INVITRO ANTIOXIDANT SCAVENGING POTENTIAL OF CARBONATED DRINK EXTRACT OF *Andrographis paniculata* PLANT

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APRIL 2024

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SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A BACHELOR OF SCIENCE DEGREE, B.Sc (HONS) IN DEPARTMENT OF BIOCHEMISTRY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY

APRIL 2024

CERTIFICATION

This is to certify that this project research was carried out by ONWORDI EMMANUELLA OBIANUJU with matriculation number, LSC1906609 under the supervision of PROF. CHUKWU E. ONYENEKE and has been read and approved as meeting the requirements of the Department of Biochemistry, Faculty of Life Sciences, University of Benin, Benin City, Edo State in partial fulfillment of the requirements for the award of a Bachelor of Science degree, B.Sc (HONS) in Biochemistry.

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(HEAD OF DEPARTMENT)

DEDICATION

This work is dedicated to Almighty God for his wisdom, grace, blessings, mercy and strength.

ACKNOWLEDGEMENT

I want to extend my sincere gratitude to God Almighty, who has been my source of strength, understanding and wisdom throughout my undergraduate program

I would also like to extend my profound gratitude to the Head of Department of Biochemistry who also happens to be my project supervisor, our very own Prof. Chukwu E. Onyeneke, for his utmost wisdom, time, guidance and discipline exerted on the students placed under him for supervision. I am indeed grateful to him for seeing to it that this project report came out successful. Special thanks to Mr. Kingsley Akpeh for diligently and dedicatedly assisting me in all the assays carried out during the experimental aspect of this research. I would also like to acknowledge the project supervisor, Dr. S.I Ojeaburu, my course adviser, Dr. Ruth Usifo and Dr. O.D Abu. And to my group members: Precious, Boluwade, Modesayo and Comfort for always carrying everyone along, never leaving anyone behind, I say a very big thank you.

And finally , to the best gift God gave me in the form of parents, to Mr. Emmanuel Ifeanyichukwu Onwordi and Mrs. Augusta Onyinyechukwu Onwordi, who through the challenges of my academic journey have always been there for me, having my back with their endless prayers and support morally and financially, and to my elder ones: Judith, Emmanuel and Theresa Onwordi for assisting me in one way or the other throughout this research and for their little contributions towards the success of this project. No words can be used to describe how extremely grateful I am to you guys. May God bless you all.

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ABSTRACT

One of the most well-known medicinal plants, *Andrographis paniculata* (family *Acanthaceae*), has been used for centuries in Asia, America, and Africa to treat a wide range of illnesses, including cancer, diabetes, high blood pressure, ulcers, leprosy, bronchitis, skin conditions, flatulence, colic, influenza, dysentery, dyspepsia, and malaria. This study evaluated the antioxidant properties of the medicinal herb, *Andrographis paniculata* with standard methods. Here, the DPPH, FRAP, TAC and hydrogen peroxide assays were carried out to determine the antioxidant activity of carbonated drink extract of *A. paniculata.* Each assay had corresponding values for the standard and the carbonated drink extract. With the exception of the DPPH assay, the IC_{50} values for the carbonated drink extract were lower compared to that of the standard (ascorbic acid). Judging from the IC_{50} values, it therefore indicated that the carbonated drink extract had more antioxidant activity than the standard, showing that the carbonated drink extract of *A. paniculata* has a better antioxidant potency.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. Background of Study

Traditionally utilized in traditional medicine for thousands of years in several places worldwide, medicinal plants are the oldest ancient type of treatment (Marrelli, 2021). They are the main providers of medicinal treatments for a range of illnesses. Their active phytoconstituents are mainly responsible for these possible therapeutic effects. Scientists that have studied plants since ancient times and found that they have therapeutic properties have well-documented society's understanding of the usefulness of plants (Bhaisare *et al.,* 2023). Over the millennia, human groups have shared actual information regarding their beneficial impacts (Khan, 2014). Since the beginning of time, they have been utilized in healthcare. Worldwide research has been done to confirm their effectiveness, and some of the results have prompted the development of plant-based medications (Sofowora *et al.,* 2013). Up until now, the focus on using medicinal herbs has been more on treating illnesses than on preventing them. Nonetheless, a significant amount of recent research on the application of medicinal plants and their components in illness prevention has been reported in various literatures (Sofowora *et al.,* 2013). Traditional medicine was defined by a World Health Organization (WHO) Expert Group as the culmination of all knowledge and practices, whether or not they can be explained, used in the diagnosis, prevention, and elimination of physical, mental, or social imbalance and solely relying on observation and hands-on learning passed down orally or in writing from generation to generation (WHO, 1976). About two thirds of people in many poor nations are thought to primarily rely on traditional healers and medicinal plants to address their basic healthcare neqeds (Okhuarobo *et al.,* 2014). Many plant

species are currently being revalued by researchers based on variance in plant species and their medicinal chemical principles, as a result of the various issues with conventional medications. An important source of drug molecules is natural products, and many contemporary medications are derived from traditional herbal medicine and utilized in modern pharmacotherapy (Patwardhan *et al.,* 2008). The extraction procedure is an essential phase in the study of the bioactive substances obtained from plant sources. Novel extraction techniques include ultrasound-assisted and supercritical fluid extraction technologies are currently being used in addition to more conventional approaches (Azwanida, 2015). The *Acanthaceae* family's genus *Andrographis* is a prime example of these therapeutic plants, having about forty species in it. Few are often used in folk medicine to treat a variety of illnesses with *A. paniculata* being the most significant of these few (Okhuarobo *et al.,* 2014). It is extensively spread due to its well-known medicinal usefulness and ability to grow in a variety of soil types (Latto *et al.,* 2006).

1.1.1. Botanical Description of *Andrographis paniculata*

The annual herb *Andrographis paniculata,* sometimes referred to as the "King of Bitters," grows up to 0.5 meters tall in branched growth. Its leaves are lanceolate, narrowly elliptic (narrow oval), or ovate-lanceolate (midway between egg- and lance-shaped), measuring 1.5-7 cm in length and 1-2.5 cm in width. Its stems are hairless and have four sides. It is a member of the *Acanthaceae* family and gets its name from the bitterness of its leaves. The white flowers of this plant possess purple streaks on them and are self pollinating. This plant is widely grown in Southern and Southeastern Asia, including India, Java, Sri Lanka, Pakistan, and Indonesia. It is also grown in North America's tropical regions, including Hong Kong, the West Indies, Jamaica, Barbados, and the Bahamas, and southwest Nigeria (Hossain *et al.,* 2014). The medicinal herb, *Andrographis paniculata* with its exceptionally bitter flavor is used to cure a variety of conditions, including

upper respiratory tract infections, colic discomfort, children's bowel problems, liver abnormalities, and the common cold (Chao and Lin, 2010). For millennia, some traditional medicine systems have successfully treated fever, the common cold, respiratory ailments, diabetes, and skin infections with the aerial section of *Andrographis paniculata* (Okhuarobo *et al.,* 2014). *Andrographis paniculata (Burm. f.) Nees* is a self-compatible, hermaphrodite, and habitual inbreeding plant. Its flowers reproduce by themselves (self pollinated) (Valdiani *et al.,* 2017).

Other scientific names include:

Andrographis paniculata (Burm. f) Nees

Andrographis paniculata var. glandulosa Trimen

Justicia paniculata (Burm. f). (Intharuksa *et al.,* 2022).

Common names used internationally include:

Arabic: Quasabhuva

Assamese: Kalmegh

Azerbaijani: Acılar Şahı, Acılar Xanı (khanı)

Bengali: Kalmegh

Chinese: Chuan Xin Lian,

English: The Creat, King of Bitters, Green Chiretta.

French: Chirette verte

Hindi: Kirayat, Kalpanath

Japanese: Senshinren.

Malaysian: Hempedubumi

Thai: Fah tha lai

(Nyeem *et al.,* 2017; Intharuksa *et al.,* 2022).

The plant is locally called 'meje-meje' in south western Nigeria (Faluyi, 2021).

Plate 1.1: Morphological characteristics of *Andrographis paniculata*. (A): Aerial part, (B): fruits and flowers, (C): close-up of the flower, and (D): fruits.

Source: Intharuksa *et al.,* (2022)

1.1.2. Origin

Andrographis paniculata is indigenous to Mainland China, India and Taiwan (Hossain *et al.,* 2014). Southeastern Asia, Sri Lanka, Cambodia, the Caribbean islands, Thailand , Indonesia, Laos, Malaysia, Vietnam and Myanmar are among the other nations where it is mostly found (Niranjan *et al.,* 2010). This plant can also be found in China, America, the West Indies, and Christmas Island, varying in phytogeographical and edaphic zones (Benoy *et al.,* 2012).

1.1.3. Taxonomy

Super Kingdom: *Eukaryota*

Kingdom: *Plantae*

Sub Kingdom: *Tracheobionta Spermatophyta*

Super Division: *Spermatophyta*

Division: *Angiosperma*

Class: *Dicotyledonae*

Sub class: *Gamopetalae*

Series: *Bicarpellatae*

Order: *Personales*

Tribe: *Justicieae*

Family: *Acanthaceae*

Genus: *Andrographis*

Species: *Andrographis paniculata (Burm. f) Nees*. (Ankita and Handique, 2010).

1.1.4. Habitat

A. paniculata is an economically significant herb in the *Andrographis* genus. It is an annual flowering herb that grows upright and branching (Hossain *et al.,* 2014). Due to their good development, gloomy and moist areas, woodlands, and wastelands are preferred. This plant grows well in hedgerows throughout hill slopes, waste ground, plane lands, farms, moist habitat, roadsides and seashores (Niranjan *et al.,* 2010). Because it is a salt-sensitive plant, stressors that significantly impact plant growth and crop productivity—such as salinity stress—limit the plant's ability to grow (Hossain *et al.,* 2021).

1.1.5. Active Compounds

Diterpenoids, polyphenols and flavonoids are the main components of the medicinal plant *Andrographis paniculata*, which is utilized in many different nations (Chao and Lin, 2010). In terms of bioactive characteristics and quantity , andrographolide is the most significant of the single chemicals isolated from *A. paniculata*. *Andrographis paniculata's* primary physiologically active component is andrographolide (AG), a naturally occurring diterpenoid. Among the effects described include immune system activation, psoriasis development prevention, and antibacterial and antiviral properties (Vetvicka and Vannucci, 2021). Neoandrographolide is anti-inflammatory, anti-infective, and anti-hepatotoxic; 14-deoxyandrographolide is immunomodulatory and anti-atherosclerotic; among the andrographolide analogues, 14-deoxy-

11,12-didehydroandrographolide is immunostimulatory, anti-infective, and anti-atherosclerotic (Chao and Lin, 2010). Among the less frequent substances found in *A. paniculata*, there are anti-inflammatory and anti-infective properties in andrograpanin; anti-inflammatory properties can be found in 14-deoxy-14,15-dehydroandrographolide; tumor suppressive properties can be found in isoandrographolide, 3,19-isopropylideneandrographolide, and 14-acetylandrographolide; and arabinogalactan proteins have anti-hepatotoxic properties (Chao and Lin, 2010).

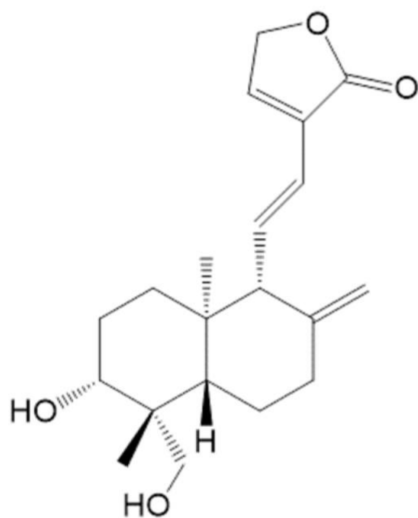


Figure 1.1: Chemical structure of 14-deoxy-11,12-didehydroandrographolide (deAND)

Source: Liu *et al*., (2020).

1.1.6. Traditional Uses of *A. paniculata*

One of the most widely utilized potential therapeutic plants in the world is *Andrographis paniculata*. Asia has long practiced traditional medicine using the aerial parts, roots, and entire plant of *A. paniculata* to treat a wide range of illnesses. Traditional medical professionals have used it to treat pyrexia, stomachaches, inflammation, and sporadic fevers (Okhuarobo *et al.,* 2014). Most commonly, leaves and roots have been used for ages in Asia and Europe for a variety of therapeutic uses as a folk cure for a wide range of illnesses or as a herbal supplement

to promote health (Ankita and Handique, 2010). Considering conventional medicine, *A. paniculata* is used as a herbal cure for a variety of conditions, including fever, herpes, diabetes, and chronic illnesses of the upper respiratory and gastrointestinal tracts (Bhaisare *et al.,* 2023).

Due to its intensely bitter flavor, *Andrographis paniculata* has been used to treat upper respiratory tract infections, colic discomfort, children's bowel complaints, liver disorders, and the common cold (Chao and Lin, 2010). For millennia, some traditional medicine systems have successfully treated fever, the common cold, respiratory ailments, diabetes, and skin infections with the aerial section of *Andrographis paniculata* (Okhuarobo *et al.,* 2014). Traditionally, this plant has been used to treat a variety of illnesses, including the common cold, diarrhea, fever from various infectious causes, jaundice, liver and cardiovascular health tonics, antioxidants, sexual dysfunction, and contraception (Hossain *et al.,* 2014).

A. paniculata is used ethnobotanically as a herbal medicine to cure snake bites, bug bites, diabetes, diarrhea, fever, and malaria in places like Bangladesh, China, Hong Kong, India, Pakistan, Philippines, Malaysia, Indonesia, and Thailand (Hossain *et al.,* 2014). The active phytochemicals in this plant can be extracted from any part of it; however, the phytoconstituent compositions vary greatly depending on the part, location, time of year, and harvest period (Hossain *et al.,* 2014).

Scientists studied the pharmacological characteristics of the *A. paniculata* plant to justify its use as a medicinal agent in the treatment of various disorders because of the extensive usage of its various sections in folk medicine, particularly in Asia (Okhuarobo *et al.*, 2014). According to Chandrasekaran *et al.,* (2010), a number of investigations revealed that this plant possessed a wide range of biological properties, including antimicrobial, cytotoxic, anti-protozoan, anti-

inflammatory, antioxidant, immunostimulant, anti-diabetic, anti-infective, anti-angiogenic, hepato-renal protective, sex hormone modulatory, liver enzymes modulatory, insecticidal, and toxicity roles.

