DPPH METHOD FOR QUANTITATIVE ASSESSMENT OF ANTIOXIDANT ACTIVITY IN GREEN LEAFY VEGETABLES

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ABSTRACT

A prominent test for quantitatively evaluating the antioxidant activity of green leafy vegetables is the DPPH (2,2-diphenyl-1-picrylhydrazyl) technique. To neutralize the DPPH radical, which has a deep purple colour, this technique depends on antioxidants' capacity to give electrons or hydrogen atoms. Antioxidants reduce DPPH to a stable yellow molecule, and spectrophotometric measurement of the drop in absorbance at 517 nm is conducted. Known for their bioactive components, including vitamins, flavonoids, and phenolics, green leafy vegetables have different antioxidant capabilities depending on the phytochemical constitution of the vegetable. The DPPH assay offers a quick, easy, and economical way to assess these capabilities while providing information about the possible health advantages of eating greens. According to this study, the maximum antioxidant activity, 34.94%, is found in Methi (*Trigonella foenum graeum*), whereas the lowest antioxidant activity, 9.5%, is found in Palak (*Spinach oleracea*). Vegetables are excellent sources of antioxidants, which are essential for battling free radicals linked to a variety of ailments. this has led to an increase in study on antioxidants and their potential benefits for health. the DPPH test is a crucial tool in these endeavours because to its dependability and simplicity. To fully realize the potential of vegetables in enhancing Human well-being, future research topics include developing standardized protocols and exploring new antioxidants.

Keywords: DPPH, Green Leafy Vegetables, Antioxidant Activity

1. INTRODUCTION

Green leafy vegetables (GLVs) have leaves, flowers, and new shoots that may be eaten. They have a high nutritional value and can be used medicinally. Phenolic acids, carotenoids, flavonoids, isothiocyanates, glucosinolates, phytosterols, and other phytochemicals are present [1]. Antioxidants, which are abundant in GLVs, function by considerably reducing or stopping the oxygen-related damage process brought on by free radicals like superoxide radical (O_2), hydroxyl radical (OH), and non-free radical species like H_2O_2 and singlet oxygen ($1O_2$) [2]. Age and several diseases, such as diabetes, cancer, heart disease, and neurological disorders, have been linked to oxidative stress, which is a chemical reaction that happens when molecules interact with oxygen to produce free radicals, which are unstable and extremely reactive chemicals that can damage the body's lipids, proteins, DNA, and cells [3]. Green leafy vegetables contain numerous minerals such as iron (Fe), calcium (Ca), phosphorus (P), Copper (Cu), Zinc (Zn), Sodium (Na) and chloride (Cl) which are vital for growth, metabolism etc [4]. Antioxidants can prevent, reduce, or eliminate oxidative damage to a target molecule [5]. Some commonly consumed leafy vegetables are Bathua (*Chenopodium album*), Fenugreek (*Trigonella foenum-graecum*), Soya (*Glycine max*), Sarso (*Brassica juncea*), Spinach (*Spinacia oleracea*), Poi (*Basella alba*), etc. [6]which are depicted in Figure 1. Researchers have estimated that every serving increase in fruit and vegetable consumption reduces the risk of cancer by 15%, cardiovascular disease by 30% and mortality by any cause by 20%. This has

been confirmed by epidemiological studies [7] Green leafy vegetables (GLV) are rich sources of many nutrients and form a major category of vegetable groups designated as 'nature's anti-ageing wonders' [8].

1.1 Bathua (*Chenopodium album*): South Asians commonly eat bathua, sometimes called lamb quarters or pigweed, a healthy green leafy vegetable, particularly in the winter. With its abundance of vital vitamins A, C, and some B-complex, as well as minerals like calcium, iron, phosphorus, magnesium, and potassium, as well as antioxidant properties (including quercetin, kaempferol, and rutin and dietary fibre, it is a great complement to a well-balanced diet [9]. Health advantages include increasing eye health, healing skin conditions, strengthening the immune system, enhancing bone health, managing blood sugar levels, helping with detoxification, helping digestive health, and having anti-inflammatory qualities, among others [10]. The intake of this plant as a food or medicinal ingredient is better during early summer as it has a good taste and abundant bioactive phenolics ([1]. The potential of bathua leaves as the food industry can utilize antioxidants as they provide a vital alternative to synthetic antioxidants [12] The active constituents can be isolated to evaluate their antioxidant and antibacterial activities thus lending credence to the biological value of this plant [13].

