

TÜRKİYE
FIRAT UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES



**INVESTIGATION OF SOME BIOCHEMICAL
PARAMETERS OF CITROBACTER FREUNDII
EXPOSED TO HEAVY METAL**

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Doctoral Thesis

DEPARTMENT OF CHEMISTRY

Program of Biochemistry

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THESIS APPROVAL

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DECLARATION

I hereby declare that I wrote this Doctoral Thesis titled “INVESTIGATION OF SOME BIOCHEMICAL PARAMETERS OF CITROBACTER FREUNDII EXPOSED TO HEAVY METAL” in consistent with the thesis writing guide of the Graduate School of Natural and Applied Sciences, Firat University. I also declare that all information in it is correct, that I acted according to scientific ethics in producing and presenting the findings, cited all the references I used, express all institutions or organizations or persons who supported the thesis financially. I have never used the data and information I provide here in order to get a degree in any way.

26 March 2021

Muhammad Salihu IBRAHIM



PREFACE

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ABSTRACT

INVESTIGATION OF SOME BIOCHEMICAL PARAMETERS OF CITROBACTER FREUNDII EXPOSED TO HEAVY METAL

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Doctoral Thesis

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In this study, *Citrobacter freundii* (*C. freundii*) was grown in an LB medium containing various concentrations of cadmium, vitamin C and selenium. The density of the bacteria in the medium, the concentration of total protein and enzyme activities were determined by spectrophotometer and the level of stress biomarkers and amino acids were determined by high performance liquid chromatography (HPLC). The bacteria were grown at 0, 10, 20, 40, 60, 75, 100 and 125 ppm cadmium (Cd). The groups containing 75, 100 and 125 ppm Cd had a statistically significant decrease in bacterial density ($p < 0.05$). Likewise, a significant increase was observed in the concentration of the bacteria after adding vitamin C (25, 50 and 75 ppm) and selenium (1.0 and 3.0 ppm) to the medium containing 75, 100 and 125 ppm Cd ($p < 0.05$). The groups containing 20, 40, 60, 75, 100 and 125 ppm Cd showed a decreased level of fat and water-soluble vitamins. Addition of 25, 50 and 75 ppm vitamin C and 1.0 and 3.0 ppm selenium showed a significant increase in the amount of these vitamins ($p < 0.05$). The groups containing 20, 40, 60, 75, 100 and 125 ppm Cd showed a decrease in the amount of reduced glutathione (GSH), stress biomarkers such as malondialdehyde (MDA), oxidized glutathione (GSSG) and 4-hydroxynonenal (4-HNE). The level of GSH increased with increasing concentrations of vitamin C and selenium, while a decrease was observed in the amount of MDA, GSSG and 4-HNE in the medium containing Cd ($p < 0.05$). The superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Red) of the groups containing 60, 75 and 100 ppm Cd were seen to significantly increase compared to the controls. A decrease in the activity of these enzymes was observed with the addition of vitamin C (25, 50 and 75 ppm) to the culture medium in a concentration dependent manner ($p < 0.05$). Levels of amino acids in *C. freundii* were also determined and the amino acid with the highest concentration was glutamic acid (38.70 ± 1.50 mg / g dw), while the amino acid with the lowest concentration was tryptophan (1.50 ± 0.08 mg / g dw). The amount of leucine, asparagine, glycine, histidine, arginine, valine, aspartic acid, proline, tyrosine, alanine, cysteine, threonine, isoleucine, phenylalanine and lysine increased with increasing concentrations of cadmium. A decrease in the amount of these amino acids was observed after addition of varying concentration of vitamin C. Also, the amount of glutamic acid, serine, glutamine, methionine and tryptophan decreased with increasing concentrations of cadmium and the amount of these amino acids increased with increasing concentrations of vitamin C. The results showed that the use of vitamin C and selenium may provide a beneficial effect on reducing or reversing the toxic effect of cadmium.

Keywords: *Citrobacter freundii*, Cadmium, Stress biomarkers, Vitamins, Amino acids

ÖZET

Ağır metale maruz kalan *Citrobacter Freundii* nin bazı biyokimyasal parametrelerinin incelenmesi

Muhammad Salihu IBRAHIM

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Bu çalışmada *Citrobacter freundii* (*C. freundii*) bakterisi değişik konsantrasyonlarda kadmiyum içeren LB besiyeri ortamında üretilirken bu besi ortamına değişik konsantrasyonlarda C vitamini ve selenyum katılarak, üretilen bakterilerdeki hücre konsantrasyonu, toplam protein miktarları ve enzim aktiviteleri spektrofotometre ile yağda ve suda çözünen vitaminler, stres biyomarkırları ve aminoasit miktarları ise yüksek performanslı sıvı kromatografisi (HPLC) ile belirlendi. Bakteriler, 0 ppm (kontrol) 10, 20, 40, 60, 75, 100, 125 ppm kadmiyum konsantrasyonlarında üretilmiştir. Kontrol grubu ile 75, 100 ve 125 ppm Cd içeren gruplar karşılaştırıldığında bakteri konsantrasyonunda istatitiki olarak anlamlı azalma görüldü ($p<0.05$). Aynı şekilde 75, 100 ve 125 ppm Cd ihtiva eden besi ortamına 25, 50 ve 75 ppm C vitamini katılmasıyla üretilen bakterilerin konsantrasyonunda anlamlı artış gözlenmiştir ($p<0.05$). 20, 40, 60, 75, 100 ve 125 ppm Cd içeren gruplar ile kontrol grubu karşılaştırıldığında yağda ve suda çözünen vitaminlerin miktarlarında azalma olur iken, 75, 100 ve 125 ppm Cd ihtiva eden besi ortamına 25, 50 ve 75 ppm C vitamini ve 1.0 ve 3.0 ppm selenyum katılmasıyla bu vitaminlerin miktarlarında anlamlı artış gözlenmiştir ($p<0.05$). Yine 20, 40, 60, 75, 100 ve 125 ppm Cd içeren gruplar ile kontrol grubu karşılaştırıldığında indirgenmiş glutatyon (GSH) miktarında azalma olduğu, stres biyomarkırlarından, malondialdehit (MDA), oksitlenmiş glutatyon (GSSG) ve 4-hidroksinonenal (4-HNE) miktarlarında ise artış gözlenirken, Cd ihtiva eden ortama artan konsantrasyonlarda C vitamini ve selenyum katılmasıyla GSH miktarında artma, MDA, GSSG ve 4-HNE miktarlarında azalma olduğu gözlenmiştir ($p<0.05$). 60, 75 ve 100 ppm Cd içeren grupların süperoksit dismutaz (SOD), katalaz (CAT), peroksidaz (POD), glutatyon peroksidaz (GSH-Px) ve glutatyon redüktaz (GSH-Red) enzim aktivitelerinin kontrole göre anlamlı artış gözlenirken, artan derişimlerde C vitamini (25, 50 ve 75 ppm) katılmasıyla bu enzimlerin aktivitelerinde azalma olduğu gözlenmiştir ($p<0.05$). *C. freundii* bakterisinde, en yüksek konsantrasyona sahip amino asit glutamik asit iken, (38.70 ± 1.50 mg/g dw) en düşük konsantrasyona sahip amino asit triptofan olarak gözlendi (1.50 ± 0.08 mg/g dw). Artan kadmiyum konsantrasyonlarına karşı Lösin, asparajin, glisin, histidin, arginin, valin, aspartik asit, pirolin, tirozin, alanin sistein, thronin, izolösin, fenil alanin ve lizin aminoasitlerin miktarlarında artma gözlenir iken, C vitamini ilavesi ile aynı amino asitlerin konsantrasyonlarında azalma gözlenmiştir. Aynı şekilde artan kadmiyum konsantrasyonuna karşı glutamik asit, serin, glutamin, metiyonin ve triptofan miktarlarında azalma olurken, C vitamini ilavesi ile bu amino asitlerin miktarlarında artma gözlenmiştir. Bu sonuçlardan kadmiyumun toksik etkisini azaltmak için C vitamini ve selenyum kullanımının yararlı olabileceği söylenebilir.

Anahtar Kelimeler: *Citrobacter freundii*, kadmiyum, stres biyomakaleri, vitaminler, amino asitler

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SYMBOLS AND ABBREVIATIONS

Abbreviations

4-HNE	: 4-Hydroxyneonal
APX	: Ascorbate Peroxidase
CAT	: Catalase
CF	: Cystic Fibrosis
DNA	: Deoxyribonucleic acid
EPA	: Environmental Protection Agency
GSH	: Reduced Glutathione
GSH-Px	: Glutathione Peroxidase
GSH-Red	: Glutathione Reductase
GSSG	: Oxidized Glutathione
GST	: Glutathione-s-transferase
HAT	: Hydrogen-based atom transfer
HIV	: Human immunodeficiency virus
HPLC	: High performance liquid chromatography
IARC	: International agency for research on cancer
LB	: Liquid Broth
MDA	: Malondialdehyde
MG	: Methyl Glyoxal
NADH	: Nicotinamide Adenine Dinucleotide
NADPH	: Nicotinamide Adenine Dinucleotide Phosphate
OS	: Oxidative Stress
PG	: Peptidoglycan
PITC	: Phenylisothiocyanide
PTC	: Phenylthiocarbonyl
PUFA	: Poly unsaturated fatty acid
ROS	: Reactive oxygen species
SEM	: Standard Error of the Mean
SET	: Single electron transfer
SOD	: Superoxide Dismutase
TEA	: Triethylamine
USEPA	: United States Environmental Protection Agency

1. INTRODUCTION

Bacteria are single-cell microscopic organisms that grow in a number of environments. They can live in diverse environments such as in soil, oceans and even inside and outside of other organisms including humans. Bacteria may be classified on the basis of their cell structure, metabolism or molecular variations such as DNA, fatty acids, pigments, antigens and quinones. Relationship of human beings with bacteria is complex. Bacteria often give us a hand of support in various occasions such as conversion of milk into yogurt or helping with our digestion. As only bacteria and some archaea have the genes and enzymes required for synthesizing vitamin B₁₂ also known as cobalamin and supplying it via the food chain, almost all animal life depends on bacteria for survival [1]. Bacteria, mostly lactic acid bacteria such as *Lactobacillus* and *Lactococcus* have been used for the preparation of fermented foods such as cheese pickles, soy sauce, sauerkraut, vinegar, wine and yogurt for thousands of years in conjunction with yeast and moulds [2, 3]. Bacteria have a remarkable ability to breakdown a number of organic compounds and have been used in the production and bioremediation of waste. In order to remove oil spills, bacteria with the ability to digest the hydrocarbons in petroleum are also used [4]. In the biological pest control, bacteria may also be used instead of pesticides. Bacteria play a pivotal function in the area of molecular biology, genetics and biochemistry because of their capability to develop rapidly and the relative ease with which they can be manipulated. By producing mutations in bacterial DNA and analyzing the resulting phenotypes, scientists are able to determine the function of genes and enzymes as well as the metabolic pathways of bacteria, which could consequently be applied on other higher organisms [5]. There are bacteria everywhere, including the whole body. Our body's bacteria weigh just as much as our skin, the largest organ. Bacteria can be dangerous, but some bacteria species are necessary to keep us alive. In our eyes, respiratory and digestive systems, the first line of defence against foreign "invaders" (pathogens) which could cause infection and other problems are bacteria. Humans have a variety of bacteria in their gut, known as the microbiome [6]. When we are born, we acquire our first bacterial habitatants and from then throughout our whole life we encounter a pleaothera of bacteria in our every day life. Some of these bacteria reside in the body and contribute to the development of a powerful immune system, while some may be pathogenic and cause a harm to us.

The bacterial species that colonize our respiratory and digestive systems help to search and control the immune system. White blood cells patrol the body in search of infections, thereby limiting the level of bacteria that grow there as well. Bacteria also provide help to us when our cells have not that capacity. For example, some complex carbohydrates and toxins are broken down by bacteria and [7]. The bacteria help avoid invading pathogens and help to repair tissue in our

intestines. Most significantly, having good bacteria in a healthy microbiome may provide us with the benefit of keeping the disease causing bad bacteria away.

Some bacteria species in our body, however, can cause disease development like cancer, diabetes, cardiovascular disease, and obesity [8]. These illnesses usually occur only when the normal microbiome is disturbed naturally or by drugs such as antibiotics. When this occurs, the bad bacteria have the chance to grow, providing a favorable environment for disease. When at low numbers, such pathogenic bacteria can live in our body without causing any problem, but when they grow to a higher levels they may cause a great damage. For example, *Staphylococcus aureus* may cause something as easy as a pimple or as severe as pneumonia or toxic shock syndrome. *Porphyromonas gingivalis* has been recently linked with pancreatic cancer and can cause gum disease. Likewise, *Klebsiella pneumonia* can cause colitis and eventually lead to colorectal cancer [9]. *Citrobacter freundii* (*C.freundii*) happen to be responsible for several infections, and indeed about 29% of all opportunistic infections have been reported to be caused by this bacterium [10]. The displacement of normal healthy microbiome with pathogenic disease-causing bacteria, may result the immune system go into overdrive, much in the same way as stress affects our body. The outcome may be common allergies or autoimmune diseases that are very disabling. Our body may suffer from constant inflammation without the right balance of bacteria. Inflammation is the alarm system of the body, signaling the leukocytes to repair a wound or do away with infection. Nevertheless, chronic inflammation can make the body more vulnerable to autoimmune and cancer conditions, such as inflammatory bowel conditions that could lead to colon cancer if uncontrolled [11].

Research suggests that efforts to create a healthier, bacteria-free environment lead to an increase in obesity, cancer, and heart disease [12]. Scientists are doing all they can to sort out how to improve our health by using probiotics (foods like yogurt with active crops and dietary supplements containing a living bacteria). In addition, environmental pollution by heavy metals has become widespread, and bacteria are now being studied for its ability to bio remediate and bio accumulate some of these toxic metals [13].

1.1. *Citrobacter freundii*

Werkman and Gillen discovered the genus of *Citrobacter* in 1932. Cultures from *C. freundii* were extracted from soil extracts and described in the same year [14]. *C. freundii* is a member of the Enterobacteriaceae family which are anaerobic gram-negative bacteria [15]. The long bacterial rod shape of the bacteria has a typical length of 1–5 μm . Most of the *C. freundii* cells usually consist of many movable flagella. *C. freundii* is a soil micro-organism, but may also be seen in variety of other places like in animals and humans, in water, and food. *C. freundii* is an opportunistic pathogen that account for several major infections. It is considered to be the cause of respiratory and urinary

tract infections. . *C. freundii* accounts for about 30% of all occasional infections [16]. Even though a bacterial pathogen; this infectious microbe plays a big part in the environment's nitrogen cycle. *C. freundii* is responsible for environmental reduction of nitrate to nitrite [17]. This change is crucial and essential step in the nitrogen cycle. Biodegradation of tannic acid used in tanneries was also examined for *C. freundii*. *C. freundii* has the potential to expand and use glycerol as its main source of carbon and fuel. The organism comprises a micro-bacterial compartment capable of processing propanediol [18]. *C. freundii* has an extraordinary ability to multiply on a single carbon and glycerol supply of energy. Glycerol is fermented with a dismutase enzyme in this process. Two paths are needed for this process. In the first course, glycerol is dehydrogenated to dihydroxyacetone by a NAD⁺ linked glycerol dehydrogenase. The dihydroxyacetone is then phosphorylated by dihydroxyacetone kinase and funneled to glycolysis. In the second pathway, glycerol is dehydrated to form 3-hydroxypropionaldehyde by the coenzyme B₁₂-dependent glycerol dehydratase. This compound is reduced by NADH-linked 1,3-propanediol dehydrogenase, regenerating NADH, to the main fermentation product 1, 3-propanediol. The DNA regulon encodes four basic enzymes these two pathways. Ironically, DNA regulon expression is triggered only when there is glycerol. Cells in *C. freundii* are can also used as a carbon source for metabolizing lactose or citrate [19].

1.2. Heavy Metals

Heavy metals known as metallic elements with a relatively high density [20]. Heavy metals contain metalloids such as arsenic, which can cause toxicity at low levels [21]. Many environmental and global public health issues have recently been attributed to environmental contamination by these metals. The use of metals in many manufacturing, farming, domestic as well as technological applications has increased exponentially [22]. Environmental sources for heavy metals include mining, agricultural, medicinal, domestic and atmospheric effluents. Pollution in the environment is significant in areas such as mining, smelters and foundries and other metal-based manufacturing sites [23]. Even though heavy metals are elements occurring naturally in the Earth's surface, most environmental contamination and human exposure are due to anthropogenic activities such as mining and smelting operations, industrial products, domestic use and metal-containing agricultural use [24, 25]. Industrial pollution can also occur by industrial waste, chemical degradation, ozone deposition, soil ion erosion and heavy metal leaching, sediment re-suspension and metal evaporation from water to soil and groundwater. It has also been recorded that natural phenomena, including weathering and volcanic eruptions, make a major contribution to heavy metal emissions. Industrial outlets such as refinery, metal production, power grids, coal combustion, petroleum combustion, nuclear power stations and high-voltage power lines, plastics, textiles, microelectronics, timber preservation and paper processing plants [26, 27]. Essential heavy metals

in plants and animals have biochemical and physiological roles. They are vital components of several important enzymes and play key roles in various oxidation-reduction reactions. Copper, for example, acts as the important cofactor for many oxidative stress-related enzymes such as: ferroxidase, catalase, superoxide dismutase, peroxidase, monoamine oxidase, cytochrome c oxidase, and dopamine β -monooxygenase [28, 29]. It is therefore an essential nutrient applied to a variety of metalloenzymes involved in the production of hemoglobin, carbohydrate metabolism, catecholamine biosynthesis, and collagen, elastin, and hair keratin cross-linking. Copper's ability to pass between an oxidized state, Cu (II), and reduced state, Cu (I), is used by redox reaction involving cuproenzymes [29]. Nevertheless, its transition from Cu (II) to Cu (I) makes it toxic, resulting in the generation of superoxide and hydroxyl radicals. In addition, excessive copper exposure was associated with cellular damage in humans that contributed to Wilson's disease. Biological function requires not only copper, but several other essential elements; however, an excess of such metals may cause damage to cell and tissue to a number of adverse effects and human diseases [30]. In biological systems, cellular organs and components such as cell membranes, mitochondria, lysosomes, endoplasmic reticulum, nuclei, certain metabolic enzymes, detoxification and cell damage repair have been documented to be impaired by heavy metals. Metal ions interact with components in cells, including DNA and nuclear proteins, causing damage to DNA and then altering conformation [31]. Several studies have shown that the production of reactive oxygen species (ROS) and oxidative stress are key factors in the toxicity and carcinogenicity of metals such as arsenic [32, 33], cadmium [34], chromium [35, 36], lead [37], and mercury [38]. These five elements have been classified as metals of significance of great public health interest due to their high level of toxicity. These are all systemic toxicants that, even at lower levels of exposure, are documented to cause multiple organ damage. These metals are often referred to as either "proven" or "probable" human carcinogens based on epidemiological and experimental evidence suggesting a link between toxicity and the prevalence of cancer in humans and animals, according to the United States Environmental Protection Agency (U.S. EPA) and the International Agency for Cancer Research (IARC). Toxicity and carcinogenicity caused by heavy metals includes many technical elements, some of which are not clarified or understood clearly. Each metal, however, is known to have unique characteristics and physico-chemical properties that give its particular mechanisms of action toxicologically [39].

1.2.1. Cadmium

Cadmium is a heavy metal that has a substantial environmental and functional effect. It is widely distributed at an average level of approximately 0.1 mg/kg in the earth's crust. Sedimentary rocks produce the highest level of cadmium compounds in the world, and marine phosphates contain approximately 15 mg of cadmium/kg. Cadmium is often used in various industrial

activities. Cadmium's major industrial uses include steel, pigment and battery production. In recent years, the use of cadmium in batteries has increased significantly, and its industrial use has decreased in developing countries in response to environmental problems. For example, the daily intake of cadmium in the United States is about 0.4 µg/kg/day, less than half of the United States oral reference dose of the EPA [40]. This reduction was connected with the introduction in some countries of general restriction of cadmium usage of strict effluent limits in the form of plating works.

1.2.2. Potential for Human Exposure to Cadmium

Key ways to be exposed to cadmium are by inhalation or cigarette smoke, and ingestion of food. It is difficult to remove from the blood. The use of primary metal factories, foodstuffs contaminated by polluted food, smoking and work in cadmium contaminated areas may expose people to cadmium. [40]. The emissions from industrial operations, including mining, smelting and battery industry, pigments, stabilizers and alloy processing provide other sources of cadmium [41]. Many food items, like leafy vegetables, onions, grains and nuts, liver and kidney, and crustaceans and mollusks, also contain trace amounts of cadmium [42]. Therefore, cadmium-rich foods can considerably increase the level of cadmium in human bodies. Sources include liver, mushrooms, shellfish, and mussels, powdered cocoa and dried sea weed. Circulatory system is a key delivery path for cadmium toxicity. The chronic exposure to cadmium particles is usually linked to alterations in pulmonary function and emphysema-compatible chest radio-graphs. A loss in olfactory function [43] was associated with occupational exposure to airborne cadmium particulates. A combination of chronic low-content cadmium toxicity with reduced bone mineral density and osteoporosis have been identified in some epidemiological studies [44, 45]. Cadmium toxicity is generally measured by testing blood or urine cadmium levels. Cadmium in urine (mostly diluted by measuring the cadmium/creatinine ratio) suggests cadmium accumulation or kidney cadmium load [46]. Around 2.3 percent of the United States population is estimated to have elevated levels of urinary cadmium (which is greater than 2 µg/g creatinine).. Cadmium levels in blood and urine are usually higher in cigarette smokers, at middle levels in those who stopped smoking and lower levels in non-smokers. In the last century, the continued use of cadmium in industrial applications has greatly increased the exposure of humans to this metal [47].

1.2.3. Toxicity and Carcinogenicity Mechanisms of Cadmium

If inhaled or swallowed, cadmium can be lethal and it is a serious irritant of the gastrointestinal and pulmonary track. Symptoms such as stomach pain, burning sensation, nausea, vomiting, salivation, muscle cramps, dizziness, shock, lack of consciousness and seizures typically

occur within 15 to 30 minutes of acute ingestion. Depending on the poisoning path, acute ingestion of cadmium may also cause gastrointestinal tract destruction, pulmonary, hepatic or kidney damage and consequently coma [48]. Chronic cadmium exposure affects nor-epinephrine, serotonin, and acetyl-choline levels depressively. Studies on Rodents have indicated that pulmonary adenocarcinomas are caused by chronic cadmium inhalation. It may also induce proliferative prostatic lesions after a systemic or a direct exposure, like adenocarcinomas. Although cadmium toxicity processes are not understood correctly, cadmium is thought to lead primarily to cell damage via the generation of ROS, that cause damage to single stranded DNA and disturbs nucleic acid and protein synthesis. Study using 2-dimensional gel electrophoresis have explained that a lot of stress response mechanisms, including heat shock, oxidative stress, intense reaction, cold shock, and S.O.S, are expressed in response to cadmium exposure. Cadmium induces cytotoxic effects in an in vitro experiment at concentrations 0.1 to 10 mM and the free radical damage to DNA [49]. In vivo experiments showed that cadmium modulates male reproduction in mice at a concentration of 1.0 mg/kg body weight. Nevertheless, compared to other carcinogenic metals, cadmium is a poor mutagen. Previous studies indicate that cadmium regulates signal transmission pathways, induces the formation of inositol by polyphosphate, increases cytosolic free levels of calcium in various cell types, and blocks calcium channels. Cadmium binds to proteins at lower concentrations (1–100 μ M), decreases DNA repair, stimulates protein degradation, up-regulates cytokines and proto-oncogenes such as c-fos, cjun, and c-myc and induces multiple gene expression, including metallothioneins, hemeoxygenases, glutathione transferases, heat-shock proteins, acute-phase reactants, and DNA polymerase β [50]. Reproductive and teratogenic effects have been shown in animal research. The inverse relation between cadmium, parental or fetal urine and the birth weight and life span during birth has been shown in limited epidemiological studies [51].