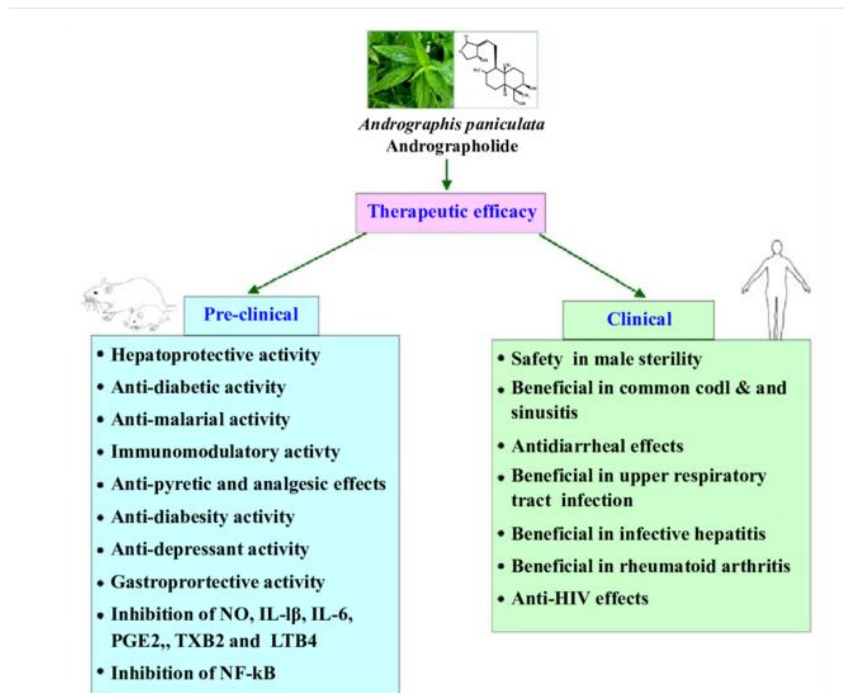


Plate 1.2: Therapeutic uses of *Andrographis paniculata.*

Source: Vikas *et al.,* (2014)

1.2. Oxidative Stress and Antioxidants

1.2.1. Oxidative Stress

Oxidative stress (OS) refers to the imbalance between the body's generation of reactive oxygen species (ROS) and the cells' capacity to quickly detoxify the intermediates or repair the resulting damage (Bardaweel *et al.,* 2018). It is a phenomena brought on by an imbalance between the generation and buildup of oxygen reactive species (ROS) in cells and tissues and the biological system's capacity to detoxify these reactive byproducts (Pizzino *et al.,* 2017), Oxidative Stress can further be defined as an imbalance favoring oxidants over antioxidants, which disrupts redox

signaling and control and/or causes molecular damage (Sies, 2020). Reactive oxygen species (ROS) include superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$). Biological systems produce these ROS as metabolic byproducts (Sato *et al.,* 2013, Navarro-Yepes *et al.,* 2014). Certain cellular processes, such as immunity, differentiation, apoptosis, protein phosphorylation, and the activation of multiple transcriptional factors, depend on appropriate ROS production and presence, which must be maintained at a low level (Rajendran *et al.,* 2014). Increased production of ROS causes damage to crucial biological components such as nucleic acids, lipids, and proteins (Wu *et al.,* 2013). Superoxide radicals (ROS) are primarily produced by mitochondria in both physiological and pathological conditions, moreover, endothelial and inflammatory cells can also produce ROS, as can cellular respiration, lipoxygenases (LOX) and cyclooxygenases (COX) during the metabolism of arachidonic acid, and cellular respiration (Al-Gubory *et al.,* 2012). To defend against ROS-induced cellular damage, cells use an antioxidant defensive mechanism that is mostly dependent on enzymatic components, such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) (Pizzino *et al.,* 2017). The pathophysiology of chronic illnesses like cancer, diabetes, neurodegenerative diseases, and cardiovascular diseases is greatly influenced by oxidative stress, prolonged exposure to elevated pro-oxidant factor levels can result in mitochondrial DNA structural abnormalities, as well as functional changes to a number of enzymes and cellular structures that can cause abnormalities in gene expression (Sharifi-Rad *et al.,* 2020).

1.2.1.1. Oxidative Stress and Human Disease

The condition known as oxidative stress arises from an imbalance between the capacity of cellular machinery to remove reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are produced and accumulate in cells and tissues, It is caused by disruptions in the

homeostatic processes that regulate pro- and antioxidant balance. ROS and RNS are byproducts of cellular respiration, which is the mechanism that results in life-sustaining cellular processes (Olufunmilayo *et al.,* 2023). Oxidative stress is a common factor in the loss of neurons linked to neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and Amyotrophic Lateral Sclerosis (ALS) (Li *et al.,* 2013). It also plays a role in brain and spinal cord damage after traumatic brain injury (TBI) and stroke (Rodríguez-Rodríguez *et al.,* 2014). It has also been discovered that the buildup of ROS and RNS contributes to mitochondrial malfunction, which results in deficiencies in energy synthesis, alterations in metal homeostasis, and the buildup of toxic protein aggregates that characterize a variety of neurodegenerative illnesses. Through apoptosis, necrosis, ferroptosis, or autophagy, these result in the activation of cell death pathways (Olufunmilayo *et al.,* 2023).

1.2.2. Free Radicals

Reactive oxygen species, or free radicals, are produced as byproducts of several bodily activities and are always present in the body (Chaudhary *et al.,* 2023). Because of the unpaired electron, they are tiny, diffusible molecules that are extremely reactive (Obeagu, 2018). Antioxidant functions eliminate free radicals from the body under normal circumstances and if these natural mechanisms are interfered with, radicals build up excessively and play a role in the onset of certain diseases (Chaudhary *et al.,* 2023). Due to their extreme instability and reactivity, these radicals can behave as oxidants or reductants by either giving or accepting an electron (Lobo *et al.,* 2010). The human body has developed an antioxidant defense mechanism that includes metal

chelation, enzymatic activity to eliminate reactive species shortly after they generate, and free radical scavenging to decrease or avoid free radical-directed oxidative damage (Chaudhary *et al.,* 2023). Antioxidants can lower the amount of reactive species in the cellular system by either increasing the expression and activities of antioxidant enzymes like glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), or by limiting the expression and activities of free radical-producing enzymes like xanthine oxidase (XO) and NAD(P)H oxidase (Aziz *et al.,* 2019). Overproduction of free radicals has the potential to harm lipids, proteins, and nucleic acids, among other macromolecules. This causes tissue damage in a number of degenerative and chronic illnesses (Martemucci *et al.,* 2022). Originally believed to be oxygen-centered radicals known as reactive oxygen species (ROS), free radicals are really a byproduct of regular cellular metabolism and also comprise a subset of reactive nitrogen species (RNS) (Obeagu, 2018). Reactive nitrogen and oxygen species (RNS/ROS) are substances that are both harmful and helpful to the organism's system. While these reactive species produce nitrosative and oxidative stress at higher concentrations, they also have positive effects and participate in various physiological processes, including immune function, cellular signaling pathways, mitogenic responses, and redox regulation (Phaniendra *et al.,* 2015). Antioxidants can lower the amount of reactive species in the cellular system by either increasing the expression and activities of antioxidant enzymes like glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), or by limiting the expression and activities of free radical-producing enzymes like xanthine oxidase (XO) and NAD(P)H oxidase (Aziz *et al.,* 2019). Our bodies produce free radicals, also known as reactive oxygen species and reactive nitrogen species, when they are exposed to diverse physiochemical circumstances, disease states, or endogenous

systems. For there to be optimal physiological function, antioxidants and free radicals must coexist in balance.

- **Adverse repercussions of free radicals:** Particularly for membrane lipids, proteins, and cell nucleic acids, free radicals represent a major risk to tissues and essential organs (Kumar and Panday, 2015). In accordance to the work of Chaudhary *et al.,* (2023), free radicals are created when ROS cause lipid peroxidation, DNA fragmentation, cell death, DNA damage, protein alteration, and membrane damage, resulting in very undesirable chsnges. Oxidative stress, caused by an excess of reactive oxygen/nitrogen species (ROS/RNS) and other related radicals, has been linked to aging and several diseases, numerous illnesses, including diabetes mellitus, cardiovascular and neurological diseases, autoimmune disorders, and different malignancies, have been linked to an excess of free radicals and the oxidative stress that results from them (Tvrdá and Benko, 2020). In addition to causing lipid peroxidation, loss of enzyme activity, mutation, and carcinogenesis when they interact with biomolecules, free radical accumulation has been linked to a number of degenerative diseases, such as diabetes, cardiovascular disease, and harmful hepatic conditions (Kumar and Panday, 2015). Numerous types of diabetes, neurodegenerative illnesses, cardiovascular diseases (CVDs), cancer, cataracts, asthma, rheumatoid arthritis, inflammation, burns, intestinal tract diseases, progerias, and ischemic and post-ischemic pathologies are among the pathological conditions that are linked to free radicals (Phaniendra *et al.,* 2015). Free radicals are essential to human health, even at low or moderate concentrations.

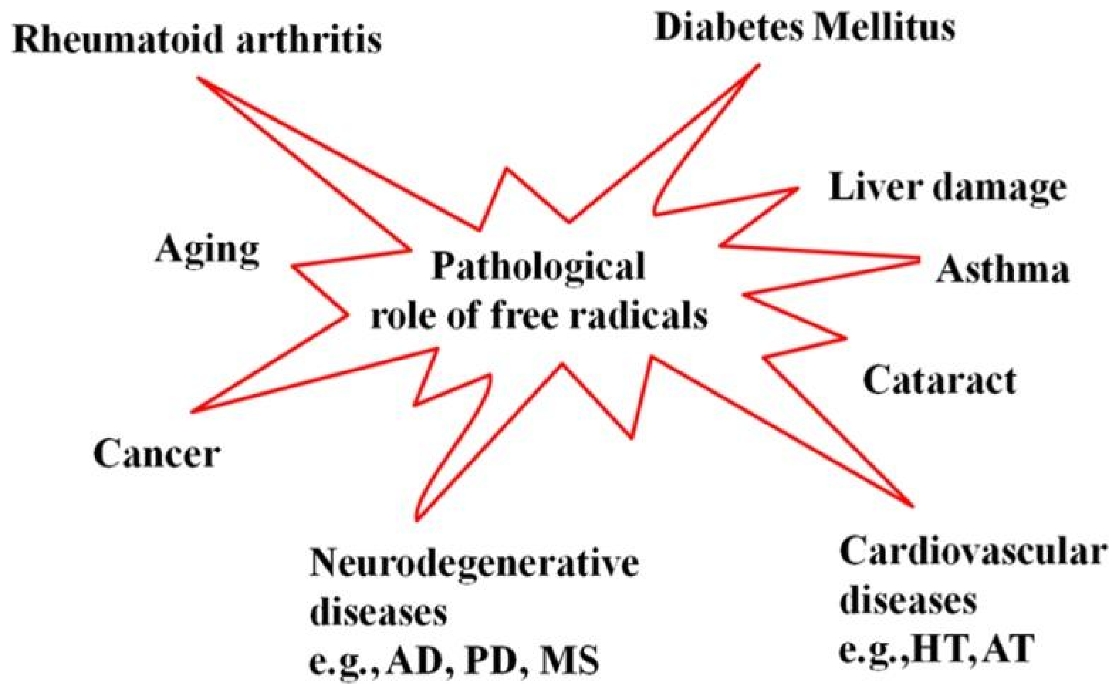


Plate 1.3: Pathological role of free radicals

Source: Phaniendra *et al.,* (2015).

- **Biochemical and physiological functions of free radicals:** The biochemistry of reactive oxygen species (ROS), which include hydrogen peroxide, superoxide anion, hydroxyl radicals, and singlet oxygen, is crucial to the aerobic metabolism of the cell, reactive nitrogen species, in particular, are widely known to have dual roles as both hazardous and advantageous species (Sisein, 2014). Oxidative stress, a process that can be a significant mediator of damage to cell structure and function, lipids, proteins, carbohydrates, and DNA, is caused by an excess of reactive oxygen species (ROS) from mitochondrial electron transport chain leakage or by overstimulating xanthine oxidase and other oxidative enzymes. In contrast, the advantageous impacts of ROS/RNS happen at extremely low concentrations and encompass physiological functions in cellular defense against pathogenic agents, gene expression, cellular proliferation, in the operation of several cellular signaling pathways, hypoxia, and respiratory burst (Sisein, 2014). The hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical are the most significant oxygen-containing free radicals in many disease conditions. They are extremely reactive species that can harm biologically significant components like DNA, proteins, carbohydrates, and lipids in the cell's nucleus and membranes (Lobo *et al.,* 2010). Important macromolecules are attacked by free radicals, which results in cell damage and disruption of homeostasis. All of the body's molecules are targets for free radicals. Lipids, nucleic acids, and proteins are the main targets among them (Lobo *et al.,* 2010).
- **Therapeutic role of free radicals:** Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the two main types of free radicals that are produced by the body's

many endogenous and external systems and although enhanced formation of these free radicals by chemotherapeutic treatments is linked to apoptosis in cancer cells, excess free radical production is known to cause various chronic disorders, including cancer, suggesting the dual nature of free radicals (Gupta *et al.,* 2020). Overexposure to ROS damages DNA, RNA, and proteins, changing a cell's genetic makeup. On the flip side, low ROS levels are necessary for several biological processes, such as immune response, cell growth, proliferation, and differentiation, as well as cell survival. Certain agents also induce cancer cells to die by producing lethal amounts of ROS (Gupta *et al.,* 2020).

