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# *In-vitro* Assessment of the Antioxidant Potentials of Cyanocobalamin (Vitamin $B_{12}$): A Comparative Study with Ascorbic Acid

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**Abstract**
Cyanocobalamin is a form of vitamin $B_{12}$, which is a water-soluble vitamin that is naturally present in some foods and available as a dietary supplement as well as prescription medication to manage some illnesses. This study aimed to examine the ability of cyanocobalamin to scavenge free radicals using *in vitro* methods in comparison to ascorbic acid (control). Biochemical tests such as 1'1 diphenyl-2 picryl hydrazine (DPPH) radical, Ferric Reducing Antioxidant Power (FRAP), $Fe^{2+}$ chelation, hydroxyl radical ($OH^{\bullet}$), nitric oxide ($NO^{\bullet}$), and inhibition of lipid peroxidation assays were carried using established procedures. The results revealed that ascorbic acid control scavenged DPPH radical ($EC_{50}$ = 1.514 mM) and $OH^{\bullet}$ radical ($EC_{50}$ = 1.562 mM) better than cyanocobalamin ($EC_{50}$ = 3.751 mM), ($EC_{50}$ = 1.942 mM) while cyanocobalamin chelated $Fe^{2+}$ ($EC_{50}$ = 3.849 mM), scavenged $NO^{\bullet}$ radical ($EC_{50}$ = 1.812 mM) and also inhibited lipid peroxidation ($EC_{50}$ = 1.787 mM) better than the ascorbic acid. No significant difference was observed ($p <$ 0.05) as both the cyanocobalamin and ascorbic acid control had similar FRAP. These findings suggest that cyanocobalamin may be utilized as a supplement to manage oxidative stress- mediated diseases.

**Key words:** cyanocobalamin, ascorbic acid, free radical, oxidative stress, antioxidant
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## Introduction

Cyanocobalamin, another name for vitamin $B_{12}$, is a water-soluble vitamin that is needed for metabolism [1]. Humans need this vitamin, which is among the eight B vitamins, since it is employed as a cofactor in the production of DNA and the metabolism of both fatty and amino acids [2]. Its involvement in the production of myelin and the development of red blood cells in the bone marrow, is critical to the proper operation of the circulatory and neurological systems [3]. The pharmaceutical sector uses cyanocobalamin, the most stable chemical form, as an active ingredient in the creation of medications and dietary supplements [3,4]. Of all vitamins, vitamin $B_{12}$ is the most chemically complex, and the only one that humans need to get from animal-derived meals or supplements [4,5].

Deficiency in vitamin $B_{12}$ has the potential to cause serious, irreversible harm, particularly to the brain and nervous system [6]. Significantly low levels of this vitamin can result in a variety of symptoms, including mouth ulcers, weakness, decreased appetite, impaired memory, poor reflexes, numbness in the hands and feet and disorientation [7]. Deficiency in babies may cause neurological impairment and anaemia if neglected [8,9]. Meat, shellfish, liver, fish, poultry, eggs, and dairy products are natural sources of vitamin $B_{12}$ [10].

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Several *in vitro* studies have demonstrated that at healthy vitamin $B_{12}$ levels, there is a reduction of superoxide ions inside the cytosol and mitochondria, indicating the direct removal of reactive oxygen species (ROS) [11,12]. In the presence of cyanocobalamin, the nervous system possesses strong ROS-scavenging properties [13]. Much of cyanocobalamin's anti-inflammatory and protective properties against oxidative DNA damage can be ascribed to its antioxidant properties, especially those associated with superoxide radical binding [14]. Vitamin $B_{12}$ may also help to maintain cellular glutathione, which can accumulate hydrogen peroxide when it is lacking [15]. As co-factors for methionine synthesis, it is possible for cyanocobalamin to indirectly exercise its antioxidant effects through its participation in the breakdown of homocysteine to methionine [16]. An excess of homocysteine has been associated with the development of various clinical conditions, such as schizophrenia, Alzheimer's disease, and various cardiovascular diseases. This also generates various reactive oxygen species [17,18]. Cyanocobalamin prevents this process by permitting homocysteine to be transformed into methionine.