1.3. Antioxidants

Antioxidant is a molecule which can survive the oxidation of others. Oxidation is a chemical reaction that converts electrons from a substrate into an oxidizing substance. Oxidation reactions can create free radicals. These radicals can actually trigger series of reactions that may eventually lead to cell destruction. These series of reactions can be avoided by antioxidants by neutralizing free radical intermediates and preventing other oxidation reactions. They do so by themselves becoming oxidized without becoming a free radical in the process, so antioxidants are also reducing agents like thiols, vitamin C or polyphenols [52]. Recent studies into the role of antioxidants as source of rancidity in biology centered on its use to prevent oxidation of unsaturated fatty acids [53]. An easy way to measure antitropic behavior is to put the fat in a covered container of oxygen and to calculate the consumption rate of oxygen. Nevertheless, the field has been revolutionized by recognizing vitamins A, C, and E as antioxidants and the importance of antioxidants in living

organisms [54]. Possible antioxidant action mechanisms were first investigated when it was understood that a material with antioxidant activity is likely to be one that is readily oxidized by it. Studies on the prevention of lipid peroxidation by vitamin E has led to the identification of antioxidants as reducers of oxidants; mostly by scavenging reactive oxygen species before they cause harm to the cells [55]. Antioxidants are divided into two major groups, according to their solubility in hydrophilic (water) or lipid (hydrophobic). In the cell cytosol and blood plasma, water-soluble antioxidants usually react with oxidants, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation [52]. Such substances can be produced or extracted from the diet in the body [56]. The different antioxidants are found in a broad variety of concentrations in the body fluids and tissues, with some of them mainly present in cells like glutathione or ubiquinone and others like uric acid more widely distributed. Some antioxidants are only available in a few species and they may be necessary for pathogens and virulence factors. The relative importance and interactions of these various antioxidants are very complex, and synergistic and interdependent effects are exerted on each other through separate metabolites and enzymes. Consequently, the activity of an antioxidant can depend on the proper functioning of other members of the antioxidant system. The degree of defense of each antioxidant also depends on its concentration, its reactivity to particular oxygen species and the status of the antioxidants with which it interacts [56]. By chelating transition metals and preventing catalyzation of free radical production in the cell, some of the compounds contribute to antioxidant protection. The capability to sequester iron, which is the role of iron-binding proteins including transferrin and ferritin, is particularly important [57]. Selenium and zinc are generally considered as antioxidant nutrients, but they have no antioxidant activity themselves and are required for some antioxidant enzymes to work.

1.4. Selenium (Se)

Selenium (Se) is a trace mineral element and also a nutrient that is essential for human health but is only needed in small quantity. However, higher intake of selenium above the safety amount is toxic to animals and humans leading to selenium poisoning [58]. Several proteins have been identified to contain selenium incorporated in their structure. There are currently at least 25 selenium-containing protein compounds, most of them enzymes, classified as generic selenoproteins [59]. Selenium deficiency can lead to human disease and problems including, cardiac problems, a compromised immune system and hypothyroidism [60]. Chronic selenium toxicity in humans have been reported leading to condition called 'selenosis', characterized by loss of hair and nail, stomach disorders, rash of the skin and irregularities of the nervous system [61]. Selenium is identified mainly by its antioxidant function exerted via enzymes such as glutathione peroxidase and thioredoxin reductase. In fact physiologically, glutathione-peroxidases, thyroid hormone deiodinases and thioredoxin reductases are the most important selenoproteins [62]. Apart from its

role as an antioxidant, selenium has a variety of other vital roles that includes acting as insulin-mimetic, immune response modulation, endocrine pathway control including biosynthesis and metabolism of thyroid hormones [63, 64]. Furthermore, the maintenance of fertility and certain anti-cancer properties have been linked with selenoproteins [65]. Selenoprotein activity may depend on a sufficient dietary selenium supply. However it should be noted that in regard to its quantity, selenium plays an ambivalent role, with high levels causing toxic effects [66].

1.5. Vitamins

A vitamin is an organic compound and an essential nutrient in limited quantities needed by an organism. Vitamins are classified by their biological and chemical function, rather than by their form. Vitamins have various functions in biochemistry. Others, like vitamin D, have hormone-like functions as mineral metabolism regulators, or growth and differentiation regulators of cells and tissues (such as some types of vitamin A). Others act as antioxidants (for example, vitamin E and vitamin C) [67]. The B-complex vitamins are the largest number of vitamins, which serve as co-factors or precursors; coenzymes assist the enzymes in their metabolism as catalysts. Vitamins can be closely related to enzymes in this situation as one of the prosthetic groups: for example, biotin is part the enzymes responsible for fatty acid production. As coenzymes, removable molecules that act to transport chemical groups or electrons between molecules, they can also be less strongly bound to enzyme catalysts. For instance, folic acid could be present in cell by carrying groups such as methyl, formyl and methylene. Such roles in enzyme substratum reactions may be the best known function of vitamins but also of other vitamin functions [68]. Vitamin B₁ (thiamine) is a cofactor for so many enzymes, including pyruvate dehydrogenase and α -ketoglutarate dehydrogenase, which are both involved in the bacterial tricarboxylic acid cycle [69]. Flavin adenine dinucleotide and Flavin mono nucleotide are the two active forms of vitamin B₂ (riboflavin). They are cofactors for enzymatic reactions in the TCA cycle and in fatty acid oxidation. This reactions are important for energy release [70]. Vitamin B₃ (niacin) is a precursor of an important coenzyme in the cellular redox reaction, nicotinamide adenine dinucleotide, which plays a key role in aerobic respiration [71]. Vitamin B₅ (pantothenic acid) is a precursor of coenzyme A, which is an essential cofactor for the TCA cycle and fatty acid oxidation [72]. Vitamin B₆ exists in a variety of forms which includes: pyridoxine, pyridoxal and pyridoxamine. These forms of vitamin B₆ are precursors of the coenzymes pyridoxal phosphate and pyridoxamine phosphate that are involved in a variety of cellular metabolic processes such as amino acid, lipid and carbohydrate metabolism [73].

For the normal development and growth of a multi-cellular organism vitamins are necessary. The fetus starts developing from the nutrients which it absorbs with the genetic blueprint inherited from its parents. This needs the presence at certain times of certain vitamins and minerals. Such

foods help to trigger chemical reactions, including skin, bone and muscle. A child may develop a disease as a result of deficiency if there is a considerable deficiency in one or more of the nutrients. There can be continuing harm to even minor defects [74]. Vitamins are mostly derived from fruit, but some are acquired by other means. For instance, intestinal microorganisms produce vitamin K and biotin, while the natural ultra-violet wavelength of the sunlight is used to synthesize a form of vitamin D in the skin. Human beings can derive certain vitamins from their precursors. For example, beta-carotene produced vitamin A and tryptophan for niacin [75].

In order to avoid deficiency, humans must consume daily vitamins but with varying schedules. Vitamins A, D, and B₁₂ are reserved in reasonable quantities, particularly in the hepatics [76] and the diet of an adult could be deficient for several months in vitamins A and D and in some instances B₁₂ for years prior to developing a deficiency disorder. Their diet may be deficient for several months and B₁₂ for years. However, vitamin B₃ is not stored in large quantities, so that stores are only allowed to last for a few weeks [76, 77]. The early symptoms of vitamin C have been common, from one month to over six months, in the experimental studies on the full deficiency of vitamin C in humans depending on prior dietary history determined by the body [78]. There is a primary deficiency if an organism is deficient in its diet to get the vitamin. A secondary deficiency can arise because of a life-style condition such as a smoking factor, excessive alcohol intake or medicines that can meddle with the absorption or use of the vitamin, or by an underlying disorder that impedes or restricts its absorption or consumption. It is unlikely that people eating a diversified diet will develop severe basic vitamin deficit. In comparison, restrictive diets can cause chronic vitamin deficiencies, often leading to painful and life-threatening diseases. Thiamine (beriberi), niacin (pellagra) [79], vitamin C (scurvy) and vitamin D (rickets) are common deficiencies in human vitamins [80].

1.6. Proteins

Proteins are polymers of amino acids connected to one another via a peptide bond. A protein is an essential nutrient for all living organisms. Protein is an important molecule for cells and tissues. Within cells, proteins perform a number of roles. Some proteins engage in structural supporting and movement, others engage in enzyme activity and others interact in external interaction. Protein collection in a cell determines its health and biological role. Proteins are responsible for almost every cell life role and often receive signals from outside the cell and intracellular response is mobilized. Indeed, individual protein functions are as distinct as their specific sequences of amino acids and complex 3D structures [81]. However, the best way to characteristically define a protein from function and nutritional perspective is its amino acid composition [82]. It is important to note that, in all living organisms, the priority role of amino acid is for protein synthesis rather than catabolism. Separate classes of proteins, grouped into complexes

or functional modules, which are gradually interconnected in cell-wide protein networks, perform distinct cellular functions. Determination of total protein is a key to understanding the general structure, properties and functional modes of these protein complexes [83]. Bacteria have acquired several systems to show proteins on their surface and then release them or even inject them into a neighboring cell in the extracellular environment. The secretion of proteins has a high adaptive value, and many functions include secreted proteins, which are also important for bacterial adaptation, tolerance and even fitness in conditions such as oxidation stress induced as a result of heavy metals or other means [84].

1.7. Enzymes

Enzymes are biological catalysts which, in living organisms, accelerate the rate of biochemical reactions. Enzymes may also be taken from cells and then used to catalyze a wide variety of processes that are commercially important. Some of the unique features of these molecules are their specificity in function and potency. A wide variety of experiments indicated the high efficiency of enzymes in catalyzing chemical reactions [85]. However, an enzyme is a protein just like other proteins in which its function depends on its structure and also its active site. The active site of an enzyme is the region where the substrate binds and reactions occur. Furthermore, the active site of an enzyme is not just a pocket that binds the substrate but it is a highly sophisticated molecular machine that responds to physical and chemical changes within the environment and can also undergo precisely timed conformational changes that facilitate its role in binding and conversion of substrate to product [86]. It should be pointed out that while many enzymes only consist of protein, a non-protein portion, called cofactor, is also present in many, which is essential for the enzyme catalytic activity. For example, tetrahydrobiopterin (THB) is a cofactor for three hydroxylase enzymes namely: phenylalanine hydroxylase, tyrosine hydroxylase and tryptophan hydroxylase where its deficiency leads to toxic accumulation of the amino acids phenylalanine, tyrosine and tryptophan respectively [87]. Another organic molecule might be a cofactor, in which case it is termed as a coenzyme, or it may be an inorganic molecule, usually a metal ion like copper, zinc or manganese. A coenzyme that binds tightly and permanently to the protein is generally referred to as the prosthetic group of the enzyme. The inactive protein part is generally referred to as an apoenzyme because the enzyme requires a cofactor for its function and the apoenzyme plus the cofactor (i.e. the active enzyme) is referred to as a holoenzyme. Importantly, in the human diet, the need for minerals and vitamins is primarily due to their functions as cofactors and coenzymes in metabolism [88].

Several studies have indicated the importance of antioxidant enzymes activity during oxidative stress [89]. The most important defense mechanism against oxidative stress-induced cell damage is exhibited by the antioxidant enzyme system. These antioxidant enzymes are highly

important proteins that take part in the catalytic conversion of ROS together with their by-products into nontoxic stable molecules [90]. Antioxidant enzymes like catalase, SOD and GPX exert a key function in the antioxidant enzyme system and changes in their activity as a result of increased ROS production gives the cell the ability to with stand the stress thereby consequently leading to the neutralization of the ROS to nontoxic molecules [91, 92].

1.7.1. Superoxide Dismutase (SOD)

Reaction catalyze by SOD: $2 \text{HO}_2 \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$

Found in all living cells. SOD is an enzyme that helps break down oxygen molecules that are potentially toxic in cells. SODs constitute the leading defense against injury mediated by the reactive oxygen (ROS). These proteins are catalyst for the dismutation into molecular oxygen of superoxide anion free radicals and reduce the amount of the anion that destroys cells at excessive concentrations [93]. SODs are metalloenzymes which use a redox activated metal to disproportionate two superoxide molecules to oxygen and hydrogen peroxide. The hydrogen peroxide is neutralized later by catalase and peroxidase enzymes. In three different instances. SODs have developed to include the Mn and Fe SODs family, the Cu/Zn SOD family using Cu for catalysis. and a special Ni SODs family [94]. A catalytic metal is necessary of SOD function. SOD mechanism of dismutation from superoxide anion into hydrogen peroxide involves the reduction and re-oxidation of the copper (Cu) and manganese (Mn) active metal in the active site of the enzyme. SODs have also a significant function to play in avoiding oxidative inactivation of nitric oxide and in this way preventing the production of peroxynitrite and endothelial and mitochondrial dysfunction [95].

1.7.2. Catalase (CAT)

Reaction catalyze by catalase: $2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$

Catalase is a tetramer of four chains of polypeptides; each with a length of over 500 amino acids. It includes four groups of iron-containing hemes that provide the enzyme with the ability to react with hydrogen peroxide. Catalase is one of the most essential antioxidant enzymes. In almost all aerobic species, it is present. In a two-step reaction. Catalase breaks down two hydrogen peroxide molecules into one oxygen molecule and two water molecules. Catalase also has one of the largest turnover numbers of all enzymes; one molecule of catalase can transform millions of molecules of hydrogen peroxide into water and oxygen each second [96]. In controlling the cellular level of hydrogen peroxide, catalase has a prime function and its catabolism of hydrogen peroxide prevents the cells from oxidative attack. One of these functions is by protecting the pancreatic β -cells from hydrogen peroxide damage [97]. Hydrogen peroxide has been involved in inactivating

tyrosine phosphatase to act as a cellular messenger in the signaling pathway for insulin secretion [98]. It has been postulated that by degrading hydrogen peroxide to water and oxygen, catalase in the liver can provide cellular defense. The absence of catalase may lead to diabetes mellitus growth [99]. Apart from catalase, there are other enzymes that can neutralize hydrogen peroxide. Glutathione peroxidase and other peroxidases such as cytochrome-C-peroxidase and NADH peroxidase are part of these enzymes. Catalase is a main enzyme which as its substrate uses hydrogen peroxide, a non-radical ROS. By decomposing hydrogen peroxide this enzyme is responsible for neutralization, thus retaining an ideal molecule level in the cell that is also important for cellular signaling processes. Because of its over expression and indirect presence in multiple diseases and illnesses, it is possible to calculate the importance of the enzyme [100].

1.7.3. Glutathione Peroxidase (GSH-Px)

Reaction catalyze by GSH-Px: $2 \text{ GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2 \text{ H}_2\text{O}$

Glutathione peroxidase is an antioxidant enzyme whose biochemical purpose is to convert lipid hydroperoxides to their alcohols and free H_2O_2 to water. GSH-Px is a selenocysteine compound class since it binds four selenium atoms and gives glutathione peroxidase catalytic action. The co-substrate of GSH-Px is glutathione. GSH is popular as an antioxidant against ROS and free radicals for its various physiological roles in xenobiotic compounds detoxification. The glutathione redox cycle is a big part of the intracellular hydroperoxide reduction. Glutathione, in reduced form (GSH), converts into GSSG (oxidized glutathione) when disulfide bridge is formed by another glutathione molecule when working with hydrogen peroxides or lipid peroxides in detoxifications of these molecules and GSH-Px enzyme catalyze the reaction [101]. In order to sustain free radical detoxification in the cell, GSSG has to be transformed back to a reduced form GSH. In the presence of NADPH, GSSG is converted back to GSH in a reaction catalyze by glutathione reductase GR [102]. GSH-Px has been reported to be the most powerful antioxidant to inhibit oxidantive stress in erythrocytes and it has essential phagocytic cells functions. A typical mechanism aimed at shielding the cell from peroxidant molecules is glutathione peroxidase, catalase and superoxide dismutase enzyme system [103].

1.7.4. Glutathione Reductase (GSH-Rd)

Reaction catalyze by GSH-Rd: $\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{ GSH} + \text{NADP}^+$.

Glutathione reductase is an enzyme which is found in both prokaryotic and eukaryotic organisms and belongs to the NADPH-dependent oxidoreductase family. The reduction of GSSG to GSH is catalyzed by glutathione reductase a crucial molecule in resisting oxidative stress and preserving the cell's reducing environment. Glutathione is at the center of one of the most effective

antioxidant processes in the cell. In preserving proper function and avoiding oxidative stress in human cells, glutathione plays a crucial role. Glutathione is capable of scavenging reactive oxygen and nitrogen species in its reduced form, thus leading to redox homeostasis regulation [104]. Glutathione reductase carry out an important function by successfully protecting cells from reactive oxygen metabolites. By catalyzing the reduction of GSSG to GSH with the subsequent oxidation of NADPH, the cellular reduced GSH pool is retained [105]. A crucial factor in correctly preserving the oxidative balance of the cell is the ratio of GSSG/GSH contained in the cell which ensures that the cell retains elevated amounts of reduced glutathione and a low level of oxidized glutathione disulfide. Glutathione reductase which catalyzes the decrease of GSSG to GSH, preserves this narrow equilibrium [106].

1.7.5. Peroxidase (POD)

Reaction catalyze by POD: $2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$

Oxidoreductases that catalyze the decrease or reduction of peroxides such as hydrogen peroxide H_2O_2 and the oxidation of a number of organic and inorganic compounds are called peroxidases. Peroxidases carry out there function through a free radical mechanism that convert several compounds in oxidized or polymerized products [107]. The peroxidase prosthetic group consists of a protein-bound heme, usually by way of histidine residue which serves as a proximal ligand. Activation of hydrogen peroxide by heme peroxidase produces high valence intermediate species that have the ability to abstract electrons from various substrates. In particular, the operation of peroxidase requires contributing electrons that attach to other substrates, such as ferricyanides and ascorbate, to transform them into harmless substance [108]. By bioremediating waste water containing phenols, cresols and chlorinated phenols, for biopulping and decolorisation of synthetic clothing azo-dyes, peroxidases have the ability to mitigate environmental contamination [109].

1.8. Stress Biomarkers

The biological state of oxidative stress (OS), as defined by the difference between prooxidant and antioxidant, has several effects, including inflammation, carcinogenesis, neurodegeneration and developmental disorders. O.S.'s critical component is lipid peroxidation. Free radicals in particular and particularly reactive oxygen (ROS) have the ability to attack poly unsaturated fatty acids (PUFAs) cell membrane, producing a family of alpha unsaturated reactive aldehydes, like 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). Emerging information points out their key role in oxidative damage generation. Such molecules, therefore, are to be treated as mediators rather than simply biomarkers of the disease. The presence of these molecules above normal is a very good indication of oxidative stress [110]. A change in the ratio of reduced and oxidized forms

of glutathione in favor of the oxidized form is also a very good indicator of stress condition. Biomarkers aid not only in the diagnosis of illness, but also in the follow-up of relapse, deterioration and post-intervention outcomes [111]. Among the numerous types of stress, endogenous oxidative and inflammatory stresses are the key drivers of various biomarkers leading to changes in the particular cellular systems they reflect [112]. Non-neutralized free radicals and ROS make a lot of damage to the cell. In various forms of stress these ROS and free radicals are scavenged by strong antioxidants in mammalian cells, including glutathione peroxidase and catalase [113]. Extremely reactive unpaired electrons found in ROS and free radicals are produced during oxidative stress and can be used as cellular biomarkers [114]. Some biomarkers assess the degree of damage and act as measures of stress or disease degradation such as MDA. Isoprostanes, likewise others such as anti-oxidant markers, indicate the status of the defense system of the body against changes caused by stress. In animals, multiple biomarkers have been identified as measures of cellular oxidative stress. These include malondialdehyde plasma and serum levels. Isoprostanes, glutathione, and enzymes that catalyze ROS reduction such as superoxide dismutase, catalase, glutathione peroxidase, and thioredoxine reductase [114, 115].

1.8.1. 4-Hydroxynonenal (4-HNE)

4-HNE is the end product of two concurrent methods, namely the production of omega-6 hydro peroxide polyunsaturated fatty acid (PUFA) during lipid peroxidation and the incorporation of the hydroxy group in combination with the breaking of the peroxidised PUFA carbon chain. Only omega-6 PUFAs, such as linoleic acid and arachidonic acid essential fatty acids, play a role in the 4-HNE production. Lipid peroxidation is a strong non-enzymatic chain reaction that continuously produces free lipid radicals for further peroxidation. PUFAs are targeted by free radicals irrespective of whether the fatty acid is in free form (e.g. in circulation, non-covalently bound to albumin) or in phospholipids in the cell membranes [116]. 4-HNE is one of the key products of lipid peroxide toxicity [117]. The rapid interaction with thioles and amino groups can explain 4-HNE's high toxicity [118]. Reactive aldehydes especially 4-HNE act as both signaling molecules for lipid peroxidation and cytotoxicants, causing a prolonged biological effect, in particular by covalent macro molecules modification. 4-HNE is considered one of the second most physiologically active free radical toxic messengers as well as one of the most significant generators of oxidative stress, the chemical aldehyde-based final product of the lipid peroxidation and one of the leading lipid peroxidizing agents [119]. Thus there is no surprise that 4-HNE is now considered to be the principal bioactive indicator of lipid peroxidation and the signaling molecule involved in the regulation of several stress-sensitive transcription factors such as the 2-related factor of nuclear erythroid 2 (NRF) activating protein 1 (AP-1), NF- μ B, and the peroxisome-proliferator-activated

receptor (PPAR) in cell proliferation and differentiation, cell survival, auto-phagy, senescence, apoptosis, and necrosis [120].

1.8.2. Malondialdehyde (MDA)

MDA is a final product produced by arachidonic acid decomposition and larger PUFAs via enzymatic or non-enzymatic processes [121]. Enzymatic MDA generation is a well known process, but its biological roles and potential dose-dependent double function have not been studied. Furthermore, MDA is more chemically active and permeable to the membrane than ROS and less toxic than 4-HNE and methylglyoxal (MG) [122]. On the other hand, the development of MDA through non-enzymatic processes is still less known given its potential therapeutic importance, since this MDA is assumed to derive under stress conditions and has a strong reaction ability with multiple biomolecules such as proteins or DNA leading to adduct formation [123, 124], and excessive output of MDA has been correlated with different pathological conditions [125].

MDA, as a bifunctional electrophilic aldehyde, its reactivity happens to be pH-dependent, and occurs as an enolate ion (conjugate bases with adjacent Carbon-Carbon double bond negative oxygen) with low physiological pH reactivity. However, as pH decreases, MDA exists as β -hydroxy-acrolein and raises its reactivity. It was also suggested that MDA may physiologically react with a lot of nucleosides (deoxy-guanosine and cytidine) to form deoxyguanosine and deoxyadenosine adducts, resulting in pyrimidopurinone. MDA is also a major contributor to the damage and mutation of DNA [126, 127].

1.8.3. Glutathione

A water-soluble tripeptide, Glutathione (GSH) is made up of glutamine, cysteine, and glycine amino acids. GSH consist a thiol group which is a powerful reduction agent that makes GSH the most abundant small intracellular molecule, reaching concentrations of millions of molar in some tissues. GSH plays a role in the detoxification of a number of electrophilic compounds and peroxides through glutathione S-transferases (GST) and glutathione peroxidases (GPx). The widespread usefulness in plants, mammals, fungi and some prokaryotic species is evident in the importance of GSH [128]. Apart from detoxification, GSH also plays a function in other cellular reactions, such as glyoxalase systems, the reduction of ribonucleotide to deoxyribonucleotides, protein control, and gene expression through thiol (i.e disulfide exchange reactions) [128]. The tripeptide may exist occur in an oxidized (GSSG) or reduced (GSH) form intracellularly. Maintaining optimum GSH: GSSG ratios in the cell are crucial to survival, thus requiring tight system control. GSH deficiency causes oxidative damage to the cell. It is not surprising that excess GSH is found in a wide variety of diseases, including cancer, neurodegenerative disorders, cystic

fibrosis, HIV, and aging. In conjunction with catalase and superoxide dismutase (SOD) action, GSH peroxidase (GPx) protects the cell from ROS damage. In the reduction reaction, GPx detoxifies peroxides with GSH serving as an electron donor, creating GSSG as an end product. In a process that requires NADPH, GSSG reduction is catalyzed by GSH reductase (GR). GR is a member of the oxidoreductase family of flavoprotein disulfide and exists as a dimer. GR is controlled at the transcription stage and also through post-translational modifications under conditions of oxidative stress. Changes in expression and activity of GR have been associated with cancer and aging [105].