1.2.2.1. Reactive Oxygen Species (ROS)

According to Jakubczyk *et al.,* (2020), molecules with at least one oxygen atom and one or more unpaired electrons are known as reactive oxygen species (ROS), they are molecules that can exist on their own. Although ROS were first identified as harmful byproducts of aerobic metabolism, it has since become clear that they play a significant signaling role in plants, regulating functions like growth, development, and particularly how they respond to biotic and abiotic environmental stimuli (Das and Roychoudhury, 2014). Free radicals such as $O^{\bullet -}_2$, OH•, and non-radicals like H_2O_2 and $^1O_2$ are the main constituents of the ROS family. The chloroplast, mitochondria, and peroxisom are where most plants produce ROS (Das and Roychoudhury, 2014). The breakdown of biomolecules such as pigments, proteins, lipids, carbohydrates, and DNA is one way that these ROS, which serve as secondary messengers in various important physiological phenomena, cause oxidative damages under a variety of environmental stress conditions, such as salinity, drought, cold, heavy metals, UV irradiation, etc., when the delicate balance between ROS production and elimination, required for normal cellular homeostasis, is upset and this will eventually result in plant cellular death (Das and Roychoudhury, 2014). Plants

in order to survive, have created an effective defense system against free radicals. This defense system is composed of two arms: (i) enzymatic antioxidants such as catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), guaiacol peroxidase (GPX), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR) and dehydroascorbate reductase (DHAR); (ii) antioxidants which are not enzymatic, such as ascorbic acid (AA), α-tocopherol, carotenoids, flavonoids, reduced glutathione (GSH) and the osmolyte proline. To scavenge ROS, these two arms collaborate (Das and Roychoudhury, 2014). In accordance with Davies *et al.,* (2009), ROS are extremely reactive molecules that can damage normal cells through a number of reduction reactions. All cells must undergo ROS detoxification in order to survive. With the goal of survival in the oxygen-rich cellular environment, living things have evolved a range of defense mechanisms to provide a balance between the formation and removal of ROS (Bardaweel *et al.,* 2018). As byproducts of regular cellular metabolism, endogenous and physiological reactive oxygen species (ROS) are typically produced primarily in the oxidative reaction process of the mitochondrial respiratory chain (Nathan and Cunningham-Bussel, 2013).

1.2.2.2. Reactive Nitrogen Species (RNS)

Nitric oxide (•NO), the source of RNS, is a crucial signaling element in a number of stress tolerance processes, some of which also include the nitrogen cycle (Saddhe *et al.,* 2019). Nearly all physiological plant processes, including seed germination, development, senescence, stomatal movement, fruit ripening, and reproduction, are influenced by nitric oxide (•NO) and its derived molecules, also known as reactive nitrogen species (Corpas, 2017). These processes also involve mechanisms of response to unfavorable environmental conditions that may be linked to nitro-oxidative stress. It is well known that RNS play two roles in plant cells: they can induce nitrosative stress and also regulate physiological functions. They are engaged in a number of

physiological processes in plants, such as the symbiosis between legumes and Rhizobium, germination of seeds, plant development and growth, reproduction, senescence and response to biotic factors (Chamizo-Ampudia *et al.,* 2017; Signorelli and Considine, 2018; Fu *et al.,* 2018). Additionally, under various abiotic stressors such drought, salinity, cold, heat, and heavy metals, plants can respond by modulating their RNS level and RNS-dependent signaling pathways (Yu *et al.,* 2014; Domingos *et al.,* 2015; Fu *et al.,* 2018). A class of substances known as reactive nitrogen species varies in their reactivity and characteristics. It appears that some of these reactive nitrogen species lack the specificity required to take part in cell signaling processes since they are very reactive and can permanently alter macromolecules through their interactions. Reactive nitrogen species have, however, lately come to be thought of as macromolecule oxidizers that cause widespread oxidative damage (Adams *et al.,* 2015).

1.2.2.3. Free Radical Generation

Many common processes, including light, heat, sonication, radiation, redox reactions, and electrolysis, can produce free radicals (Wang *et al.,* 2021). Through several endogenous sources, free radicals raise the quantity of reactive oxygen and nitrogen species in the biological system, increasing overall oxidative stress (Tripathi *et al.,* 2022). Overdosing on drugs, industrial air pollution, heavy metals that are poisonous, ionizing radiation, alcohol, smoke, pesticides, and UV radiation are the primary contributors of free radical production (Tripathi *et al.,* 2022). The pathogenesis and progression of neurodegenerative disease and other neurological defects are caused by an excess of free radicals generated inside the cell, which increases reactive oxygen and nitrogen species and causes oxidative damage. An increase in oxidative damage also alters various cellular pathways and processes, including mitochondrial impairment, DNA damage response, cell cycle arrest, and inflammatory response (Tripathi *et al.,* 2022). Both the body's

natural, vital metabolic activities and external factors including exposure to X-rays, ozone, cigarettes, air pollution, and industrial toxins can produce free radicals and other reactive oxygen species (ROS). They are constantly formed in the cells as a result of both nonenzymatic and enzymatic processes (Lobo *et al.,* 2010).

1.3. Antioxidants

Chemical oxidation is prevented or delayed by antioxidants (Chaudhary *et al.*, 2023). Though the original research on antioxidants primarily concentrated on their utility in keeping unsaturated fats from going rancid, the discovery of vitamins E, C, and A in living things has contributed to our understanding of antioxidant activity (Rahaman *et al*., 2023). Antioxidants are substances that, when present in less quantities than the oxidizable substrate, significantly lessen or postpone the substrate's oxidation (Chaudhary *et al.,* 2023). Lipids, proteins, and DNA are shielded from oxidative stress by antioxidants such glutathione (GSH), thioredoxin, ascorbic acid, and enzymes like catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx). Among these listed, natural antioxidants found in diet include tocopherols, ascorbic acid, carotenoids, flavonoids, and amino acids (Ali *et al.,* 2020). The body of the organism has developed an alternative endogenous system to offset the production of reactive oxygen intermediates. The two main categories for the endogenous system include enzymatic and non-enzymatic antioxidant systems. Antioxidants' primary job in the body is to eliminate reactive species (Kurutas, 2016). The preventive role of antioxidants in food products against oxidative deterioration and in the body against oxidative stress-directed aberrant processes is the reason for the increased interest in antioxidants among the general public, health professionals, and food scientists (Chaudhary *et al.,* 2023). Antioxidants are commonly classified as either enzymatic or non-enzymatic, having various kinds of chemicals with various sites of action and final effects

among them (Flieger *et al.,* 2021). They have the ability to chelate metals, work as synergists, inhibit enzymes, donate electrons, donate hydrogen, and scavenge radicals (Sharifi-Rad *et al.,* 2022). Natural antioxidants present in food consumption, such as tocopherols, ascorbic acid, carotenoids, flavonoids, amino acids, phospholipids, and sterols, are important in scavenging both free radical and non-radical oxidants and shielding cells from oxidative stress and damage (Ali *et al.,* 2020).

As earlier mentioned, enzymatic and non-enzymatic antioxidants are two types of antioxidants. Free radicals are eliminated by the breakdown of the enzymatic antioxidant. The breakdown of reactive oxygen species (ROS) in plant cells is catalyzed by these antioxidant enzymes like catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD). Non-enzymatic antioxidants have two distinct modes of action: (1) they directly detoxify radicals and ROS, and (2) they decrease the substrates that antioxidant enzymes need to function. Ascorbate/ascorbic acid, glutathione, tocopherol, carotenoids, favonoids, proline, raffnose family oligosaccharides, and sugar alcohols make up these non-enzymatic antioxidant defense systems (Ramadoss *et al.,* 2021).

1.4. Antioxidant Systems

1.4.1. Enzymatic Antioxidant Systems

A. **Superoxide dismutases (SODs):** The enzyme known as superoxide dismutase is widely distributed throughout the body and is responsible for catalyzing the dismutation of superoxide (Jeeva *et al.,* 2015). They belong to a family of closely related enzymes that catalyze the conversion of hydrogen peroxide and oxygen from the superoxide anion.

Extracellular fluids and nearly all aerobic cells contain SOD enzymes (Johnson *et al.,* 2005). One natural antioxidant enzyme that is essential for efficiently reducing oxidative stress is superoxide dismutase (SOD) (Liu *et al.,* 2014). The human body generates a staggering amount of reactive oxygen species, including superoxide, hydrogen peroxide, and hydroxyl radicals, the most lethal kind of radical to the tissue being hydroxyl radicals which can destroy neighboring cells (Jeeva *et al.,* 2015). There are three types of SOD enzymes: manganese SOD is found in the mitochondria, while copper-zinc containing enzymes are primarily found in the cytoplasm. Extracellularly, a third kind exists (Ramasarma, 2007). Depending on the metal cofactor, superoxide dismutase belongs to three main families: Fe and Mn types (SOD2) bind either iron or manganese; the Ni type (SOD3) binds nickel; and Cu/Zn (SOD1) binds copper and zinc (Liu *et al.,* 2014). A natural antioxidant enzyme that plays a major role in reducing the oxidative burden is mitochondrial SOD2 (Hubackova *et al.,* 2012). Most mammalian cells' total cellular SOD activity is accounted for by SOD1, which is mostly found in the cytoplasm (Liu *et al.,* 2014).

B. Catalases: The antioxidant enzyme catalase neutralizes the effects of intracellular hydrogen peroxide by acting as a catalyst to convert hydrogen peroxide to oxygen and water (Jeeva *et al.,* 2015). Since most catalase is destroyed during tissue manipulation, it is impossible to determine the precise amount of catalase present in the cytoplasm (Aly and Shahin, 2010). In peroxisomes of eukaryotic cells, catalase—which is heme-containing—catalyzes the dismutation of two hydrogen peroxide molecules into water and an oxygen molecule and although the hydrogen peroxide Km is high, the process proceeds very quickly. It has been suggested that acatalasemia, a hereditary deficit of

catalase activity, is a benign trait (Fujii *et al.,* 2022). However, more research suggests that individuals or animals lacking the enzyme catalase actually display a situation that makes oxidative damage and aging more difficult (Goth *et al.,* 2013). Many regular metabolic activities produce hydrogen peroxide as a hazardous byproduct; in order to avoid damage, hydrogen peroxide must be swiftly changed into less hazardous molecules. According to Lobo *et al*., (2010), cells commonly employ catalase to swiftly catalyze the breakdown of hydrogen peroxide into less harmful gaseous oxygen and water molecules.

C. **Glutathione peroxidase (GPx):** Phylogenetically related enzymes include the GPx family (Brigelius and Maiorino, 2013). GPxs can catalyze H_2O_2 or organic hydroperoxides into water or the equivalent alcohols by using glutathione as a reductant (Han *et al.,* 2010). Members of the GPx family have anti-oxidative functions at various cellular components. The proteins gpx1 and gpx2 are extensively dispersed in the cytosol and mitochondria respectively, gpx3 is located in the plasma, and gpx4 is membrane-associated and appears to protect membranes against oxidative stress. (Liu *et al.,* 2014).

D. **Thioredoxin (Trx):** As an endogenous antioxidant system, the thioredoxin antioxidant system, which is made up of NADPH, thioredoxin reductase (TrxR), and Trx, is crucial in the fight against oxidative stress. These thioredoxin antioxidants reduce methionine sulfoxide reductases and ribonucleotide reductases, which aid in the repair of DNA and proteins (He *et al.,* 2017). Furthermore, it has been discovered that Trx systems participate in the immunological response (Lu and Holmgren, 2014). Cells' Trx systems can sustain the redox level by utilizing the thiol and selenol groups (He *et al.,* 2017).

E. **Glutathione reductase:** The GSR gene in humans codes for the enzyme glutathione reductase (GR), also referred to as glutathione-disulfide reductase (GSR). It catalyzes the

conversion of glutathione disulfide (GSSG) to glutathione (GSH) in its sulfhydryl form, which is an essential component for preventing oxidative stress and preserving the cell's reducing environment (Deponte, 2013). By converting glutathione disulphide (GSSG) to its reduced form (GSH), the flavin enzyme glutathione reductase functions as an antioxidant to shield cells from oxidative stress. Additionally, it plays a significant part in drug metabolism and detoxification processes, particularly in the liver. This is because liver microsomes have the cytochrome P-450 system, which offers detoxifying activities (Isik and Soydan, 2023). One of the most significant recognized goals of GR enzyme-catalyzed processes is maintaining the GSH/GSSG ratio in the cellular environment (Kocaoglu *et al.,* 2019).

1.4.2. Non enzymatic Antioxidant Systems

A. **Ascorbic acid:** Ascorbate, sometimes referred to as vitamin C or L-threo-hexenon-1,4-lactone, is a well-known, soluble, non-enzymatic antioxidant that is widely distributed in plant cells' cytoplasm, chloroplasts, vacuoles, mitochondria, and cell walls (Shao *et al.,* 2007). Because of its ability to donate electrons to a variety of enzymatic and non-enzymatic activities, it is also regarded as one of the most potent antioxidants (Foyer and Noctor 2011). Plants' antioxidative metabolism is efficiently regulated by the exogenous delivery of ascorbic acid through foliar spraying, root feeding, and seed pre-treatment (Ramadoss *et al.,* 2021). According to a number of studies (Raza *et al.,* 2013; Bybordi, 2012; Agami, 2014), ascorbic acid regulates the metabolism of antioxidant defense in a variety of crop plants grown under varying stress conditions, including salinity in okra, canola, and barley, and drought in canola (Shafq *et al.,* 2014). This demonstrates how the antioxidant defense system and several antioxidant enzymes are activated. Ascorbate

peroxidase (APXs), an enzyme that contains iron, dismutases H_2O_2 into H_2O and molecular oxygen using ascorbic acid as an electron donor (van Doorn and Ketsa, 2014). By influencing metabolism, osmolytes, antioxidants, and the transcriptional control of catalases and heat shock proteins in tomatoes, ascorbic acid applied exogenously can produce both heat stress tolerance and chilling tolerance (Alayaf 2020; Elkelish *et al.,* 2020).

B. **Glutathione (GSH):** The structure of glutathione was not identified until after 1935, despite its identification in 1888. Long hydrophilic groups are present in glutathione, a ubiquitous tripeptide also known as γ-glutamyl-cysteinyl-glycine (γ-Glu-Cys-Gly; GSH) (Ramadoss *et al*., 2021). One of the most important factors in keeping redox homeostasis in a plant cell is the balance between two forms of glutathione, glutathione disulfide (GSSG) and tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) (Foyer and Noctor, 2011). Because of its core nucleophilic cysteine residue, which detoxifies H_2O_2 and scavenges $^1O_2$, $O_2^{\bullet -}$, and OH• without the need for enzymes, GSH has a significant potential for reductive reactions (Ramadoss *et al.,* 2021). GSH can bind to reactive electrophiles by glutathionylation or react with ROS to form GSSG, which shields lipids, proteins, and nucleic acids from oxidative stress (Foyer and Noctor, 2011). Through the ascorbate-glutathione cycle, GSH also promotes the regeneration of additional possible water-soluble antioxidants, such as ascorbic acid. Additionally, GSH improves resistance to a number of abiotic stressors, including salt, drought, high and low temperatures, and metal toxicity (Hasanuzzaman and Fujita, 2013). GSH inhibits lipid peroxidation, lowers oxidative stress, and safeguards the plasma membrane (Ramadoss *et al.,* 2021).

