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Antioxidant Potentials of *Citrus limon* Juice Extract - An Invitro Analysis.

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Abstract - *Citrus limon* is a medicinal fruit of the family *Rutaceae* which can function as direct antioxidants and free radical scavengers and have the capacity to modulate enzymatic activities and inhibit cell proliferation. Free radicals contribute to more than one hundred disorders in humans. The extract of *Citrus limon* was screened for antioxidant activity using 1,1-diphenyl-2-picryl hydroxyl (DPPH) assay, 2,2'-azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) cation decolorization test, scavenging capacity towards hydrogen peroxide (H_2O_2) radical, and Ferric Reducing Antioxidant Power (FRAP) assay. The extract exhibited high antiradical activity against DPPH, ABTS, and hydrogen peroxide. The Ferric Reducing Antioxidant Power (FRAP) increased with increasing concentration of the sample. The antioxidant activity of the sample was comparable with that of the standard ascorbic acid.

Keywords - *Citrus limon*, Oxidative Stress, Antioxidants, Vitamin C, ROS, DPPH, FRAP, ABTS.

1.0. Introduction

Fruits are an important source of various vitamins, minerals, and fibers for humans. However, they differ in many aspects, including the content of vitamins, minerals, and fibers as well as their antioxidant capacity. Fruits contain various antioxidants which include ascorbic acid, carotenoids, and phenolics (Qamar et al., 2021).

Some studies show that the antioxidants contained in certain fruits are bioavailable (Rafique et al., 2020). Therefore, these fruits can be considered as natural sources of antioxidants. It is true that a deliberate increase in the consumption of these fruits will increase the intake of natural antioxidants, which may provide an alternative to the intervention of the aging process by protecting against oxidative damage.

Different fruits may provide different protection against oxidative stress since they are different in their antioxidant capacity. It is hypothesized that fruits with high antioxidant capacity are more effective in reducing oxidative damage associated with the aging process than those with low antioxidant capacity (Qamar et al., 2021).

Fruits that are rich in antioxidants help in lowering the incidence of degenerative diseases such as arthritis, cancer, heart diseases, atherosclerosis, inflammation, brain dysfunction, and acceleration of the ageing process (Qamar et al., 2021).

Antioxidants are substances that, when present at low concentrations, are able to prevent or delay oxidative damage of lipids, proteins, and nucleic acids by reactive oxygen species. These reactive oxygen species are mainly reactive free radicals such as superoxide, hydroxyl, peroxyl, alkoxyl and non-radicals such as hydrogen peroxide, hypochlorous (Qamar et al., 2021). Antioxidants are also known as scavengers. They scavenge radicals by inhibiting initiation and breaking chain propagation or suppressing the formation of free radicals by binding to the metal ions, reducing hydrogen peroxide and quenching superoxide and singlet oxygen (Bensid et al., 2022).

The most abundant antioxidants in fruits are Vitamin C, polyphenols, Vitamin A, B, and E, whereas carotenoids can be found in some fruits to a lesser extent. These polyphenols, most of which are flavonoids, are present mainly in ester and glycoside forms (Maheshwari et al., 2022).

However, the ability to utilize oxygen has provided the human system with the ability to metabolize fats, proteins, and carbohydrates for energy production. Oxygen is a very reactive atom that is capable of becoming part of potentially damaging molecules known as free radicals. Free radicals are capable of attacking healthy cells of the body, causing them to lose their structure and function (Jimoh, 2022).

Free radicals are electrically charged molecules, i.e., they have an unpaired electron, which causes them to seek out and capture electrons from other substances to neutralize themselves. Although the initial attack causes the free radical to be neutralized, another free radical is formed as a result of this neutralization, causing a chain reaction to occur. And until subsequent free radicals are neutralized, thousands of free radical reactions can occur within seconds of the first reaction (Jimoh, 2022).

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Cell damage caused by free radicals appears to be a major factor in aging and degenerative diseases of aging such as cancer, cardiovascular diseases, cataracts, immune system decline, and brain dysfunction (Sies et al., 2022). Overall, free radicals have been implicated in the pathogenesis of at least 50 diseases (Swartz et al., 2022). Fortunately, free radicals' formation is controlled naturally by antioxidants. Antioxidants are capable of stabilizing or deactivating free radicals before they attack cells. Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being. It is when the availability of antioxidants is not sufficient that cellular damage can become cumulative and debilitating (Bensid et al., 2022).

1.1. Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS) is a term used to describe all highly reactive, oxygen-containing molecules, including free radicals (Bensid *et al*., 2022). Types of ROS include the hydroxyl radical, the superoxide anion radicals, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage (Bensid *et al*., 2022).

ROS are produced by several mechanisms. Most of the oxidants produced by cells occur as:

A consequence of normal aerobic metabolism: approximately 90% of the oxygen utilized by the cell is consumed by the mitochondrial electron transport system.

- Oxidative burst from phagocytes (white blood cells) as part of mechanism by which bacteria and viruses are killed, and by which foreign proteins (antigens) are denatured.
- Xenobiotic metabolism, i.e., detoxification of toxic substances. Consequently, things like vigorous exercise which accelerates cellular metabolism; chronic inflammation, infections, and other illnesses; exposure to allergens and the presence of leaky gut syndrome; and exposure to drugs or toxins such as cigarette smoke, pollution, and insecticides may all contribute to an increase in the body's oxidant load (Bensid *et al*., 2022).