1.2 Methi (*Trigonella foenum-graecum*): Methi, often known as fenugreek, is a leafy vegetable and adaptable herb used in traditional medicine and food worldwide. Because of its high concentration of bioactive compounds, vitamins C and K, folates, and minerals including calcium, magnesium, potassium, and iron, its leaves and seeds are nutrient-rich and offer several health advantages [14]. Health benefits include promoting healthy digestion due to the fibre content, controlling blood sugar levels, lowering cholesterol, controlling blood pressure, preventing atherosclerosis, improving bone health, having anti-inflammatory properties, boosting immunity, improving skin and hair health, reducing the risk of neurodegenerative diseases like Alzheimer's, and stabilising mood [15] this plant has been widely used for more than 2500 years due to its food and medicinal properties as a herbal remedy. In essence, plant extracts and oils are not only used for antioxidant stability in foods, drinks, cosmetics creams and other goods but they are also used as multi-purpose additives such as flavouring, anti-microbial and anti-fungal agents. [16].

1.3 Soya (*Anthenum graveolens*): A common aromatic herb used in both traditional medicine and cooking is soya, sometimes referred to as dill leaves. Rich in minerals (calcium, magnesium, potassium, phosphorus, iron), vitamins A, B, and C, antioxidants, and bioactive chemicals, it is a great complement to a balanced diet [17]. Health benefits include increased immunity, improved heart health, congestion relief, reduced asthma symptoms, increased metabolism, anti-inflammatory qualities, and support for women's health. Consuming soy regularly can improve health and stave off several chronic illnesses [18]. The presence of many phytochemicals in *Anethum graveolens* L., including alkaloids, terpenoids, steroids, phenols, tannins, saponins, cardiac glycosides, terpenes, flavonoids, phenolic acid, and coumarins, has been confirmed by several scientific studies [19]. *Anethum graveolens* extracts show antimicrobial activity against *Bacillus cereus, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Listeria monocytogenes, Yersinia enterocolitica, Salmonella choleraesuis, Salmonella typhi, Shigella flexneri, S. typhimurium, Pseudomonas aeruginosa* and *Mycobacterium* [20].

1.4 Sarso (*Brassica juncea*): A nutrient-dense leafy vegetable with both culinary and therapeutic uses is sarso, often known as mustard greens. Mustard greens are rich in minerals like calcium, magnesium, potassium, phosphorus, iron, vitamin A, vitamin C, vitamin K, and folate. They are also powerful antioxidants and have been used for a long time in traditional medicine and cuisines all over the world. [21] Improved immunity, heart health, digestion, skin and hair care, anti-inflammatory qualities, prevention of neurodegenerative illnesses like Parkinson's and Alzheimer's, mood regulation, and protection against chronic diseases are just a few of the health advantages [22] The unique and pungent flavour of *B. juncea* is mainly caused by allyl isothiocyanate, a major volatile and spicy ingredient in mustard. The pungent taste is generated when the enzyme myrosinase degrades sinigrin, the glucosinolate present in *B. juncea*, to release allyl isothiocyanate. *B. juncea* is rich in secondary metabolites, such as flavonoids, polyphenols, and sulfur compounds [23]

1.5 Palak (*Spinacia oleracea*): The nutrient-dense leafy green vegetable spinach is rich in minerals (iron, magnesium, calcium, potassium, and zinc), antioxidants, phytochemicals, and numerous B-complex vitamins, including folate [24]. Because of its many health benefits, including heart, bone, digestive, detoxifying, anti-inflammatory, and cognitive enhancement, as well as its ability to support a healthy pregnancy and guard against chronic diseases, it has been a staple food in many civilizations [25]. Spinach, especially raw, is a very good source of folic acid, Spinach leaves are rich in vitamins C and E, which are antioxidants. These are supposed to lower risks of heart disease, stroke and cancer. The high amount of vitamin A in spinach may protect against eye degeneration. Potassium helps prevent and regulate high blood pressure [26].

