.9326	3.5426
		6	-.00500	1.40398	.997	-3.2426	3.2326
		7	.23500	1.40398	.871	-3.0026	3.4726

*. The mean difference is significant at the 0.05 level.

MDA

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	2	4.0000	1.41421	1.00000	-8.7062	16.7062	3.00	5.00
2	3	15.6667	2.08167	1.20185	10.4955	20.8378	14.00	18.00
3	2	6.5000	3.53553	2.50000	-25.2655	38.2655	4.00	9.00
4	2	6.5000	3.53553	2.50000	-25.2655	38.2655	4.00	9.00
5	2	5.5000	.70711	.50000	-.8531	11.8531	5.00	6.00
6	3	5.3333	3.21455	1.85592	-2.6521	13.3187	3.00	9.00
7	2	5.5000	2.12132	1.50000	-13.5593	24.5593	4.00	7.00

8	2	5.0000	.00000	.00000	5.0000	5.0000	5.00	5.00
Total	18	7.1667	4.40921	1.03926	4.9740	9.3593	3.00	18.00

Multiple Comparisons

Dependent Variable:VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1	2	-11.66667*	2.26078	.000	-16.7040	-6.6293
		3	-2.50000	2.47656	.337	-8.0181	3.0181
		4	-2.50000	2.47656	.337	-8.0181	3.0181
		5	-1.50000	2.47656	.558	-7.0181	4.0181
		6	-1.33333	2.26078	.568	-6.3707	3.7040
		7	-1.50000	2.47656	.558	-7.0181	4.0181
		8	-1.00000	2.47656	.695	-6.5181	4.5181
	2	1	11.66667*	2.26078	.000	6.6293	16.7040
		3	9.16667*	2.26078	.002	4.1293	14.2040
		4	9.16667*	2.26078	.002	4.1293	14.2040
		5	10.16667*	2.26078	.001	5.1293	15.2040
		6	10.33333*	2.02210	.000	5.8278	14.8389
		7	10.16667*	2.26078	.001	5.1293	15.2040
		8	10.66667*	2.26078	.001	5.6293	15.7040
	3	1	2.50000	2.47656	.337	-3.0181	8.0181
		2	-9.16667*	2.26078	.002	-14.2040	-4.1293
		4	.00000	2.47656	1.000	-5.5181	5.5181
		5	1.00000	2.47656	.695	-4.5181	6.5181
		6	1.16667	2.26078	.617	-3.8707	6.2040
		7	1.00000	2.47656	.695	-4.5181	6.5181
		8	1.50000	2.47656	.558	-4.0181	7.0181
	4	1	2.50000	2.47656	.337	-3.0181	8.0181
		2	-9.16667*	2.26078	.002	-14.2040	-4.1293

	3	.00000	2.47656	1.000	-5.5181	5.5181
	5	1.00000	2.47656	.695	-4.5181	6.5181
	6	1.16667	2.26078	.617	-3.8707	6.2040
	7	1.00000	2.47656	.695	-4.5181	6.5181
	8	1.50000	2.47656	.558	-4.0181	7.0181
5	1	1.50000	2.47656	.558	-4.0181	7.0181
	2	-10.16667*	2.26078	.001	-15.2040	-5.1293
	3	-1.00000	2.47656	.695	-6.5181	4.5181
	4	-1.00000	2.47656	.695	-6.5181	4.5181
	6	.16667	2.26078	.943	-4.8707	5.2040
	7	.00000	2.47656	1.000	-5.5181	5.5181
	8	.50000	2.47656	.844	-5.0181	6.0181
6	1	1.33333	2.26078	.568	-3.7040	6.3707
	2	-10.33333*	2.02210	.000	-14.8389	-5.8278
	3	-1.16667	2.26078	.617	-6.2040	3.8707
	4	-1.16667	2.26078	.617	-6.2040	3.8707
	5	-.16667	2.26078	.943	-5.2040	4.8707
	7	-.16667	2.26078	.943	-5.2040	4.8707
	8	.33333	2.26078	.886	-4.7040	5.3707
7	1	1.50000	2.47656	.558	-4.0181	7.0181
	2	-10.16667*	2.26078	.001	-15.2040	-5.1293
	3	-1.00000	2.47656	.695	-6.5181	4.5181
	4	-1.00000	2.47656	.695	-6.5181	4.5181
	5	.00000	2.47656	1.000	-5.5181	5.5181
	6	.16667	2.26078	.943	-4.8707	5.2040
	8	.50000	2.47656	.844	-5.0181	6.0181
8	1	1.00000	2.47656	.695	-4.5181	6.5181
	2	-10.66667*	2.26078	.001	-15.7040	-5.6293
	3	-1.50000	2.47656	.558	-7.0181	4.0181
	4	-1.50000	2.47656	.558	-7.0181	4.0181

5	-.50000	2.47656	.844	-6.0181	5.0181
6	-.33333	2.26078	.886	-5.3707	4.7040
7	-.50000	2.47656	.844	-6.0181	5.0181

*. The mean difference is significant at the 0.05 level.