The search for effective antioxidants has become increasingly vital due to the role of oxidative stress in the pathogenesis of various chronic diseases, including cancer, cardiovascular diseases, and neurodegenerative disorders [19,20,21]. Antioxidants counteract oxidative stress by neutralizing free radicals, thus preventing cellular damage [22]. Among the numerous antioxidant vitamins, ascorbic acid (Vitamin C) is well-recognized for its potent free radical scavenging abilities [23,24,25]. However, the antioxidant potential of cyanocobalamin (Vitamin B12), has not been extensively explored. The findings of this study provided insight into the efficacy of cyanocobalamin as an antioxidant, with potential implications for its use in preventing oxidative damage in biological systems.

## Materials and methods

*Chemicals and Reagents*

In this study, analytical-grade chemicals and reagents were used. The following products were bought from Chemie GmH (Steinheim, Germany) and Sigma Aldrich, through a chemical vendor in Nigeria. Iron sulphate ($FeSO_4$), hydrogen peroxide ($H_2O_2$), trichloroacetic acid (TCA), and cyanocobalamine (Vitamin $B_{12}$). Naphtyl ethylenediamine dihydrochloride (NEDD), 2,4-dinitrophenyl hydrazine (DNPH), 1'1-diphenyl-2-picryl hydrazine (DPPH), and sulphanilamide.

### *In vitro* Antioxidant Assays

*Determination of DPPH radical scavenging ability*

One millilitre (1 mL) of diluted cyanocobalamin was combined with 0.4 mM 1-diphenyl-2-picryl hydrazine (DPPH) radicals in a methanolic solution. After 30 minutes of incubation in the dark, the mixture's absorbance value was measured at 516 nm using a spectrophotometer. Likewise, 2 mL of DPPH solution was employed as the control in the absence of the test samples. The samples' capacity to use DPPH to scavenge free radicals was contrasted with the control [22].

**Calculation:**

$$\% \text{ scavenging ability} = (Abs_{ref} - Abs_{sam}) / Abs_{ref} \times 100$$

Where, $Abs_{ref}$ =Absorbance of Reference
$Abs_{sam}$ = Absorbance of Sample

*Determination of the Ferric Reducing Antioxidant Power (FRAP)*

The reducing property of cyanocobalamin was ascertained based on its capacity to reduce $Fe^{3+}$ to $Fe^{2+}$. Sodium phosphate buffer (2.5 mL, 200 Mm, pH 6.6) and 2.5 mL of 1% w/v potassium ferricyanide were combined with 2.5 mL aliquots. After 20 minutes of incubation at 50°C, 2.5 mL of 10% w/v trichloroacetic acid was added to the mixture. For ten minutes, this

mixture was centrifuged at 8000 rpm. Then, 1 mL of 0.1% w/v ferric chloride and water was added to 5 mL of the supernatants obtained. After measuring the absorbance of the mixture at 700 nm, the ascorbic acid equivalent deduced was used to calculate the ferric-reducing power [23].

**Calculation:**

Reducing property = $(Abs_{ref} – Abs_{sample}) / (Abs\ ref \times 100)$
Where, $Abs_{ref}$ = Absorbance of Reference
$Abs_{sample}$ = Absorbance of Sample/Standard or control
Therefore, Percentage scavenging ability of sample ($Per_{sam}$) in mgAAE/g will be
$(Per_{sam} * Conc._{std}) / (Per_{std}*Conc._{sam}\ AMW)$
Where, $Per_{std}$ = Percentage scavenging ability of Standard or control (Ascorbic acid)
$Per_{sam}$ = Percentage scavenging ability of Sample
$Conc._{std}$ = Stock Concentration of Standard or control in mmol
$Conc._{sam}$ = Stock Concentration in mmol
AMW = Molecular Weight of Ascorbic acid

*Determination of $Fe^{2+}$ chelating ability*

A modified version of the Puntel *et al*. [24] was employed to evaluate cyanocobalamin's ability to chelate $Fe^{2+}$. Freshly prepared $FeSO_4$ (150 µL, 500 µM) was added to reaction solutions containing cyanocobalamin. Thereafter, 168 µL of 0.1 MTris-HCl (pH 7.4) and 218 µL of saline solution were used. After five minutes of incubation, 13 µL of 0.25% 1,10 phenanthroline (w/v) was added to the reaction mixtures. The absorbance at 510 nm was measured using a UV/Visible spectrophotometer. The capacity to chelate $Fe^{2+}$ was then calculated.