1.6 Poi (*Basella alba*): Poi, also known as Malabar spinach, is a fast-growing, nutrient-rich leafy vegetable commonly found in tropical regions. It is a climbing vine with succulent stems and leaves, making it a unique addition to diets worldwide. It is rich in vitamin A, vitamin C, and several B-complex vitamins like folate, minerals like (calcium, iron, magnesium, potassium, and manganese), and high in fibre [27]. Some health benefits include better digestion, increased immunity to improve the brain's health, skin, eyes, hair, bones, and heart, anti-inflammatory qualities, and natural hydration [28]. It exhibits androgenic potential, antiviral, antibacterial, antioxidant, antidiabetic, anti-inflammatory, antidepressant, antiulcer (as it cures aphtha- the mouth ulcers), wound healing, nephron- and hepatoprotective activity [29].

Antioxidants, which scavenge free radicals and combat undesirable health issues brought on by oxidative stress, may thus be found in plants and natural components. Antioxidants are substances that neutralize oxidative damage to biological processes by providing electrons to free radicals and veiling them as harmless. Most often, free radicals are linked to oxidative stress. When oxygen and certain chemicals mix, free radicals are produced. Once these radicals are present, they pose a risk of harming vital components of the cell, including proteins, DNA, and the cell membrane. By reacting with free radicals, antioxidants can neutralize them and prevent damage before it begins [30]. Antioxidants are a significant component of health protection. According to scientific research, it may lower the chance of developing chronic illnesses including cancer and heart disease. [31]. These days, a variety of antioxidants including natural, synthetic, dietary, and endogenous antioxidants are present in food and are crucial for food preservation [32]. These are:

1. Natural Antioxidants [33]:
    - These are naturally occurring compounds found in plants, animals, and microorganisms that prevent oxidative stress.
    - Functions: (a)Neutralize free radicals in biological systems. (b) Enhance the nutritional value of foods. (c) Protect against chronic diseases like cancer, heart disease, and neurodegenerative disorders.
    - Examples: Polyphenols (flavonoids, tannins), Carotenoids (beta-carotene, lutein), Vitamin C (ascorbic acid), Vitamin E (tocopherols and tocotrienols).

2. Synthetic Antioxidants [34]:
    - These are artificially manufactured antioxidants used in food, pharmaceuticals, and cosmetics to prevent oxidation and increase shelf life.
    - Functions: (a) Delay oxidation in processed foods. (b) Stabilise products in the cosmetic and pharmaceutical industries.
    - Examples: Butylated hydroxytoluene (BHT), Propyl gallate (PG), Tertiary-butylhydroquinone (TBHQ).

3. Dietary Antioxidants [35]:
    - These are antioxidants obtained through diet, particularly from foods rich in vitamins, minerals, and bioactive compounds.
    - Functions: (a) Boost immune function and protect cells from oxidative stress. (b) Reduce the risk of chronic diseases through regular intake. (c) Act as cofactors for antioxidant enzymes.
    - Examples: (a) Vitamins: Vitamin C, Vitamin E, Beta-carotene. (b) Minerals: Selenium, Zinc. (c) Plant Compounds: Polyphenols, flavonoids, and anthocyanins from fruits, vegetables, and nuts.

4. Endogenous Antioxidants [36]:
    - These are antioxidants naturally produced by the body to combat free radicals and maintain cellular balance.
    - Functions: (a) Repair damaged cells and neutralize free radicals. (b) Support detoxification processes. (c) Maintain redox homeostasis in cells.
    - Examples: (a) Enzymatic Antioxidants: Superoxide dismutase (SOD), catalase, glutathione peroxidase. (b) Non-Enzymatic Antioxidants: Glutathione, uric acid, coenzyme Q10.

The antioxidant activity may act in 4 ways [37]:

1. Free Radical Scavenging: Antioxidants neutralize free radicals by donating an electron or a hydrogen atom.
2. This stabilizes the free radicals without becoming a reactive species themselves.