1.9. Amino Acids

Amino Acids are organic compounds that contain amine ($-NH_2$) and carboxyl ($-COOH$) functional groups, along with a side chain (R) unique from each other in their structure. Although the lateral chains of some amino acids include other elements, carbon, hydrogen, oxygen and nitrogen are the main elements of the amino acid. Approximately 500 amino acids (although only 20 exist in the genetic code) are recognized and can be identified in several ways [129]. Amino acids exert a pivotal function in the synthesis of both protein building blocks and intermediates. A wide variety of chemical flexibility is reflected by the 20 amino acids found in proteins. The exact structure of a given protein's amino acid and the sequence of those amino acids are determined by the sequence of bases in the gene encoding the protein. Another importance of amino acids is that the biological function of a protein is determined by its amino acids chemical properties. Proteins control nearly all cell functions, in addition to catalysing all (or most) of the reactions in living cells. Furthermore, proteins provide the information needed to determine how this protein can fold into a three-dimensional structure and the stability of the resulting structure inside their amino acid sequences. Amino acids take part in a variety of processes, including neurotransmitter transport and biosynthesis, in addition to their function as residues in proteins. Many important amino acids, both protein and non-protein, have biological functions. For example, glutamate (standard glutamic acid) and gamma-amino-butyric acid (non-standard gamma-amino acid) are the key exciting and inhibitory neurotransmitters in the human brain [130], respectively. Hydroxyproline is synthesized from proline, which is an important constituent of the connective tissue collagen. Glycine is a precursor to porphyrin biosynthesis used in red blood cells. Carnitine is used for the transport of lipids. In humans, nine proteinogenic amino acids are considered "necessary" as they cannot be synthesized by the human body from other compounds and must therefore be obtained from food. For certain ages or medical conditions, certain individuals may be conditionally necessary. Essential amino acids between organisms may also vary. Amino acids are necessary especially in nutrition because of their biological significance and are mostly used in nutritional additives, fertilizers and

food technology. Industrial applications include drug manufacturing, bio-degradable plastics, and chiral catalysts [131].

1.9.1. Essential Amino Acids

Necessary amino acids, also known as indispensable amino acids, are a group of amino acids which humans and other vertebrates are unable to synthesize from metabolic intermediates. These amino acids should be provided via an exogenous means since the human body has no mechanism for synthesizing these amino acids [132]. Amino acids are categorized in diet as either essential or non-essential. These characterizations were the result of early human nutrition experiments that revealed the need for specific amino acids for growth or stabilize nitrogen even though there is a sufficient quantity of alternative amino acids. Out of the 20 amino acids, nine are classified as essential amino acids which include; phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, leucine and lysine [133].

1.9.2. Non-Essential Amino Acids

Amino acids that can be omitted from a diet are classified as non-essential. also known as dispensable amino acids and the human body is able to synthesize certain amino acids using only essential amino acids. Alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine are the nonessential amino acids. However, amino acids such as arginine and histidine can be regarded conditionally essential because the body does not synthesize them in adequate amounts during certain physiological processes of development, including pregnancy, juvenile growth or recovery from trauma [134]. Furthermore, studies have indicated that at least 6 of the 11 non-essential amino acids are regarded as conditionally essential since there are physiological and/or pathological conditions where they are needed for nutrition such as phenylketonuria in the inborn error of metabolism where tyrosine can no longer be produced and thus must be ingested [135].

1.9.3. Amino Acids In The Bacteria

Proteins and the cell wall peptidoglycan (PG) are the two major types of amino acid containing polymers in bacteria. Prokaryotic proteins contain 20 specific L-amino acids in different proportions. These proteins have either regulatory or catalytic roles and are involved in bacterial membranes in different binding and transport functions. Many proteins are bacterial exotoxins. The sidechains of polypeptides and peptidoglycan cross-links contain both L-and D-amino acids, including several rare non-protein amino acids. The different type of amino acids found in microorganisms makes their determination and recognition useful for isolated bacteria to be

distinguished and classified. Amino acids can also be beneficial as chemical markers to identify the presence of bacteria without previous cultivation in complex matrices such as mammalian body fluids and tissues. In addition to protein, the bacterial cell wall is a major source of amino acids in bacteria. For chemotaxonomic differentiation of bacteria, the amino acid composition and variations between the peptide compositions of isolated peptidoglycans were used extensively. Bacterial cell walls also contain less common constituents of amino acids (including ornithine, D-D, L-L, and mesodiaminopimelic acid, D-alanine, and D-glutamic acid) that can be used as chemical indicators for bacterial presence [136].

PG is the base of alternating muramic acid and glucosamine units with a side chain of pentapeptides or tetrapeptides. Certain peptides also connect these side chains. The cross-bridge and its connection between bacterial species are quite variable; it can constitute identical sequence of peptides as the side-chain or, alternatively, a completely new peptide. For instance, there are very similar pentapeptide side chains (L-Alanine-D-isoglutamine-L-lysine-D-alanine-D-alanine) in both *Streptococcus pyogenes* and *Staphylococcus aureus*. The bridge crossing in *S. Pyogenes* is a dipeptide of L-alanine; it is a pentapeptide of *S.aureus* [137].

Alternating L-and D-amino acids are the side chains. The first place is usually held by L-alanine, D-glutamic acid position 2 (or one of its equivalents, glutamine or isoglutamine), L-lysine or mesodiaminopimelic acid position 3, and D-alanine position 4 (and position 5 if present). The presence of aromatic amino acids that are seldom found in cell walls (e.g., tryptophan or histidine) can easily detect protein pollutants in isolated cell walls. There are almost exclusively L-amino acids in eukaryote proteins (including yeast) and prokaryotes. The PG layer is multi-lamellar in the Gram positive bacterial cell envelopes, whereas it is a monolayer in Gram negative bacteria. Gram-positive bacteria have a higher total content of D-alanine and D-glutamic acid, and higher concentrations of D-alanine / L-alanine and D-glutamic acid / L-glutamic acid compared with Gram-negative bacteria. Differences in D/L amino acid ratios can easily distinguish both Gram positive and negative bacteria from yeasts. D-amino acids are also rarely found in plants or mammals, so markers of D-amino acids are also useful for bacterial peptidoglycan detection [138]. D-alanine was used in environmental samples and mammalian tissues to detect bacteria. In the year 2000, Guangu and Shao-Min predicted a two and three amino acid sequences of *C. freundii* Beta-lactamase from its amino acid composition. These amino acid sequences that made up the Beta-lactamase provide the bacteria with the ability to resist antibiotics especially those that consist of a Beta-lactam ring such as penicillin and cephalosporin [139].

1.10. Aims and Objectives of This Research Work

The main aims of this research were as follow:

1. To induce stress in *C. freundii* by the use of cadmium in variable concentrations.

2. To determine the changes in the bacterial growth and density with and without cadmium.
3. To determine the levels of fat soluble vitamins and water soluble vitamins with and without cadmium.
4. To determine the level and changes in the concentration of stress biomarkers namely; malondialdehyde (MDA) and 4-hydroxyneonal (4-HNE) with and without cadmium
5. To determine the concentrations of reduced and oxidized glutathione with and without cadmium and also to determine the ratio between reduced and oxidized glutathione (GSH/GSSG).
6. To determine the level of total protein in the bacteria.
7. To determine changes in enzyme activity before and after cadmium addition.
8. To determine the level of amino acids with and without cadmium.

2. MATERIALS AND METHODS

In this study, *C. freundii* was obtained and cultured in Department of Chemical Engineering Faculty of Engineering, Firat University, Elazig, Turkey.

2.1. Equipments and Chemicals

Analytical-grade solvents used for sample preparation were redistilled before use as impurities in the solvent were interfered with the sample peak in the HPLC chromatogram. Solvents for liquid chromatography were of HPLC grade. All solvents used as the mobile phase in HPLC were filtered through a membrane filter system and degassed using an ultrasonic bath. HPLC was performed with the SHIMADZU Prominence-İ LC- 2030C 3D Model and PDA detector. For spectrophotometric readings, a UV-VIS spectrophotometer was used.

2.2. Bacteria processing

2.2.1. Step 1: LB Preparation

We prepared 250 mL LB of 6 groups. Each group is divided into 5 flasks containing 50 mL each. For the 250 mL LB preparation, we used:

Yeast bacto = 1.25g

Peptone = 2.5g

NaCl (sodium chloride) = 2.5g

2.2.2. Step 2: Cadmium Preparation

Cadmium was prepared from cadmium chloride (CdCl_2).

2.2.3. Step 3: Sterilization

For the sterilization process, we first covered each flask with a cotton wool then covered the top with aluminum foil and the media was sterilized in an autoclave at 1.2 bars pressure at 121°C. Then the media was placed in a alcohol cleansed laminar flow cabine and stayed there for 30 minutes under UV light.

2.2.4. Step 4: Inoculation

After cooling down in the laminar flow cabine, 0.5 μL of *C. freundii* was inoculated into each flask.

2.2.5. Step 5: Incubation

For the incubation process, a shaking incubator with a temperature at 37 °C and 150 rpm was used and the incubation continued for 16-18 hours.

2.2.6. Step 6: Spectrophotometric reading of cell density and measurement of pH

The cell density of 16-18 hours incubated cultures were measured with a spectrophotometer set to 600 nm wavelength, using fresh LB as control. To 0.4 mL cultures in plastic tubes 1.6 mL of the centrifuged LB supernatant was added to make a total volume of 2.0 mL.

2.3. Step 7: Analysis of Selected Parameters

Samples from each group were collected, centrifuged and the microorganism were washed twice with distilled water and were used for further experiments.

2.3.1. Sample Preparation for Fat Soluble Vitamins and 4-Hydroxynonenal (4-HNE)

6.0 mL of ethanol was added to each tube, vortexed, and then a total 7.5-minutes ultrasonic treatment was used for cell breakage. After ultrasonic breakage, each sample was divided into 3 tubes (2 mL each), then 1.0 mL of n-hexane was added to each tube and then centrifuged for 6 minutes. The n-hexane phase was extracted and the procedure was repeated twice. The n-hexane phase was taken into the glass tubes and then vacuumed to evaporate the liquid. Then 1.0 mL of methanol was added to each tube and mixed by vortexing, then 1.0 mL of the samples was taken into HPLC vials for HPLC reading. An ODS-2 column (25 cm, 4.6 mm ID, 5 μ m) was used for the determination of vitamin A, E, β -carotene, lycopene and 4-HNE. Methanol-acetonitrile-water mixture (33:63:4 v/v) at a flow rate of 1.0 mL/min was used as a mobile phase. HPLC peaks for vitamin E were measured at 296 nm, vitamin A at 326 nm, β -carotene at 436 nm, lycopene at 460 nm and 4-HNE at 232 nm [140-142].

2.3.2. The Analysis of Water Soluble Vitamins

3.0 mL of distilled water was added to each tube and mixed by vortexing and then the ultrasonic breakage followed. The total time for the ultrasonication was 7.5 minutes, with interruptions at every 1.5 minutes for cooling down the ultrasonicator. After ultrasonic breakage, 1.0 mL of 0.5 M perchloric acid was added to each tube and then mixed by vortexing. The tubes were placed in a sonicator for 5 minutes and then centrifuged for 10 minutes at 6000 rpm. The clear portion was then taken into HPLC vials for HPLC analysis. Vitamin B₁ was measured at 247 nm, B₂ at 269 nm, B₃ at 262 nm, B₅ at 212 nm, B₆ at 291 nm, B₉ at 273 nm, B₁₂ at

361 nm [143, 144] and vitamin C at 244 nm [145]. For vitamin C mobile phase, we used 0.50 grams of potassium dihydrogen phosphate (KH_2PO_4) in 1.0 liter of distilled water, then adjusted the pH to 3.4 by using *o*-phosphoric acid (H_3PO_4). For vitamin B complex mobile phase, we prepared a total 1.0 liter of solution by using 250 mL methanol, 750 mL distilled water, 0.75 grams of heptanes-1-sulfonic acid sodium salt and 0.75 mL tri-ethylamine (TEA). The mixture was adjusted to pH 2.8 by using *o*-phosphoric acid (H_3PO_4).

2.3.3. Sample Preparation for the Analysis of Total Protein and Enzymes

5.0 mL of tris buffer was added to each tube and mixed by vortexing and then ultrasonic breakage was applied as explained in 2.2.9.. After ultrasonic breakage, samples were centrifuged at 6000 rpm for 10 minutes and the supernatants were kept for analysis.

2.3.4. Total Protein Analysis

The total protein analysis was carried out by using Lowry protein determination assay [146]. The assay was carried out by diluting the LB to 1.0 mL with H_2O and adding 0.9 mL of solution A (2.0 g/L potassium sodium tartrate ($\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$) and 100 g/L sodium carbonate (Na_2CO_3) in 0.5 M NaOH) before incubation for 10 minutes at 50°C. Following this, the samples were cooled down to room temperature and then 1 mL of solution B (0.2 g/L $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$) and 0.1 g/L copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 0.1 M NaOH) was added and left for 10 minutes. Finally, 3.0 mL of solution C (Folin–Ciocalteu phenol reagent in H_2O (1:16 v/v)) was added before incubation for 10 minutes at 50 °C. A standard curve was made from bovine serum albumin (BSA). Six test tubes were prepared from the BSA stock (0.25 mg/mL).; 0, 20, 100, 200, 500, 1000 μL was added and completed to a total volume 1.0 mL with saline, tubes having the protein concentrations of 0; 0.005; 0.025; 0.05; 0.125, respectively. Five mL of C solution was added to each tube containing 1.0 mL of bacteria samples and standard solutions, the volume of which was completed to 1.0 mL. After 10 minutes at room temperature, 0.5 mL of a 1:1 diluted Folin-Ciocalteu solution was added to each tube. It was kept at room temperature for 30 minutes and the absorbances were read against the blank at 750 nm. The absorbance values were plotted against the concentrations and protein contents of the samples were calculated using the standard protein curve with the same method .

2.3.5. Glutathione Peroxidase Assay

GSH-Px activity was measured by reading the difference in absorbance at 340 nm during the oxidation of NADPH to NADP. For glutathione peroxidase activity determination, phosphate buffer is used. We prepared 50 mM phosphate buffer of 100 mL volume (60 mL A + 40 mL B +

0.208 g EDTA. A= 426 mg Na₂HPO₄ and B= 275 mg KH₂PO₄) and adjust the pH to 7.5. We prepared 3 mM of NADPH (10 mg NADPH dissolved in 2.0 mL of buffer). We prepared 150 mM GSH (50 mg GSH was dissolved in 1.0 mL buffer). 1M NaN₃ was also prepared (65 mg sodium azide was dissolved in 1.0 mL buffer). 50 mM of H₂O₂ was also prepared (15 µL of H₂O₂ was taken and the volume was completed with 5.0 mL of buffer). For sample tube, we added 2.66 mL phosphate buffer + 0.1 mL GSH + 0.1 mL NADPH + 0.01 mL GSH-Rd + 0.01 mL NaN₃ + 0.02 mL sample. However, the procedure is the same for blank except the addition of sample. The tubes were then mixed and incubated for 30 minutes at room temperature of 25°C. After 30 minutes of incubation, 0.1 mL of H₂O₂ was added and absorbance was taken at 340 nm and the second absorbance was taken after 30s [147].

2.3.6. Glutathione Reductase Assay

Glutathione reductase catalyzes the reduction of GSSG by NADPH to GSH. Enzyme activity is determined by the absorbance difference of NADPH oxidized during the reaction at 37 °C at 340 nm wavelength. 1.0 M Tris buffer at pH: 8.0 was prepared containing Tris acid 8.8 g, Trisbase 5.4 g and EDTA 0.14 g completed to 100 mL with distilled water. 10.0 mM FAD was also prepared (For the 100 mM stock FAD solution, 0.8 mg FAD was prepared in 10 mL distilled water. This stock FAD solution was diluted 1:10 to prepare 10 mM FAD). 0.033 M GSSG was prepared (210 mg of GSSG was dissolved in 10 mL of distilled water). 2mM of NADPH was also prepared (17 mg of NADPH was dissolved in 10 mL of distilled water). In order to assay GSH-Rd activity, we added 50 µL of tris buffer + 100 µL of sample + 100 µL of FAD then we incubated for 10 minutes at 37°C then we added 100 µL GSSG and we incubated again for 10 minutes at 37°C then we finally added 50 µL NADPH and then we read absorbance at 340 nm and second absorbance was taken after 30s [148].

2.3.7. Catalase Assay

Catalase activity was determined using the method described by Aeibi [149]. We prepared 50mM of phosphate buffer of 100 mL volume (60 mL A + 40 mL B + 0.208 g EDTA. A= 426 mg Na₂HPO₄ and B= 275 mg KH₂PO₄) and adjust the pH to 6.8. We also prepared 50 mL of 1M HCL (4.1 mL of 37% HCL was taken and filled to the 50 mL mark with distilled water). For catalase activity determination, 2.45 mL of phosphate buffer and 50 µL of sample was mixed and incubated for 2 minutes at 37°C then we added 0.5 mL HCL and we read at absorbance at 240 nm.

2.3.8. Superoxide Dismutase

Superoxide dismutase activity was determined by using the pyrogallol autoxidation method [150]. For this assay, tris buffer was used. 0.05 M of tris buffer was prepared (pH 8.5). 20 mL of 10 mM pyrogallol was also prepared (we took 25.2 mg of pyrogallol and added 1.0 mL of 0.1 M HCL then we complete the volume to 20 mL with distilled water). In order to assay SOD activity, we mixed 2.8 mL of tris buffer + 100 μ L pyrogallol + 150 μ L sample then we read the absorbance at 420 nm. The second absorbance was taken after 30s.

2.3.9. Peroxidase Activity

Peroxidase activity was carried out by using the Pyrogallol oxidation method [151]. 50 mM of 100 mL phosphate buffer was prepared (0.681g KH_2PO_4 and 0.71g Na_2HPO_4) and the pH was adjusted to 6.8. The reaction was carried out by mixing 2.0 mL of 50 mM phosphate buffer, 1.0 mL of 10mM pyrogallol, 0.5 mL enzyme and 1.0 mL of 5.0 mM H_2O_2 . The reaction mixture was incubated at 25 $^\circ\text{C}$ for 5 minutes and the enzymatic reaction was terminated with 1.0 mL of 1.25 N H_2SO_4 . The increasing of absorbance at 420 nm was then recorded.

2.3.10. Sample Preparation for the Analysis of Stress Biomarkers

3.0 mL of distilled water was added to each tube and mixed by vortexing and then ultrasonic breakage was carried out. Each sample was exposed to ultrasonic breakage as given in 2.2.9. After ultrasonic treatment 1.0 mL of 0.5 M perchloric acid was added to each tube and then mixed briefly with a vortex. The tubes were placed in a sonicator for 5 minutes and then centrifuged for 10 minutes at 6000 rpm. The supernatant was then taken into HPLC vials for HPLC analysis. Column Exsil 100-5 ODS at flow rate of 1.0 mL/min (25 cm, 4.6 mm ID, 5 μm) was used. HPLC peaks for GSH and GSSG were measured at 210 nm [152] while that of MDA was measured at 254 nm [153].

2.3.11. Amino Acid Analysis: Derivatization

For the amino acid analysis, both standard and hydrolyzed samples were neutralized by the addition of 20.0 μL of a 2:2:1 mixture of ethanol: water: TEA (v/v) then followed by vortex mixing. The samples were then dried in a vacuum. The derivatization process was carried out by adding 20 μL of a 7:1:1:1 mixture of ethanol: water: TEA: PITC (v/v) then followed by vortexing. The reaction between PITC and the hydrolysate leads to the formation of phenylthiocarbamyl (PTC) amino acids complex which was allowed to complete for 20 minutes at room temperature. Samples were dried again completely in a vacuum then stored in a freezer for analysis [154].

2.3.12. Chromatographic Analysis of Amino Acids

The column temperature was maintained at 40°C. The PTC amino acids were separated and eluted by a gradient resulting from mixing eluents A 0.07 M CH₃COONa.3H₂O (pH 6.4 with H₃PO₄) and B 6:4 acetonitrile: water (v/v). Both eluents were sparged with ultra-pure helium gas for 10 minutes before use. The flow rate was 0.8 mL/min throughout and the gradient consisted of the following profiles as shown in Table 2.1. The PTC amino acids eluted from column were detected at 254 nm. The column was regenerated and equilibrated with eluent B for 10 minutes. A new and freshly reconstituted sample was injected, either by manual injector or auto sampler/injector, and analyzed every 35 minutes [154, 155].

Table 2.1. Gradient program used for the separation of Phenylthiocarbamyl-amino acids

Time (minute)	Flow rate (mL/min)	% Eluent A	% Eluent B
0.01	0.8	90	10
12.00	0.8	70	30
16.00	0.8	65	35
16.01	0.8	50	50
25.00	0.8	100	0
30.00	0.8	50	50
30.01	0.8	10	90
35.00	0.8	10	90

2.3.13. Statistical Analysis

All experiments were carried out in triplicates. The mean standard deviation was calculated and the findings were subjected to the Variance Analysis. The SPSS 10.0 for Windows has been used to evaluate variation and LSD has been done in several comparisons at P<0.05. All data are expressed as ± standard error of the mean (SEM).

3. RESULTS

3.1. *C. freundii* Optimum Growth Condition

The most suitable temperature for *C. freundii* was found to be 37°C and it revealed maximum growth at pH 8 after 18 hours incubation (Figure 3.1).

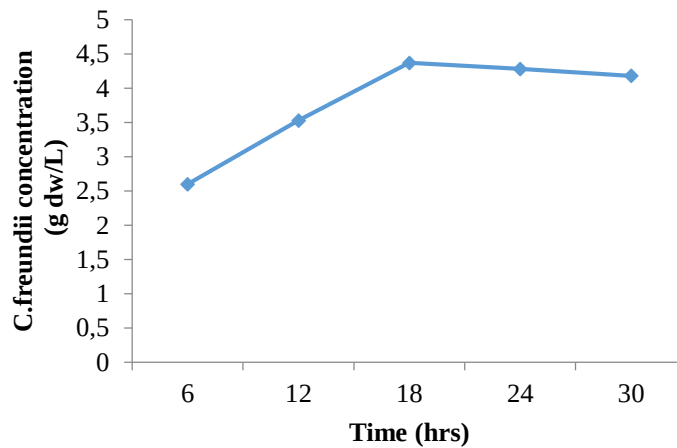


Figure 3.1. *C. freundii* concentration at different time interval.

3.2. Changes in *C. freundii* Concentration Under Cadmium Stress

The effect of cadmium on the growth of *C. freundii* was monitored by growing the bacterium in the presence of 10, 20, 40, 60, 75, 100 and 125 ppm cadmium or in the absence of cadmium (which served as control). The presence of cadmium significantly decreased the growth of the bacterium (Table 3.1 and Figure 3.2).

Table 3.1. *C. freundii* concentration at different Cadmium concentration

Cadmium (ppm)	<i>C. freundii</i> (g dw /L)
0 (control)	4.37 ± 0.04
10	4.34 ± 0.03
20	4.18 ± 0.01
40	4.08 ± 0.10
60	3.86 ± 0.01
75	3.55 ± 0.05
100	2.60 ± 0.08
125	2.38 ± 0.13

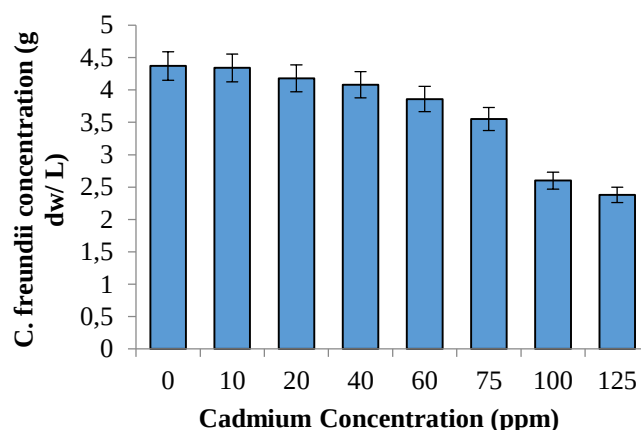


Figure 3.2. *C. freundii* concentrations at varying cadmium concentration

3.3. Vitamin C Effect on the Growth of *C. freundii* Under Cadmium Stress

Vitamin C is a strong antioxidant that has the ability to protect organisms from oxidative stress. Several studies have indicated a positive effect of vitamin C in abolishing the toxic effect of cadmium [156]. The table 3.2 and figure 3.3 shows the results of vitamin C effect on the growth of *C. freundii* under cadmium stress.