1.1.1. Endogenous Sources of ROS

ROS can be divided into two groups: free radicals and non-radicals. Molecules containing one or more unpaired electrons and thus giving reactivity to the molecule are called free radicals. When two free radicals share their unpaired electrons, non-radical forms are created. The three major ROS that are of physiological significance are superoxide anion, hydroxyl radical, and hydrogen peroxide (Acar *et al*., 2022). Superoxide anion is formed by the addition of one electron to the molecular oxygen (Saleh *et al*., 2022). This process is mediated by nicotine adenine dinucleotide phosphate [NAD(P)H] oxidase or xanthine oxidase or by mitochondrial electron transport system. The superoxide anion is produced majorly in the mitochondria, the organelle of the cell to produce adenosine triphosphate. Normally, electrons are transferred through mitochondrial electron transport chain for reduction of oxygen to water, but approximately 1 to 3% of all electrons leak from the system and produce superoxide. NAD(P)H oxidase is found in polymorphonuclear leukocytes, monocytes, and macrophages. Upon phagocytosis, these cells produce a burst of superoxide that led to bactericidal activity. Superoxide is converted into hydrogen peroxide by the action of superoxide dismutase (SODs, EC 1.15.1.1) (Sindhu *et al*., 2022). Hydrogen peroxide easily diffuses across the plasma membrane. Hydrogen peroxide is also produced by xanthine oxidase, amino acid oxidase, and NAD(P)H oxidase (Hurst *et al*., 2022) and in peroxisomes by consumption of molecular oxygen in metabolic reactions (Acar *et al*., 2022).

Hydroxyl radical is the most reactive of ROS and can damage proteins, lipids, and carbohydrates and DNA. It can also start lipid peroxidation by taking an electron from polyunsaturated fatty acids (Hurst *et al*., 2022).

Granulocytic enzymes further expand the reactivity of hydrogen peroxide via eosinophil peroxidase and myeloperoxidase (MPO). In activated neutrophils, hydrogen peroxide is consumed by MPO. In the presence of chloride ion, hydrogen peroxide is converted to hypochlorous acid (HOCl). HOCl is highly oxidative and plays an important role in killing of the pathogens in the airways (Ebokaiwe *et al*., 2022). However, HOCl can also react with DNA and induce DNA–Protein interactions and produce pyrimidine oxidation products and add chloride to DNA bases (Szluc-Kleilbik and Klink, 2022). Eosinophil peroxidase and MPO also contribute to the oxidative stress by modification of proteins by halogenations, nitration, and protein cross-links via tyrosyl radicals (Tornberg-Belanger *et al*., 2022).

Other oxygen-derived free radicals are the peroxyl radicals. Simplest form of these radicals is hydroperoxyl radical and has a role in fatty acid peroxidation. Free radicals can trigger lipid peroxidation chain reactions by abstracting a hydrogen atom from a side-chain methylene carbon (Sindhu *et al.,* 2022). The lipid radical then reacts with oxygen to produce peroxyl radical. Peroxyl radical initiates a chain reaction and transforms polyunsaturated fatty acids into lipid hydroperoxides.

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Lipid hydroperoxides are very unstable and easily decompose to secondary products, such as aldehydes (such as 4-hydroxy-2,3-nonenal) and malondialdehydes (MDAs). Isoprostanes are another group of lipid peroxidation products that are generated via the peroxidation of arachidonic acid and have also been found to be elevated in plasma and breath condensates of asthmatics (Williams *et al*., 2022). Peroxidation of lipids disturbs the integrity of cell membranes and leads to rearrangement of membrane structure.

Hydrogen peroxide, superoxide radical, oxidized glutathione (GSSG), MDAs, isoprostanes, carbonyls, and nitro tyrosine can be easily measured from plasma, blood, or bronchoalveolar lavage samples as biomarkers of oxidation by standardized assays (Acar *et al*., 2022).

1.1.2. Exogenous Sources of ROS

Cigarette Smoke

Cigarette smoke contains many oxidants and free radicals and organic compounds, such as superoxide and nitric oxide (Salem *et al*., 2022). In addition, inhalation of cigarette smoke into the lung also activates some endogenous mechanisms, such as accumulation of neutrophils and macrophages, which further increase the oxidant injury.

Ozone Exposure

Ozone exposure can cause lipid peroxidation and induce influx of neutrophils into the airway epithelium. Short-term exposure to ozone also causes the release of inflammatory mediators, such as MPO, eosinophil cationic proteins and also lactate dehydrogenase and albumin (Broit *et al*., 2022). Even in healthy subjects, ozone exposure causes a reduction in pulmonary functions (Choi *et al*., 2022) have shown that particulate matter (mixture of solid particles and liquid droplets suspended in the air) catalyzes the reduction of oxygen.

Hyperoxia

Hyperoxia refers to conditions of higher oxygen levels than normal partial pressure of oxygen in the lungs or other body tissues. It leads to greater production of reactive oxygen and nitrogen species (Vang *et al*., 2022).

Ionizing Radiation

Ionizing radiation, in the presence of oxygen, converts hydroxyl radical, superoxide, and organic radicals to hydrogen peroxide and organic hydroperoxides. These hydroperoxide species react with redox active metal ions, such as Fe and Cu, via Fenton reactions and thus induce oxidative stress (Choi *et al*., 2022). Reddy *et al*. (2022) showed that fibroblasts that were exposed to alpha particles had significant increases in intracellular superoxide anion and hydrogen peroxide production via plasma membrane-bound NADPH oxidase.

Signal transduction molecules, such as extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38, and transcription factors, such as activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and p53, are activated, which result in the expression of radiation response–related genes (Liu *et al*., 2022).

Ultraviolet A (UVA) photons trigger oxidative reactions by excitation of endogenous photosensitizers, such as porphyrins, NADPH oxidase, and riboflavins. 8-Oxo-7,8-dihydroguanine (8-oxoGua) is the main UVA-mediated DNA oxidation product formed by the oxidation of hydroxyl radical, 1-electron oxidants, and singlet oxygen that mainly reacts with guanine (Wei *et al*., 2022). The formation of guanine radical cation in isolated DNA has been shown to efficiently occur through the direct effect of ionizing radiation (Nakagawa *et al*., 2022). After exposure to ionizing radiation, intracellular level of glutathione (GSH) decreases for a short term but then increases again (Tian *et al*., 2022).

Heavy Metal Ions

Heavy metal ions, such as iron, copper, cadmium, mercury, nickel, lead, and arsenic, can induce generation of reactive radicals and cause cellular damage via depletion of enzyme activities through lipid peroxidation and reaction with nuclear proteins and DNA (Jeong *et al*., 2022).