3. Prevention of Lipid Peroxidation: Free radicals attack lipids in cell membranes, causing lipid peroxidation, which damages cell structures. Antioxidants like vitamin E interrupt the chain reaction by stabilizing lipid radicals.
4. Metal Ion Chelation: Some antioxidants (e.g., flavonoids, citric acid) chelate metal ions like Fe^{2+} and Cu^{2+}, which catalyze free radical formation through the Fenton reaction. This reduces the production of ROS.
5. Reduction of Hydroperoxides: Antioxidants like glutathione peroxidase reduce hydroperoxides to non-toxic 5. Repair and Regeneration of Damaged Molecules

Steps of antioxidant mechanism [38]:

- Antioxidants can slow lipid oxidation by inactivating or scavenging free radicals, thus inhibiting initiation and propagation reactions. The antioxidant molecule is represented by AH, in which 'H' is the active hydrogen.

$$ROO\cdot + AH \longrightarrow ROOH + A\cdot$$
$$RO\cdot + AH \longrightarrow ROOH + A\cdot$$
$$R\cdot + AH \longrightarrow RH + A\cdot$$

- Antioxidants donate hydrogen atoms to lipid radicals and produce more stable lipid derivatives and antioxidant radicals. Antioxidant radicals are capable of participating in termination reactions with peroxy, oxy, and other antioxidant radicals.
-

$$ROO\cdot + A\cdot \longrightarrow ROOA$$
$$RO\cdot + A\cdot \longrightarrow ROA$$
$$A\cdot + A\cdot \longrightarrow AA$$

- Formation of antioxidant dimmers (dimerisation) This effectively stops the autocatalytic free radical chain mechanism as long as the antioxidant is present in its nonradical form.

The free radical 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), which is frequently used to test compounds' capacity to act as hydrogen donors or free radical scavengers and to assess antioxidant activity, provides a quick, easy, and affordable way to measure the antioxidant capacity of food [39].

2. DPPH Method

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method evaluates the antioxidant activity of a compound, extract, or material. This method measures the ability of antioxidants to scavenge free radicals, precisely the DPPH radical, which is a stable nitrogen-centred free radical The DPPH radical has a deep violet colour in solution, with a strong absorption peak at 517 nm in UV-Vis spectrophotometry As a result, the absorbance from the DPPH radical to the DPPH-H form decreases, which leads to a yellow colour depending on how many electrons are captured which is depicted in Fig. 1 [40].

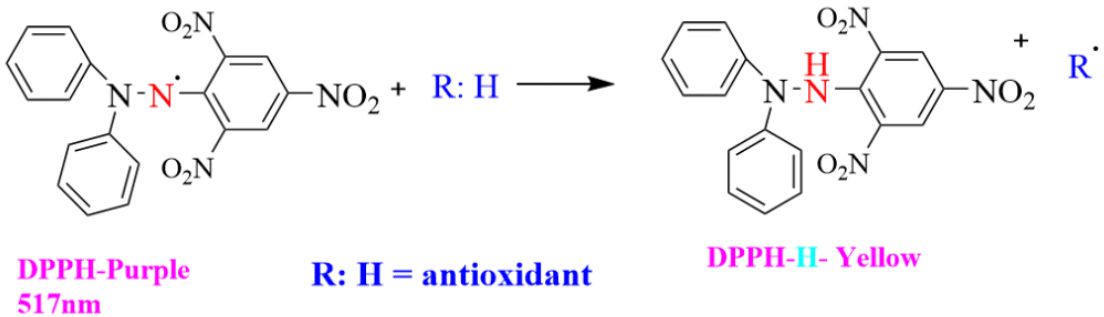

Fig 1. DPPH Method Mechanism.

Importance of Antioxidant Mechanisms [41]i.e. (a) Prevents Oxidative Damage (b) Reduces Risk of Chronic Diseases (c) Delays Aging: Protects cells from age-related oxidative stress and (d) Boosts Immune Function: Maintains healthy immune responses by reducing inflammation.

3. MATERIALS AND METHODS

This work thoroughly examines the DPPH assessment procedure, including sample preparation, DPPH Solution formulation, and crucial measurement parameters. Standardisation is stressed to ensure consistent and dependable results, underscoring its importance in enhancing the assay's credibility.

All materials were used as of A grade of compound-2,2- diphenyl -1- picrylhydrazyl, fresh green leaves of *Trigonella foenum-graeum*, *Brassica juncea*, *Chenopodium album*, *Anethum graveolens*, *Spinach oleracea*, *Basella alba* (Fig. 2), Distilled Water, Ethanol, Spectrophotometer, Centrifuge, Test tubes, Eppendorf tubes.

Fig 2. Green Leafy Vegetables.

3.1 Preparation of Plant Extract:

Examine fresh green leafy vegetables for any signs of Microbial infection, Deterioration or Distortion, to get rid of any microbes that can't be removed with the tap water, wash the leaves that do not harm with water first, and then wipe them with ethanol, allow it to dry at ambient temperature 37°C. Make a mortar paste out of every plant leaf. Filter this paste and transfer pure plant extract in 2ml Eppendorf tube for centrifugation (Fig. 3).