Table 3.2: Results showing the changes in growth pattern of *C. freundii* under cadmium stress after addition of 25, 50 and 75 ppm vitamin C

Treatment	<i>C. freundii</i> (g dw /L)
Control	4.37 ± 0.04
0 ppm Cd + 25 ppm Vit C	4.64 ± 0.01
0ppm Cd + 50 ppm Vit C	4.77 ± 0.03
0ppm Cd + 75 ppm Vit C	4.82 ± 0.02
75ppm Cd	3.55 ± 0.05
75ppm Cd + 25 ppm Vit C	3.88 ± 0.02
75ppm Cd + 50 ppm Vit C	4.10 ± 0.03
75 ppm Cd + 75 ppm Vit C	4.30 ± 0.06
100 ppm Cd	2.60 ± 0.08
100 ppm Cd + 25 ppm Vit C	2.70 ± 0.06
100 ppm Cd + 50 ppm Vit C	2.80 ± 0.01
100 ppm Cd + 75 ppm Vit C	3.04 ± 0.09
125 ppm Cd	2.38 ± 0.13
125ppm Cd + 25 ppm Vit C	2.65 ± 0.01
125 ppm Cd + 50 ppm Vit C	2.75 ± 0.01
125 ppm Cd + 75 ppm Vit C	3.00 ± 0.01

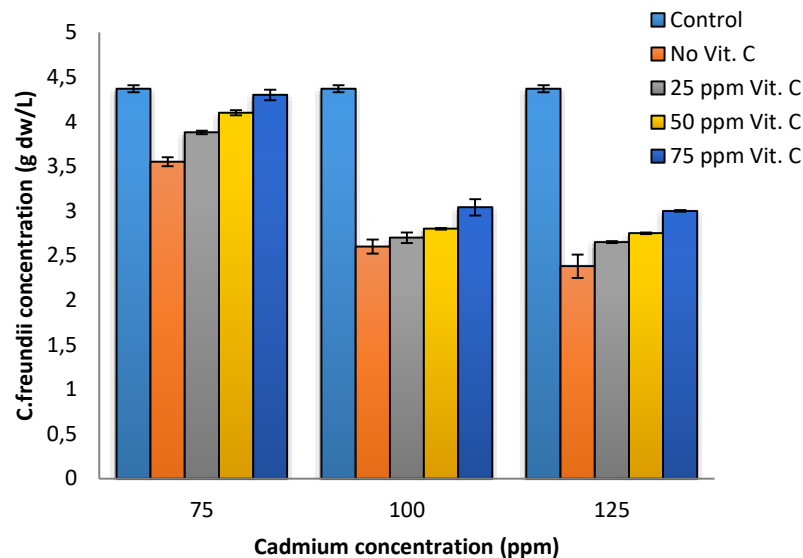


Figure 3.3. *C. freundii* concentrations at varying cadmium concentration with addition of 25, 50 and 75 ppm vitamin C.

3.4. Effect of Cadmium and Vitamin C on Fat Soluble Vitamins in *C. freundii*

For effective functioning of all organisms, fat soluble vitamins are very vital. The table 3.3 and figures 3.4 - 3.7 shows the level of fat soluble vitamins in *C. freundii* at different cadmium concentrations.

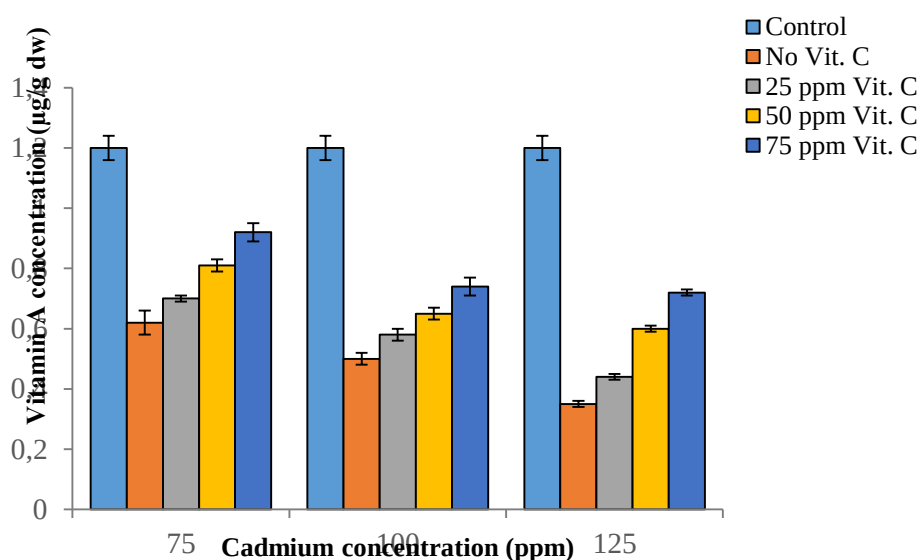
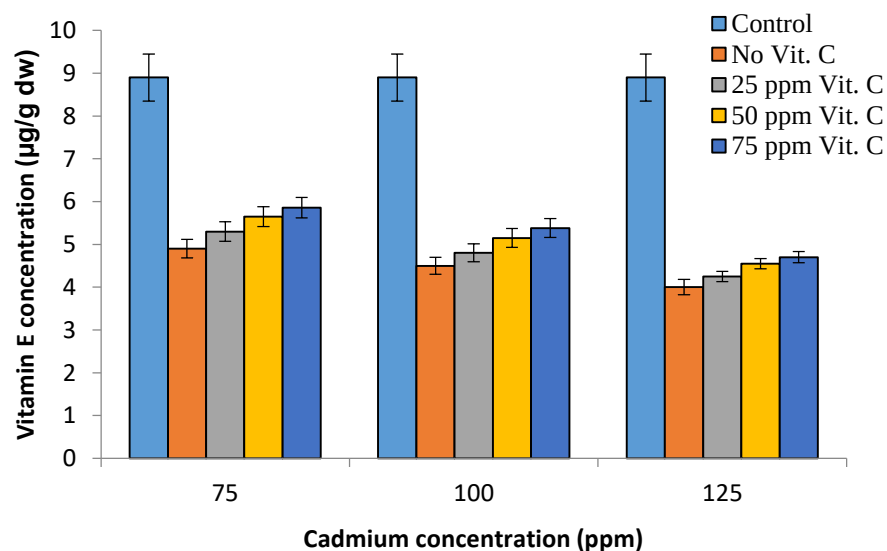


Figure 3.4. Effect of cadmium and vitamin C on the level of vitamin A in *C. freundii*.

Table 3.3: Effect of cadmium and vitamin C on Fat soluble vitamins levels ($\mu\text{g/g dw}$) in *C. freundii*

	Vitamin A	Vitamin E	β -carotene	Lycopene
Control	1.20 ± 0.04	8.90 ± 0.55	0.85 ± 0.06	0.82 ± 0.05
10 ppm Cd	1.10 ± 0.06	8.00 ± 0.50	0.73 ± 0.05	0.75 ± 0.04
20 ppm Cd	1.00 ± 0.07	7.00 ± 0.45	0.65 ± 0.04	0.70 ± 0.04
40 ppm Cd	0.80 ± 0.06	6.00 ± 0.40	0.60 ± 0.04	0.65 ± 0.03
60 ppm Cd	0.70 ± 0.05	5.20 ± 0.30	0.56 ± 0.03	0.60 ± 0.03
75 ppm Cd	0.62 ± 0.04	4.90 ± 0.22	0.50 ± 0.03	0.56 ± 0.02
75 ppm Cd + 25 ppm C	0.70 ± 0.01	5.30 ± 0.23	0.53 ± 0.02	0.59 ± 0.02
75 ppm Cd + 50 ppm C	0.81 ± 0.02	5.65 ± 0.23	0.57 ± 0.02	0.62 ± 0.03
75 ppm Cd + 75 ppm C	0.92 ± 0.03	5.86 ± 0.24	0.61 ± 0.03	0.65 ± 0.03
100 ppm Cd	0.50 ± 0.02	4.50 ± 0.20	0.47 ± 0.02	0.50 ± 0.02
100 ppm Cd + 25 ppm C	0.58 ± 0.02	4.80 ± 0.21	0.50 ± 0.02	0.53 ± 0.02
100 ppm Cd + 50 ppm C	0.65 ± 0.02	5.15 ± 0.22	0.54 ± 0.02	0.56 ± 0.02
100 ppm Cd + 75 ppm C	0.74 ± 0.03	5.38 ± 0.22	0.58 ± 0.02	0.59 ± 0.03
125 ppm Cd	0.35 ± 0.01	4.00 ± 0.18	0.43 ± 0.02	0.45 ± 0.01
125 ppm Cd + 25 ppm C	0.44 ± 0.01	4.25 ± 0.12	0.46 ± 0.02	0.47 ± 0.02
125 ppm Cd + 50 ppm C	0.60 ± 0.01	4.55 ± 0.12	0.50 ± 0.03	0.50 ± 0.02
125 ppm Cd + 75 ppm C	0.72 ± 0.02	4.70 ± 0.13	0.55 ± 0.02	0.55 ± 0.03

**Figure 3.5.** Effect of cadmium and vitamin C on the level of vitamin E in *C. freundii*.

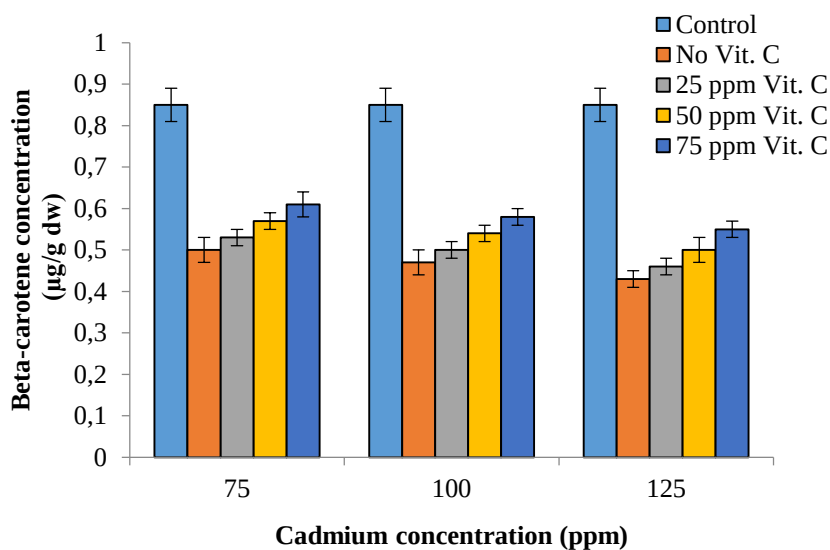


Figure 3.6.Effect of cadmium and vitamin C on the level of beta-carotene in *C. freundii*.

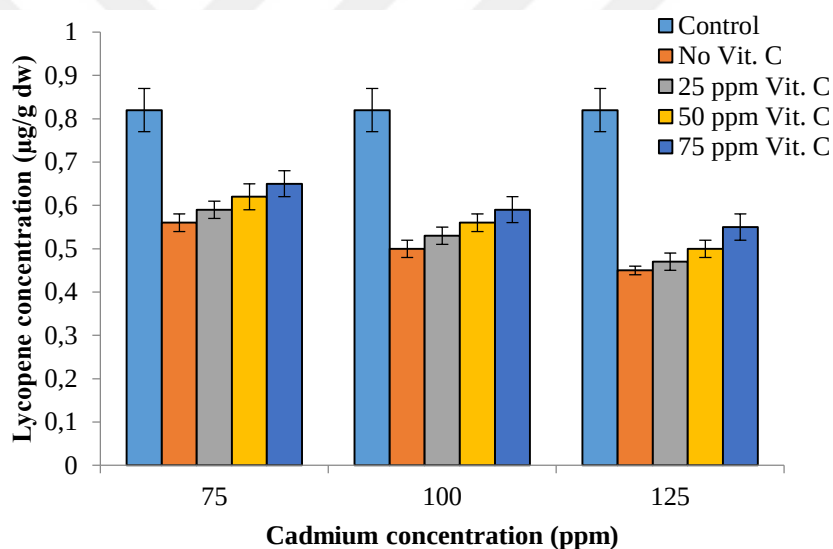


Figure 3.7. Effect of cadmium and vitamin C on the level of lycopene in *C. freundii*.

3.5. Effect of Cadmium and Vitamin C on Water Soluble Vitamins in *C. freundii*

Vitamins are important micronutrients that are often precursors to enzymes that all living cells require for biochemical reactions. Water soluble vitamins serves as coenzymes that typically transport specific chemical groups [157]. The table 3.4 and figures 3.8 – 3.15 shows the level of water soluble vitamins in *C. freundii* at different concentrations of cadmium.

Table 3.4: Effect of cadmium and vitamin C on the level of water soluble vitamins ($\mu\text{g/g dw}$) in *C. freundii*

Treatment	Vitamin C	Vitamin B ₁	Vitamin B ₂	Vitamin B ₃	Vitamin B ₅	Vitamin B ₆	Vitamin B ₉	Vitamin B ₁₂
Control	31.30 $\pm$ 2.00	16.00 $\pm$ 1.00	12.00 $\pm$ 0.80	262.00 $\pm$ 12.00	170.00 $\pm$ 8.00	320.00 $\pm$ 15.00	3.60 $\pm$ 0.20	2.00 $\pm$ 0.08
10 ppm Cd	25.20 $\pm$ 1.60	13.00 $\pm$ 0.80	10.20 $\pm$ 0.70	232.00 $\pm$ 11.00	155.00 $\pm$ 6.50	300.00 $\pm$ 14.00	3.00 $\pm$ 0.14	1.80 $\pm$ 0.07
20 ppm Cd	14.73 $\pm$ 1.10	10.80 $\pm$ 0.60	8.60 $\pm$ 0.60	205.00 $\pm$ 9.50	142.00 $\pm$ 6.00	285.00 $\pm$ 13.00	2.70 $\pm$ 0.12	1.65 $\pm$ 0.06
40 ppm Cd	11.50 $\pm$ 0.80	8.70 $\pm$ 0.45	7.20 $\pm$ 0.55	175.00 $\pm$ 8.00	130.00 $\pm$ 5.00	260.00 $\pm$ 12.00	2.50 $\pm$ 0.10	1.50 $\pm$ 0.06
60 ppm Cd	9.00 $\pm$ 0.60	7.00 $\pm$ 0.40	5.50 $\pm$ 0.48	156.00 $\pm$ 7.30	120.00 $\pm$ 4.50	225.00 $\pm$ 11.20	2.20 $\pm$ 0.09	1.35 $\pm$ 0.04
75 ppm Cd	7.00 $\pm$ 0.50	5.40 $\pm$ 0.35	4.00 $\pm$ 0.32	130.00 $\pm$ 7.00	110.00 $\pm$ 4.30	210.00 $\pm$ 10.30	2.00 $\pm$ 0.08	1.10 $\pm$ 0.03
75 ppm Cd + 25 ppm C	8.50 $\pm$ 0.47	6.40 $\pm$ 0.30	5.00 $\pm$ 0.31	152.00 $\pm$ 6.50	125.00 $\pm$ 4.20	227.00 $\pm$ 9.50	2.50 $\pm$ 0.11	1.30 $\pm$ 0.03
75 ppm Cd + 50 ppm C	10.00 $\pm$ 0.54	7.30 $\pm$ 0.40	5.80 $\pm$ 0.33	161.00 $\pm$ 6.70	134.00 $\pm$ 4.40	238.00 $\pm$ 10.00	3.00 $\pm$ 0.13	1.43 $\pm$ 0.04
75 ppm Cd + 75 ppm C	12.00 $\pm$ 0.68	8.60 $\pm$ 0.44	6.70 $\pm$ 0.35	172.00 $\pm$ 6.80	145.00 $\pm$ 4.80	247.00 $\pm$ 10.10	3.60 $\pm$ 0.15	1.51 $\pm$ 0.04
100 ppm Cd	4.50 $\pm$ 0.30	3.70 $\pm$ 0.27	3.00 $\pm$ 0.20	110.00 $\pm$ 6.60	100.00 $\pm$ 4.10	195.00 $\pm$ 9.60	1.70 $\pm$ 0.07	0.90 $\pm$ 0.02
100 ppm Cd + 25 ppm C	6.50 $\pm$ 0.41	4.50 $\pm$ 0.20	3.50 $\pm$ 0.10	135.00 $\pm$ 4.50	117.00 $\pm$ 4.10	207.00 $\pm$ 8.50	2.00 $\pm$ 0.09	1.00 $\pm$ 0.02
100 ppm Cd + 50 ppm C	8.00 $\pm$ 0.52	5.30 $\pm$ 0.22	4.20 $\pm$ 0.14	144.00 $\pm$ 4.60	126.00 $\pm$ 4.30	216.00 $\pm$ 8.70	2.25 $\pm$ 0.10	1.10 $\pm$ 0.03
100 ppm Cd + 75 ppm C	10.00 $\pm$ 0.63	6.00 $\pm$ 0.26	5.00 $\pm$ 0.22	155.00 $\pm$ 4.90	136.00 $\pm$ 4.50	225.00 $\pm$ 8.90	2.50 $\pm$ 0.11	1.22 $\pm$ 0.04
125ppm Cd	2.50 $\pm$ 0.20	2.80 $\pm$ 0.18	1.00 $\pm$ 0.06	90.00 $\pm$ 6.10	80.00 $\pm$ 3.90	170.00 $\pm$ 9.00	1.45 $\pm$ 0.05	0.75 $\pm$ 0.01
125 ppm Cd + 25 ppm C	3.50 $\pm$ 0.16	3.80 $\pm$ 0.14	1.30 $\pm$ 0.08	115.00 $\pm$ 4.20	92.00 $\pm$ 3.80	182.00 $\pm$ 8.10	1.65 $\pm$ 0.06	0.85 $\pm$ 0.02
125 ppm Cd + 50 ppm C	5.00 $\pm$ 0.24	4.90 $\pm$ 0.19	1.60 $\pm$ 0.09	132.00 $\pm$ 4.60	101.00 $\pm$ 4.00	191.00 $\pm$ 8.30	1.78 $\pm$ 0.07	0.92 $\pm$ 0.02
125 ppm Cd + 75 ppm C	6.50 $\pm$ 0.28	5.80 $\pm$ 0.23	2.00 $\pm$ 0.10	147.00 $\pm$ 4.80	110.00 $\pm$ 4.40	202.00 $\pm$ 8.40	1.86 $\pm$ 0.08	1.10 $\pm$ 0.03

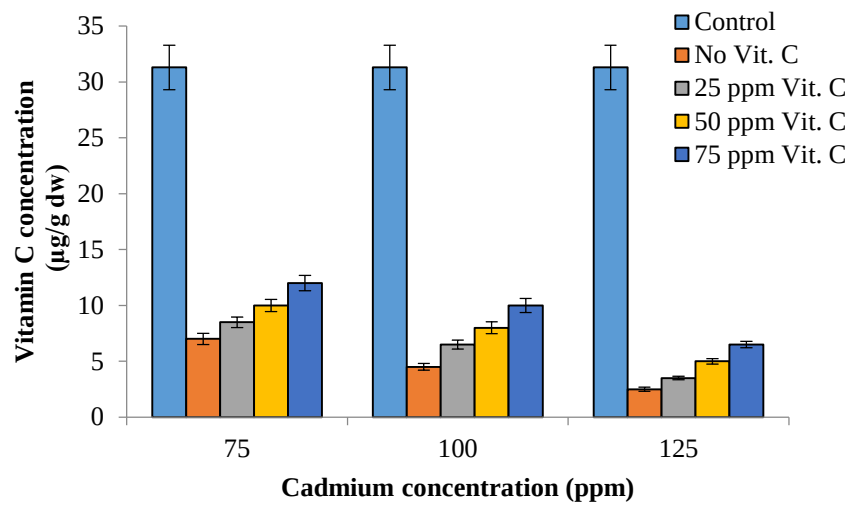


Figure 3.8. Effect of cadmium and vitamin C on the level of vitamin C in *C. freundii*

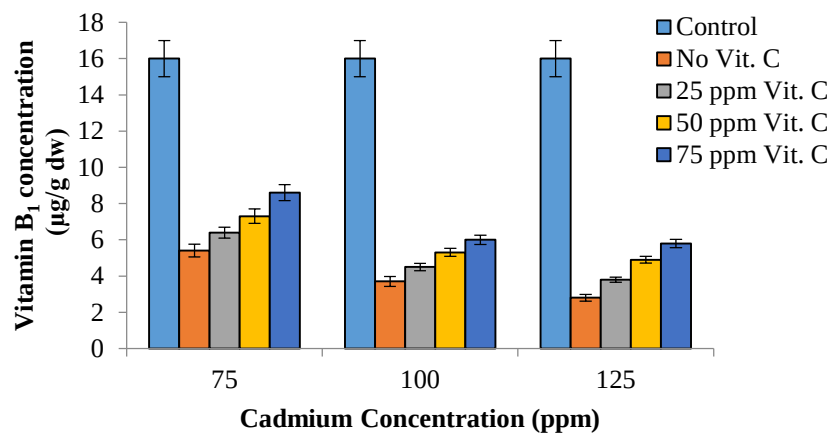


Figure 3.9. Effect of cadmium and vitamin C on the level of vitamin B₁ in *C. freundii*

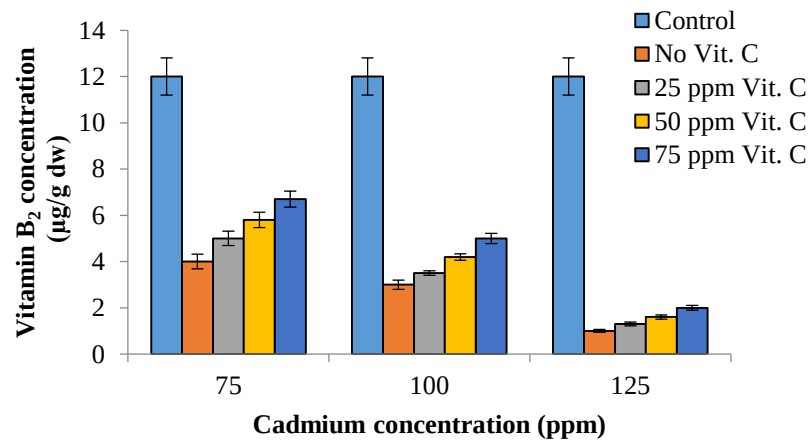


Figure 3.10. Effect of cadmium and vitamin C on the level of vitamin B₂ in *C. freundii*

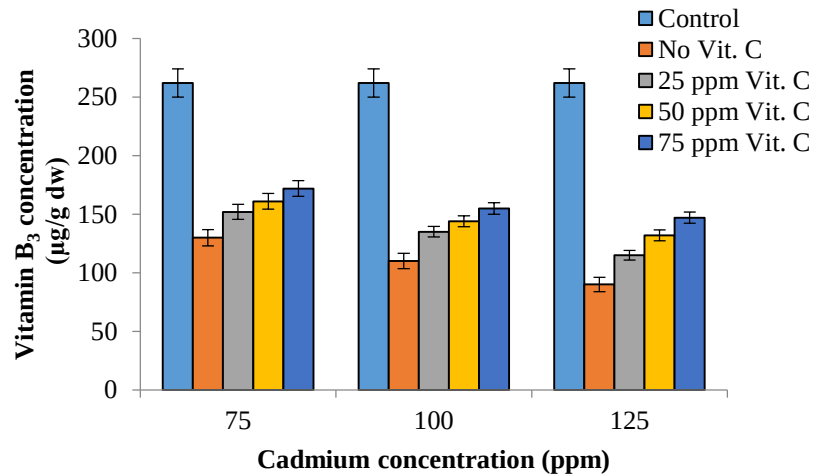


Figure 3.11. Effect of cadmium and vitamin C on the level of vitamin B₃ in *C. freundii*