**Calculation:**

% $Fe^{2+}$ scavenging ability = $(Abs_{ref} – Abs_{sam}) / Abs_{ref} \times 100$
Where, $Abs_{ref}$ =Absorbance of Reference
$Abs_{sam}$ = Absorbance of Sample

*Determination of hydroxyl radical ($OH^{•}$) scavenging ability*

To a reaction mixture containing cyanocobalamin, 40 µL of 20 mM hydrogen peroxide, 400 µL of 0.1 M phosphate buffer, 40 µL of 20 mM deoxyribose, and 40 µL of 500 mM $FeSO_4$ were added. Afterwards, distilled water was added to the volume until 800 µL was attained. After 30 minutes of incubation at 37°C, the reaction was halted by adding 0.5 mL of 2.8% trichloroacetic acid (TCA) solution. Then, 400 µL of 0.6% thiobarbituric acid (TBA) solution was subsequently added. The tubes were then submerged in water for about twenty minutes. Using a spectrophotometer, the absorbance was measured at 532 nm. Subsequently, a percentage (%) estimate of the $OH^{•}$ radical scavenging ability was calculated [25].

**Calculation:**

% $OH^{•}$ scavenging ability = $(Abs_{ref} – Abs_{sam}) / Abs_{ref} \times 100$
Where, $Abs_{ref}$ =Absorbance of Reference
$Abs_{sam}$ = Absorbance of Sample

*Determination of nitric oxide ($NO^{•}$) scavenging ability*

Sodium nitroprusside (300 µL. 5 Mm) and 1 mL of the cyanocobalamin were mixed in test tubes and incubated for 150 minutes at 25°C. Then after150 minutes, 0.5 mL of Griess reagent—which was used after 12 hours of preparation—and 5 mL of distilled water containing 5% ortho-phosphoric acid were added. This was the same as 1% sulphanilamide and 0.01% naphtyl ethylenediamine. Solubility was measured at 546 nm [26].

**Calculation:**

% $NO^{•}$ scavenging ability = $(Abs_{ref} – Abs_{sam}) / Abs_{ref} \times 100$
Where, $Abs_{ref}$ =Absorbance of Reference
$Abs_{sam}$ = Absorbance of Sample

*Determination of lipid peroxidation assay*

In a Teflon glass homogenizer, one hundred *Drosophila melanogaster* (fruit flies) were homogenized in cold saline (1/10w/v) using around ten up-and-down strokes at a rate of about 1200 rev/min. After centrifuging the homogenate for 10 minutes at 3000 rpm, the supernatant was obtained for lipid peroxidation assay [27]. The modified protocol of Oboh *et al.* [28] was utilized to conduct the lipid peroxidation experiment. Briefly, 50 µL of supernatant was combined with a reaction mixture which included 15 µL of freshly prepared 250 µM $FeSO_4$, cyanocobalamin (0–50 µL), and 15 µL of 0.1M pH 7.4 Tris–HCl buffer. Water was added to the volume to reach 150 µL before incubation was carried out for one hour at 37 °C. Next, 150 µL of 8.1% sodium dodecyl sulfate (SDS) was added to the reaction mixture. Next, 300 µL of acetic acid/HCl (pH 3.4) and 300 µL of 0.8% thiobarbituric acid (TBA) in successive steps. The amount of thiobarbituric acid reactive species (TBARS) that were formed after one hour of incubation at 100 °C was measured at 532 nm. Afterward, malondialdehyde (MDA) produced (% of control) was used to quantify the lipid peroxidation using MDA as the benchmark.