1.2. Oxidative Stress

Free radicals oxidize many biological structures, damaging them. This is known as oxidative damage, a major cause of aging, cancer, atherosclerosis, chronic inflammatory processes and cataracts, which are the most characteristics. It can also be defined as a state in which oxidation exceeds antioxidant system in the body secondary to a loss of the balance between them (Maznan *et al*., 2022). This also mean an imbalance between the speed of production and destruction toxic molecules, leading to an increase in cellular concentration of free radicals.

Oxidative stress has been implicated in over hundred disease conditions, such as cancer, cardiovascular disease, aging and neurodegenerative diseases (Hassan *et al*., 2022). The innate defense in the human body may not be enough for severe oxidative stress. Hence, certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in other to balance the ROS.

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Increased cellular level of oxidative stress can arise as a result of many factors, including exposure to alcohol, trauma, cold, infections, poor diet, toxins, radiations, or strenuous physical activity (Acar *et al*., 2022).

1.2.1. Oxidative Stress and Human Disease

Oxidative damage of DNA, proteins, and other macromolecules has been implicated in the pathogenesis of a wide variety of diseases, most notably heart disease and cancer (Seckin *et al*., 2022). A growing body of animal and epidemiological studies as well as clinical intervention trials suggest that antioxidants may play pivotal role in preventing or slowing the progression of both heart disease and some forms of cancer (Crocetto *et al*., 2022).

Heart Disease

Several factors such as high cholesterol levels, hypertension, cigarette smoking, and diabetes, are believed to promote atherosclerosis, a growing body of evidence suggests a critical step in its development is the oxidation of low-density lipoprotein (LDL) within the arterial wall (Sarandol *et al*., 2022). This theory is supported by several epidemiological studies which link low intakes of dietary antioxidants to an increased frequency of heart disease (Violi *et al*., 2022). Additionally, an inverse relationship between heart disease and plasma antioxidant levels has been reported. (Wells *et al*., 2022)

Antioxidants have been shown to prevent LDL oxidation in vitro and retard the progression of atherosclerosis in animal models (Li *et al*., 2022). Several human studies found supplemental vitamin E increased vitamin E levels in LDL, increased the resistance of LDL oxidation, and decreased the rate of LDL oxidation. It has been estimated that dietary increase in antioxidant vitamins may reduce the risk of heart disease by 20-30% (Li *et al*., 2022).

Cancer

It is estimated that diet may account for as much as 35% of all human cancers (Li *et al*., 2022). Epidemiological evidence consistently relates low antioxidant intake or low blood levels of antioxidants with increased cancer risk (Fassunke *et al*., 2022). In fact, low dietary intake of fruits and vegetables doubles the risk of most types of cancers (Wahabi *et al*., 2022). Oxidants are capable of stimulating cell division, which is a critical factor in mutagenesis. When a cell with a damaged DNA strand divide, cell metabolism and duplication become deranged. Thus, a mutation can arise which in turn is an important factor in carcinogenesis. It is believed that antioxidants exert their protective effect by decreasing oxidative damage to DNA and by decreasing abnormal increase in cell division. Both cigarette smoking and chronic inflammation-two of the major causes of cancer-have strong free radical components in their mechanism of action. Over 100 studies have reported that reduction in cancer risk is associated with a diet high in vitamin C (Odogwu *et al*., 2022).

Pulmonary Disorders

The respiratory tract has a large surface area therefore making it a major target for free radical attack. Besides, air pollution a major source of ROS (Bellas *et al*., 2022). Studies suggest that free radicals may be involved in the development of pulmonary disorders such as asthma (Bellas *et al*., 2022). Cellular damage caused by free radicals is thought to be partly responsible for the bronchial inflammation characteristics of this disease. It has been suggested that increasing antioxidants intake may help to reduce oxidant stress and help to prevent or minimize the development of asthma symptoms (Bellas *et al*., 2022). Vitamin E, vitamin C, and beta carotene supplementation has been associated with improved pulmonary function (Cole *et al*., 2022). Some evidence suggests glutathione, or possibly N-acetyl cysteine, which is a precursor to glutathione, may be helpful in protecting against pulmonary damage as well (Bland *et al*., 2022).

Other major pathologies that may involve free radicals include neurological disorders and cataracts (Marques, 2022). Neural tissue may be particularly susceptible to oxidative damage because it receives a disproportionately large percentage of oxygen, and it has a high concentration of polyunsaturated fatty acids which are highly prone to oxidation (Seok *et al*., 2022). Formation of cataracts is believed to involve damage to lens protein by free radical, causing the lens to lose its transparency. Some evidence suggests that cataract progression may be slowed with regular consumption of supplemental antioxidants, vitamin E, vitamin C, and the carotenoids (Selhub *et al*., 2022)

1.2.2. Oxidative Stress and the Mitochondria

The mitochondria are referred to as the energy power houses of the cell. Because of their critical role in producing the energy that drives every physiologic process, mitochondrial function is an area of intense interest and study today. It has been suggested that certain chronic illnesses related to muscle pain and chronic fatigue, e.g., myofascial pain syndrome (MPS), fibromyalgia syndrome, and chronic fatigue immunodeficiency syndrome (CFIDS), are disorders in which there is an aberration or dysfunction of mitochondrial energy (Almey *et al*., 2022). It has been suggested that mitochondrial dysfunction is related to damage caused by ROS produced as a consequence of increased oxidative stress and insufficient antioxidant defenses (Ho *et al*., 2022). Levels of ROS produced within the mitochondria are reported to increase with age. Therefore, oxidative damage to

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mitochondria would also appear to increase with age. This damage results in a decrease in energy production by some of the cell's mitochondria. Mitochondrial function is supported by a broad spectrum of nutritional modulators including antioxidants and antioxidant support systems. Two important modulators that appear to clinically benefit mitochondrial function are N-acetyl carnitine, which assists fatty acid transport into the mitochondria, and N-acetyl cysteine, which stimulates mitochondrial glutathione synthesis and acts as an antioxidant (Woo *et al*., 2022).

1.3. Antioxidants

The human body is equipped with a variety of antioxidants that serve to counterbalance the effect of oxidants. These can be divided into 2 categories: enzymatic and non-enzymatic.