C. **Tocopherols:** Tocopherols are an essential component of biological membranes and are referred to as lipophilic antioxidants. They come in several forms, with α-tocopherol, also known as vitamin E, being said to have the strongest antioxidant action, followed by β-, γ-, and δ-tocopherols (Ramadoss *et al.,* 2021). Under different abiotic stress situations, α-tocopherol significantly reduces oxidative damage and increases the amount of water and nutrients available to sustain plant growth, development, and production (Shao *et al.,* 2007). Tocopherols can react with substances like RO• and ROO• to stop the propagation step of lipid peroxidation and protect lipid and other membrane components in chloroplasts from ROS, maintaining the integrity of PS II (Ramadoss *et al.,* 2021). Tocopherols mediate membrane shape, fuidity, and permeability in addition to their antioxidant properties (Wang and Quinn, 2000). Numerous earlier studies came to the conclusion that changes in tocopherol content either directly or indirectly significantly affect an organism's ability to withstand abiotic stress (Ramadoss *et al.,* 2021). There are two distinct methods via which abiotic stress modifies tocopherol in plants. The first step, known as stress tocopherol synthesis, intensifies in order to eliminate ROS and prevent oxidative damage. In the second step, a critical stress state that breaks down tocopherol causes net tocopherol loss since synthesis is unable to make up for the lost tocopherol. The lack of tocopherol prevents fast production from making up for it, which causes lipid peroxidation and ultimately cell death. As indicated by MunneBosch, (2005), the first and second processes are visible for genotypes that are stress resistant and sensitive, respectively. The charge transfer mechanism serves as the basis for these antioxidant capabilities' singlet oxygen quenching.

D. **Carotenoids:** The presence of oxygen, light, and heat influences the synthesis and accumulation of carotenoids in plants, which in turn influences oxidation, color deterioration, and accumulation (Ramadoss *et al.,* 2021). Along with being a class of lipophilic antioxidants originating from the isoprenoid pathway, carotenoids are also recognized for their ability to absorb light in the visible spectrum between 400 and 550 nm (Sharma *et al.,* 2012). Moreover, they transfer the energy to molecules of chlorophyll. As essential components of the PS I system, carotenoids scavenge $^1O_2$ and maintain the stability of the thylakoid membrane and the light-harvesting complex (Farré *et al.,* 2010; Ramel *et al.,* 2012). In numerous ways, carotenoids shield photosynthetic equipment from photooxidative stress. The presence of carotenoids in β-carotene (reaction centers) and the antenna system (lutein and neoxanthin) protects the photosynthetic apparatus from light stress, which causes the light-harvesting complex (antenna system) to become overexcited (Ramadoss *et al.,* 2021).

E. **Sugar Alcohols and the Raffinose Family of Oligosaccharides (RFOS):** A class of soluble sugars called the raffinose family of oligosaccharides (RFOs) is produced from sucrose's α-galactosyl (Raja *et al.,* 2016). Abiotic stress in crop plants causes the most frequent trisaccharide, raffinose, to actively accumulate as verbascose, ajugose, stachyose, and the tetra saccharide stachyose (Raja *et al.,* 2015; Ramadoss and Shunmugam, 2014; Li *et al.,* 2020). RFO and sugar alcohols are examples of carbohydrates that help keep redox equilibrium and shield cells from oxidative damage (Keunen *et al.,* 2013). Additionally, it has been observed that RFO directly contributes to ROS detoxification, RFO transform to their oxidized radical forms during this detoxification process and are further regenerated by reacting with other antioxidants such as favonoids or ascorbic acid

(Van den Ende and Valluru, 2009). The first member of this family is raffnose, and the others are stachyose and verbascose (Gangola *et al.,* 2013). According to Keunen *et al.,* (2013), raffnose and galactinol, which is a precursor of RFO production, are crucial defenses against oxidative stress in plants.

F. Flavonoids: A broad and common class of plant phenolics known as flavonoids, comprising around 5000 distinct favonoids classified into six primary subclasses—favones, favanols, favanones, favanols, anthocyanidins, and isofavones—are secondary metabolites (Ramadoss *et al.,* 2021). It has long been known that flavonoids respond to interactions with their environment in a variety of ways (Agati and Tattini, 2010). When plants are subjected to different abiotic stress situations, their production of flavonoids is increased (Agati *et al*. 2011). Reactive oxygen species are typically formed by various stresses, and it has been hypothesized that flavonoids were also produced to counteract stress-induced oxidative damage. Certain plant circumstances cause antioxidant enzymes to become inactive, which increases the production of flavonoids and creates a secondary ROS scavenging system in plants exposed to prolonged abiotic stress conditions (Ramadoss *et al.,* 2021). The production of flavonoids is increased in response to UV radiation and a variety of abiotic stress tolerances (Agati *et al.,* 2011). It is suggested that flavonoids are accumulated or produced to counteract stress-induced oxidative damage in plants because the majority of abiotic stimuli produce reactive oxygen species (Ramadoss *et al.,* 2021).

G. Uric acid: Purine nucleotide metabolism produces uric acid as its byproduct, and an increase in the body's uric acid concentration causes hyperuricemia, which in turn causes gout. Allantoin is produced non-enzymatically by uric acid, which is a strong antioxidant

that interacts with reactive oxygen species (ROS) (Mikami and Sorimachi, 2017). Uric acid's antioxidant qualities have been used in medical treatment. For instance, an increase in ROS production is thought to be responsible for the start of Parkinson's disease. Parkinson's disease patients also exhibit a drop in serum uric acid concentration (Schwarzschild *et al.,* 2008). In a previous study, inosine—which is metabolized to uric acid—was given orally to Parkinson disease patients in an effort to increase the body's uric acid content and improve antioxidant capacity. The results demonstrated that inosine administration orally can slow the progression of Parkinson disease (Schwarzschild *et al.,* 2008). Uric acid's antioxidant potential may help treat some illnesses (Mikami and Sorimachi, 2017).

1.5. Plant Sources of Antioxidants

Antioxidants can be found naturally in a wide range of plant materials, including fruits, vegetables, seeds, herbs, and spices (Lourenço *et al.,* 2019). Foods and medicinal plants are rich sources of natural antioxidants. According to Xu *et al.,* (2017), these naturally occurring antioxidants, particularly polyphenols and carotenoids, have a variety of biological benefits, such as anti-inflammatory, anti-aging, anti-atherosclerosis, and anticancer properties. To investigate new sources of antioxidants and encourage their use in functional foods, medications, and food additives, it is essential to extract and evaluate antioxidants from food and medicinal plants in an efficient manner (Xu *et al.,* 2017). Foods and medical plants, including fruits, vegetables, cereals, mushrooms, drinks, flowers, spices, and conventional medicinal herbs, are the primary sources of exogenous antioxidants (Li *et al.,* 2014).

Polyphenols (phenolic acids, flavonoids, anthocyanins, lignans, and stilbenes), carotenoids (xanthophylls and carotenes), and vitamins (E and C) make up the majority of these naturally occurring antioxidants derived from plant materials (Baiano and del Nobile, 2015). These naturally occurring antioxidants, particularly polyphenols and carotenoids, have a variety of biological effects, including anti-inflammatory, antibacterial, antiviral, anti-aging, and anticancer ones (Xu *et al.,* 2017).

Foods and medications, particularly those that contain oils and fats, frequently use both natural and synthetic dietary antioxidants to prevent oxidation (Lobo *et al.,* 2010). Propyl gallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butyl hydroquinone (TBHQ) are the synthetic antioxidants most frequently mentioned in the food industry. Furthermore, fruits and vegetables are frequently treated with 2,4-dichlorophenoxyacetic acid (2,4-DA), 4-phenylphenol (OPP), and 2-naphthol (2NL) (Lourenço *et al.,* 2019).

Plant-based natural antioxidants can be categorized into three primary groups: carotenoids, vitamins, and phenolic chemicals (Lourenço *et al.,* 2019). Some phenolic compounds have significant impacts on the flavors and textures of food products in addition to being the main plant chemicals with antioxidant activity. They also have antibacterial and antifungal properties (Soto-Vaca *et al.,* 2012). The structures of phenolic compounds range widely, from simple molecules like caffeic acid, gallic acid, vanillin, and ferulic acid to polyphenols like tannins and flavonoids (Abbas *et al.,* 2017). The two most crucial vitamins in terms of vitamins are E and C. The first vitamin is lipid soluble and is made up of a mixture of four tocopherols and four tocotrienols, including four isomers (α, β, γ, and δ). Of these, only α-tocopherol is absorbed by the body. Legumes and cereal grains are the primary sources of it

(Boschin and Arnoldi, 2011; Hussain *et al.,* 2012). Many fruits and vegetables naturally contain vitamin C, which is soluble in water. Fruits and vegetables also contain the majority of carotenoids. The primary carotenoids having antioxidant activity include lutein, β-carotene, α-carotene, and lycopene (Lourenço *et al.,* 2019). Apart from their antioxidant properties, they could also be employed as food coloring agents (Böttcher *et al.,* 2015).

1.6. Mechanism of Action of Antioxidants

Antioxidants are thought to work through two main processes (Lobo *et al.,* 2010). The first is a chain-breaking mechanism wherein the system's free radical receives an electron from the main antioxidant. By quenching the chain-initiating catalyst, the second process eliminates reactive oxygen species (ROS) and their initiators, also known as secondary antioxidants. According to Lobo *et al.,* (2010), antioxidants can influence biological systems through a variety of ways, such as co-antioxidants, electron donation, metal ion chelation, or the control of gene expression. Antioxidant chemicals function chemically through a variety of pathways, including single electron transfer (SET), hydrogen atom transfer (HAT), and the capacity to chelate transition metals (Santos-Sánchez *et al.,* 2019). The two main techniques to increase antioxidant capacity are 1) hydrogen atom transfer (HAT), which transfers a hydrogen ion from a stable molecule to the antioxidant, enabling it to scavenge reactive oxygen species (ROS); and 2) single electron transfer (SET), which relies on the antioxidant's ability to reduce specific molecules and compounds by transferring an electron (Siddeeg *et al.*, 2021). These techniques differ in how they work.

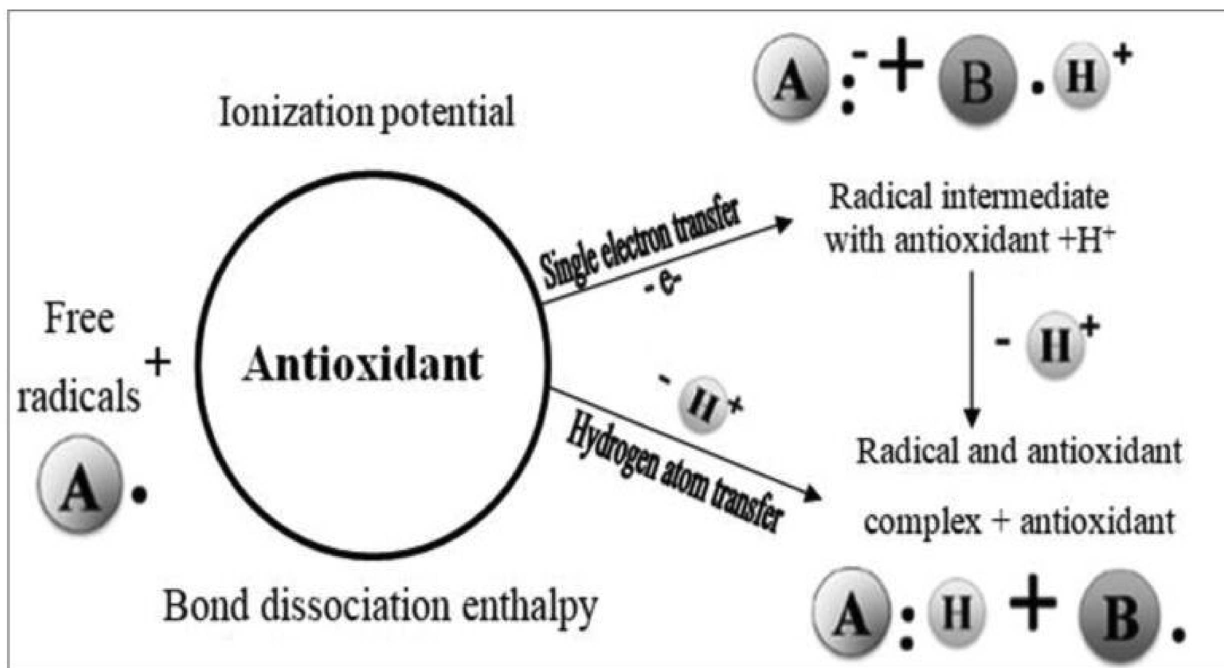


Plate 1.4: Mode of action of antioxidant reacting with free radicals.

Source: Siddeeg *et al.,* (2021).

Ionization and deprotonation potentials, two important antioxidant functional group properties, are the primary determinants of antioxidant reactivity in the SET experiment. Acidic pH causes an increase in the protonation of the antioxidant, which lowers the ionization potential and inhibits the antioxidant's overall capacity to reduce (Siddeeg *et al.,* 2021). Conversely, under the fundamental situation, proton dissociation increases, boosting the antioxidant reducing capacities (Prior *et al.,* 2005). Furthermore, because of the time required for solvent stabilization and reaction completion, the SET reaction proceeds more slowly than the HAT (Karadag *et al.,* 2009). Nevertheless as the reaction shown below illustrates, the HAT-based approach assesses the antioxidant's capacity for salvaging ROS by measuring its capacity to donate hydrogen atom(s).

$$ROO\bullet + AH/ArOH \rightarrow ROOH + A\bullet/ArO\bullet(HAT)$$

Generally speaking, the HAT assay procedure is mostly based on the bond dissociation enthalpy (energy) of the antioxidant's hydrogen-donating group (Siddeeg *et al.,* 2021). Thus, a faster and more stable HAT reaction will result from the antioxidant's donating group releasing hydrogen ions at a lesser rate. HAT is solvent- and pH-dependent, similar to SET. HAT test, unlike SET, is derived from kinetic curves and is faster compared to SET (Siddeeg *et al.*, 2021).

1.7. Aim of Study

The idea of this study is to assess the carbonated drink extract of the Andrographis paniculata plant's capacity to scavenge antioxidants.