**Calculation:**

$$\% \text{ MDA inhibition} = (Abs_{ref} - Abs_{sam}) / Abs_{ref} \times 100$$

Where, $Abs_{ref}$ =Absobance of Reference
$Abs_{sam}$ = Absorbance of Sample

*Data Analysis*

All analysis were carried out in triplicates and presented as mean ± standard deviation (SD) of *n* = 3. The least significant difference (LSD) and one-way analysis of variance (ANOVA) were used to determine whether significant differences existed between the mean of different treatments at $p \leq 0.05$ [29]. Linear regression analysis was used to get the $EC_{50}$ and $IC_{50}$ values.

**Results**

*In-vitro free radical scavenging abilities of cyanocobalamin*

The capacity of cyanocobalamin to scavenge DPPH free radicals is shown in Figure 1. The results showed that cyanocobalamin scavenged DPPH radicals in a dose-dependent manner as compared to the ascorbic acid control. Table 1 shows that the DPPH radical scavenging activity of cyanocobalamine ($EC_{50}$ = 3.751 mM) was substantially lower than that of the ascorbic acid control ($EC_{50}$ = 1.514 mM) at $p$ value of < 0.05. The representation of cyanocobalamin's Ferric-Reducing Antioxidant Power (FRAP) in milligrams of ascorbic acid equivalent per gram (mgAAE/g) is shown in Figure 2. There was no significant difference in FRAP values between cyanocobalamin (0.61 mgAAE/g) and ascorbic acid (0.62 mgAAE/g). Cyanocobalamin's $Fe^{2+}$ chelating capabilities is shown in Figure 3. Comparing the $EC_{50}$ values of ascorbic acid ($EC_{50}$ = 0.046 mg/mL) and cyanocobalamin ($EC_{50}$ = 0.046 mg/mL), cyanocobalamin had a significantly ($p < 0.05$) higher $Fe^{2+}$ chelating activity. Figure 4 illustrates how well ascorbic acid and cyanocobalamin scavenged hydroxyl radicals ($OH^{\bullet}$). In a dose-dependent way, the hydroxyl radical ($OH^{\bullet}$) generated from deoxyribose in the Fenton reaction was scavenged by cyanocobalamin. However, throughout the range of concentrations used, ascorbic acid ($EC_{50}$ = 1.562 mg/mL) had significantly higher hydroxyl radical ($OH^{\bullet}$) scavenging ability than cyanocobalamin ($EC_{50}$ = 1.942 mg/mL) (Table 3). Figure 5 shows the nitric oxide radicals (NO•) scavenging abilities of ascorbic acid and cyanocobalamin. Based on their $EC_{50}$ values (Table 4), Cyanocobalamin ($EC_{50}$ = 1.812 mg/mL) demonstrated a considerably better capability to scavenge $NO^{\bullet}$ radicals than the ascorbic acid control ($EC_{50}$ = 3.342 mg/mL) at $p$ <0.05. Lastly, Figure 6 demonstrates the abilities of cyanocobalamin and ascorbic acid to inhibit malondialdehyde. The findings show that cyanocobalamin prevented $Fe^{2+}$-induced lipid peroxidation by malondialdehyde inhibition. Cyanocobalamin ($EC^{50}$ = 1.787 mg/mL)

considerably prevented malondialdehyde generated by $Fe^{2+}$ better than the ascorbic acid ($EC^{50}$ = 1.728 mg/mL) as indicated by their $IC_{50}$ values (Table 5).