1.3.1. Enzymatic Antioxidants

The major enzymatic antioxidants of the lungs are superoxide dismutase-SODs (EC 1.15.1.11), catalase (EC 1.11.1.6), and glutathione peroxidase GSH-Px (EC 1.11.1.9). In addition to these major enzymes, other antioxidants, including heme oxygenase-1 (EC 1.14.99.3), and redox proteins, such as thioredoxins (TRXs, EC 1.8.4.10), peroxiredoxins (PRXs, EC 1.11.1.15), and glutaredoxins, have also been found to play crucial roles in the pulmonary antioxidant defenses (Altuner *et al*., 2022).

1.3.2. **Non-Enzymatic Antioxidants**

Non-enzymatic antioxidants include low-molecular-weight compounds, such as vitamins (vitamins C and E), β-carotene, uric acid, and GSH, a tripeptide (L-γ-glutamyl-L-cysteinyl-L-glycine) that comprise a thiol (sulfhydryl) group (Altuner *et al*., 2022).

1.3.2.1. Vitamin C

Water-soluble vitamin C (ascorbic acid) provides intracellular and extracellular aqueous-phase antioxidant capacity primarily by scavenging oxygen free radicals. It converts vitamin E free radicals back to vitamin E. Its plasma levels have been shown to decrease with age (Giamperi *et al*., 2022).

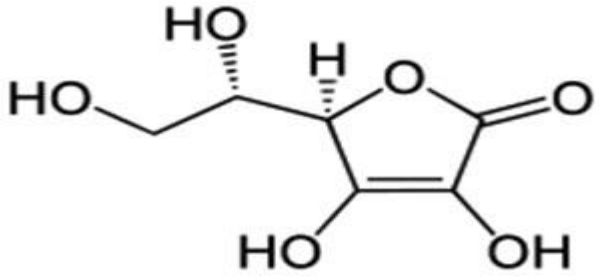
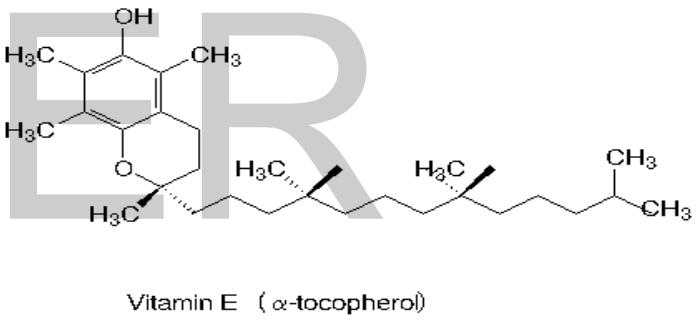


Figure 1. Structure of Vitamin C

1.3.2.2. Vitamin E

Lipid-soluble vitamin E is concentrated in the hydrophobic interior site of cell membrane and is the principal defense against oxidant-induced membrane injury. Vitamin E donates electron to peroxyl radical, which is produced during lipid peroxidation. α-Tocopherol is the most active form of vitamin E and the major membrane-bound antioxidant in cell. Vitamin E triggers apoptosis of cancer cells and inhibits free radical formations (Pluta *et al*., 2022).



Vitamin E (α-tocopherol)

Figure 2. Structure of Vitamin E

1.3.2.3. Glutathione

Glutathione (GSH) is highly abundant in all cell compartments and is the major soluble antioxidant. GSH/GSSG ratio is a major determinant of oxidative stress. GSH shows its antioxidant effects in several ways (Wang *et al*., 2022). It detoxifies hydrogen peroxide and lipid peroxides via action of GSH-Px. GSH donates its electron to hydrogen peroxide to reduce it into water and oxygen. GSSG is again reduced into GSH-by-GSH reductase that uses NAD(P)H as the electron donor. GSH-Pxs are also important for the protection of cell membrane from lipid peroxidation. Reduced glutathione donates protons to membrane lipids and protects them from oxidant attacks (Pisoschi *et al*., 2022).

GSH is a cofactor for several detoxifying enzymes, such as GSH-Px and transferase. It has a role in converting vitamin C and E

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back to their active forms. GSH protects cells against apoptosis by interacting with proapoptotic and antiapoptotic signaling pathways (Wang *et al*., 2022). It also regulates and activates several transcription factors, such as AP-1, NF-κB, and Sp-1.

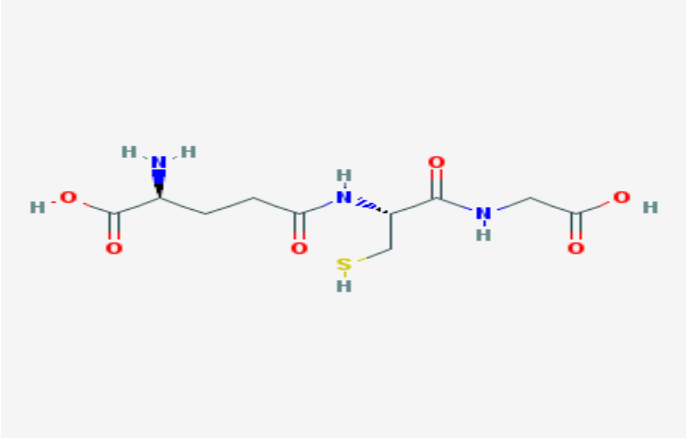

Figure 3. Structure of Glutathione

1.3.2.4. Carotenoids (β-carotene)

Carotenoids are pigments found in plants. Primarily, β-carotene has been found to react with peroxyl, hydroxyl, and superoxide radicals (Gong *et al*., 2022). Carotenoids show their antioxidant effects in low oxygen partial pressure but may have pro-oxidant effects at higher oxygen concentrations (Lee *et al*., 2022). Both carotenoids and retinoic acids (RAs) are capable of regulating transcription factors (Russell *et al*., 2022). β-Carotene inhibits the oxidant-induced NF-κB activation and interleukin (IL)-6 and tumor necrosis factor-α production. Carotenoids also affect apoptosis of cells. Antiproliferative effects of RA have been shown in several studies. This effect of RA is mediated mainly by retinoic acid receptors and vary among cell types. In mammary carcinoma cells, retinoic acid receptor was shown to trigger growth inhibition by inducing cell cycle arrest, apoptosis, or both (Wang *et al*., 2022).