1.8. Objectives

I. To identify the different antioxidants contained in the carbonated drink extract of *Andrographis paniculata* by employing assays which involve hydrogen peroxide scavenging, DPPH, FRAP and TAC.

II. To conduct a comparative analysis of *Andrographis paniculata* extracts (Aqueous and ethanol) in order to determine each one's relative antioxidant potency.

III. To examine the possible health advantages of ingesting *Andrographis paniculata*.

IV. To ensure the quality and potency of the *Andrographis paniculata* carbonated drink extract, indicating its effectiveness in providing antioxidant protection.

V. To write a thorough report that includes a summary of the study's findings, statistical analysis, and data interpretation.

VI. To provide a contribution to the scientific investigation about the possible uses of *Andrographis paniculata* in functional foods and beverages and its antioxidant qualities.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Materials.

2.1.1. Sample Collection and Authentication

Fresh leaves of the plant *Andrographis paniculata* were procured from Benin City, Edo state. At the Herbarium Unit of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Edo state, Nigeria, the plant was subsequently identified by Dr. O. Timothy and verified by Prof. Akinnibosun Henry Adewale (FLS, MRSB; London), with voucher specimen number UBH-A599.

2.1.2. Equipments and Instruments

VII.	Analytical weighing balance (PA213)	OHAUS Corp, Pioneer, U.S.A.
VIII.	Beakers	Pyrex, England
IX.	Burette(100mL, 200mL, 500mL, 1000mL)	Pyrex, England
X.	Conical flask(100mL, 200mL, 500mL)	Pyrex, England
XI.	Desiccator	Pyrex, England
XII.	Disposable gloves	Uni gloves, Nigeria
XIII.	Electric blender	Binatone Elects. Int'l Ltd, H.Kong
XIV.	Foil paper	Tower Group Ltd, Nigeria
XV.	Hot plate	Infiniti forever, INF-HP8070
XVI.	Magnetic stirrer (79-1)	JINOTECH Instruments, China
XVII.	Measuring cylinder	Pyrex, England

XVIII.	Micro pipette	Erba Lachemas Brno, Czech Republic.
XIX.	Oven (DHG 90 30A)	Hinotek, China
XX.	Pasteur pipette	Alpha Laboratories Ltd, U.K.
XXI.	Pipette	Pyrex, England
XXII.	Plastic containers (4L calibration)	OK plastics, Nigeria
XXIII.	Refrigerator (HR-137)	Haier Thermocool, Nigeria
XXIV.	Test tubes	Pyrex, England
XXV.	Test tube racks	
XXVI.	Separating funnel	Pyrex, England
XXVII.	Spectrophotometer (20D S23A)	Techmel and Techmel, U.S.A
XXVIII.	Vortex XH-B	Hinotech, China
XXIX.	Whatman filter paper	Whatman Int'l Ltd, England

2.1.3. Chemicals and Reagents

i.	Absolute Ethanol	G.G. Chemical Factory Ltd. Guangdua, China.
ii.	Ammonium molybdate tetrahydrate.	Otto Chemie Pvt, Ltd. Mumbai.
iii.	Ascorbic acid	Brenntag Chemicals Ltd, Nigeria.
iv.	Carbonated drink (7up)	Seven up bottling company (SBC), Nigeria.
v.	Distilled water	
vi.	EDTA- Ethylene diamine tetra acetic acid	Turraco Industrial Ltd, Nigeria.
vii.	Ferric chloride	Haihang Industry, Co. Ltd, China.
viii.	Ferrous sulphate	Haihang Industry Co. Ltd, China.

ix. Hydrogen chloride — Brenntag Chemicals Ltd, Nigeria.

x. Hydrogen peroxide — Brenntag Chemicals Ltd, Nigeria.

xi. Methanol — British Drug House Chemicals Ltd, Poole England.

xii. Sodium carbonate — Danto Chemicals Ltd, Nigeria.

xiii. Sodium hydrogen carbonate — Brenntag Chemicals Ltd, Nigeria.

xiv. Sodium hydroxide — Brenntag Chemicals Ltd, Nigeria.

xv. Sulphuric acid — British Drug House Chemicals Ltd. Poole England.

xvi. 2,4,6-Tris(2 pyridyl)-s-triazine (TPTZ)

2.2. Methods

2.2.1. Sample Preparation

After being cleared of dust and cut into pieces, fresh *Andrographis paniculata* leaves were allowed to air dry at room temperature in a shaded area. After that, the dehydrated plant material was ground up with an electric blender and put into plastic storage containers.

2.2.2. Extraction

A precise 200g portion of the ground sample (plant materials) was weighed and macerated for 72 hours with 1.5L of carbonated drink (7 up) while being continuously stirred. Muslin cloth was used to filter the resultant extracts before being placed in sanitized plastic containers. Following a 72-hour stirring period, the previously utilized ground sample was again mixed in 1L (1000mL) of carbonated drink (7 up), filtered, and combined with the previously obtained extract. The filtrates were then concentrated using the decantation method, after which the necessary extract was left behind with the majority of the liquid removed and the mixture, dried further in an oven.

After that, the extracts were kept in a desiccator until they were required to carry out the necessary experiments.

2.2.3. Invitro Antioxidant Assays

2.2.3.1. DPPH (2,2-Diphenyl-1-picrylhydrazyl) Radical Scavenging Assay

Principle: A well-liked, quick, simple, and reasonably priced method for measuring antioxidant qualities is 2,2-Diphenyl-1-picrylhydrazyl (DPPH), which uses free radicals to determine whether a substance has the ability to act as a hydrogen provider or a free radical scavenger (FRS) (Baliyan *et al.,* 2022). Its method for scavenging free radicals provides the first strategy to assess an extract's, compound's, or any biological source's antioxidant capability. The simplest approach involves mixing the potential chemical or extract with DPPH solution, and then measuring the absorbance after a predetermined amount of time (Kedare and Singh, 2011). Blois (1958) developed this method with the goal of measuring the antioxidant activity in a similar way using a stable free radical called α, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M = 394.33). (Kedare and Singh, 2011). The DPPH assay is predicated on antioxidants' capacity to scavenge radicals from the purple-colored DPPH in MeOH. After reacting with a hydrogen donor, the free radical DPPH was reduced to the corresponding stable diamagnetic molecule, hydrazine (yellow in color). The level of discolouration reveals the antioxidant chemicals' or extracts' capacity to scavenge hydrogen by donating energy.

Procedure: The DPPH radical scavenging assay, as reported by Jha *et al.,* (2018), was used to examine the carbonated drink extract's capacity to scavenge free radicals. In summary, a 0.1 mM DPPH solution in methanol was generated, and 2.4 mL of this solution was combined with 1.6 mL of extract in methanol to provide a total volume of 3 mL at varying concentrations (15 to 960

μg/mL) in each test tube. After giving the reaction mixture a vigorous vortex, it was either left in the dark or incubated for 30 minutes at room temperature (27°C ±2) with full foil masking. The ability of the sample to donate hydrogen atoms was assessed by measuring how much 2,2-diphenyl-1-picrylhydrazyl (DPPH) in the methanol solution changed in color. When antioxidants are present, the violet/purple color that DPPH creates in methanol solution fades to yellow hues, signaling a successful outcome and a drop in absorbance measurements. The absorbance of the combination was measured at 517 nm using a spectrophotometer.Ascorbic acid was utilized as the reference or positive control, while tubes containing reagents but no sample were used as the blank (negative control). Using the following formula, the percentage DPPH radical scavenging activity was determined:

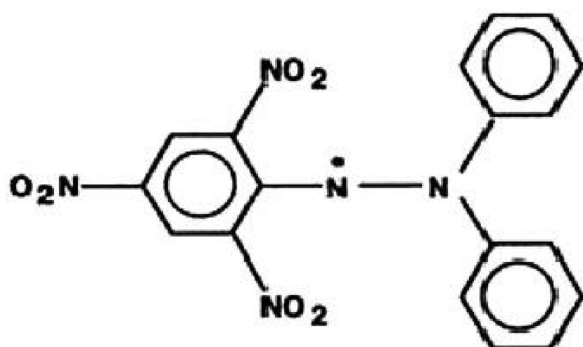
%DPPH radical scavenging activity (%RSA) = $\{(A_0-A_1)/A_0\}\times100$

Where

A_0= the absorbance of the control

A_1= the absorbance of the extractives/standard.

Then % of inhibition was plotted against concentration and from the graph, IC_{50} was calculated. All experiments were done in triplicates for each concentration.



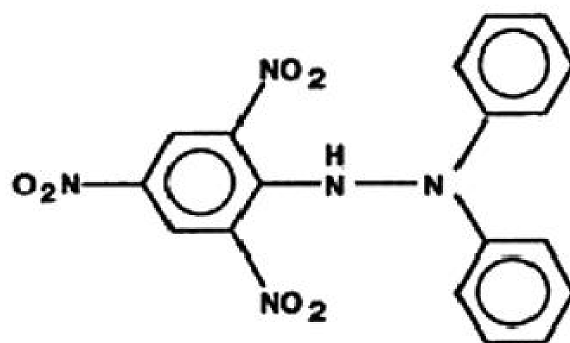


Fig 2.1: DPPH radical and its stable form

Source: Kadare and Singh, (2011).

2.2.3.2. FRAP (Ferrous reducing antioxidant power) Assay

Principle: The ferrous reducing antioxidant capacity assay's basic idea is that antioxidants in the sample quickly reduce ferric-tripyridyltriazine (Fe^{m}-TPTZ), resulting in the formation of ferrous-

tripyridyltriazine (Fe^{m}-TPTZ), a blue-colored product. A modified version of Benzie and Strain's (1996) procedure was used to perform the Ferric Reducing Antioxidant Power (FRAP) assay.

Procedure: Exactly 1 mL of the extract at concentrations of 100–600µM was added to 1.5 ml of freshly made FRAP solution [25 ml of 300 mM acetate buffer pH 3.6, 2.5 ml of 10 mM 2,4,6–tripyridyls–triazine (TPTZ) in 40 mM HCl, and 2.5 mL of 20 mM ferric chloride ($FeCl_3.6H_2O$) solution]. After 30 minutes of incubation at 37°C, the reaction mixtures' increased absorbance at 593 nm was measured. Ascorbic acid functioned as the positive control and $FeSO_4$ as the calibration curve. Then, FRAP values for the extracts were extrapolated from the standard curve.

2.2.3.3. Hydrogen Peroxide (H_2O_2) Scavenging Activity Assay

Principle: A comprehensive kit for measuring hydrogen peroxide is the colorimetric H_2O_2 assay. Utilizing an acidic solution of sorbitol, ammonium ion sulfate, and Tylenol orange dye, it employs a color reagent that reacts to give a purple hue proportional to the amount of H_2O_2 present in the sample. The precise mechanism is unknown, although it most likely entails coordinated reactions between the dye molecule and H_2O_2.

Procedure: The extract's ability to scavenge hydrogen peroxide radicals was assessed using the Laughton *et al*., (1989) technique. In short, tubes holding different concentrations (25 to 1600µg/mL) of sample extracts or standard were filled to 4mL with phosphate buffer solution (50mmol L-1, pH 7.4) and then 0.6mL of H_2O_2 solution was added. The identical process was carried out again, but this time without sample extracts—ascorbic acid was used as a blank or control. After 10 to 30 minutes of incubation at 27°C(±2), the mixtures' absorbance was measured at 230nm in comparison to the blank, which consisted of H_2O_2 and phosphate buffer.

As the concentration of the standard antioxidant increased, the intensity of the hydrogen peroxide color diminished.

Using the formula, the proportion of radical scavenging activity was determined;

scavenging activity (%) = $\{(A_0 - A_1)/A_0\}\times100$

Where

A_0 is the absorbance of the control

A_1 is the absorbance of the extractives .

2.2.3.4. Determination of Total Antioxidant Capacity

Principle: This test relies on samples reducing Mo(VI) to Mo(V) and the creation of green Mo(V) at acidic pH levels.

Procedure: The method outlined by Prieto *et al.,* (1999) was used to assess the samples' total antioxidant capacity (TAC). During the procedure, 3mL of a reaction mixture containing 0.6M sulfuric acid, 28mM sodium phosphate, and 1% ammonium molybdate was combined with 0.5mL of sample at various concentrations (25 to 1600µg/mL) and put into test tubes. To finish the reaction, the test tubes were incubated at 95°C for 30 minutes. Using a spectrophotometer, the sample absorbance was measured at 695 nm against a blank solution after cooling to room temperature (27°C ±2). The standard was ascorbic acid. Precisely 3mL of the reaction mixture and the proper volume of the same solvent used for the standard were the usual components of a blank solution. The absorbance at 695 nm was also measured when the blank was incubated for 30 minutes at 95°C. A rise in the reaction mixture's absorbance signifies an increase in its overall antioxidant capacity.

The total antioxidant activity may also be expressed as the number of gram equivalent of ascorbic acid. % scavenging activity and IC50 may also be calculated.

2.3. Statistical Analysis

The results were presented as mean ± S.E.M and were analyzed statistically using one way Analysis of Variance test (ANOVA) followed by tukey test using SPSS.

CHAPTER THREE

RESULTS

3.1. Invitro Antioxidant Scavenging Potential of Carbonated Drink Extract of *Andrographis paniculata*

3.1.1. DPPH Scavenging Activity of Carbonated Drink Extract of *A. paniculata*

Table 3.1 shows the result of the invitro DPPH radical scavenging power of carbonated drink extract of *A. paniculata* and that of the standard, ascorbic acid.

Table 3.1: Invitro scavenging activity of DPPH of carbonated drink extract of A. paniculata and ascorbic acid.

Concentration (mg/mL)	DPPH Percentage Inhibition	
	Ascorbic acid	**Carbonated drink extract**
0.2	95.67±0.20[a]	21.37±2.00[b]
0.4	95.90±0.20[a]	20.93±1.00[b]
0.6	94.91±0.30[a]	23.77±4.00[b]
0.8	95.75±0.10[a]	17.70±2.00[b]
1.0	97.37±0.20[a]	20.32±4.00[b]
IC_{50}	**0.71[a]**	**10.17[b]**

Values are expressed as mean±SEM. Values with different alphabets in the same row are significantly different (*P*<0.05).

Table 3.1 evidently shows that with decreasing concentration, the carbonated drink extract of this herbal plant enhanced the Inhibitory activity of DPPH radicals. However, the extract's percentage of DPPH radical inhibition was significantly less than that of ascorbic acid standard. The IC_{50} value for the standard is significantly lower than that of the sample. The high percentage inhibition of the standard as well as it's low IC_{50} value suggest that it has a better DPPH scavenging ability than the sample (carbonated drink extract of *A. paniculata*).