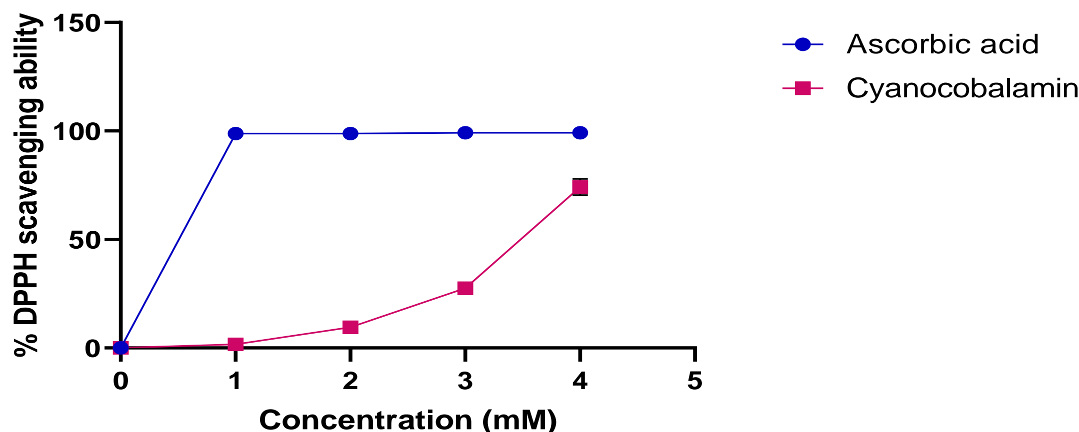


**Figure 1: DPPH$^{\cdot}$ scavenging abilities of ascorbic acid and cyanocobalamin**

**Table 1: $EC_{50}$ of DPPH$^{\cdot}$ radical scavenging abilities of ascorbic acid and cyanocobalamin (mM)**

| Sample | $EC_{50}$ (mM) |
|---|---|
| Ascorbic acid | $1.514 \pm 0.050^{a}$ |
| Cyanocobalamin | $3.751 \pm 0.050^{b}$ |

Values represent means of triplicate. Values with the same alphabet along the same column are not significantly different ($p > 0.05$). **$EC_{50}$ = Effective concentrations at 50% of cyanocobalamine and ascorbic acid to exhibit radical scavenging potentials**