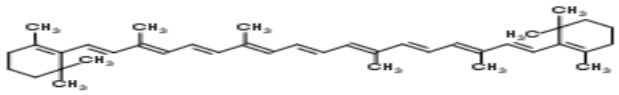

Figure 4. Structure of β-carotene

1.4.1. *Citrus limon*

Lemon is an important medicinal plant of the family Rutaceae (Liang *et al*., 2022). It is cultivated mainly for its alkaloids, which are having anticancer activities and the antibacterial potential in crude extracts of different parts (viz., leaves, stem, root and flower) of Lemon against clinically significant bacterial strains has been reported (Jangi *et al*., 2022). Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities (Byun *et al*., 2022). Flavonoids can function as direct antioxidants and free radical scavengers and have the capacity to modulate enzymatic activities and inhibit cell proliferation (Tanaka *et al*., 2022). In plants, they appear to play a defensive role against invading pathogens, including bacteria, fungi and viruses (Wang *et al*., 2022). Flavonoids are generally present in glycosylated forms in plants, and the sugar moiety is an important factor determining their bioavailability.

Figure 5. Lemon (*Citrus limon*)

1.4.2. Scientific Classification

- Kingdom – Plantae, Angiosperms, Eudicots, Rosids
- Order – Sapindales
- Family – Rutaceae
- Genus – Citrus
- Species – *C. limon*
- Binomial name – *Citrus limon*

1.4.3. Botanical distribution

Lemon grows on small, thorny trees which reaches a height of 10 to 20 feet. The leaves of the lemon are dark green in color, and they are arranged alternately on the stem. The lemon has a white, fragrant flower with five petals. This flower comes from a lemon cultivar called 'Pink Lemonade'. The leaves of this cultivar are variegated, and the fruit is striped. Lemons are oval

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citrus fruits with smooth porous skin (Woodayagiri *et al*., 2022). Some fruits have a pointed tip on the bottom of the fruit while other lemons are rounded at the base. Some kinds of lemons are quite larger than other lemon varieties and resemble elongated grapefruits. Lemon has many varieties few of which includes Bush lemon, Eureka, Lisbon, Ponderosa, Variegated Pink, Verna, Villafranca, Yen Ben and Yuzu. The color range of lemon fruit is from greenish yellow to bright yellow. Lemons look very similar to limes, but lemons tend to be a little larger and are yellow when ripe, where limes are green (Woodayagiri *et al*., 2022).

1.4.4. Geographical Distribution

The lemon is both a small evergreen tree which is native to Asia as well as tree's oval yellow fruit. Throughout the world, the fruit can be used for culinary and non-culinary purposes. Primarily it is used for its juice through the pulp and zest is also used mainly in cooking and baking. The top producers of lemon include India, Mexico, Argentina, Brazil, Spain, Peoples Republic of China, United States, Turkey, Iran, Italy and South Africa (Retallack, 2022).

1.4.5. Nutritional Value

The following is the nutritional value of 100 grams of raw lemon without peel;

- Energy – 121 kJ (29 kcal)
- Carbohydrates – 9.32g
- Sugars - 2.50g
- Dietary fibre – 2.8g
- Fat – 0.30g
- Protein – 1.10g
- Thiamine (Vit.B1) – 0.040mg (3%)
- Riboflavin (Vit.B2) – 0.020mg (1%)
- Niacin (Vit.B3) – 0.100mg (1%)
- Pantothenic acid (B5) – 0.190mg (4%)
- Vitamin B6 – 0.080mg (6%)
- Folate (Vit. B9) – 11μg (3%)
- Vitamin C – 53.0mg (88%)
- Calcium – 26mg (3%)
- Iron –0.60mg (5%)
- Magnesium – 8mg (2%)
- Phosphorus – 16mg (2%)
- Potassium – 138mg (3%)
- Zinc – 0.06mg (1%)

1.4.6. Phytochemicals

Lemons are a rich source of vitamin C, providing 64% of the Daily Value in a 100 g serving. Other essential nutrients, however, have insignificant content. Lemons contain numerous phytochemicals, including polyphenols, terpenes, and tannins (Rauf *et al*., 2022). Lemon juice contains slightly more citric acid than lime juice (about 47g/l), nearly twice the citric acid of grapefruit juice, and about five times the amount of citric acid found in orange juice (AliAbadi *et al*., 2022).

1.4.7. Culinary Uses of Lemon

Lemon juice, rind, and peel are used in a wide variety of foods and drinks. The whole lemon is used to make marmalade, lemon curd and lemon liqueur. Lemon slices and lemon rind are used as a garnish for food and drinks. Lemon zest, the grated outer rind of the fruit, is used to add flavor to baked goods, puddings, rice, and other dishes (Rabi *et al*., 2022).

Juice

Lemon juice is used to make lemonade, soft drinks, and cocktails. It is used in marinades for fish, where its acid neutralizes amines in fish by converting them into nonvolatile ammonium salts, and meat, where the acid partially hydrolyzes tough collagen fibers, tenderizing the meat, but the low pH denatures the proteins, causing them to dry out when cooked. Lemon juice is frequently used in the United Kingdom to add to pancakes. Lemon juice is also used as a short-term preservative on certain foods that tend to oxidize and turn brown after being sliced (enzymatic browning), such as apples, bananas, and avocados, where its acid denatures the enzymes (Rabi *et al*., 2022).

Peel

In Morocco, lemons are preserved in jars or barrels of salt. The salt penetrates the peel and rind, softening them, and curing them so that they last almost indefinitely. The preserved lemon is used in a wide variety of dishes. Preserved lemons can also be found in Sicilian, Italian, Greek, and French dishes (Rabi *et al*., 2022).

Leaves

The leaves of the lemon tree are used to make a tea and for preparing cooked meats and seafoods (Rabi *et al*., 2022).

1.4.8. Industrial Uses

Lemons were the primary commercial source of citric acid before the development of fermentation based processes (Flores-Valdes *et al*., 2022).