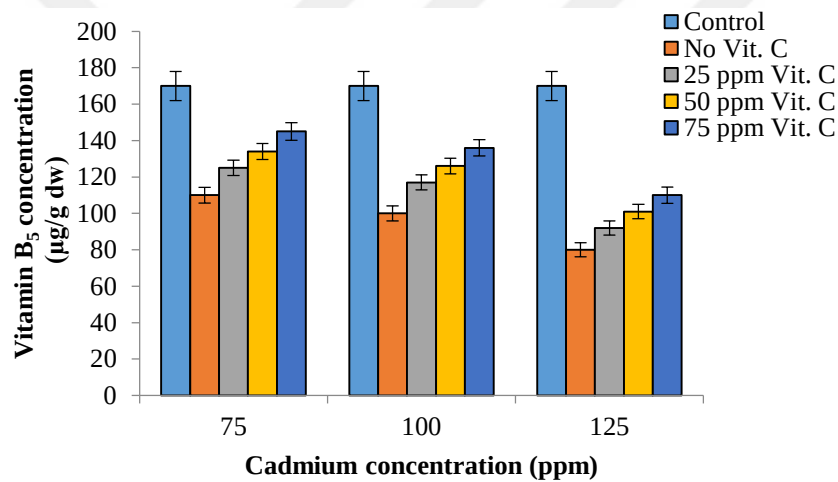


Figure 3.12. Effect of cadmium and vitamin C on the level of vitamin B₅ in *C. freundii*

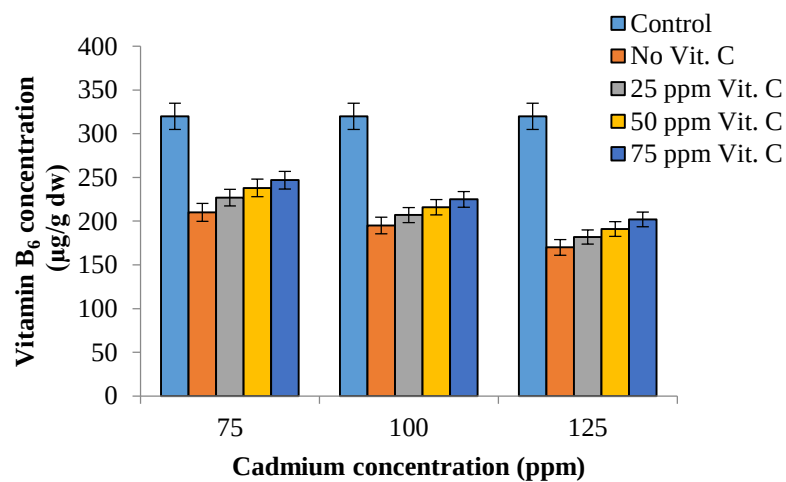


Figure 3.13. Effect of cadmium and vitamin C on the level of vitamin B₆ in *C. freundii*

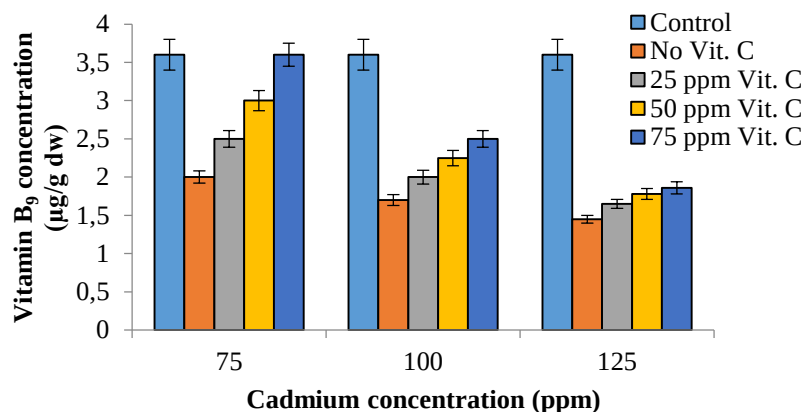


Figure 3.14. Effect of cadmium and vitamin C on the level of vitamin B₉ in *C. freundii*

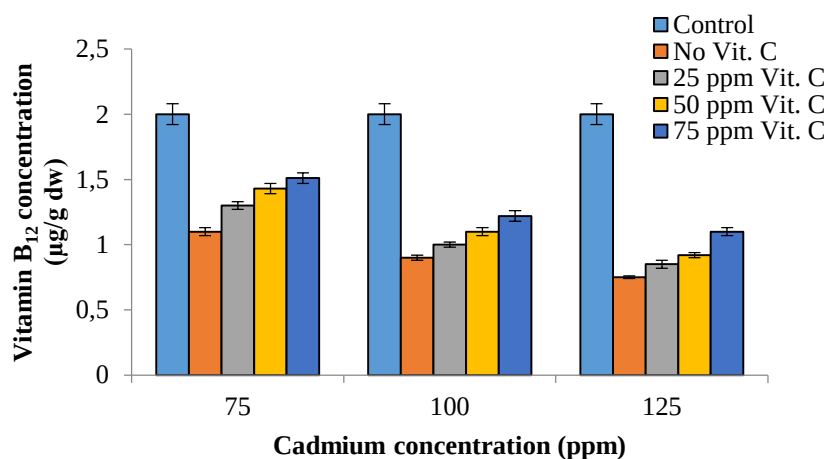


Figure 3.15. Effect of cadmium and vitamin C on the level of vitamin B₁₂ in *C. freundii*

3.6. Effect of Cadmium and Vitamin C on Total Protein and Enzymes Activity in *C. freundii*

The table 3.5 and figures 3.16 – 3.21 shows the amount of total protein and enzymes activity in *C. freundii* after adding varying concentrations of cadmium and vitamin C.

3.7. Effect of Cadmium and Vitamin C on Stress Biomarkers in *C. freundii*

Stress biomarkers are identified as molecules that are altered through association with ROS in the microenvironment and also with molecules of the antioxidant system that subsequently change in response to increased redox stress[114]. Stress biomarkers are important in disease evaluation and also in health-promoting outcome of antioxidants[158]. The table 3.6 and figures 3.22 – 3.26 shows the level of stress biomarkers in *C. freundii* at different cadmium concentrations.

Table 3.5. Total protein and enzymes activity results in *C. freundii* after addition of cadmium and vitamin C.

Treatment	Total Protein mg protein/g, dw	SOD U/g, dw	GSH-Px U/g, dw	GSH-Red U/g, dw	POD U/g, dw	CAT U/g, dw
Control (0 ppm Cd)	23.77±1.25	82.27±4.89	22.89±1.20	14.72±0.98	39.75±1.92	174.53±11.20
0 ppm Cd + 25 ppm C vit	24.06±1.27	80.00±4.58	22.49±1.12	14.04±0.92	38.65±1.80	168.25±11.06
0 ppm Cd + 50 ppm C vit	27.88±1.30	78.00±4.24	20.02±1.06	13.64±0.76	35.76±1.73	160.48±10.90
0 ppm Cd + 75 ppm C vit	29.33±1.34	70.00±4.17	17.03±0.98	12.88±0.72	32.87±1.65	152.43±10.60
60 ppm Cd	18.12±1.13	120.40±6.16	42.19±2.05	26.47±1.35	81.93±4.52	223.99±12.80
60 ppm Cd+ 25 ppm C vit	19.98±1.17	110.56±5.78	36.12±1.52	22.27±1.20	67.86±3.25	205.94±11.88
60 ppm Cd + 50 ppm C vit	21.32±1.20	101.60±5.10	31.87±1.40	20.16±1.14	61.02±3.02	190.65±11.20
60 ppm Cd + 75 ppm C vit	22.20±1.24	93.72±4.94	28.90±1.35	19.06±1.10	56.53±2.91	172.33±10.75
75 ppm Cd	13.26±0.92	135.60±6.84	75.66±3.95	38.08±1.82	101.46±5.06	235.58±13.10
75 ppm Cd + 25 ppm C vit	14.48±0.94	125.25±6.20	67.05±3.30	34.10±1.24	91.88±4.82	216.95±12.80
75 ppm Cd + 50 ppm C vit	16.41±0.97	117.20±5.79	60.85±3.10	30.01±1.26	80.34±4.50	202.62±11.70
75 ppm Cd + 75 ppm C vit	17.10±0.95	107.36±5.46	52.40±3.01	27.52±1.32	72.24±4.10	188.05±11.30
100 ppm Cd	8.99±0.62	150.0±8.50	108.63±5.29	54.50±2.45	122.22±5.73	250.12±13.85
100 ppm Cd + 25 ppm C vit	10.37±0.68	140.40±8.10	98.42±5.26	48.95±2.38	105.63±5.15	223.86±12.95
100 ppm Cd + 50 ppm C vit	12.00±0.75	129.62±6.33	87.25±4.78	43.70±2.12	95.15±4.80	207.52±12.00
100 ppm Cd + 75 ppm C vit	13.41±0.77	118.81±6.05	79.70±4.49	38.64±1.86	88.15±4.65	192.13±11.48

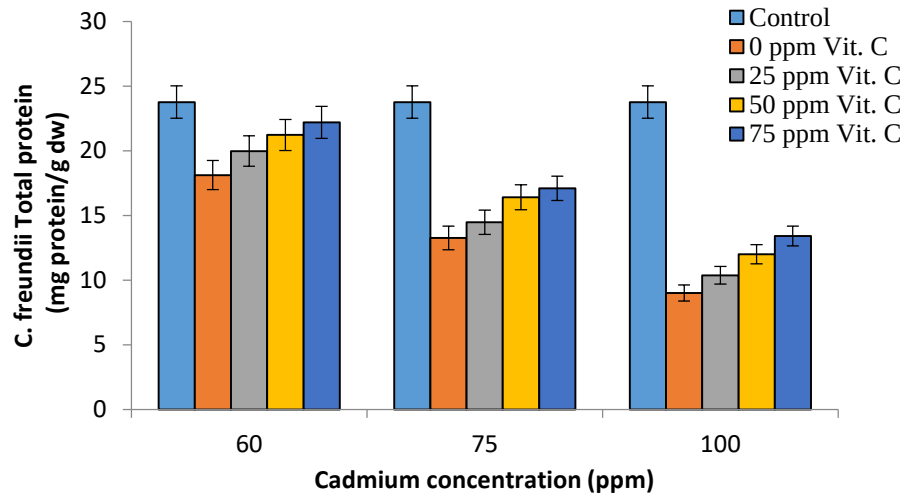


Figure 3.16. Effect of cadmium and vitamin C on total protein concentration in *C. freundii*

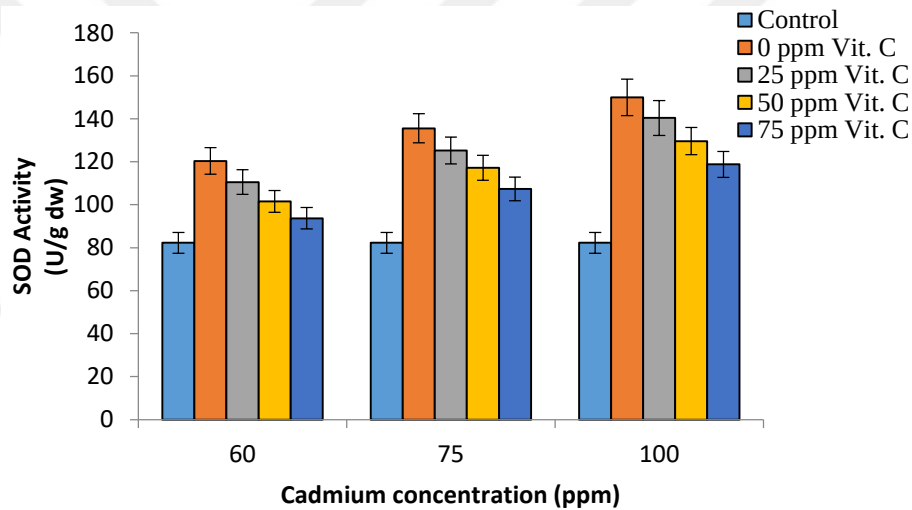


Figure 3.17. Effect of cadmium and vitamin C on SOD activity in *C. freundii*

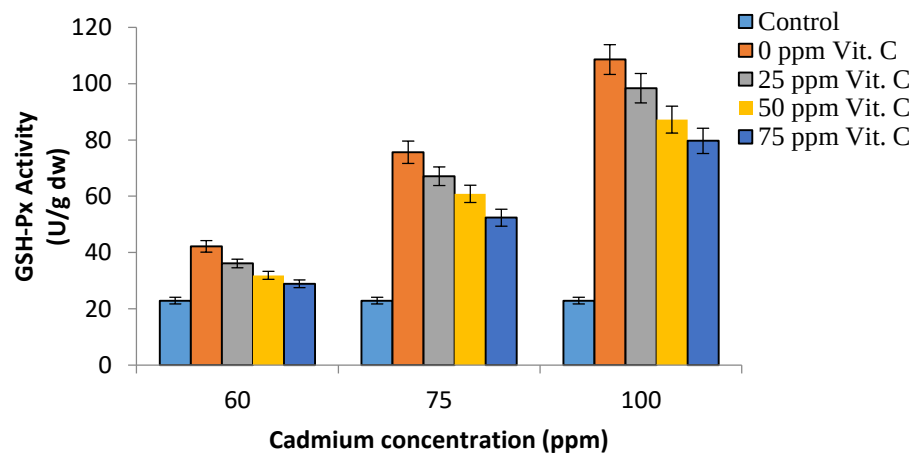


Figure 3.18. Effect of cadmium and vitamin C on GSH-Px activity in *C. freundii*

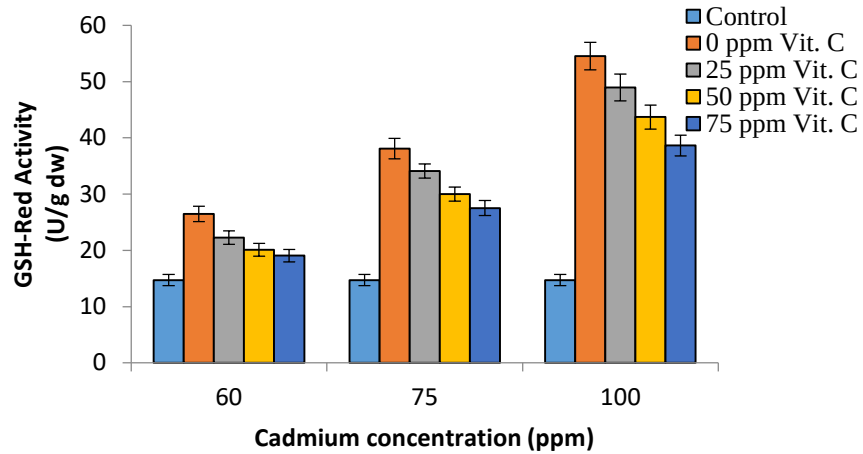


Figure 3.19. Effect of cadmium and vitamin C on GSH-Red activity in *C. freundii*

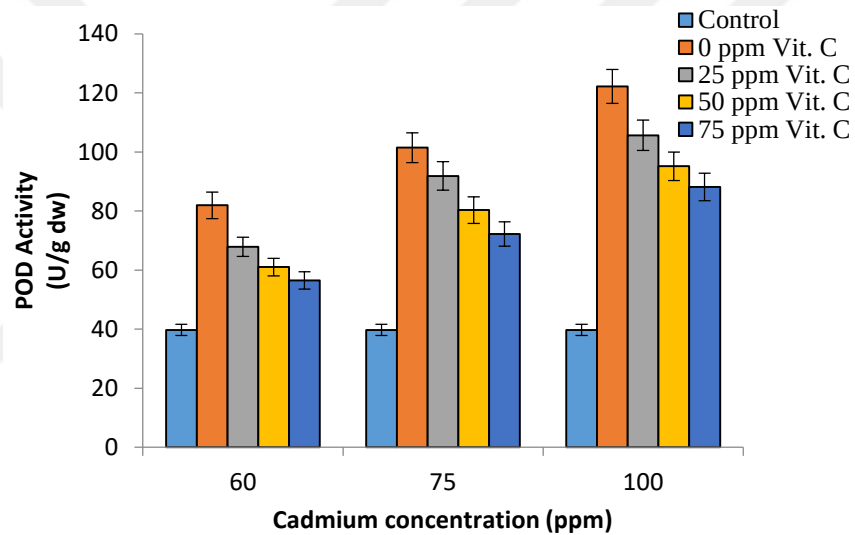


Figure 3.20. Effect of cadmium and vitamin C on POD activity in *C. freundii*

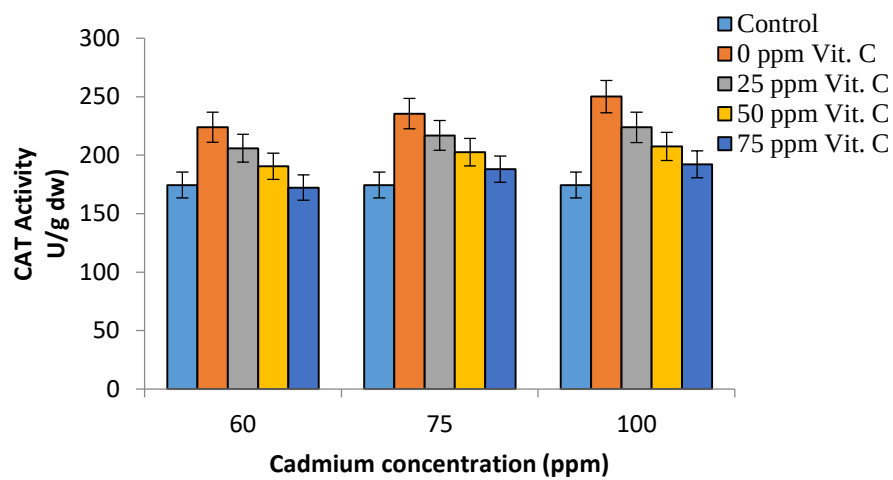


Figure 3.21. Effect of cadmium and vitamin C on CAT activity in *C. freundii*

Table 3.6: Effect of cadmium and vitamin C on the level of stress biomarkers in *C. freundii*.

	MDA (µg/g dw)	GSH (µg/g dw)	GSSG (µg/g dw)	GSH/GSSG	4-HNE (µg/g dw)
Control	2.90 ± 0.12	2311.00 ± 50.00	124.00 ± 8.00	18.64	1.24 ± 0.06
10 ppm Cd	4.80 ± 0.20	1860.00 ± 43.00	164.00 ± 9.50	11.34	1.85 ± 0.10
20 ppm Cd	6.00 ± 0.30	1327.00 ± 35.00	216.00 ± 11.00	6.14	2.60 ± 0.15
40 ppm Cd	8.60 ± 0.55	1010.00 ± 30.00	250.00 ± 11.50	4.04	4.10 ± 0.18
60 ppm Cd	10.20 ± 0.65	850.00 ± 22.00	295.00 ± 12.00	2.88	5.80 ± 0.26
75 ppm Cd + 0 ppm C vit	12.10 ± 0.80	760.00 ± 20.00	360.00 ± 11.00	2.11	7.90 ± 0.32
75 ppm Cd + 25ppm C vit	9.16 ± 0.58	896.00 ± 19.00	295.00 ± 11.00	3.04	6.80 ± 0.30
75 ppm Cd + 50 ppm C vit	8.00 ± 0.54	945.00 ± 20.50	255.00 ± 10.50	3.71	6.00 ± 0.25
75 ppm Cd + 75 ppm C vit	6.50 ± 0.48	990.00 ± 21.00	207.00 ± 10.00	4.78	5.30 ± 0.21
100 ppm Cd + 0 ppm C vit	14.50 ± 1.00	640.00 ± 17.00	390.00 ± 13.00	1.64	10.20 ± 0.44
100 ppm Cd + 25 ppm C vit	11.61 ± 0.78	810.00 ± 18.00	338.00 ± 12.00	2.40	9.20 ± 0.38
100 ppm Cd + 50 ppm C vit	9.67 ± 0.70	855.00 ± 18.50	283.00 ± 11.60	3.02	8.30 ± 0.32
100 ppm Cd + 75ppm C vit	8.10 ± 0.56	905.00 ± 20.00	227.00 ± 10.30	3.99	7.50 ± 0.25
125 ppm Cd + 0 ppm C vit	16.60 ± 1.10	550.00 ± 15.00	500.00 ± 14.50	1.10	12.60 ± 0.50
125 ppm Cd + 25 ppm C vit	13.50 ± 0.92	635.00 ± 16.00	437.00 ± 14.00	1.45	11.40 ± 0.57
125 ppm Cd + 50 ppm C vit	11.00 ± 0.83	698.00 ± 17.50	385.00 ± 12.40	1.81	10.10 ± 0.52
125 ppm Cd + 75 ppm C vit	9.60 ± 0.74	740.00 ± 18.00	310.00 ± 11.60	2.39	9.00 ± 0.44

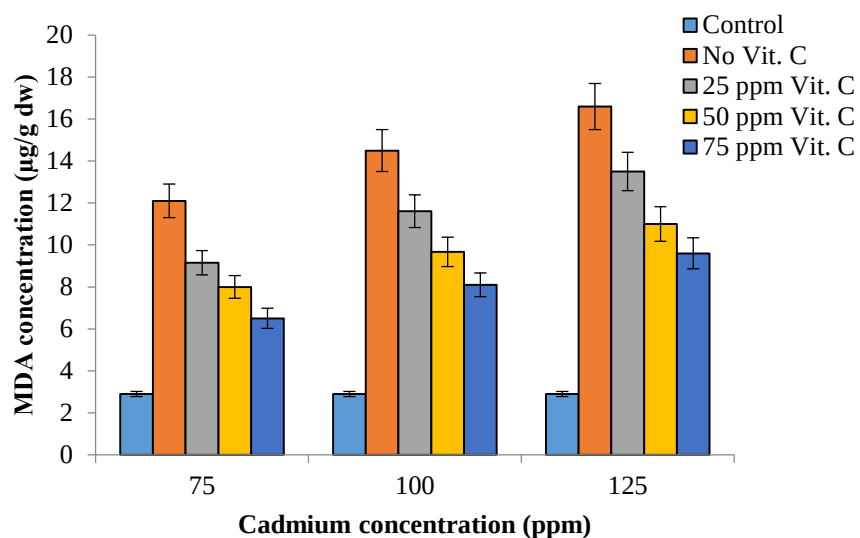


Figure 3.22. Effect of cadmium and vitamin C on the level of MDA in *C. freundii*

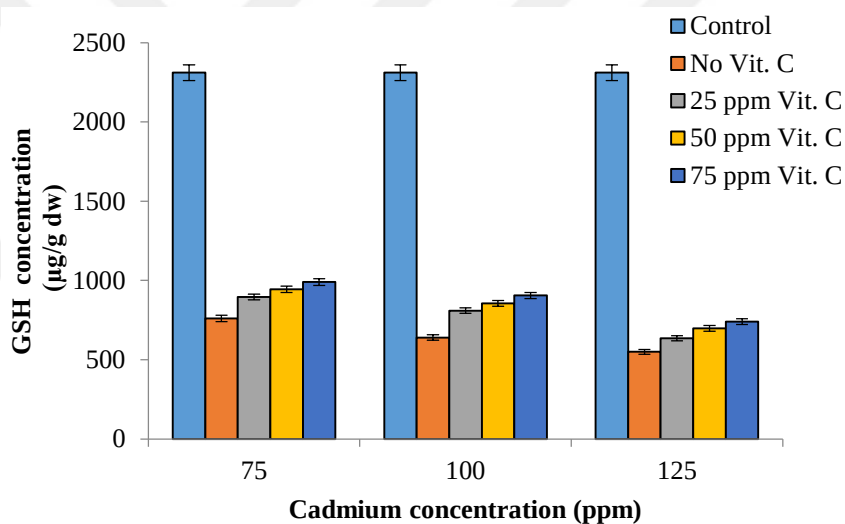


Figure 3.23. Effect of cadmium and vitamin C on the level of GSH in *C. freundii*