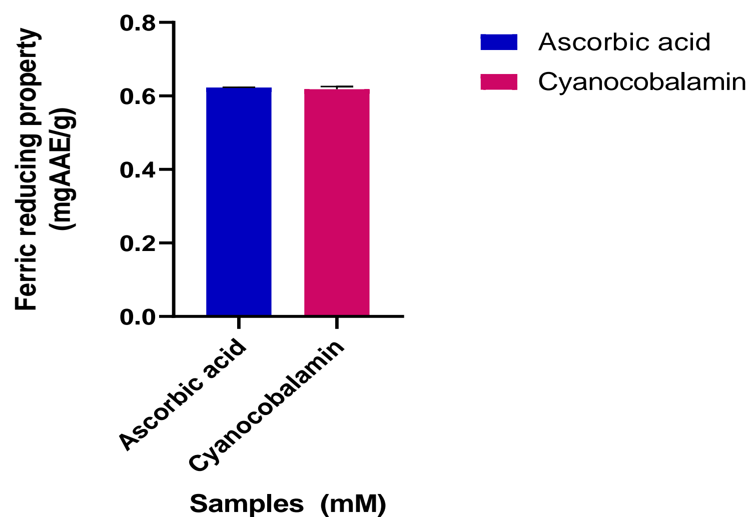


**Figure 2: Ferric Reducing Antioxidant Power of ascorbic acid and cyanocobalamin**

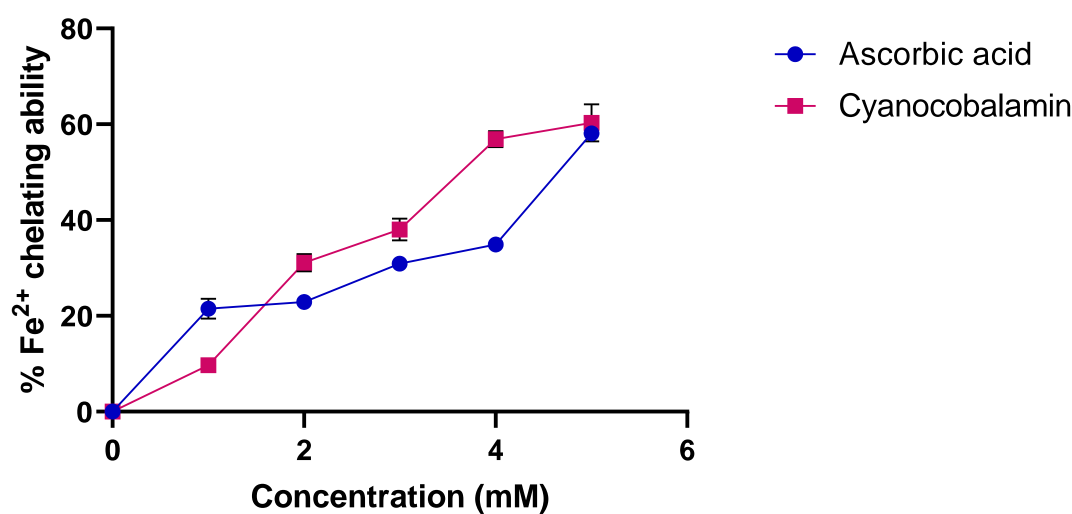


**Figure 3:** **$Fe^{2+}$ chelating abilities of ascorbic acid and cyanocobalamin**

**Table 2:** **$EC_{50}$ of the $Fe^{2+}$ chelating abilities of ascorbic acid and cyanocobalamin (mM)**

| Sample | **$EC_{50}$ (mM)** |
|---|---|
| Ascorbic acid | 4.664 ±0.050[a] |

| Cyanocobalamin | 3.849 ±0.051[b] |
|---|---|

Values represent means of triplicate. Values with the same alphabet along the same column are not significantly different ($p > 0.05$). **$EC_{50}$ = Effective concentrations at 50% of cyanocobalamine and ascorbic acid to exhibit radical scavenging potentials**

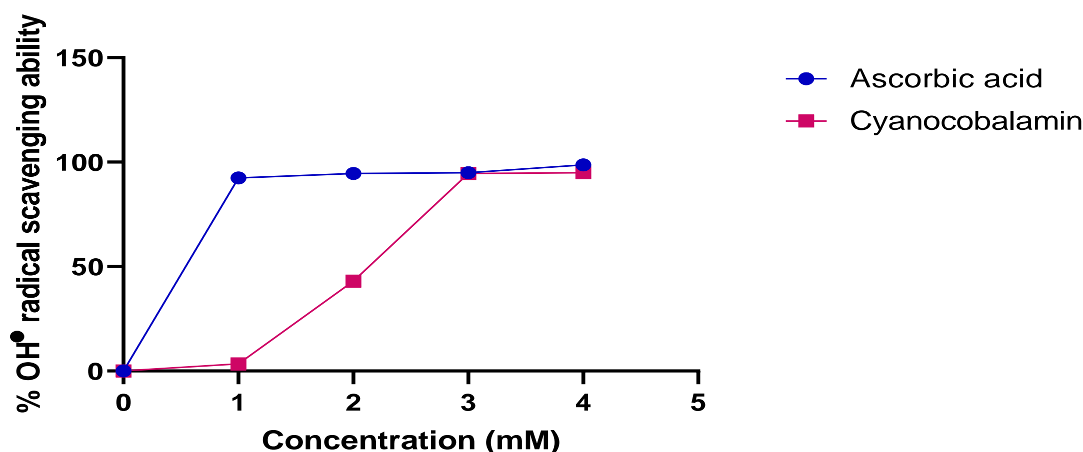


**Figure 4:** **$OH^•$ radical scavenging abilities of ascorbic acid and cyanocobalamin**

**Table 3:** **$EC_{50}$ of $OH^•$ radical scavenging abilities of ascorbic acid and cyanocobalamin (mM)**

| Sample | **$EC_{50}$ (mM)** |
|---|---|
| Ascorbic acid | 1.562 ± 0.040[a] |
| Cyanocobalamin | 1.942 ± 0.040[b] |

Values represent means of triplicate. Values with the same alphabet along the same column are not significantly different ($p > 0.05$). **$EC_{50}$ = Effective concentrations at 50% of cyanocobalamine and ascorbic acid to exhibit radical scavenging potentials**