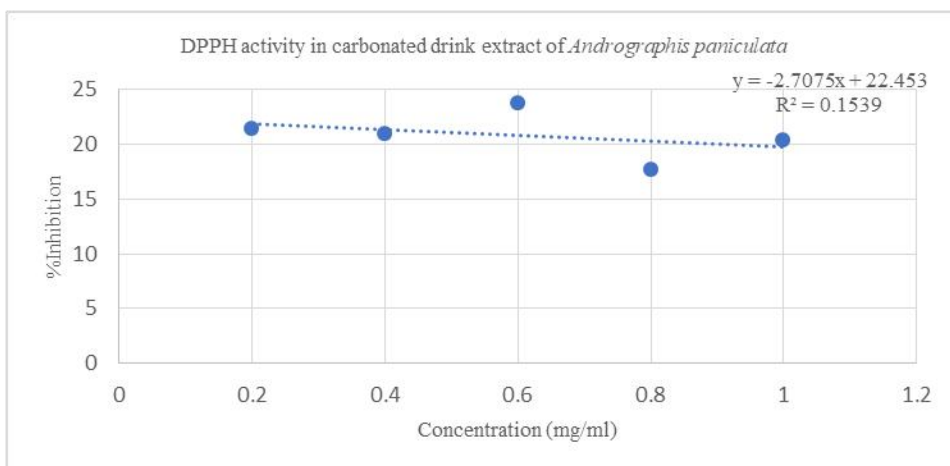


Fig 3.1: Inhibitory activity of DPPH of carbonated drink extract of *A. paniculata*

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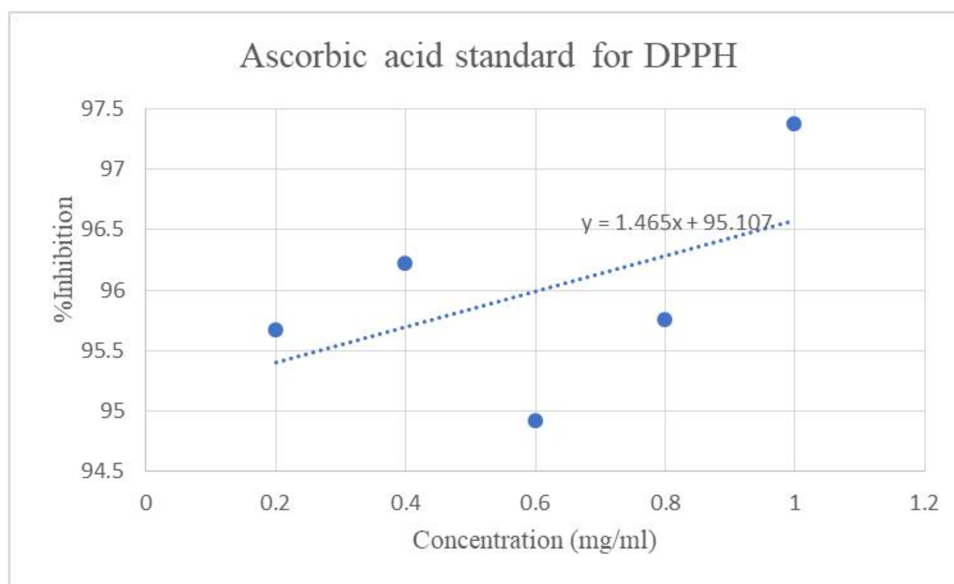

Fig 3.2: Ascorbic acid inhibitory activity of DPPH

3.1.2. FRAP Scavenging Activity of Carbonated Drink Extract of A. paniculata

Table 3.2 and Table 3.3 show the result of the invitro FRAP scavenging power of the carbonated drink extract of *A. paniculata* and that of the standard, ascorbic acid.

Table 3.2: Invitro scavenging activity of FRAP by carbonated drink extract of *A. paniculata* (sample).

Concentration (mg/mL)	**FRAP Percentage Inhibition**
1.008	25.79±0.40[a]
1.036	23.75±2.00[a]
1.049	22.79±1.00[a]
1.034	23.90±1.00[a]
1.043	23.19±1.00[a]
IC_{50}	**0.678[a]**

Table 3.3: Control (Ascorbic acid) values for FRAP scavenging activity.

Concentration (mg/mL)	FRAP Percentage Inhibition
1.004	38.95±1.00[b]
0.997	39.40±0.10[b]
1.021	37.87±1.30[b]
1.006	38.80±0.40[b]
0.995	39.51±1.00[b]
IC_{50}	**0.825[a]**

Values are expressed as mean±SEM. Comparing the two tables, values with different alphabets are significantly different ($P<0.05$).

Tables 3.2 and 3.3 show quite evidently that at varying extrapolated concentrations, the percentage inhibition values for FRAP scavenging activity of carbonated drink extract of *A. paniculata* are lower compared to that of the standard/control (ascorbic acid). Same applies for the IC_{50} values. Judging from this, it shows that the sample is more potent in scavenging free radicals at lower concentrations than the standard, even if the standard shows a higher percentage inhibition in the assay. This is indicated by the sample's IC_{50} value, indicating that the sample is more effective in scavenging free radicals even though its total inhibition percentage is lower and less of the chemical is needed to provide a noticeable inhibitory effect.

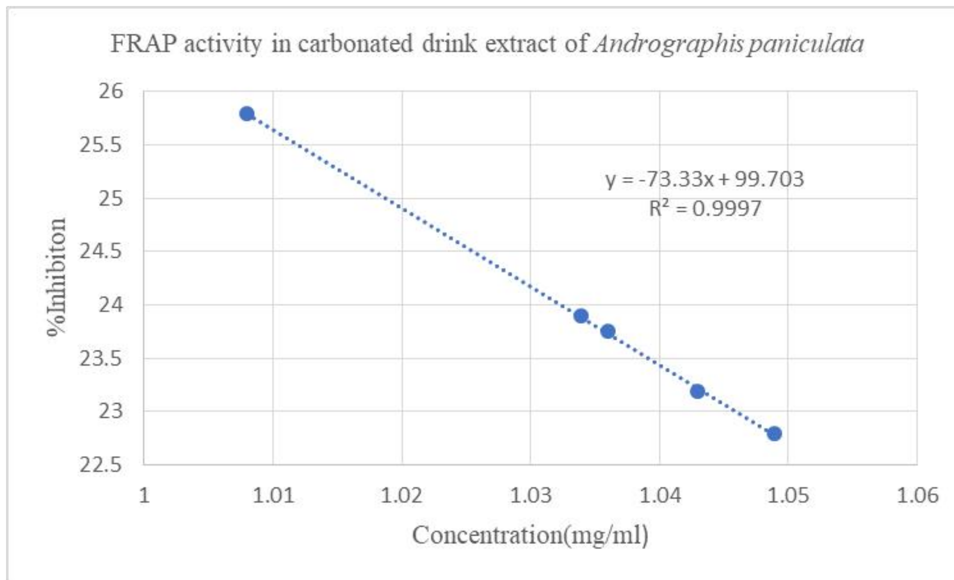

Fig 3.3: Inhibitory activity of FRAP of carbonated drink extract of *A. paniculata*

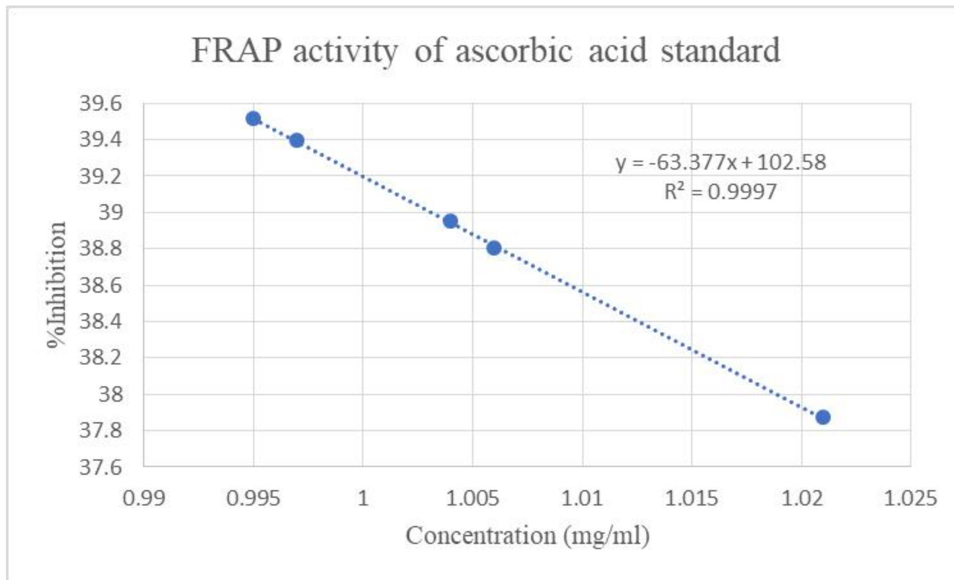

Fig 3.4: Ascorbic acid inhibitory activity of FRAP

3.1.3. Total Antioxidant Activity of Carbonated Drink Extract of *A. paniculata*

Table 3.4 shows the resulting values of the invitro TAC test of the carbonated drink extract of *A. paniculata* and that of the standard, ascorbic acid.

Table 3.4: Invitro TAC assay of carbonated drink extract of *A. paniculata* and ascorbic acid

Concentration (mg/mL)	**TAC Percentage Inhibition**	
	Ascorbic acid	**Carbonated drink extract**
0.2	91.91±0.10[a]	35.96±2.00[b]
0.4	91.88±0.10[a]	40.09±3.00[b]
0.6	91.81±0.10[a]	71.98±5.00[b]
0.8	91.90±0.10[a]	76.66±6.00[b]
1.0	92.03±0.10[a]	74.76±3.00[b]
IC_{50}	**0.65[a]**	**0.41[a]**

Values are expressed as mean ± SEM. Values with different alphabets in the same row are significantly different ($P<0.05$).

Judging from the above values in Table 3.4, the standard, ascorbic acid has a much higher percentage inhibition than the sample (carbonated drink extract of *A. paniculata*) at varying concentrations. Same applies to the IC_{50}. The high percentage inhibition

values show that ascorbic acid has very good antioxidant activity. Also, the low IC50 value of the sample also suggests that the carbonated drink extract of A. paniculata has good antioxidant abilities as well.

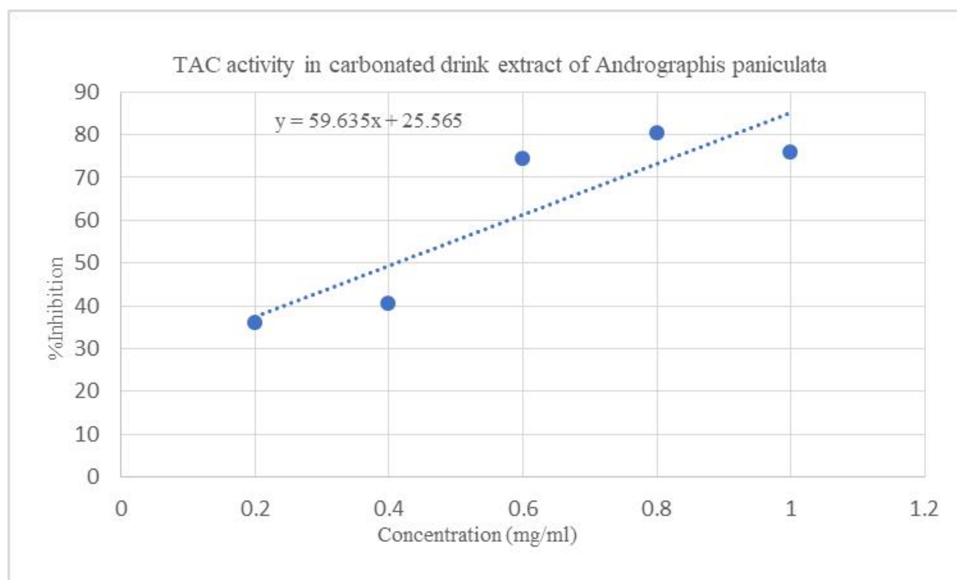

Fig 3.5: TAC activity of carbonated drink extract of *A. paniculata*

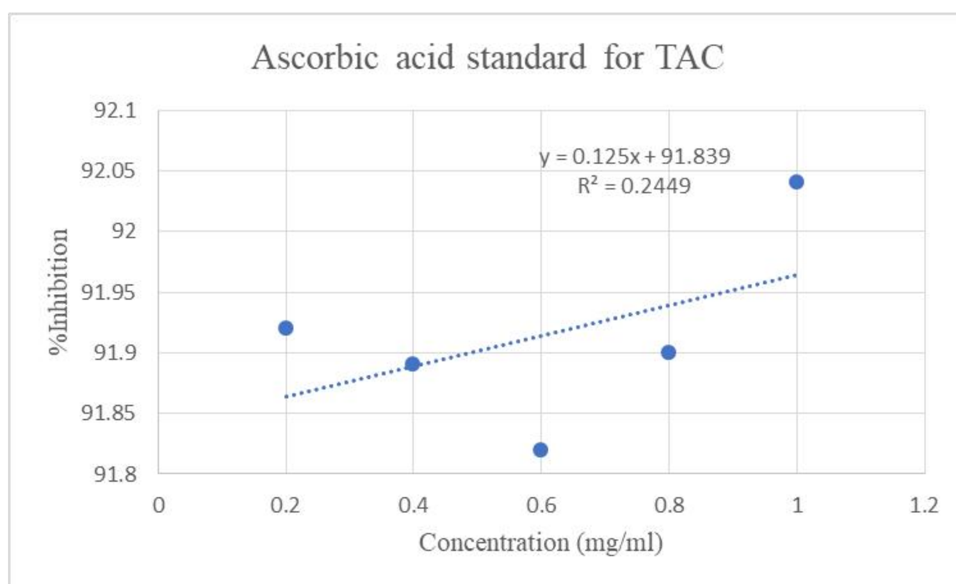

Fig 3.6: Ascorbic acid TAC activity

3.1.4. Hydrogen Peroxide Scavenging Activity of Carbonated Drink Extract of *A. paniculata*

Table 3.5 shows the results of the invitro analysis for the hydrogen peroxide scavenging potential of carbonated drink extract of *A. paniculata.*