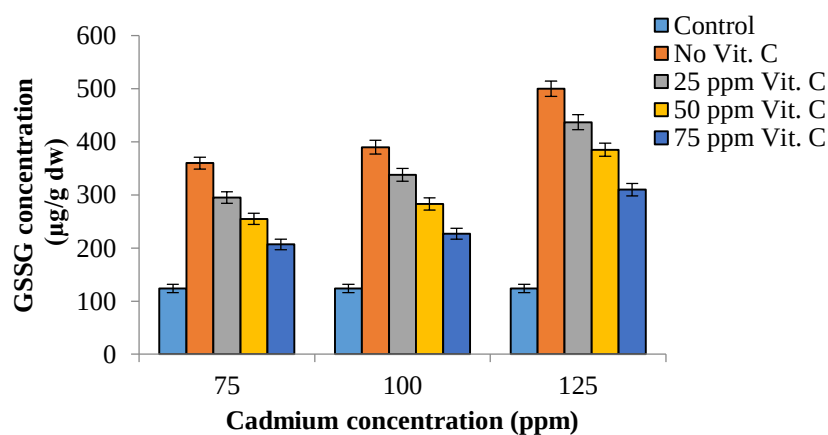


Figure 3.24. Effect of cadmium and vitamin C on the level of GSSG in *C. freundii*

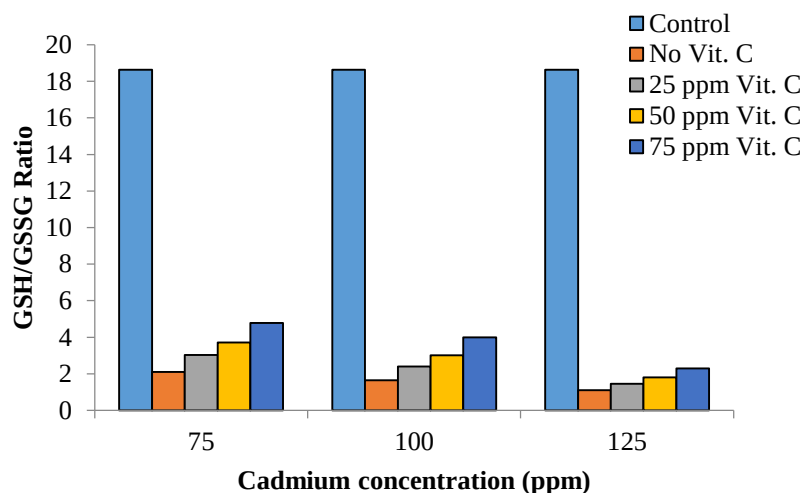


Figure 3.25. Effect of cadmium and vitamin C on the ratio of GSH/GSSG in *C. freundii*

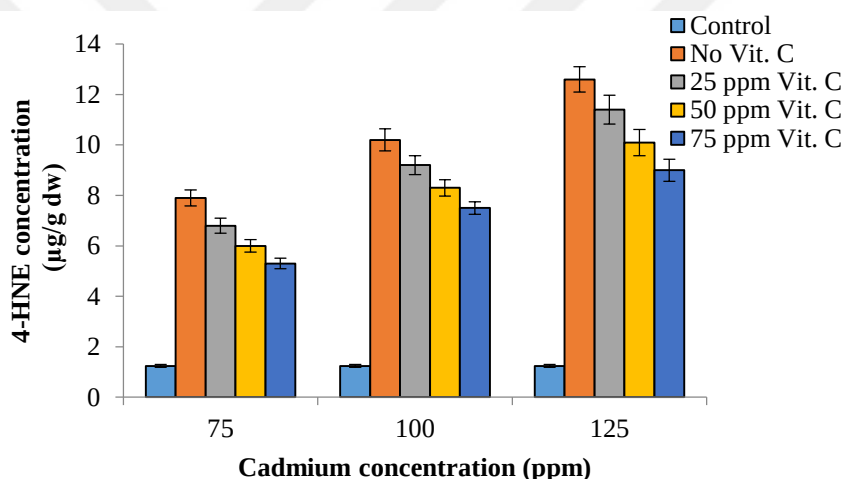


Figure 3.26. Effect of cadmium and vitamin C on the level of 4-HNE in *C. freundii*

3.8. Selenium Effect on the Growth of *C. freundii* Under Cadmium Stress

In this study, we added two different concentrations of selenium to the culture medium and we monitored the growth of the bacterium in the presence of cadmium with and without selenium. The table 3.7 and figure 3.27 shows the results we obtained.

3.9. Selenium Effect on the Level of Fat Soluble Vitamins in Cadmium Stressed *C. freundii*

The table 3.8 and figures 3.28 – 3.31 shows the results of fat soluble vitamins level in *C. freundii* with and without cadmium after addition 1.0 ppm and 3.0 ppm selenium.

Table 3.7. Results showing the changes in growth pattern of *C. freundii* under cadmium stress after addition of 1.0 ppm and 3.0 ppm selenium

Treatment	<i>C. freundii</i> (g dw /L)
Control	4.37 ± 0.04
0 ppm Cd + 1.0 ppm Se	4.50 ± 0.06
0 ppm Cd + 3.0 ppm Se	4.58 ± 0.01
75 ppm Cd	3.55 ± 0.05
75 ppm Cd + 1.0 ppm Se	3.75 ± 0.02
75 ppm Cd + 3.0 ppm Se	4.02 ± 0.09
100 ppm Cd	2.60 ± 0.08
100 ppm Cd + 1.0 ppm Se	2.68 ± 0.05
100 ppm Cd + 3.0 ppm Se	2.90± 0.01
125 ppm Cd	2.38 ± 0.01
125 ppm Cd + 1.0 ppm Se	2.51 ± 0.01
125 ppm Cd + 3.0 ppm Se	2.74 ± 0.06

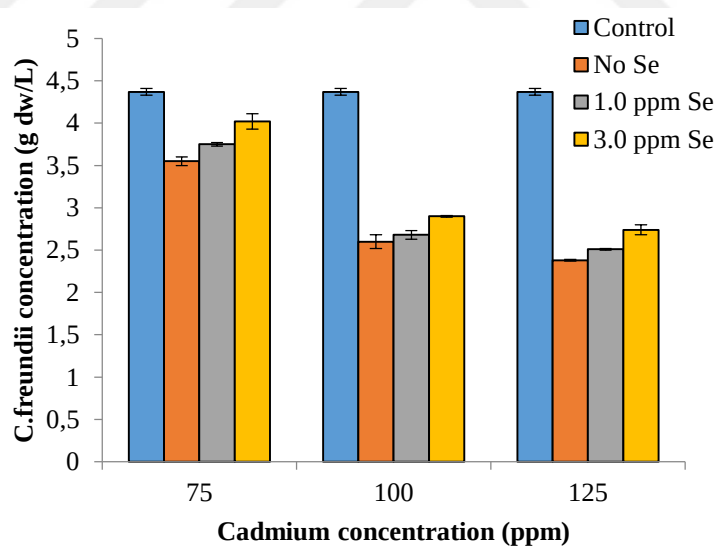


Figure 3.27. *C. freundii* level at varying cadmium concentration with addition of 1.0 ppm and 3.0 ppm selenium.

Table 3.8. The results of fat soluble vitamins ($\mu\text{g/g dw}$) with and without cadmium in *C. freundii* with 1.0 ppm and 3.0 ppm selenium added.

Treatment	Vitamin A	Vitamin E	β -carotene	Lycopene
Control	1.20 ± 0.04	8.90 ± 0.55	0.85 ± 0.04	0.82 ± 0.05
0 ppm Cd + 1.0 ppm Se	1.25 ± 0.04	9.05 ± 0.59	0.87 ± 0.04	0.83 ± 0.05
0 ppm Cd + 3.0 ppm Se	1.50 ± 0.05	9.70 ± 0.61	0.92 ± 0.05	0.87 ± 0.05
75 ppm Cd	0.62 ± 0.04	4.90 ± 0.22	0.50 ± 0.03	0.56 ± 0.02
75 ppm Cd + 1.0 ppm Se	0.67 ± 0.01	5.15 ± 0.22	0.52 ± 0.02	0.58 ± 0.02
75 ppm Cd + 3.0 ppm Se	0.85 ± 0.02	5.75 ± 0.24	0.58 ± 0.03	0.63 ± 0.03
100 ppm Cd	0.50 ± 0.02	4.50 ± 0.20	0.47 ± 0.02	0.50 ± 0.02
100 ppm Cd + 1.0 ppm Se	0.56 ± 0.02	4.70 ± 0.21	0.50 ± 0.02	0.52 ± 0.02
100 ppm Cd + 3.0 ppm Se	0.70 ± 0.02	5.25 ± 0.23	0.56 ± 0.03	0.57 ± 0.02
125 ppm Cd	0.35 ± 0.01	4.00 ± 0.18	0.43 ± 0.02	0.45 ± 0.01
125 ppm Cd + 1.0 ppm Se	0.41 ± 0.01	4.18 ± 0.20	0.45 ± 0.02	0.46 ± 0.01
125 ppm Cd + 3.0 ppm Se	0.65 ± 0.02	4.60 ± 0.22	0.52 ± 0.02	0.52 ± 0.02

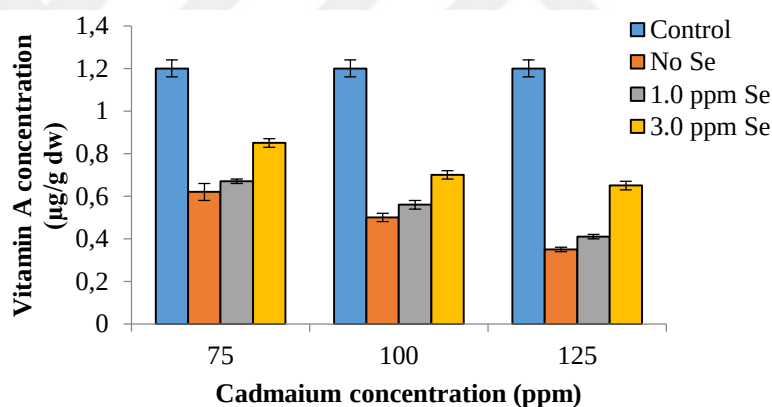


Figure 3.28. Selenium and cadmium effect on the level of vitamin A in *C. freundii*

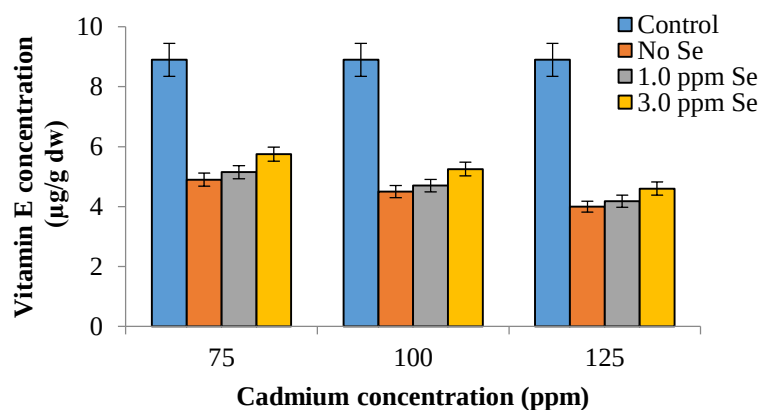


Figure 3.29. Selenium and cadmium effect on the level of vitamin E in *C. freundii*

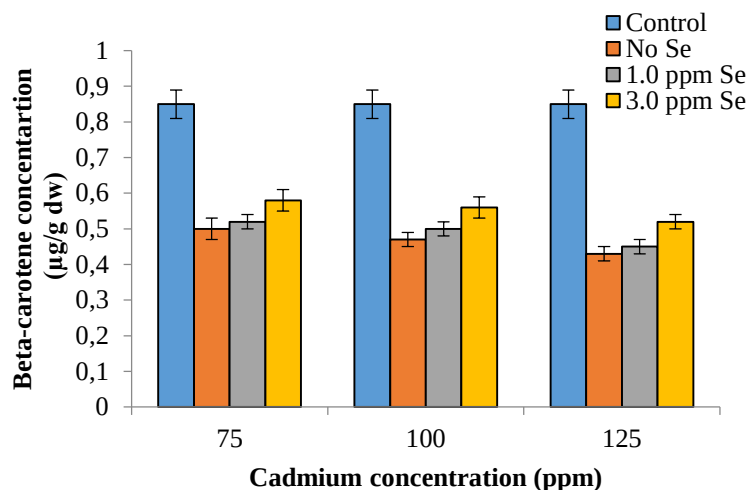


Figure 3.30. Selenium and cadmium effect on the level of beta-carotene in *C. freundii*

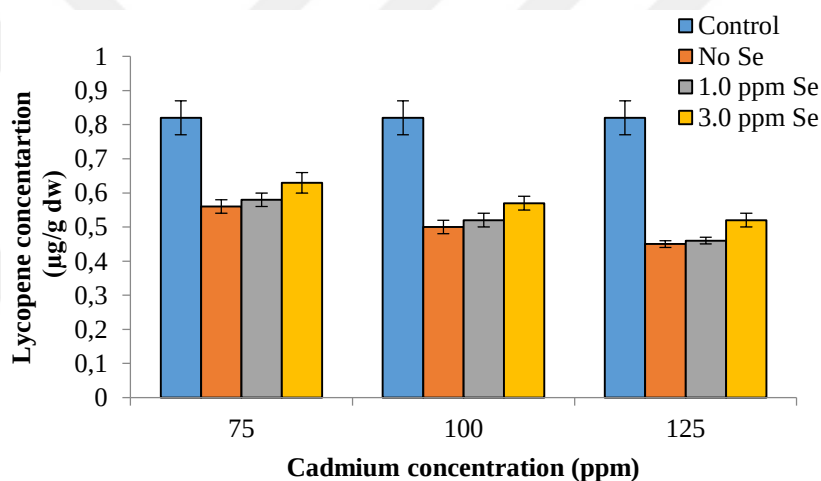


Figure 3.31. Selenium and cadmium effect on the level of lycopene in *C. freundii*

3.10. Selenium Effect on the Level of Water Soluble Vitamins in Cadmium Stressed *C. freundii*

The table 3.9 and figures 3.32 – 3.39 shows the levels of the water soluble vitamins in *C. freundii* with and without cadmium after addition 1.0 ppm and 3.0 ppm selenium.

Table 3.9. The results of water soluble vitamins ($\mu\text{g/g dw}$) with and without cadmium in *C. freundii* with 1.0 ppm and 3.0 ppm selenium added.

Treatment	Vitamin C	Vitamin B ₁	Vitamin B ₂	Vitamin B ₃	Vitamin B ₅	Vitamin B ₆	Vitamin B ₉	Vitamin B ₁₂
Control	31.30 $\pm$ 2.00	16.00 $\pm$ 1.00	12.00 $\pm$ 0.80	262.00 $\pm$ 12.00	170.00 $\pm$ 8.00	320.00 $\pm$ 15.00	3.60 $\pm$ 0.20	2.00 $\pm$ 0.08
0 ppm Cd + 1.0 ppm Se	33.50 $\pm$ 2.30	17.40 $\pm$ 0.76	12.90 $\pm$ 0.68	270.00 $\pm$ 12.10	181.00 $\pm$ 7.10	328.00 $\pm$ 15.20	3.90 $\pm$ 0.27	2.19 $\pm$ 0.09
0 ppm Cd + 3.0 ppm Se	41.00 $\pm$ 2.70	18.60 $\pm$ 0.82	15.10 $\pm$ 0.71	295.00 $\pm$ 12.30	205.00 $\pm$ 7.40	348.00 $\pm$ 15.50	5.30 $\pm$ 0.30	2.52 $\pm$ 0.10
75 ppm Cd	7.00 $\pm$ 0.50	5.40 $\pm$ 0.35	4.00 $\pm$ 0.32	130.00 $\pm$ 7.00	110.00 $\pm$ 4.30	210.00 $\pm$ 10.30	2.00 $\pm$ 0.08	1.10 $\pm$ 0.03
75 ppm Cd + 1.0 ppm Se	8.10 $\pm$ 0.41	6.00 $\pm$ 0.28	4.80 $\pm$ 0.30	145.00 $\pm$ 6.10	120.00 $\pm$ 4.20	218.00 $\pm$ 9.30	2.35 $\pm$ 0.10	1.24 $\pm$ 0.03
75 ppm Cd + 3.0 ppm Se	9.50 $\pm$ 0.48	7.40 $\pm$ 0.35	6.00 $\pm$ 0.34	167.00 $\pm$ 6.80	138.00 $\pm$ 4.50	239.00 $\pm$ 9.90	3.25 $\pm$ 0.14	1.45 $\pm$ 0.04
100 ppm Cd	4.50 $\pm$ 0.30	3.70 $\pm$ 0.27	3.00 $\pm$ 0.20	110.00 $\pm$ 6.60	100.00 $\pm$ 4.10	195.00 $\pm$ 9.60	1.70 $\pm$ 0.07	0.90 $\pm$ 0.02
100 ppm Cd + 1.0 ppm Se	6.00 $\pm$ 0.37	4.30 $\pm$ 0.20	3.35 $\pm$ 0.10	127.00 $\pm$ 4.50	110.00 $\pm$ 4.00	200.00 $\pm$ 8.40	1.90 $\pm$ 0.08	0.96 $\pm$ 0.02
100 ppm Cd + 3.0 ppm Se	8.20 $\pm$ 0.51	5.40 $\pm$ 0.22	4.40 $\pm$ 0.12	150.00 $\pm$ 4.80	130.00 $\pm$ 4.40	220.00 $\pm$ 8.80	2.30 $\pm$ 0.10	1.15 $\pm$ 0.03
125 ppm Cd	2.50 $\pm$ 0.20	2.80 $\pm$ 0.18	1.00 $\pm$ 0.06	90.00 $\pm$ 6.10	80.00 $\pm$ 3.90	170.00 $\pm$ 9.00	1.45 $\pm$ 0.05	0.75 $\pm$ 0.01
125 ppm Cd + 1.0 ppm Se	3.40 $\pm$ 0.15	3.75 $\pm$ 0.15	1.24 $\pm$ 0.07	110.00 $\pm$ 4.10	89.00 $\pm$ 3.70	178.00 $\pm$ 8.00	1.58 $\pm$ 0.05	0.80 $\pm$ 0.02
125 ppm Cd + 3.0 ppm Se	5.20 $\pm$ 0.24	5.00 $\pm$ 0.20	1.70 $\pm$ 0.09	138.00 $\pm$ 4.60	104.00 $\pm$ 4.20	195.00 $\pm$ 8.40	1.79 $\pm$ 0.07	1.00 $\pm$ 0.03

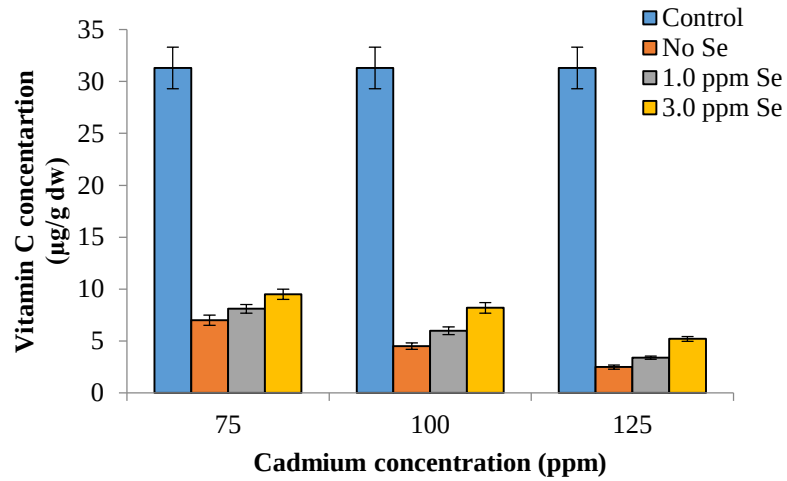


Figure 3.32. Effect of cadmium and selenium on the level of Vitamin C in *C. freundii*

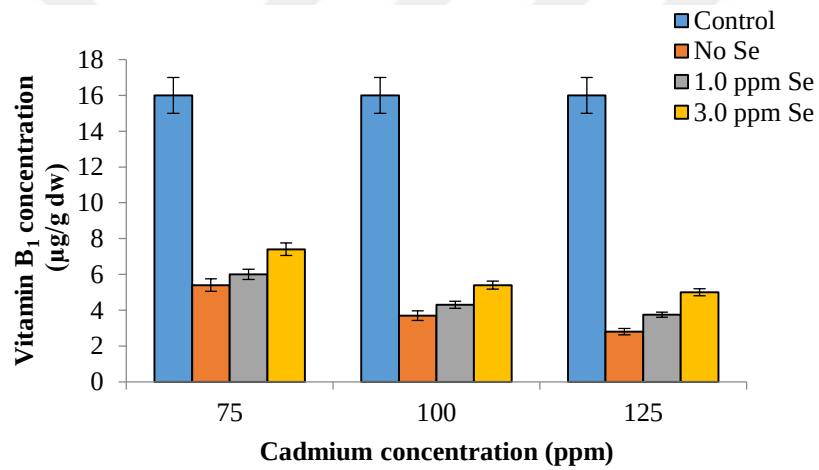


Figure 3.33. Selenium and cadmium effect on the level of vitamin B₁ in *C. freundii*

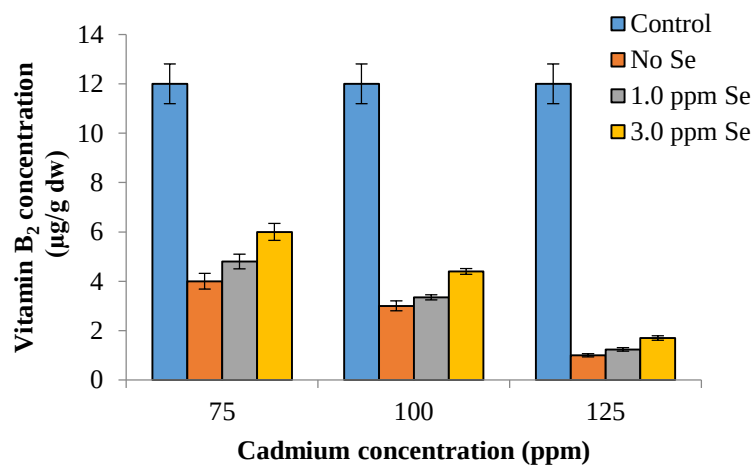


Figure 3.34. Selenium and cadmium effect on the level of vitamin B₂ in *C. freundii*

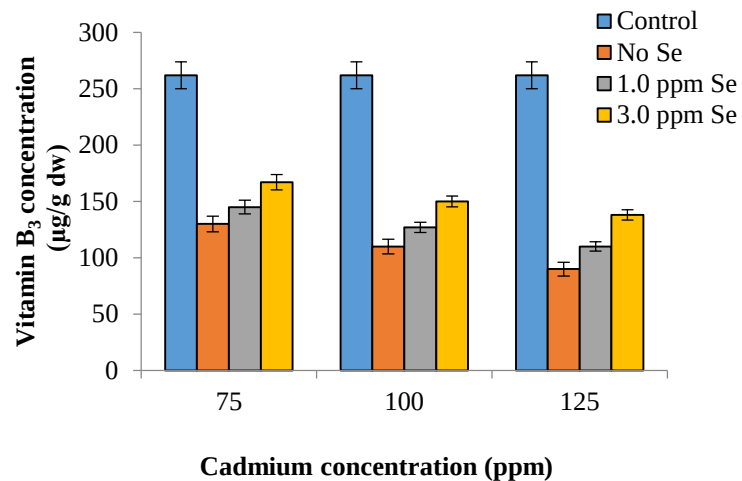


Figure 3.35. Selenium and cadmium effect on the level of vitamin B₃ in *C. freundii*

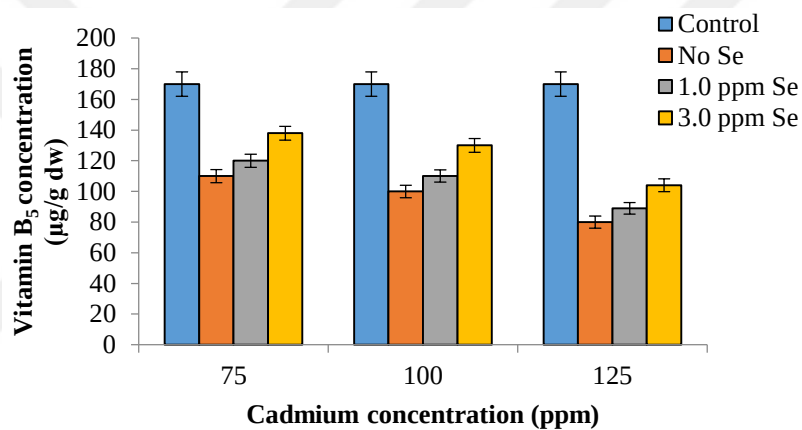


Figure 3.36. Selenium and cadmium effect on the level of vitamin B₅ in *C. freundii*