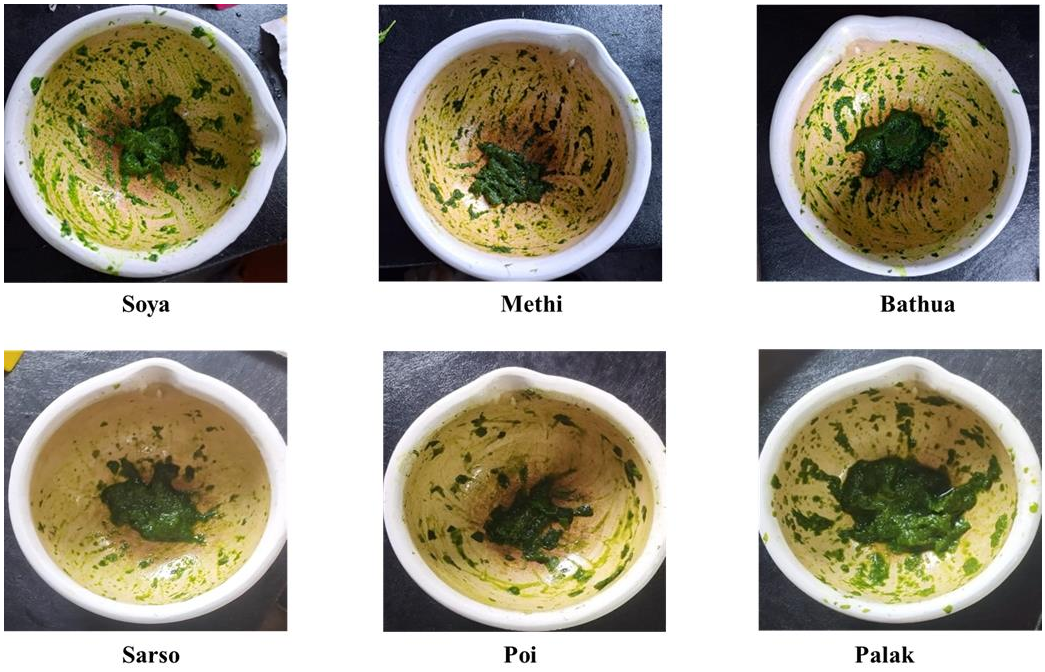

Fig 3. Green Leafy Vegetables Extracts.

3.2 Prepare a stock solution of DPPH:

To create a stock solution dissolve 20 mg of DPPH in 100 ml of methanol. The concentration should be 0.5mM, or 0.2mg/ml. Depending on the anticipated activity of the material being examined, the precise concentration may change.

3.3 Set up the control:

Make a control solution with just the solvent exactly 3ml of DPPH solution and no plant sample. This will act as a standard against which to compare.

3.4 Prepare Test Solution:

Take aliquots of 10 microliter of the sample solution in 2ml test tube and mix with 2.99 ml DPPH solution.

3.5 Incubation:

To enable the reaction between DPPH and the plant sample, let the test solution and the control incubate in the dark at room temperature for a predetermined amount of time typically about half an hour.

3.6 Measure the absorbance:

Using a spectrophotometer determine each test solution is absorbance at a particular wavelength following incubation usually around 517nm measure the control solution's absorbance as well.

3.7 Calculate the DPPH activity:

By comparing the absorbance of the test solution with the control, one can ascertain the sample's DPPH activity, and the sample's ability to scavenge DPPH radicals indicated by the decreases in absorbance. Higher antioxidant activity is interrelated with a larger absorbance decline.

The following formula can be used to determine absorbance:

$$\textbf{Antioxidant \% scavenging activity} = [(Ac - As)/ Ac] \times 100$$

Where: Ac= Absorbance of control; As= Absorbance of each sample prepared in DPPH

4. RESULTS AND DISCUSSION

We were given the antioxidant activity of different green leafy vegetables in Table 1.

Table 1. Antioxidant activity of different samples.