As a cleaning agent

The juice of the lemon may be used for cleaning. A halved lemon dipped in salt or baking powder is used to brighten copper cookware. The acid dissolves the tarnish, and the abrasives assist the cleaning. As a sanitary kitchen deodorizer, the juice can

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deodorize, remove grease, bleach stains, and disinfect; when mixed with baking soda, it removes stains from plastic food storage containers. The oil of the lemon's peel also has various uses. It is used as a wood cleaner and polish, where its solvent property is employed to dissolve old wax, fingerprints, and grime. Lemon oil and orange oil are also used as a nontoxic insecticide treatment.
A halved lemon is used as a finger moistener for those counting large amounts of bills, such as tellers and cashiers (Patel *et al*., 2022)

1.4.9. Medicinal Uses
Medicinal uses of lemon include;
- Blood purifier
- Blood sugar balance
- Treat throat infection
- Reduce asthma symptoms
- Prevent kidney stone (Patel *et al*., 2022).

1.5. Justification of Study
Natural sources of nutrients have been proven to be the richest sources of nutrients than those from artificial sources such as nutrients from supplements, which are manmade sources of antioxidants. The estimated numbers of fruits used as a source of nutrient and for medicinal purpose varies. *Citrus limon* (lemon) is a common fruit consumed daily without knowing its health benefit, particularly as an antioxidant. Hence, it is essential to determine its essentiality in human health.

1.6. Objective of Study
i. To measure the antioxidant properties of aqueous extract of *Citrus limon* using DPPH, ABTS, FRAP, hydrogen peroxide radicals and the ascorbic acid assay analysis under standard room temperature.
ii. To compare the antioxidant activity of the extract against that of standard ascorbic acid.

1.7. Specific Objectives of the Study
To evaluate the antioxidant properties of the aqueous extract of *Citrus limon* through in-vitro analysis by the use of various spectrophotometric methods.

2.0 Materials

Reagents
2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Horse Radis Peroxidase, 3,5 dichloro hydroxybenzene sulfonic acid, 4 amino antipyrine, and 2,4,6-Tris(2-pyridyl)-s-triazine are products of Santa Cruz Biotechnology, Dallas, Texas, USA.

Ethanol, Disodium monophosphate, Potassium dihydrogen Phosphate Potassium Persulphate, Ferric Chloride and Ferrous Sulphate are products of Loba Chemie, India.

Plant Materials
Fresh fruits of *Citrus limon* were purchased from the local market at Challenge in Ilorin, Nigeria in March 2022.

2.1. Methods

2.1.1. Sample Extraction
The lemon fruits were washed and cut transversely. A fork was used to remove the seeds and the fruits were squeezed to collect the juice. The juice was then sieved using a muslin cloth to remove the pulp.

2.1.2. DPPH Radical Scavenging Activity
This test was measured as described by Ergun (2022) with some modifications. 20µl of lemon extract and standard (ascorbic acid) solutions with varying concentrations; (10, 20, 50, 100,150 and 200 µl/ml in phosphate buffer) was added to wells of a microplate and 160ul of phosphate buffer pH 7.2 was added. 40ul of a DPPH solution (0.2mM in ethanol) was subsequently added. After 15 min of reaction at room temperature, the absorbance of the solution was measured at 517 nm. The free radical scavenging activity of each sample and standard fraction was determined by comparing its absorbance with that of a blank solution (Buffer alone, no sample or standard). The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = (A_0 - A_1)/A_0 \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.1.3. ABTS Radical Scavenging Activity
ABTS radical-scavenging activity of the extract was determined according to Ergun (2022) with some modifications. The $ABTS^{\bullet+}$ radical was produced by the reaction between 5 ml of 14 mM ABTS solution and 5 ml of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution, stored in the dark at room temperature for 16 h. Before use, this solution was diluted with ethanol to get an absorbance of 0.700 ± 0.020 at 734 nm. 20ul of lemon extract and standard (ascorbic acid) solutions with varying concentrations; (10, 20, 50, 100, 150 and 200 µl/ml in phosphate buffer) was added to wells of a microplate and 160ul of phosphate buffer pH 7.2 was added.

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40ul of the ABTS +cation radical solution was subsequently added. After 15 min of reaction at room temperature, the absorbance of the solution was measured at 734 nm. The free radical scavenging activity of each sample and standard fraction was determined by comparing its absorbance with that of a blank solution (Buffer alone, no sample or standard).

ABTS scavenging activity (%) = $(A_0 – A_1) /A_0 \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample or standard.

2.1.4. Hydrogen Peroxide Scavenging Activity

The hydrogen peroxide scavenging assay was carried out following the procedure of Ungur *et al*. (2022) with modifications. A solution of H_2O_2 (43 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). 50ul of lemon extract and standard (ascorbic acid) solutions with varying concentrations; (10, 20, 50, 100 and 150 µl/ml in phosphate buffer) was added to wells of a 2ml microtitre plate and 100ul of phosphate buffer pH 7.4 was added. 50ulof H_2O_2 solution was subsequently added, and the reaction mixture was incubated for 15 minutes. 600ul of Phosphate buffer was added to each sample and standard to make up a volume of 1ml. The amount of Hydrogen peroxide radical remaining after incubation with samples was measured by adding 300ul of a chromogen (150 mM potassium phosphate buffer, pH 7.0, containing 0.25 mM 4-aminoantipyrine, 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid and 0.5 U/l Horse Radish Peroxidase). The absorbance of the resulting red quinonimine dye is measured at 520nm. The peroxide radical scavenging activity of each sample fraction was determined by comparing its absorbance with that of a blank solution (no sample).

H_2O_2 scavenging activity (%) = $(A_0 – A_1) /A_0 \times 100$

Where A_0 is the absorbance of the blank, and A_1 is the absorbance of the sample.