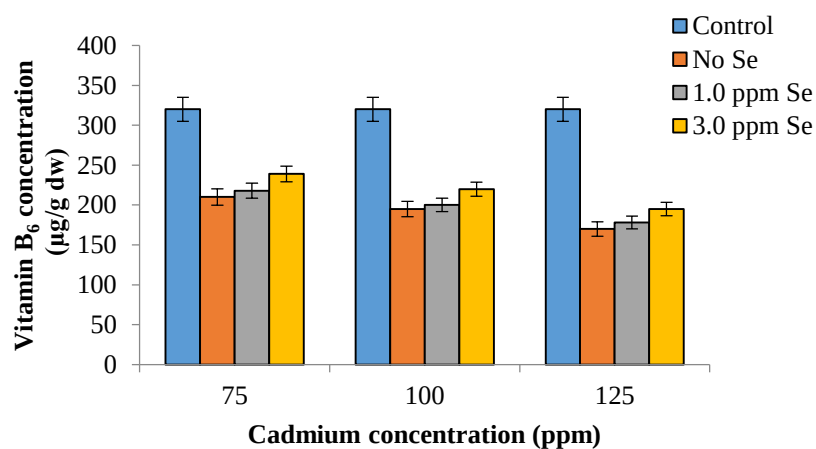


Figure 3.37. Selenium and cadmium effect on the level of vitamin B₆ in *C. freundii*

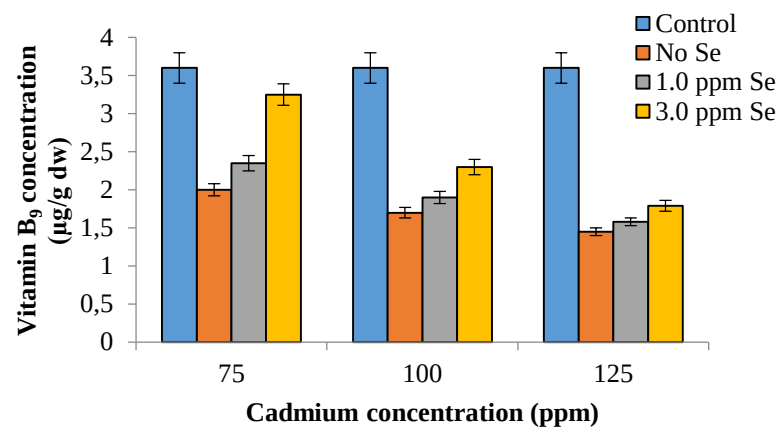


Figure 3.38. Selenium and cadmium effect on the level of vitamin B₉ in *C. freundii*

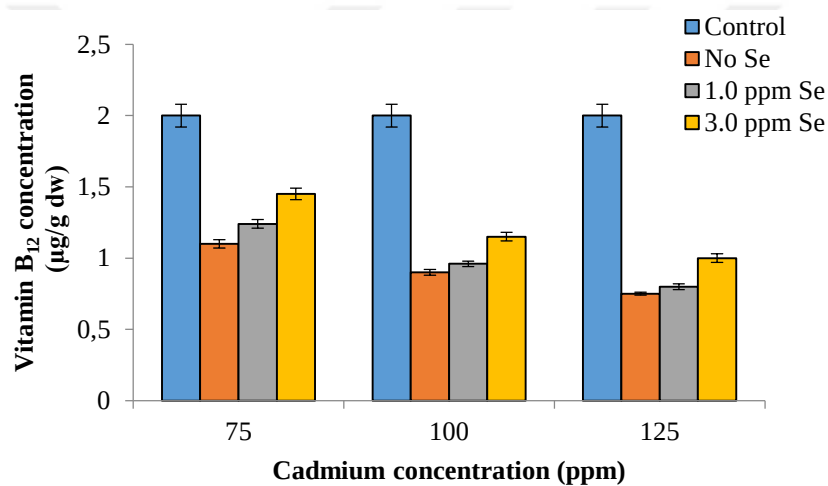


Figure 3.39. Selenium and cadmium effect on the level of vitamin B₁₂ in *C. freundii*

3.11. Selenium Effect on the Level of Stress Biomarkers in Cadmium Stressed *C. freundii*

The table 3.10 and figures 3.40 – 3.44 shows the results of stress biomarkers in *C. freundii* with and without cadmium after addition 1.0 ppm and 3.0 ppm selenium.

Table 3.10. Selenium and cadmium effect on the level of stress biomarkers ($\mu\text{g/g dw}$) in *C. freundii*

Treatment	MDA	GSH	GSSG	GSH/ GSSG	4-HNE)
Control	2.90 ± 0.12	2311.00 ± 50.00	124.00 ± 8.00	18.64	1.24 ± 0.06
0 ppm Cd + 1.0 ppm Se	2.58 ± 0.11	2380.00 ± 51.00	116.00 ± 7.10	20.52	1.15 ± 0.05
0 ppm Cd + 3.0 ppm Se	2.47 ± 0.10	2450.00 ± 53.00	100.00 ± 6.30	24.50	1.00 ± 0.04
75 ppm Cd	12.10 ± 0.80	760.00 ± 20.00	360.00 ± 11.00	2.11	7.90 ± 0.32
75 ppm Cd + 1.0 ppm Se	10.10 ± 0.55	825.00 ± 18.40	310.00 ± 10.80	2.66	6.90 ± 0.30
75 ppm Cd + 3.0 ppm Se	8.90 ± 0.52	890.00 ± 19.00	275.00 ± 10.20	3.23	5.90 ± 0.26
100 ppm Cd	14.50 ± 1.00	640.00 ± 17.00	390.00 ± 13.00	1.64	10.20 ± 0.44
100 ppm Cd + 1.0 ppm Se	12.80 ± 0.64	750.00 ± 18.20	345.00 ± 11.70	2.17	9.35 ± 0.37
100 ppm Cd + 3.0 ppm Se	11.10 ± 0.61	845.00 ± 18.50	291.00 ± 10.50	2.90	8.10 ± 0.30
125 ppm Cd	16.60 ± 1.10	550.00 ± 15.00	500.00 ± 14.50	1.10	12.60 ± 0.50
125 ppm Cd + 1.0 ppm Se	14.60 ± 0.70	605.00 ± 15.80	445.00 ± 13.20	1.33	11.50 ± 0.56
125 ppm Cd + 3.0 ppm Se	13.00 ± 0.65	710.00 ± 16.30	385.00 ± 12.40	1.84	10.10 ± 0.51

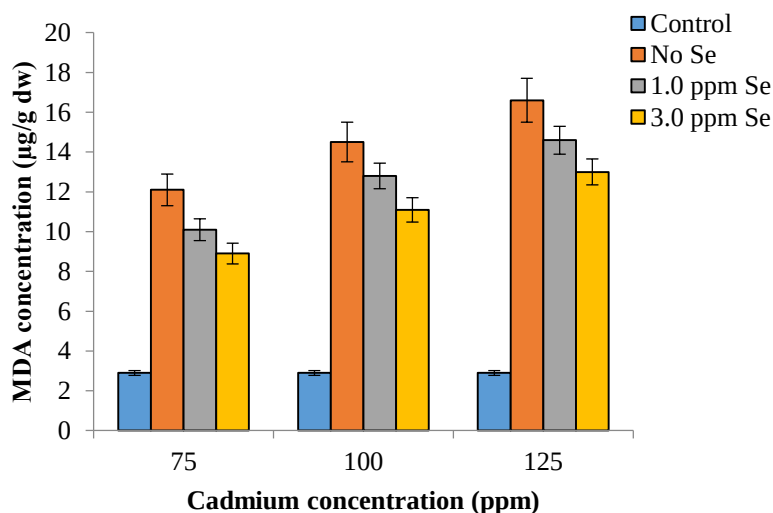


Figure 3.40. Selenium and cadmium effect on the level of MDA in *C. freundii*

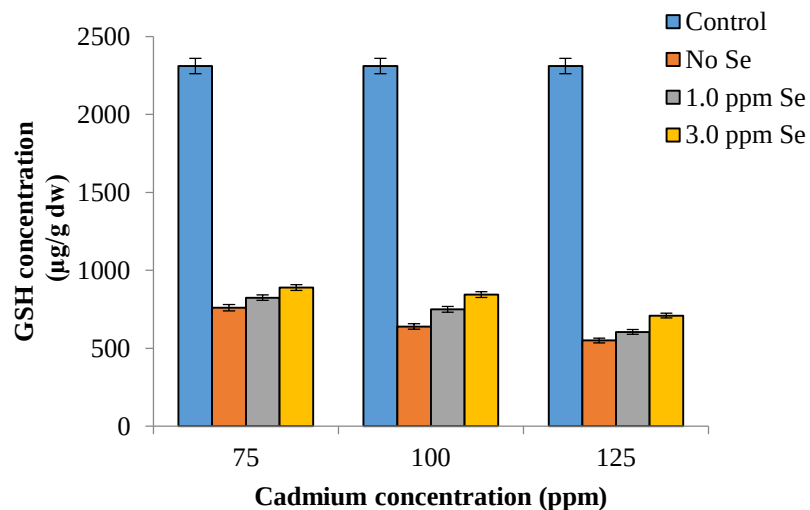


Figure 3.41. Selenium and cadmium effect on the level of GSH in *C. freundii*

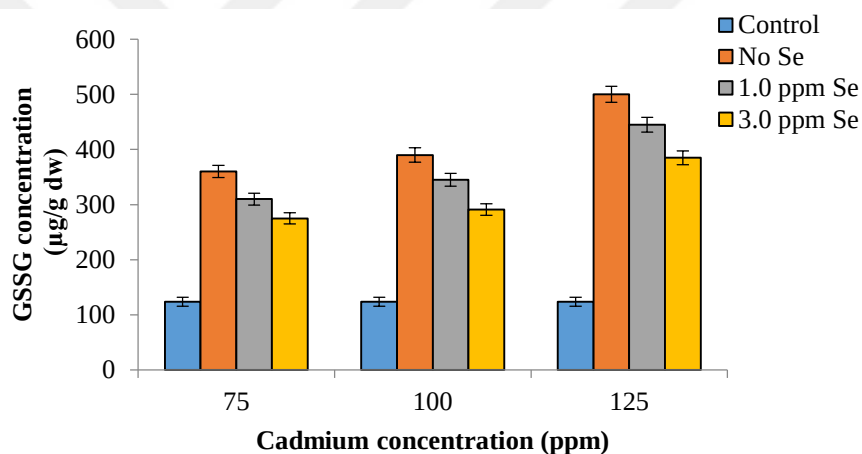


Figure 3.42. Selenium and cadmium effect on the level of GSSG in *C. freundii*

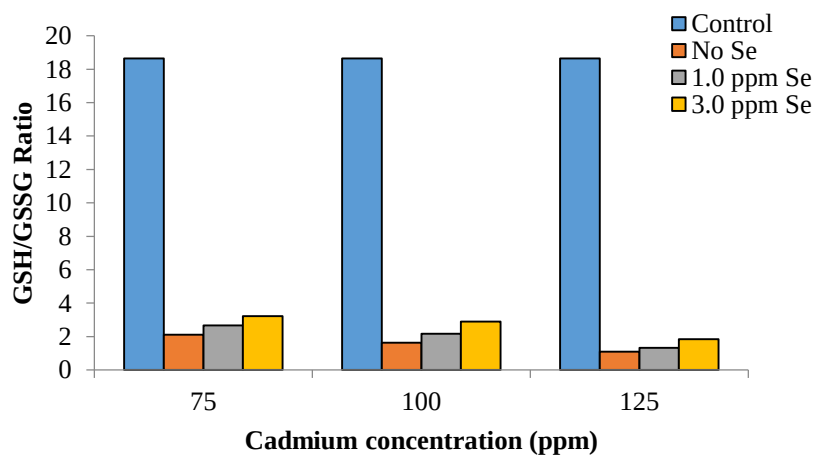


Figure 3.43. Selenium and cadmium effect on the ratio of GSH: GSSG in *C. freundii*

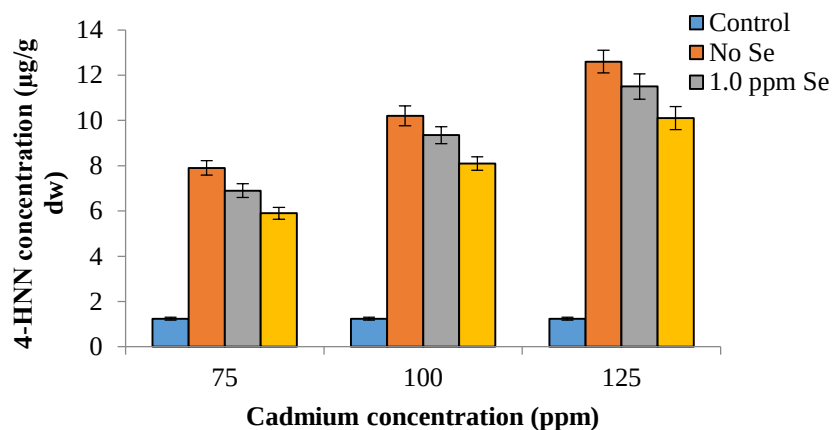


Figure 3.44. Selenium and cadmium effect on the level of 4-HNE in *C. freundii*

3.12. Amino Acid Composition in *C. freundii*

The table 3.11 and figures 3.45 shows the amino acid composition in *C. freundii* without cadmium in the culture medium.

Table 3.11. The composition of amino acids in *C. freundii*.

Amino Acids	Concentration (mg/g dw)
leucine	25.10 ± 1.05
Glutamic acid	38.70 ± 1.50
Asparagine	14.10 ± 0.55
Glycine	9.60 ± 0.40
Serine	19.60 ± 0.90
Glutamine	7.90 ± 0.35
Histidine	5.00 ± 0.25
Arginine	5.20 ± 0.25
Valine	8.40 ± 0.40
Aspartic acid	30.00 ± 1.20
Proline	12.00 ± 0.50
Tyrosine	9.50 ± 0.40
Alanine	27.60 ± 0.90
Methionine	4.95 ± 0.20
Cysteine	1.85 ± 0.08
Threonine	22.50 ± 0.85
Isoleucine	10.00 ± 0.45
Phenylalanine	12.50 ± 0.50
Tryptophan	1.50 ± 0.08
Lysine	20.40 ± 0.95

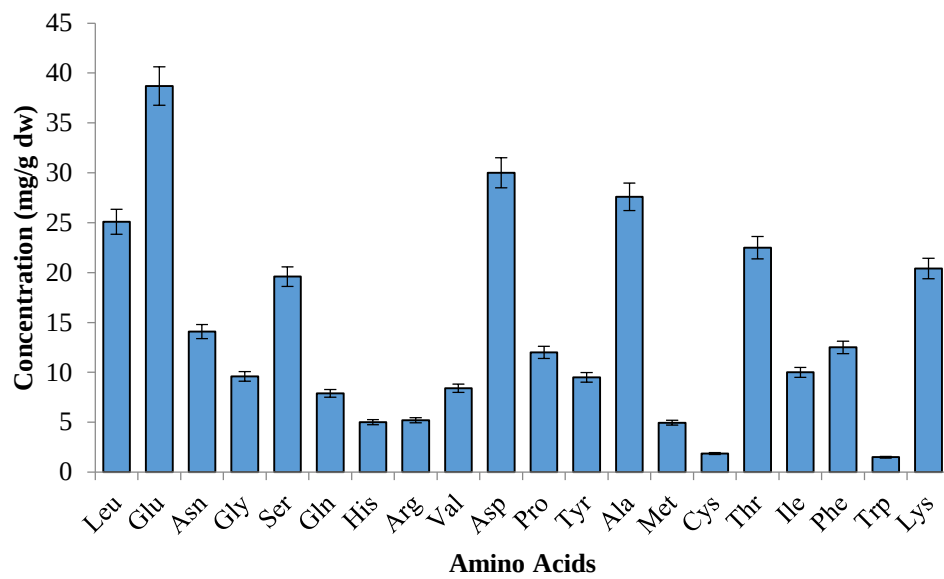


Figure 3.45. Amino acid composition of *C. freundii*

3.13. Amino Acid Composition in *C. freundii* After Adding Varying Concentrations of Cadmium and Vitamin C

The table 3.12 and Figures 3.46 - 3.65 below show the composition of amino acids in *C. freundii* at varying concentrations of cadmium and vitamin C.

Table 3.12. The concentration of amino acids (mg/g dw) in *C. freundii* after adding varying concentrations of cadmium and vitamin C.

Treatment	Leucine	Glutamic Acid	Asparagine	Glycine	Serine	Glutamine	Histidine
Control	25.10±1.05	38.70±1.50	14.10±0.55	9.60±0.40	19.60±0.90	7.90±0.35	5.00±0.25
0 ppm Cd + 25 ppm C Vit	28.60±1.10	42.00±1.60	16.60±0.60	11.00±0.40	21.90±0.95	9.10±0.40	6.00±0.35
0 ppm Cd + 50 ppm C Vit	27.50±1.00	41.10±1.50	15.70±0.55	10.50±0.40	20.70±0.90	8.70±0.35	5.50±0.25
0 ppm Cd + 75 ppm C Vit	26.40±1.00	40.20±1.50	15.00±0.50	10.00±0.45	20.20±0.85	8.20±0.30	4.90±0.25
75 ppm Cd + 0 ppm C Vit	27.30±1.05	35.70±1.40	15.50±0.55	11.20±0.45	17.80±0.80	7.00±0.30	6.20±0.30
75 ppm Cd + 25 ppm C Vit	25.10±0.95	38.80±1.50	14.30±0.50	10.10±0.40	19.90±0.90	8.20±0.40	5.50±0.30
75 ppm Cd + 50 ppm C Vit	24.60±0.90	37.90±1.45	13.60±0.45	9.70±0.35	19.30±0.90	7.80±0.35	5.10±0.20
75 ppm Cd + 75 ppm C Vit	23.80±0.90	37.20±1.40	13.00±0.45	9.10±0.35	18.60±0.85	7.10±0.30	4.90±0.20
100 ppm Cd + 0 ppm C Vit	29.50±1.10	32.90±1.30	16.90±0.60	12.50±0.45	16.20±0.70	5.70±0.25	7.50±0.35
100 ppm Cd + 25 ppm C Vit	27.35±1.05	35.70±1.40	15.60±0.50	11.30±0.40	18.10±0.75	6.90±0.30	6.60±0.30
100 ppm Cd + 50 ppm C Vit	26.60±1.00	34.80±1.40	14.80±0.50	10.60±0.45	17.70±0.70	6.30±0.25	6.10±0.25
100 ppm Cd + 75 ppm C Vit	25.80±0.90	34.00±1.45	14.30±0.45	9.80±0.40	17.10±0.75	5.70±0.20	5.70±0.20
125 ppm Cd + 0 ppm C Vit	31.80±1.15	30.20±1.20	18.30±0.70	13.60±0.45	14.40±0.65	4.90±0.20	8.50±0.40
125 ppm Cd + 25 ppm C Vit	29.50±0.95	32.80±1.30	16.80±0.65	12.30±0.40	15.90±0.70	5.50±0.20	7.60±0.35
125 ppm Cd +50 ppm C Vit	28.80±0.90	32.00±1.30	16.00±0.45	11.60±0.45	15.10±0.70	5.00±0.15	7.10±0.35
125 ppm Cd + 75 ppm C Vit	28.00±0.95	31.30±1.35	15.40±0.45	11.00±0.40	14.60±0.65	4.60±0.15	6.50±0.30

Continue Table 3.12

Treatment	Arginine	Valine	Aspartic acid	Proline	Tyrosine	Alanine
Control	5.20±0.25	8.40±0.40	30.00±1.20	12.00±0.50	9.50±0.40	27.60±0.90
0 ppm Cd + 25 ppm C Vit	6.20±0.35	9.50±0.45	32.60±1.25	13.60±0.50	11.00±0.50	29.70±1.00
0 ppm Cd + 50 ppm C Vit	5.80±0.30	9.00±0.40	31.70±1.15	13.00±0.45	10.40±0.45	29.20±1.00
0 ppm Cd + 75 ppm C Vit	5.30±0.25	8.60±0.40	30.80±1.10	12.50±0.40	9.80±0.40	28.80±1.10
75 ppm Cd + 0 ppm C Vit	6.55±0.35	9.50±0.40	32.50±1.15	13.50±0.55	10.50±0.45	29.60±1.00
75 ppm Cd + 25 ppm C Vit	5.75±0.30	8.60±0.40	30.10±1.15	12.40±0.40	9.60±0.40	27.60±0.90
75 ppm Cd + 50 ppm C Vit	5.20±0.25	8.20±0.35	29.60±1.10	11.90±0.40	8.90±0.40	26.90±0.85
75 ppm Cd + 75 ppm C Vit	4.80±0.20	7.80±0.30	29.10±1.10	11.20±0.35	8.20±0.35	26.30±0.80
100 ppm Cd + 0 ppm C Vit	7.60±0.35	10.60±0.45	35.00±1.20	15.00±0.55	11.80±0.50	31.70±1.00
100 ppm Cd + 25 ppm C Vit	6.80±0.30	9.65±0.40	32.40±1.10	13.80±0.50	10.70±0.50	29.60±0.90
100 ppm Cd + 50 ppm C Vit	6.20±0.30	9.00±0.40	31.80±1.05	13.20±0.50	10.20±0.45	28.90±0.85
100 ppm Cd + 75 ppm C Vit	5.80±0.25	8.50±0.35	31.10±1.00	12.70±0.40	9.80±0.40	28.20±0.85
125 ppm Cd + 0 ppm C Vit	8.80±0.40	12.00±0.50	37.50±1.20	16.40±0.60	13.10±0.50	33.80±1.00
125 ppm Cd + 25 ppm C Vit	7.90±0.40	11.00±0.40	35.10±1.10	15.20±0.50	12.10±0.45	31.90±0.80
125 ppm Cd + 50 ppm C Vit	7.40±0.35	10.40±0.45	33.90±1.25	14.60±0.45	11.70±0.40	31.10±0.90
125 ppm Cd + 75 ppm C Vit	7.00±0.30	9.90±0.40	33.00±1.20	14.00±0.45	11.10±0.40	30.60±0.85

Continue Table 3.12

Treatment	Methionine	Cysteine	Threonine	Isoleucine	Phenylalanine	Tryptophan	Lycine
Control	4.95±0.20	1.85±0.08	22.50±0.85	10.00±0.45	12.50±0.50	1.50±0.08	20.40±0.95
0 ppm Cd + 25 ppm C Vit	5.50±0.25	2.10±0.10	24.60±1.00	11.10±0.50	14.10±0.60	1.90±0.09	22.60±1.00
0 ppm Cd + 50 ppm C Vit	5.00±0.25	1.90±0.10	23.70±1.10	10.80±0.50	13.60±0.50	1.80±0.08	22.00±1.05
0 ppm Cd + 75 ppm C Vit	4.60±0.25	1.70±0.09	23.00±1.00	10.30±0.45	13.00±0.45	1.70±0.06	21.50±1.00
75 ppm Cd + 0 ppm C Vit	4.00±0.20	2.30±0.10	24.50±0.90	11.20±0.40	13.80±0.50	1.30±0.06	22.40±0.90
75 ppm Cd + 25 ppm C Vit	4.60±0.25	2.00±0.10	22.40±0.90	10.30±0.40	12.60±0.55	1.60±0.07	20.40±1.00
75 ppm Cd + 50 ppm C Vit	4.20±0.20	1.90±0.10	21.80±0.90	9.80±0.40	12.10±0.40	1.50±0.07	19.70±0.90
75 ppm Cd + 75 ppm C Vit	3.90±0.15	1.80±0.06	21.20±0.90	9.20±0.40	11.70±0.45	1.40±0.04	19.10±0.90
100 ppm Cd + 0 ppm C Vit	3.60±0.10	2.70±0.13	26.50±0.90	12.20±0.50	15.10±0.55	1.10±0.04	24.50±1.00
100 ppm Cd + 25 ppm C Vit	4.00±0.20	2.40±0.10	24.50±0.95	11.30±0.35	14.00±0.45	1.20±0.05	22.60±0.80
100 ppm Cd + 50 ppm C Vit	3.80±0.15	2.10±0.09	23.80±1.00	10.80±0.35	13.60±0.40	1.00±0.04	22.10±0.80
100 ppm Cd + 75 ppm C Vit	3.50±0.10	1.90±0.06	23.20±1.00	10.20±0.40	13.00±0.40	0.85±0.02	21.70±0.75
125 ppm Cd + 0 ppm C Vit	3.10±0.15	3.00±0.15	28.40±0.90	13.50±0.50	16.30±0.60	1.00±0.02	26.70±1.00
125 ppm Cd + 25 ppm C Vit	3.50±0.15	2.70±0.10	26.50±0.85	12.20±0.55	15.00±0.50	1.10±0.03	24.70±0.90
125 ppm Cd + 50 ppm C Vit	3.30±0.10	2.40±0.10	25.80±1.00	11.50±0.40	14.40±0.50	1.00±0.02	24.00±0.80
125 ppm Cd + 75 ppm C Vit	3.10±0.10	2.10±0.10	25.20±1.00	10.90±0.35	13.90±0.45	0.90±0.02	23.50±0.80