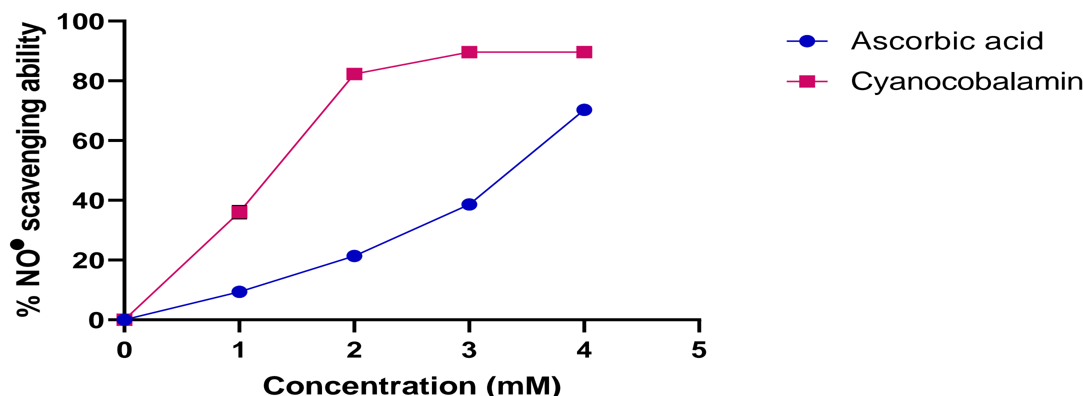


**Figure 5:** **NO• radical scavenging abilities of ascorbic acid and cyanocobalamin**

**Table 4:** **$EC_{50}$ of NO• radical scavenging abilities of ascorbic acid and cyanocobalamin (mM)**

| Sample | **$EC_{50}$ (mM)** |
|---|---|
| Ascorbic acid | 3.342 ± 0.060[a] |
| Cyanocobalamin | 1.812 ± 0.055[b] |

Values represent means of triplicate. Values with the same alphabet along the same column are not significantly different ($p > 0.05$). **$EC_{50}$ = Effective concentrations at 50% of cyanocobalamine and ascorbic acid to exhibit radical scavenging potentials**

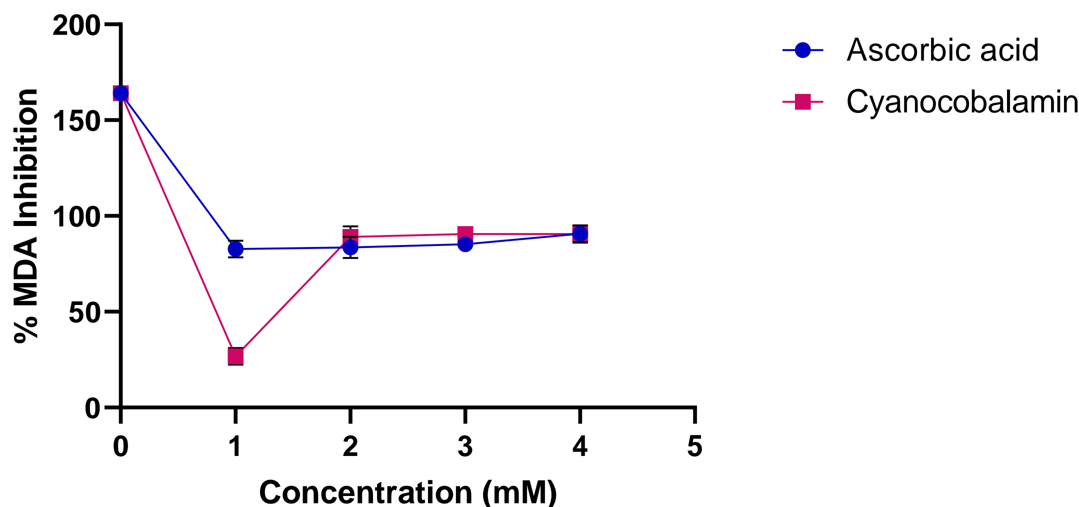


**Figure 6:** **% Malondialdehyde inhibition by ascorbic acid and cyanocobalamin**

**Table 5: $IC_{50}$ of the % malondialdehyde inhibition by ascorbic acid and cyanocobalamin (mM)**

| Sample | $IC_{50}$ (mM) |
|---|---|
| Ascorbic acid | 1.728 ± 0.010[a] |
| Cyanocobalamin | 1.787 ± 0.010[b] |