2.1.5. Ferric Reducing Antioxidant Power (FRAP) Assay

The ability to reduce ferric ions was measured using the method described by Lee *et al*. (2022) with modifications. The FRAP reagent was generated by mixing 300 mM sodium acetate buffer (pH 3.6), 10.0 mM (tripyridyl triazine) TPTZ solution and 20.0 mM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 in volume. 20ul lemon extract and standards at different concentrations (10, 20, 50, 100 and 150 µl/ml) was then added to 300ul FRAP reagent and the reaction mixture was incubated at 37 °C for 30 min. The increase in absorbance at 593 nm was measured. Fresh working solutions of $FeSO_4$ were used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalents per gram of sample (DW).

2.1.6. Ascorbic Acid Assay

Principle: Phosphomolybdate is stoichiometrically reduced by ascorbic acid in the presence of inorganic phosphorous to give a characteristic molybdenum blue color which absorb light at 660 nm.

Reagents

All reagents were prepared in distilled water. 1) Sodium molybdate (3.33 %). 2) H_2SO_4 (0.25 N). 3) disodium monophosphate (0.25 mM).

Procedure

20µl of Trichloroacetic acid (TCA) (5 % w/v) was added to 1ml of lemon extract and centrifuged at 3500rpm for 10 minutes at 4ºC to obtain the supernatant. To 0.5 ml supernatant, 0.2 ml sodium molybdate (0.66 %), 0.2 ml H_2SO_4 (0.05 N) and 0.1 sodium phosphate (0.025mM) was added and incubated at 60 ºC in a water bath for 40 mins. The reaction mixture was cooled under running water and centrifuged again at 4000 rpm for 5 mins. The absorbance of the clear supernatant was read at 660 nm with appropriate blank. Ascorbic acid standard in ug scale was used to compare the results and the result expressed in µg ascorbic acid per ml of sample.

3.0. Results and Discussion

3.1. DPPH Radical Scavenging Activity

The reactivity of *Citrus limon* extract was analyzed with DPPH, a stable free radical. The addition of the extract to DPPH solution induces a rapid color change, which indicates the formation of a stable diamagnetic molecule. As DPPH picks up one electron in the presence of a free radical scavenger, the absorption decreases and the resulting discoloration is stoichiometrically related to the number of electrons gained (Bitsie *et al*., 2022). The DPPH radical scavenging (%) activity is shown in Figure 6., antioxidant reaction with DPPH could neutralize the excessive free radicals by transfer of either an electron or a hydrogen atom to DPPH. *Citrus limon* extract exerted an inhibition of 62.25% and that of standard was 77.02% at 200µl/ml.

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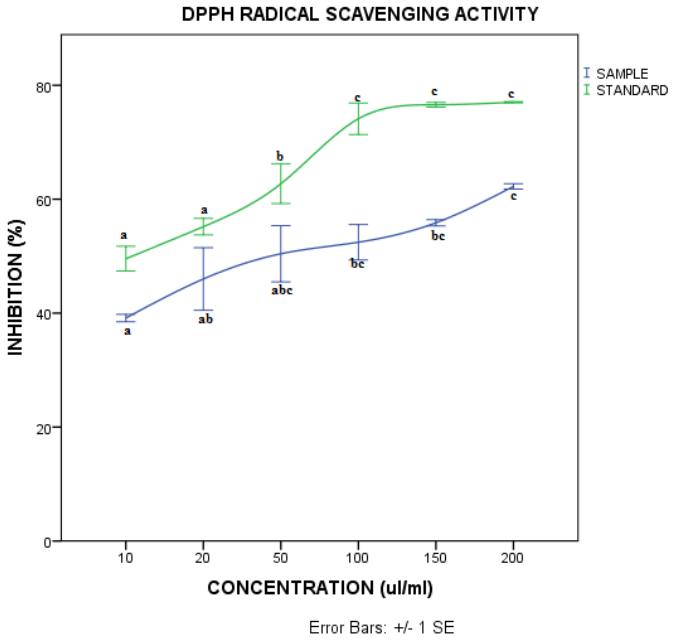
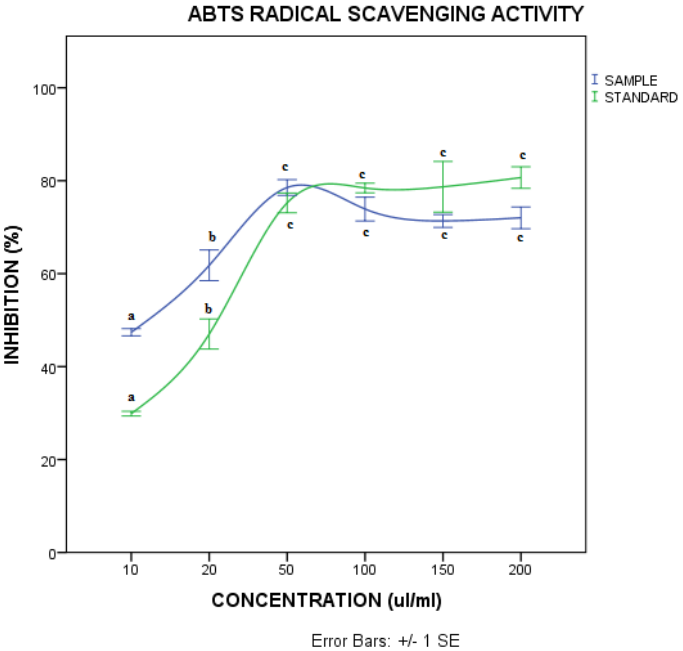


Figure 6. DPPH Radical Scavenging Activity of Lemon

3.2. ABTS Radical Scavenging Activity

ABTS$^{\bullet+}$ scavenging assay, which employs a specific absorbance (734nm) at a wavelength remote from the visible region and requires a short reaction time, can be used as an index that reflects the antioxidant activity of the test samples (Wu *et al*., 2022). In Figure 7., *Citrus limon* extract was found to be effective in scavenging radicals and the increase was concentration dependent. At 50µg/ml, the inhibition of the extract was found to be highest at 78.53% and that of standard was 75.23%. This shows that *C. limo*n extract presents a good ability to scavenge the ABTS radical.