Table 3.5: Invitro H_2O_2 assay of carbonated drink extract of *A. paniculata* (sample)

Concentration (100mg/mL)	**H_2O_2 Percentage Inhibition**
20	44.07
40	47.46
60	49.15
IC_{50} (mg/mL)	**2.95**

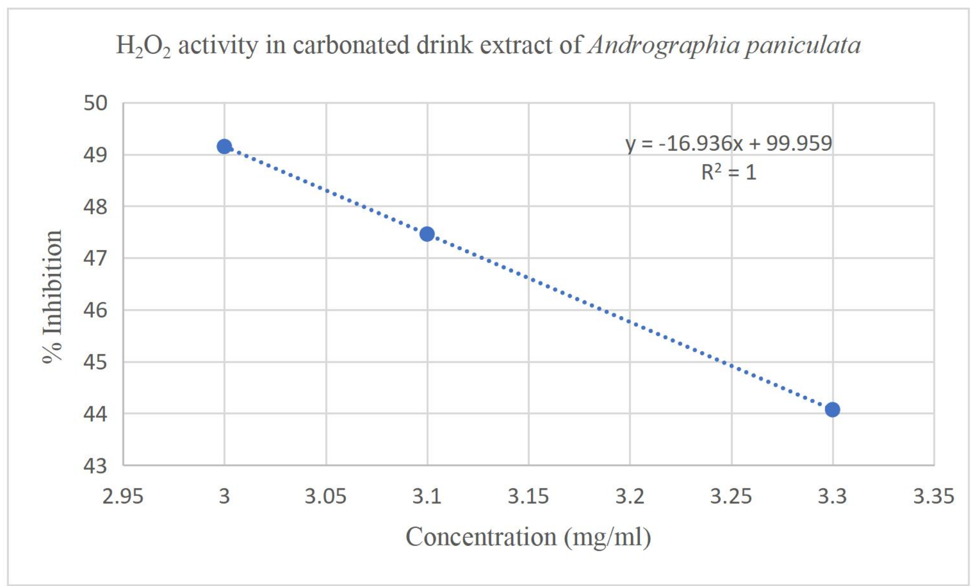

Fig 3.7; Hydrogen peroxide activity of carbonated drink extract of *A. paniculata*

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1. Discussion

The most widely used antioxidant assay for plant extract is the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay (Nagarajan *et al.,* 2017). The DPPH assay evaluates antioxidants' capacity to scavenge the stable free radical 2.2-diphynyl 1-picrylhydrazyl. With lowering concentrations, the carbonated drink extract of the herbal plant used in this research, encouraged the inhibition/neutralization of DPPH radicals. The extract's percentage of DPPH radical inhibition was significantly lesser than that of ascorbic acid, the standard. This suggests that ascorbic acid has a very high inhibitory capacity and that vitamin C is an incredibly potent antioxidant compound. As a result, the extract appears to have a lower inhibitory capacity when compared to ascorbic acid. This however doesn't imply that the extract does not possess antioxidant capacities, only that when compared to the standard, it has a lower activity.

The term "IC_{50}" refers to the concentration of an antioxidant-containing material required to scavenge half of the initial DPPH radicals. A substance's ability to scavenge DPPH is directly correlated with its IC_{50} value; a lower value indicates stronger antioxidant activity. The standard and extract from this research have IC_{50} values of 0.71mg/mL and 10.17mg/mL, respectively. This demonstrates that the extract can be a potent free radical scavenger and is a strong source of antioxidants.

The percentage inhibition of the standard is higher than that of the sample, the IC_{50} value is lower than the sample. This further supports the conclusion that the standard has better

antioxidant activity. Therefore, based on both the IC_{50} values and the percentage inhibition, the standard exhibits higher or better antioxidant activity compared to the sample extract.

The antioxidant's ability to donate electrons and its reducing activity are assessed using the ferric reducing power assay. Ascorbic acid decreased Fe^{3+}-TPTZ more strongly than the extract did, resulting in Fe^{2+}-TPTZ. It's stronger reduction suggests higher antioxidant capacity compared to the extract, implying that it has greater potential to neutralize free radicals and oxidative stress. Judging from the IC_{50} values however, the ascorbic acid (0.825) has a higher value than the sample (0.678). This suggests that the extract is more effective at inhibiting the oxidative process or scavenging free radicals at lower concentrations, thus indicating that the extract may contain compounds with potent antioxidant properties, even though its reducing power in the FRAP assay might be lower than that of ascorbic acid. This therefore shows that *A. paniculata* carbonated drink extract is a good source of antioxidants and has the ability to react with free radicals and act as an electron donor.

The total antioxidant capacity assay displays the antioxidant content in a certain extract volume. It measures the overall antioxidant capacity of a sample. The percentage values indicate the antioxidant activity relative to a standard or control, offering insight into the sample's ability to neutralize free radicals. The results of this study showed that the extract had a lower percentage of inhibition when compared to ascorbic acid (standard), infering that the standard has a much higher antioxidant capacity compared to the sample. On the other hand, the sample has a lower IC_{50} value (0.41) than that of the standard (6.65). This suggests that the extract contains compounds with potent antioxidant properties than the standard. None the less, ascorbic acid shows good antioxidant activity.

For the hydrogen peroxide scavenging assay, the essence of carrying out the assay is to quantify the concentration of hydrogen peroxide in a sample which can be important for various applications, such as in medical diagnostics, environmental analysis, or industrial processes. In this study, the invitro analysis showed less than 50% percentage inhibition in a hydrogen peroxide assay carried out for the carbonated drink extract. This implies that this particular extract has a low ability to inhibit the production or activity of hydrogen peroxide, indicating lower antioxidant capacity in it. The IC_{50} as well was somewhat on a high side, 2.95 meaning that this concentration of the extract is needed to inhibit hydrogen peroxide activity at 50%.

In general, with the exception of DPPH, the IC_{50} values for the extract (FRAP and TAC) were lower than that of the standard. This suggests that the sample has a higher antioxidant effectiveness compared to the standard. The sample therefore contains potent antioxidant compounds.

4.2. Conclusion

Andrographis paniculata carbonated drink extract exhibited antioxidative properties based on both the percentage inhibition values obtained from the in vitro test and the IC_{50} values. Thus, this work demonstrates the antioxidative properties of *A. paniculata's* carbonated drink extract, which includes ability to scavenge superoxide radicals, efficiently reduce ferric iron to ferrous state, and neutralize free radicals. Although this study did not test *A. paniculata's* efficacy against specific disorders, a number of other investigations (discussed in chapter 1) demonstrate that it is a useful therapeutic agent. I propose that additional research be done on this plant in order to determine its many other functions in addition to its antioxidant capacity.

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APPENDIX

Appendix I: Results from invitro antioxidant assay (DPPH) for carbonated drink extract of *A. paniculata* .

Sample ID	DPPH						
	Abs1	Abs2	Abs3	Average absorbance	AO	%	% DPPH
Ca1	0.682	0.735	0.745	0.7207	0.917	100	21.4104
Ca2	0.709	0.744	0.722	0.7250	0.917	100	20.9378
Ca3	0.731	0.734	0.633	0.6990	0.917	100	23.7732
Ca4	0.718	0.805	0.741	0.7547	0.917	100	17.7027
Ca5	0.709	0.745	0.738	0.7307	0.917	100	20.3199

Appendix II: Results from invitro antioxidant assay (FRAP) for carbonated drink extract of *A. paniculata.*

Sample ID	FRAP						
	Abs1	Abs2	Abs3	Average absorbance	AO	%	% FRAP
Ca1	1.063	1.048	1.064	1.0583	1.426	100	25.7831
Ca2	1.093	1.123	1.046	1.0873	1.426	100	23.7494
Ca3	1.084	1.101	1.118	1.1010	1.426	100	22.7910
Ca4	1.100	1.078	1.078	1.0853	1.426	100	23.8897
Ca5	1.112	1.084	1.090	1.0953	1.426	100	23.1884

Appendix III: Results from the invitro antioxidant assay (TAC) for the carbonated drink extract of *A. paniculata.*

Sample ID	**TAC**						
	Abs1	**Abs2**	**Abs3**	**Average absorbance**	**AO**	**%**	**% TAC**
Ca1	0.161	0.157	0.175	0.1643	0.105	100	36.105
Ca2	0.165	0.171	0.192	0.1760	0.105	100	40.341
Ca3	0.361	0.286	0.575	0.4073	0.105	100	74.223
Ca4	0.365	0.359	0.874	0.5327	0.105	100	80.288
Ca5	0.346	0.395	0.559	0.4333	0.105	100	75.769

Appendix IV: Results from the invitro antioxidant assay (Hydrogen peroxide) for carbonated drink extract of *A. paniculata.*

Sample ID	**H_2O_2**					
	A1	**A2**	**A2 - A1**	**AO**	**%**	**% H_2O_2**
C1	15	18.3	3.3	5.9	100	44.0678
C2	4	7.1	3.1	5.9	100	47.4576
C3	34	37.0	3.0	5.9	100	49.1525

Appendix V: Table showing the standard values for FRAP used for the extrapolation.

FRAP standard							
Concentration (mg/mL)	**Abs1**	**Abs2**	**Abs3**	**Average absorbance**	**AO**	**%**	**% inhibition**
50	0.266	0.188	0.263	0.2390	1.726	100	86.1530
100	0.465	0.449	0.426	0.4467	1.726	100	74.1213
200	0.466	0.468	0.458	0.4640	1.726	100	73.1170
300	0.464	0.461	0.454	0.4597	1.726	100	73.3681
400	0.496	0.441	0.446	0.4610	1.726	100	73.2908
500	0.449	0.441	0.429	0.4397	1.726	100	74.5268
600	0.437	0.445	0.447	0.4430	1.726	100	74.3337

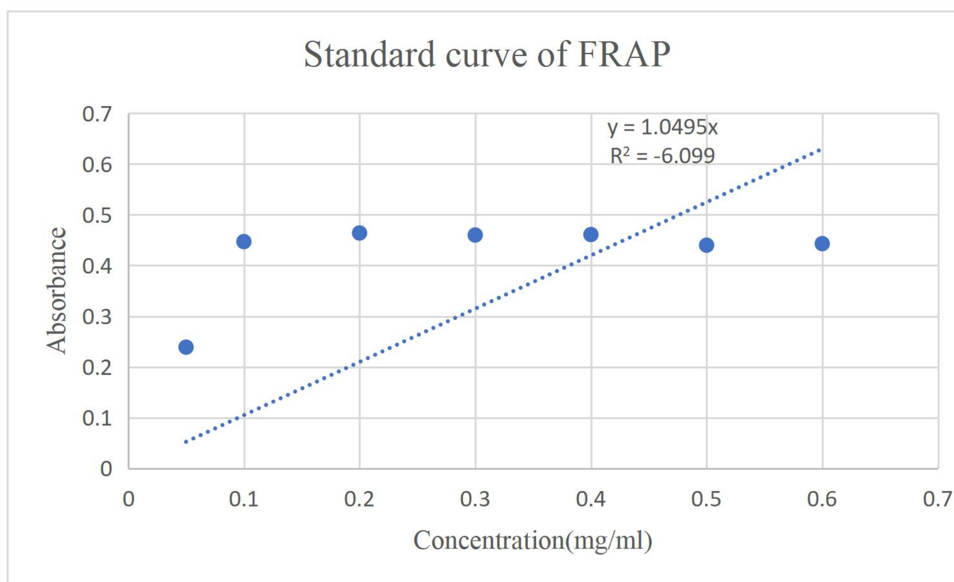

Figure 7: Plot of standard curve of FRAP scavenging activity of carbonated drink extract of *A. paniculata.*

Appendix VI: Values from one way ANOVA test and tukey test for DPPH and TAC

Output Created		22-APR-2024 17:36:52
Comments		
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	Split File	<none>
	N of Rows in Working Data File	15
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics for each analysis are based on cases with no missing data for any variable in the analysis.
Syntax		ONEWAY CAR ASCA TACASCA TACCAR BY GROUPS /STATISTICS DESCRIPTIVES /MISSING ANALYSIS /CRITERIA=CILEVEL(0.95) /POSTHOC=TUKEY ALPHA(0.05).
Resources	Processor Time	00:00:00.03
	Elapsed Time	00:00:00.05

Descriptives						
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean
						Lower Bound
CAR	FIRST	3	21.3767	3.62938	2.09543	12.3608
	SECOND	3	20.9367	1.92547	1.11167	16.1535
	THIRD	3	23.7733	6.23338	3.59884	8.2888
	FOURTH	3	17.7000	4.91733	2.83902	5.4847
	FIFTH	3	20.3200	2.07885	1.20022	15.1559
	Total	15	20.8213	4.01393	1.03639	18.5985
ASCA	FIRST	3	95.6700	.26153	.15100	95.0203
	SECOND	3	95.8900	.35679	.20599	95.0037
	THIRD	3	94.9167	.44523	.25706	93.8106
	FOURTH	3	95.7500	.21071	.12166	95.2266
	FIFTH	3	97.3733	.41669	.24058	96.3382
	Total	15	95.9200	.88064	.22738	95.4323
TACASCA	FIRST	3	91.9133	.10693	.06173	91.6477
	SECOND	3	91.8867	.15503	.08950	91.5016
	THIRD	3	91.8167	.17243	.09955	91.3883
	FOURTH	3	91.9033	.12897	.07446	91.5830
	FIFTH	3	92.0367	.22591	.13043	91.4755
	Total	15	91.9113	.15624	.04034	91.8248
TACCAR	FIRST	3	35.9667	3.59023	2.07282	27.0480
	SECOND	3	40.0900	4.65733	2.68891	28.5206
	THIRD	3	71.9833	9.26674	5.35015	48.9635
	FOURTH	3	76.6600	9.82366	5.67169	52.2567
	FIFTH	3	74.7633	5.90082	3.40684	60.1049
	Total	15	59.8927	19.53521	5.04397	49.0744