S.NO.	PLANT NAME (Local & English Name)	PLANT SCIENTIFIC NAME	PLANT FAMILY NAME	ABSORBANCE AT 517 nm	% OF ANTIOXIDANT ACTIVITY
Control	DPPH Solution	----------	---------	0.778	----------
1.	Methi (Fenugreek)	*Trigonella foenum graeum*	Fabaceae	0.608	34.94%
2.	Sarso (Mustard green)	*Brassica juncea*	Brassicaceae	0.442	23.52%
3.	Bathua (Goosefoot)	*Chenopodium album*	Amaranthaceae	0.376	21.85%
4.	Soya (Dill leaves)	*Anethum graveolens*	Apiaceae	0.512	34.19%
5.	Palak (Spinach)	*Spinach oleracea*	Amaranthaceae	0.704	9.5%
6.	Poi (Malabar spinach)	*Basella alba*	Basellaceae	0.564	27.50%

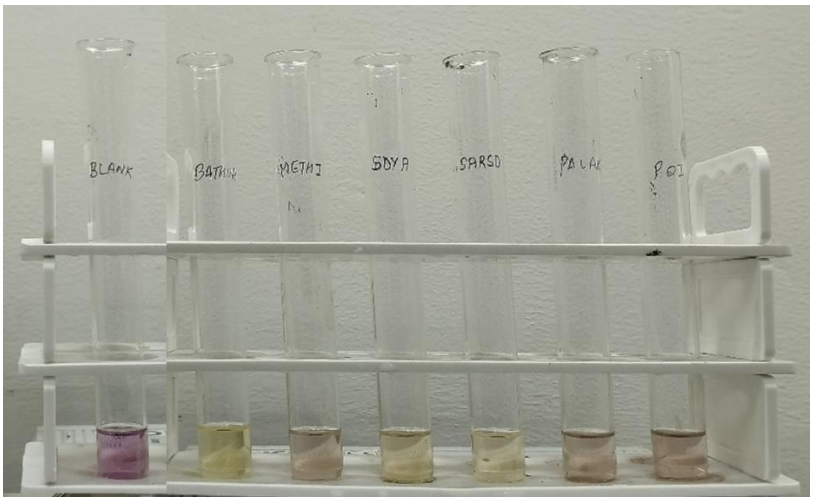

Figure 4. Antioxidant activity of Green Leafy Vegetables

The antioxidant activity of green leafy vegetables is illustrated in Fig. 4. As examined from the Table 1 *Trigonella foenum graeum* commonly called fenugreek shows 0.608 absorbances at 517 nm which shows the highest scavenging activity 34.94% neutralised free radicals produced by DPPH in quantitative estimation, it shows more antioxidant activity because of the presence of secondary metabolites polyphenols, flavonoids, alkaloids and saponins. Along with *Anethum graveolens* or soya shows 34.19% antioxidant activity because of synergistically and individually neutralised free radicals. *Spinach oleracea* shows less antioxidant activity compared to other leafy vegetables, because of its high-water content and lower concentrations of potent phenolics and essential oils. The sequence of the antioxidant activity of different green leafy vegetables in decreasing order is Methi>Soya>Poi>Sarso>Bathua>Palak

We must rely on plant-based products for medical purposes in the expanding planets. Due to their low cost and less harmful effects. The majority of people today use plant products or herbal drugs, and this dependence is only increasing over the next few years. Presenting some current developments in the use of medicinal plants to treat chronic illnesses like diabetes, cancer, heart diseases, inflammation and neurological problems in the goal of an issue (42). About 35 thousand plant species have been examined by the National Cancer Institute for possible anti-cancer activity (43).

The antioxidant analysis of green leafy vegetables such as Bathua, Methi, Soya, Sarso, Palak, and Poi, highlights their role as essential dietary components (44). These vegetables are excellent sources of natural antioxidants, contributing to both preventive and therapeutic aspects of nutrition. Including a variety of these vegetables in the diet ensures an optimal intake of bioactive compounds, providing protection against oxidative stress and endorsing overall health and well-being.

5. ACKNOWLEDGEMENT

The authors are thankful to Titan Biotech Ltd and Sisco Research Laboratories Pvt. Ltd. (SRL) for their generous contribution of chemicals used in research. Their continuous support facilitated this project's critical experiments and data collection.

Source of Support: The author(s) received no financial support for the research, authorship, and/or publication of this article.

Conflict of Interest: The author(s) declared no potential conflicts of interest for the research, authorship, and/or publication of this article.

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