Values represent means of triplicate. Values with the same alphabet along the same column are not significantly different ($p > 0.05$). **$IC_{50}$ = Inhibitory concentrations at 50% of cyanocobalamine and ascorbic acid to inhibit malondialdehyde**

**Discussion**

When pro-oxidant substances, including reactive oxygen species (ROS), are present in greater quantities than the antioxidant buffering capacity that is available, oxidative stress occurs [30,31]. Free radicals are constantly neutralized by endogenous antioxidants in eukaryotic cells [32]. Infections, smoking, and exposure to certain chemicals or medications are among the factors that can generally lead to oxidative stress [32]. On the other hand, exogenous antioxidants such as cyanocobalamin, coumarins, flavonoids, selenium, and vitamins C and E can help lower it [33]. Animal-based foods are thought to be the main source of vitamin $B_{12}$ (Cyanocobalamin) for humans [34]. Although cyanocobalamin can be synthesized by certain bacteria in the human intestinal flora, its bioavailability in this scenario is restricted [34]. Foods high in vitamin $B_{12}$ include dairy products, eggs, cattle, lamb, and liver. The quantity and Caliber of protein ingested affects its availability and absorption [35]. Its antioxidant qualities may be able to lessen superoxide anions, which may be responsible for oxidative stress-related disorders such as oxidative DNA damage and inflammation [36, 37, 38, 39]. Cyanocobalamin was tested for its ability to scavenge 1'1-diphenyl-2-picryl hydrazine (DPPH), possess ferric reducing antioxidant power, chelate iron ($Fe^{2+}$), scavenge hydroxyl radical ($OH^{\bullet}$), scavenge nitric oxide ($NO^{\bullet}$) and inhibit $Fe^{2+}$- induced lipid peroxidation using ascorbic acid as control. Ascorbic acid significantly (at $p < 0.05$) scavenged DPPH radicals better than cyanocobalamin, it also scavenged hydroxyl radicals more effectively than cyanocobalamine. Both ascorbic acid and cyanocobalamin possessed ferric reducing antioxidant potentials but no significant difference was noticed between their potentials. Cyanocobalamin chelated iron ($Fe^{2+}$), scavenged nitric oxide ($NO^{\bullet}$) and inhibited $Fe^{2+}$ induced lipid peroxidation in *Drosophila melanogaster* (Fruit flies) better than the ascorbic acid. These findings are consistent with some reports that demonstrated that, through a variety of mechanisms of action, supplementing with physiologically relevant concentrations of cyanocobalamin scavenges free radicals and also lowers superoxide levels in the cytosol and mitochondria [15]. Furthermore, through the maintenance of glutathione, vitamin $B_{12}$ may indirectly promote ROS scavenging [11]. This process most likely entails a complex web of events that is yet poorly understood. Though ascorbic acid remains a powerful and versatile antioxidant, cyanocobalamin showed promising potential, particularly in iron chelation, nitric oxide scavenging, and lipid peroxidation inhibition in the present study.

**Conclusion**

This study provides a comparative evaluation of the antioxidant activities of cyanocobalamin (Vitamin $B_{12}$) and ascorbic acid (Vitamin C)

using a variety of *in vitro* assays. The findings in this study reveals that while cyanocobalamin exhibited significant antioxidant activities when compared with ascorbic acid, its efficacy varies depending on the nature of free radicals or oxidative stress markers being assessed. These findings suggest that cyanocobalamin could be a valuable addition to the repertoire of antioxidant agents used in the prevention and management of oxidative stress-related diseased conditions. Hence, supplementing a diet with cyanocobalamin may aid in the management of these diseases. Further research, particularly *in vivo* studies, is necessary to fully elucidate the therapeutic potential of cyanocobalamin and its possible applications in clinical settings.

**Data Availability**

All data generated and analyzed during this study are all included in this publication

**Conflicts of Interest**

All authors declare that they have no conflicts of interest

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