Descriptives				
		95% Confidence Interval for Mean		
		Upper Bound	Minimum	Maximum
CAR	FIRST	30.3926	18.76	25.52
	SECOND	25.7198	18.87	22.68
	THIRD	39.2579	20.07	30.97
	FOURTH	29.9153	12.21	21.70
	FIFTH	25.4841	18.76	22.68
	Total	23.0442	12.21	30.97
ASCA	FIRST	96.3197	95.37	95.85
	SECOND	96.7763	95.50	96.20
	THIRD	96.0227	94.48	95.37
	FOURTH	96.2734	95.55	95.97
	FIFTH	98.4085	96.98	97.81
	Total	96.4077	94.48	97.81
TACASCA	FIRST	92.1790	91.82	92.03
	SECOND	92.2718	91.71	92.00
	THIRD	92.2450	91.63	91.97
	FOURTH	92.2237	91.76	92.01
	FIFTH	92.5978	91.80	92.25
	Total	91.9979	91.63	92.25
TACCAR	FIRST	44.8853	33.12	40.00
	SECOND	51.6594	36.36	45.31
	THIRD	95.0032	63.30	81.74
	FOURTH	101.0633	70.75	88.00
	FIFTH	89.4218	69.65	81.22
	Total	70.7109	33.12	88.00

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
CAR	Between Groups	57.090	4	14.273	.847	.527
	Within Groups	168.473	10	16.847		
	Total	225.563	14			
ASCA	Between Groups	9.633	4	2.408	19.677	.000
	Within Groups	1.224	10	.122		
	Total	10.857	14			
TACASCA	Between Groups	.076	4	.019	.715	.600
	Within Groups	.266	10	.027		
	Total	.342	14			
TACCAR	Between Groups	4839.190	4	1209.798	24.025	.000
	Within Groups	503.554	10	50.355		
	Total	5342.744	14			

Post Hoc Tests

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
CAR	FIRST	SECOND	.44000	3.35135	1.000	-10.5896	11.4696
		THIRD	-2.39667	3.35135	.948	-13.4262	8.6329
		FOURTH	3.67667	3.35135	.804	-7.3529	14.7062
		FIFTH	1.05667	3.35135	.997	-9.9729	12.0862
	SECOND	FIRST	-.44000	3.35135	1.000	-11.4696	10.5896
		THIRD	-2.83667	3.35135	.910	-13.8662	8.1929
		FOURTH	3.23667	3.35135	.864	-7.7929	14.2662
		FIFTH	.61667	3.35135	1.000	-10.4129	11.6462
	THIRD	FIRST	2.39667	3.35135	.948	-8.6329	13.4262
		SECOND	2.83667	3.35135	.910	-8.1929	13.8662
		FOURTH	6.07333	3.35135	.418	-4.9562	17.1029
		FIFTH	3.45333	3.35135	.836	-7.5762	14.4829
	FOURTH	FIRST	-3.67667	3.35135	.804	-14.7062	7.3529
		SECOND	-3.23667	3.35135	.864	-14.2662	7.7929
		THIRD	-6.07333	3.35135	.418	-17.1029	4.9562
		FIFTH	-2.62000	3.35135	.930	-13.6496	8.4096
	FIFTH	FIRST	-1.05667	3.35135	.997	-12.0862	9.9729
		SECOND	-.61667	3.35135	1.000	-11.6462	10.4129
		THIRD	-3.45333	3.35135	.836	-14.4829	7.5762
		FOURTH	2.62000	3.35135	.930	-8.4096	13.6496
ASCA	FIRST	SECOND	-.22000	.28565	.934	-1.1601	.7201
		THIRD	.75333	.28565	.136	-.1868	1.6934

		FOURTH	-.08000	.28565	.998	-1.0201	.8601
		FIFTH	-1.70333*	.28565	.001	-2.6434	-.7632
	SECOND	FIRST	.22000	.28565	.934	-.7201	1.1601
		THIRD	.97333*	.28565	.042	.0332	1.9134
		FOURTH	.14000	.28565	.987	-.8001	1.0801
		FIFTH	-1.48333*	.28565	.003	-2.4234	-.5432
	THIRD	FIRST	-.75333	.28565	.136	-1.6934	.1868
		SECOND	-.97333*	.28565	.042	-1.9134	-.0332
		FOURTH	-.83333	.28565	.089	-1.7734	.1068
		FIFTH	-2.45667*	.28565	.000	-3.3968	-1.5166
	FOURTH	FIRST	.08000	.28565	.998	-.8601	1.0201
		SECOND	-.14000	.28565	.987	-1.0801	.8001
		THIRD	.83333	.28565	.089	-.1068	1.7734
		FIFTH	-1.62333*	.28565	.001	-2.5634	-.6832
	FIFTH	FIRST	1.70333*	.28565	.001	.7632	2.6434
		SECOND	1.48333*	.28565	.003	.5432	2.4234
		THIRD	2.45667*	.28565	.000	1.5166	3.3968
		FOURTH	1.62333*	.28565	.001	.6832	2.5634
TACASCA	FIRST	SECOND	.02667	.13310	1.000	-.4114	.4647
		THIRD	.09667	.13310	.945	-.3414	.5347
		FOURTH	.01000	.13310	1.000	-.4280	.4480
		FIFTH	-.12333	.13310	.880	-.5614	.3147
	SECOND	FIRST	-.02667	.13310	1.000	-.4647	.4114
		THIRD	.07000	.13310	.983	-.3680	.5080
		FOURTH	-.01667	.13310	1.000	-.4547	.4214
		FIFTH	-.15000	.13310	.790	-.5880	.2880
	THIRD	FIRST	-.09667	.13310	.945	-.5347	.3414
		SECOND	-.07000	.13310	.983	-.5080	.3680
		FOURTH	-.08667	.13310	.963	-.5247	.3514
		FIFTH	-.22000	.13310	.500	-.6580	.2180

	FOURTH	FIRST	-.01000	.13310	1.000	-.4480	.4280
		SECOND	.01667	.13310	1.000	-.4214	.4547
		THIRD	.08667	.13310	.963	-.3514	.5247
		FIFTH	-.13333	.13310	.849	-.5714	.3047
	FIFTH	FIRST	.12333	.13310	.880	-.3147	.5614
		SECOND	.15000	.13310	.790	-.2880	.5880
		THIRD	.22000	.13310	.500	-.2180	.6580
		FOURTH	.13333	.13310	.849	-.3047	.5714
TACCAR	FIRST	SECOND	-4.12333	5.79398	.949	-23.1918	14.9451
		THIRD	-36.01667*	5.79398	.001	-55.0851	-16.9482
		FOURTH	-40.69333*	5.79398	.000	-59.7618	-21.6249
		FIFTH	-38.79667*	5.79398	.000	-57.8651	-19.7282
	SECOND	FIRST	4.12333	5.79398	.949	-14.9451	23.1918
		THIRD	-31.89333*	5.79398	.002	-50.9618	-12.8249
		FOURTH	-36.57000*	5.79398	.001	-55.6385	-17.5015
		FIFTH	-34.67333*	5.79398	.001	-53.7418	-15.6049
	THIRD	FIRST	36.01667*	5.79398	.001	16.9482	55.0851
		SECOND	31.89333*	5.79398	.002	12.8249	50.9618
		FOURTH	-4.67667	5.79398	.923	-23.7451	14.3918
		FIFTH	-2.78000	5.79398	.988	-21.8485	16.2885
	FOURTH	FIRST	40.69333*	5.79398	.000	21.6249	59.7618
		SECOND	36.57000*	5.79398	.001	17.5015	55.6385
		THIRD	4.67667	5.79398	.923	-14.3918	23.7451
		FIFTH	1.89667	5.79398	.997	-17.1718	20.9651
	FIFTH	FIRST	38.79667*	5.79398	.000	19.7282	57.8651
		SECOND	34.67333*	5.79398	.001	15.6049	53.7418
		THIRD	2.78000	5.79398	.988	-16.2885	21.8485
		FOURTH	-1.89667	5.79398	.997	-20.9651	17.1718

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

Homogeneous Subsets

CAR		
Tukey HSD[a]		
		Subset for alpha = 0.05
GROUPS	N	1
FOURTH	3	17.7000
FIFTH	3	20.3200
SECOND	3	20.9367
FIRST	3	21.3767
THIRD	3	23.7733
Sig.		.418

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ASCA				
Tukey HSD[a]				
		Subset for alpha = 0.05		
GROUPS	N	1	2	3
THIRD	3	94.9167		
FIRST	3	95.6700	95.6700	
FOURTH	3	95.7500	95.7500	
SECOND	3		95.8900	
FIFTH	3			97.3733

Sig.		.089	.934	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

TACASCA		
Tukey HSD[a]		
		Subset for alpha = 0.05
GROUPS	N	1
THIRD	3	91.8167
SECOND	3	91.8867
FOURTH	3	91.9033
FIRST	3	91.9133
FIFTH	3	92.0367
Sig.		.500

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

TACCAR			
Tukey HSD[a]			
		Subset for alpha = 0.05	
GROUPS	N	1	2
FIRST	3	35.9667	
SECOND	3	40.0900	

THIRD	3		71.9833
FIFTH	3		74.7633
FOURTH	3		76.6600
Sig.		.949	.923

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Appendix VII: Values from one way ANOVA test and tukey test for FRAP

```
STATISTICS DESCRIPTIVES
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  /CRITERIA=CILEVEL(0.95)
  /POSTHOC=TUKEY ALPHA(0.05).
```

Oneway

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	Cases Used	Statistics for each analysis are based on cases with no missing data for any variable in the analysis.
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	Elapsed Time	00:00:00.02

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
						Lower Bound	Upper Bound
ASCA	FIRST	3	38.9533	1.42339	.82179	35.4174	42.4892
	SECOND	3	39.3967	.21079	.12170	38.8730	39.9203
	THIRD	3	37.8700	2.17676	1.25676	32.4626	43.2774
	FOURTH	3	38.8000	.75664	.43684	36.9204	40.6796
	FIFTH	3	39.5100	1.32895	.76727	36.2087	42.8113
	Total	15	38.9060	1.29206	.33361	38.1905	39.6215
FRAP	FIRST	3	25.7867	.62740	.36223	24.2281	27.3452
	SECOND	3	23.7500	2.72213	1.57162	16.9879	30.5121
	THIRD	3	22.7900	1.19000	.68705	19.8339	25.7461
	FOURTH	3	23.8867	.88912	.51333	21.6780	26.0954
	FIFTH	3	23.1867	1.03196	.59580	20.6231	25.7502
	Total	15	23.8800	1.64997	.42602	22.9663	24.7937

Descriptives			
		Minimum	Maximum
ASCA	FIRST	37.31	39.80
	SECOND	39.22	39.63
	THIRD	36.50	40.38
	FOURTH	37.95	39.40
	FIFTH	38.06	40.67
	Total	36.50	40.67
FRAP	FIRST	25.39	26.51
	SECOND	21.25	26.65
	THIRD	21.60	23.98
	FOURTH	22.86	24.40
	FIFTH	22.02	23.98
	Total	21.25	26.65

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
ASCA	Between Groups	5.077	4	1.269	.694	.613
	Within Groups	18.295	10	1.829		
	Total	23.372	14			
FRAP	Between Groups	15.963	4	3.991	1.802	.205
	Within Groups	22.150	10	2.215		
	Total	38.114	14			

Post Hoc Tests

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
ASCA	FIRST	SECOND	-.44333	1.10438	.994	-4.0779	3.1913
		THIRD	1.08333	1.10438	.858	-2.5513	4.7179
		FOURTH	.15333	1.10438	1.000	-3.4813	3.7879
		FIFTH	-.55667	1.10438	.985	-4.1913	3.0779
	SECOND	FIRST	.44333	1.10438	.994	-3.1913	4.0779
		THIRD	1.52667	1.10438	.651	-2.1079	5.1613
		FOURTH	.59667	1.10438	.981	-3.0379	4.2313
		FIFTH	-.11333	1.10438	1.000	-3.7479	3.5213
	THIRD	FIRST	-1.08333	1.10438	.858	-4.7179	2.5513
		SECOND	-1.52667	1.10438	.651	-5.1613	2.1079
		FOURTH	-.93000	1.10438	.911	-4.5646	2.7046
		FIFTH	-1.64000	1.10438	.593	-5.2746	1.9946
	FOURTH	FIRST	-.15333	1.10438	1.000	-3.7879	3.4813
		SECOND	-.59667	1.10438	.981	-4.2313	3.0379
		THIRD	.93000	1.10438	.911	-2.7046	4.5646
		FIFTH	-.71000	1.10438	.964	-4.3446	2.9246
	FIFTH	FIRST	.55667	1.10438	.985	-3.0779	4.1913
		SECOND	.11333	1.10438	1.000	-3.5213	3.7479
		THIRD	1.64000	1.10438	.593	-1.9946	5.2746
		FOURTH	.71000	1.10438	.964	-2.9246	4.3446
FRAP	FIRST	SECOND	2.03667	1.21519	.488	-1.9626	6.0360
		THIRD	2.99667	1.21519	.175	-1.0026	6.9960

		FOURTH	1.90000	1.21519	.549	-2.0993	5.8993
		FIFTH	2.60000	1.21519	.276	-1.3993	6.5993
	SECOND	FIRST	-2.03667	1.21519	.488	-6.0360	1.9626
		THIRD	.96000	1.21519	.928	-3.0393	4.9593
		FOURTH	-.13667	1.21519	1.000	-4.1360	3.8626
		FIFTH	.56333	1.21519	.989	-3.4360	4.5626
	THIRD	FIRST	-2.99667	1.21519	.175	-6.9960	1.0026
		SECOND	-.96000	1.21519	.928	-4.9593	3.0393
		FOURTH	-1.09667	1.21519	.890	-5.0960	2.9026
		FIFTH	-.39667	1.21519	.997	-4.3960	3.6026
	FOURTH	FIRST	-1.90000	1.21519	.549	-5.8993	2.0993
		SECOND	.13667	1.21519	1.000	-3.8626	4.1360
		THIRD	1.09667	1.21519	.890	-2.9026	5.0960
		FIFTH	.70000	1.21519	.976	-3.2993	4.6993
	FIFTH	FIRST	-2.60000	1.21519	.276	-6.5993	1.3993
		SECOND	-.56333	1.21519	.989	-4.5626	3.4360
		THIRD	.39667	1.21519	.997	-3.6026	4.3960
		FOURTH	-.70000	1.21519	.976	-4.6993	3.2993

Homogeneous Subsets

ASCA		
Tukey HSD[a]		
		Subset for alpha = 0.05
GROUPS	N	1

THIRD	3	37.8700
FOURTH	3	38.8000
FIRST	3	38.9533
SECOND	3	39.3967
FIFTH	3	39.5100
Sig.		.593

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

FRAP

Tukey HSD[a]

GROUPS	N	Subset for alpha = 0.05
		1
THIRD	3	22.7900
FIFTH	3	23.1867
SECOND	3	23.7500
FOURTH	3	23.8867
FIRST	3	25.7867
Sig.		.175

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.