Figure 7. ABTS Radical Scavenging Activity of Lemon**.**



3.3. Hydrogen Peroxide Radical Scavenging Activity

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} to form hydroxyl radical and this may be the origin of many of its toxic effects (Romero-Marquez *et al*., 2022). Hydrogen peroxide scavenging activity of the extract is presented in Figure 8., the extract exerted a concentration dependent scavenging. *C. limon* extract showed a maximum activity of 15.61% inhibition and that of standard with an activity of 49.54% at different concentration of 50µg/ml and 150 µg/ml.

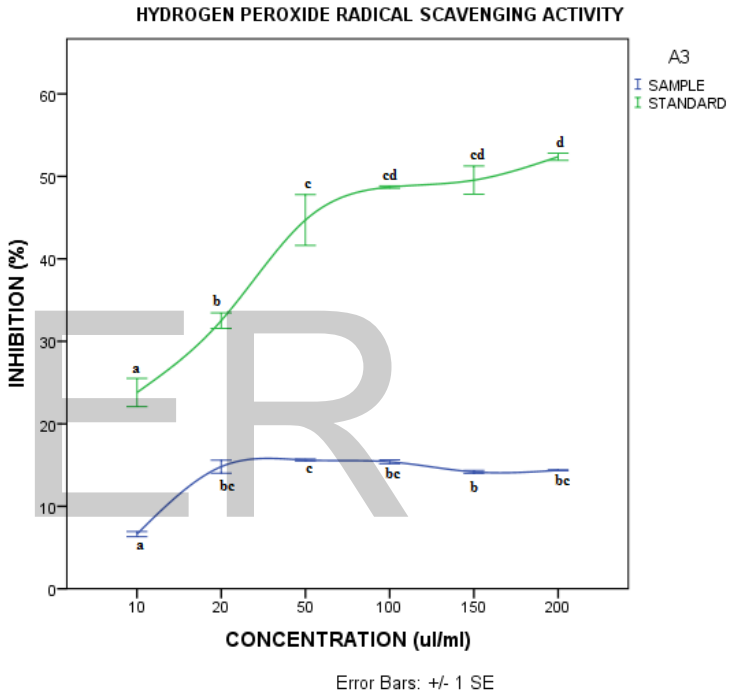


Figure 8. Hydrogen Peroxide Radical Scavenging Activity of Lemon.

3.4. Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe^{3+}-TPTZ] complex and producing a colored ferrous tripyridyltriazine [Fe2+-TPTZ] (Belani and Kaur, 2022). Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom (Basar *et al*., 2022). Frap assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction (Guo *et al*., 2022). In this assay, the trend for ferric ion reducing activities of *C. limon* and standard are shown in Fig 9. The absorbance of *C. limon* clearly increased with increase in concentration, due to theformation of the Fe^{2+}-TPTZ complex

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with increasing concentration.

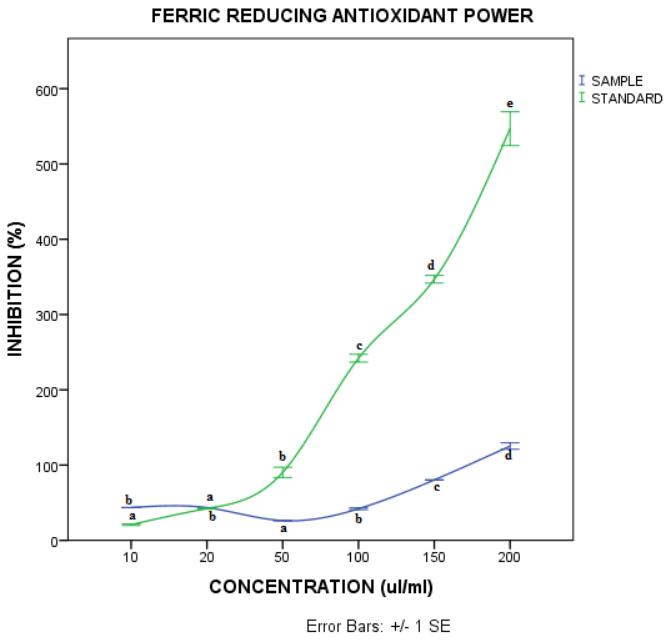

Figure 9. Ferric Reducing Antioxidant Power of Lemon

3.5. Ascorbic Acid Assay

Ascorbic acid is a good antioxidant and has been known to neutralize ROS in the human system. The ascorbic content of *C. limon* is 120.6µg/ml and the absorbance were shown to increase with increase in concentration in Fig 10. It is bio-available and is consequently water-soluble antioxidant vitamin in cells.

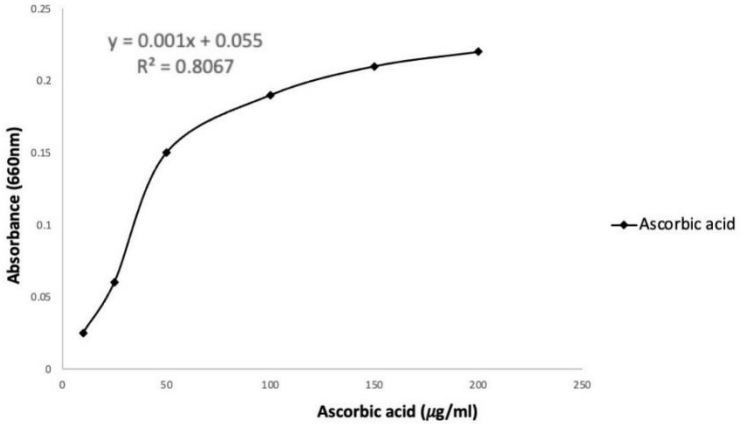

Figure 10. Ascorbic Acid Absorbance

2.2. Statistical Analysis

All numerical data were subjected to statistical analysis. The group mean ± S.E.M. were calculated for each data and significant differences between were evaluated using one analysis of variance (ANOVA). Post-test analysis were done using the Duncan Multiple Range at 95% confidence interval.

Conclusion

At the end of the assays, *Citrus limon* extract was found to be an effective scavenger of ABTS, DPPH, H_2O_2 and FRAP activity. It has reducing power. This indicate this fruit is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stress.

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