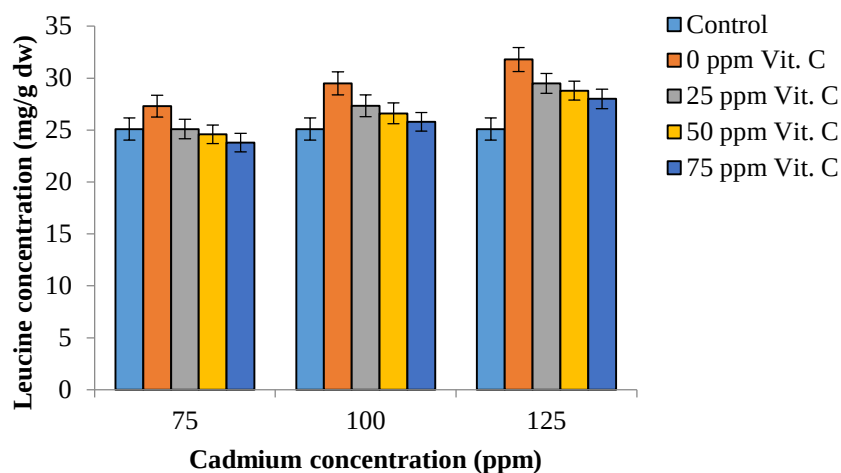


Figure 3.46. Effect of cadmium and vitamin C on the level of leucine in *C. freundii*

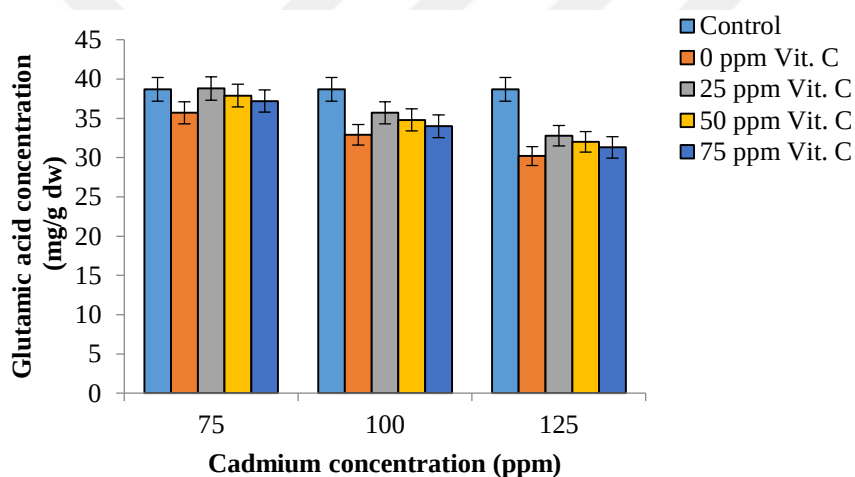


Figure 3.47. Effect of cadmium and vitamin C on the level of glutamic acid in *C. freundii*

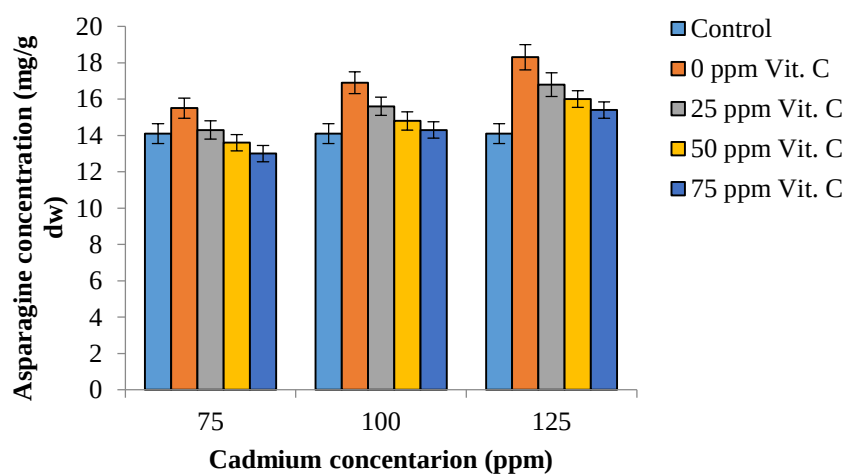


Figure 3.48. Effect of cadmium and vitamin C on the level of asparagine in *C. freundii*

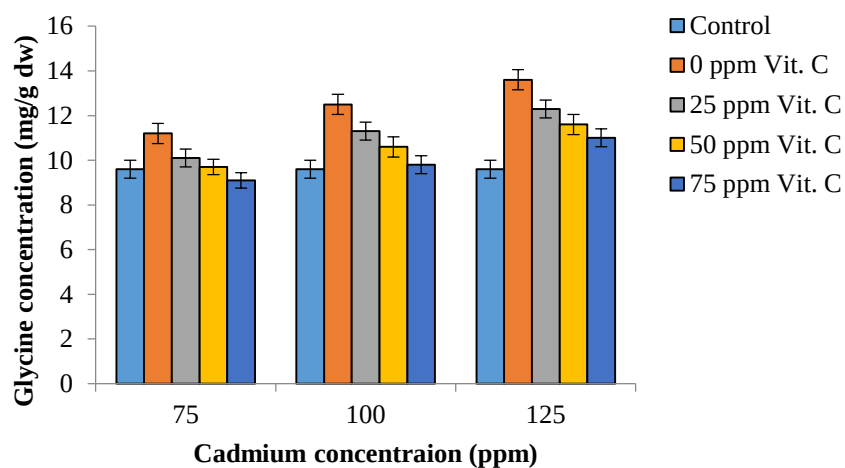


Figure 3.49. Effect of cadmium and vitamin C on the level of glycine in *C. freundii*

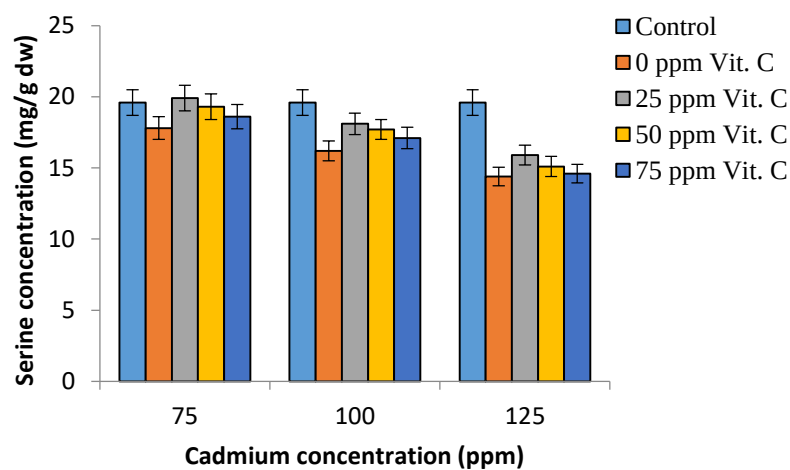


Figure 3.50. Effect of cadmium and vitamin C on the level of serine in *C. freundii*

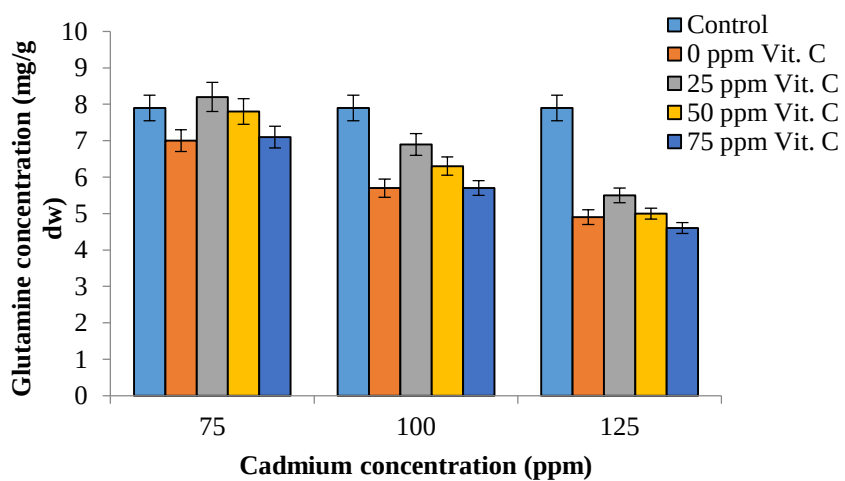


Figure 3.51. Effect of cadmium and vitamin C on the level of Glutamine in *C. freundii*

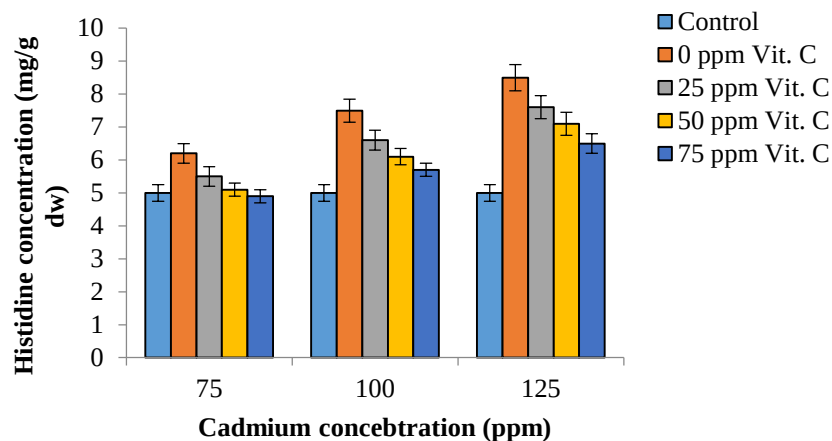


Figure 3.52. Effect of cadmium and vitamin C on the level of histidine in *C. freundii*

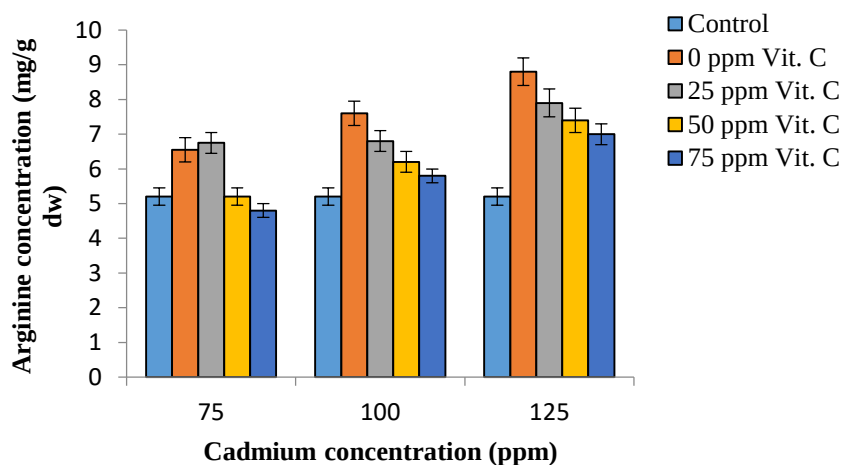


Figure 3.53. Effect of cadmium and vitamin C on the level of arginine in *C. freundii*

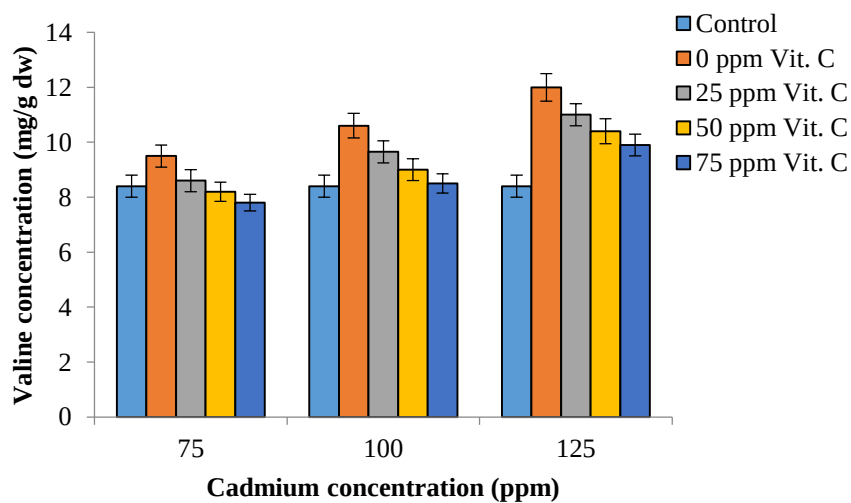


Figure 3.54. Effect of cadmium and vitamin C on the level of valine in *C. freundii*

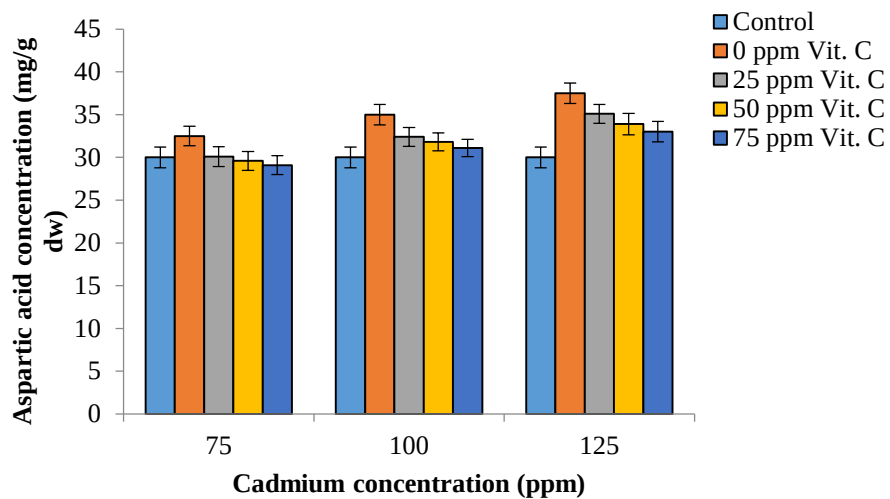


Figure 3.55. Effect of cadmium and vitamin C on the level of aspartic acid in *C. freundii*

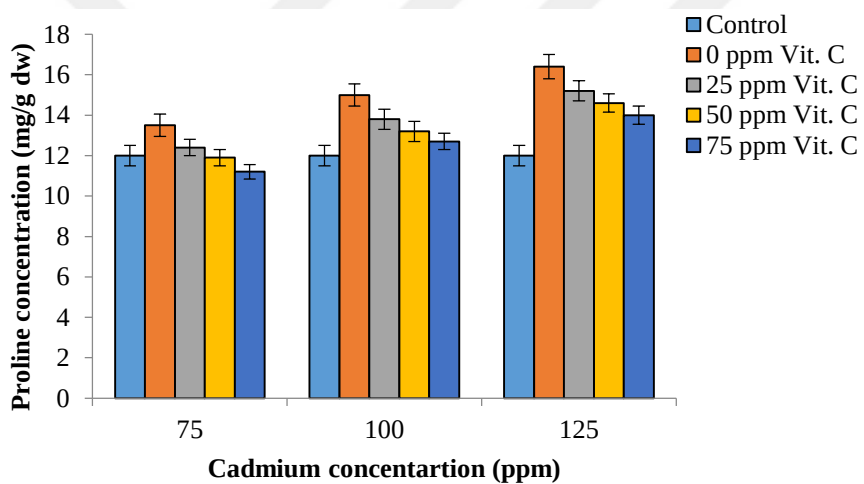


Figure 3.56 Effect of cadmium and vitamin C on the level of proline in *C. freundii*

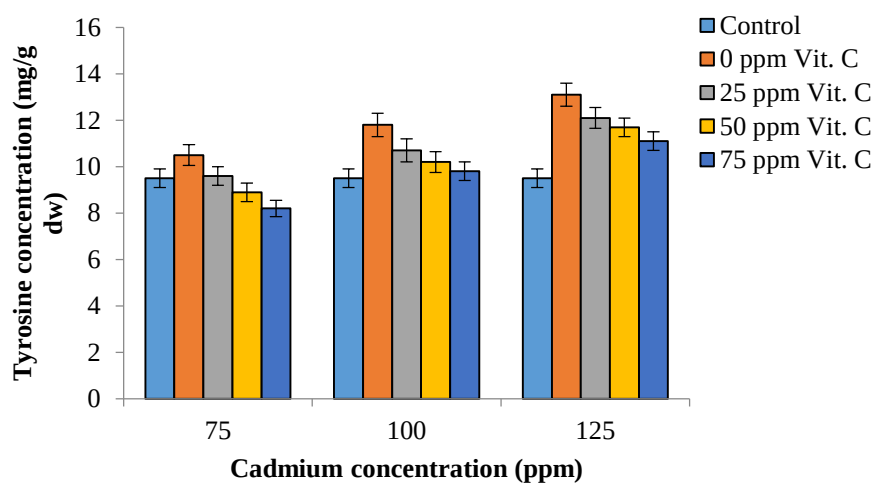


Figure 3.57. Effect of cadmium and vitamin C on the level of tyrosine in *C. freundii*

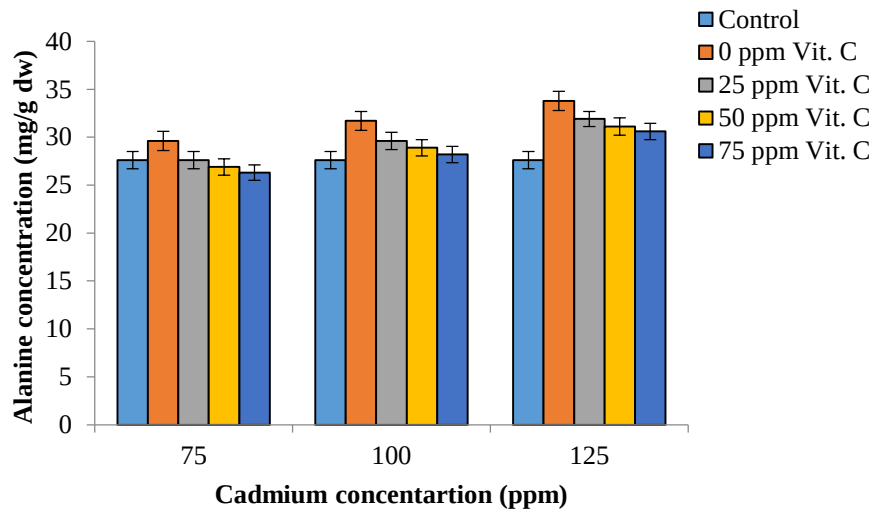


Figure 3.58. Effect of cadmium and vitamin C on the level of alanine in *C. freundii*

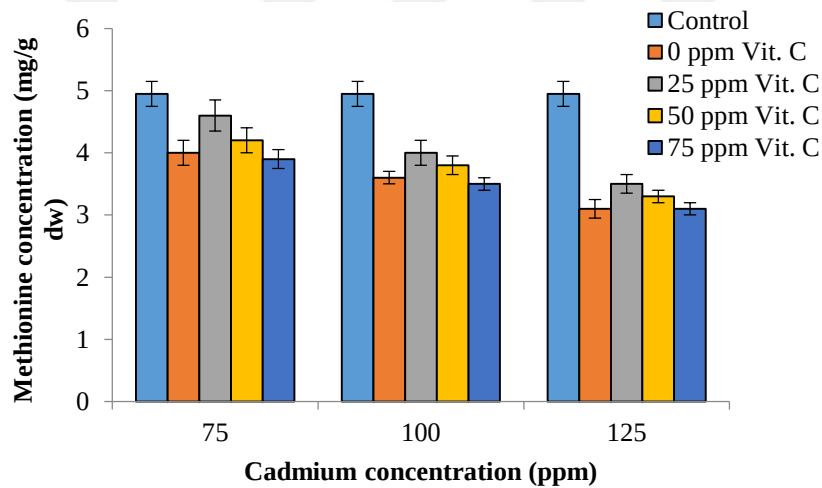


Figure 3.59. Effect of cadmium and vitamin C on the level of methionine in *C. freundii*

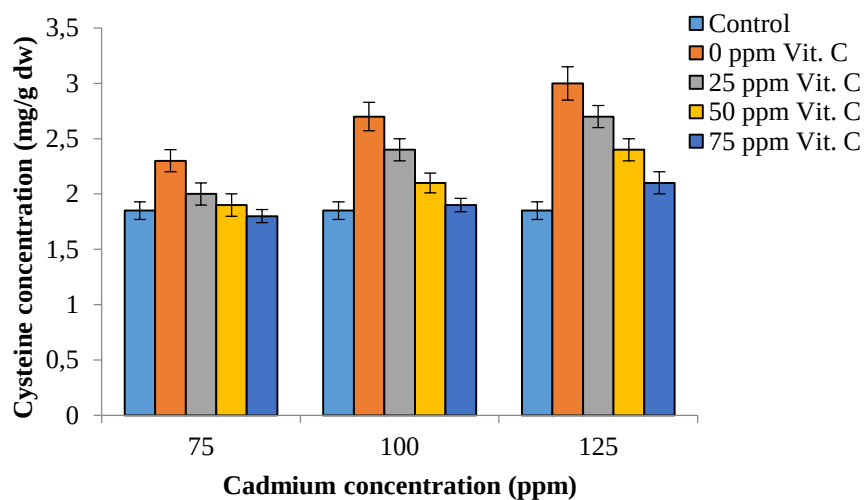


Figure 3.60. Effect of cadmium and vitamin C on the level of cysteine in *C. freundii*

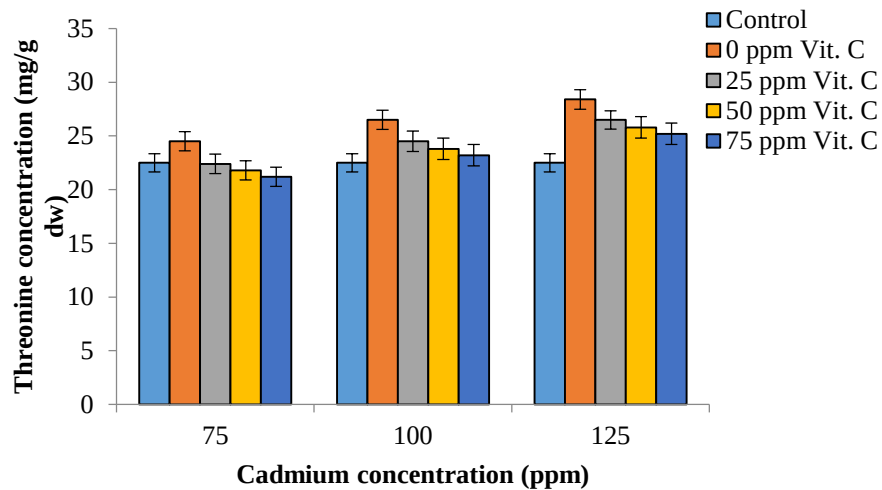


Figure 3.61. Effect of cadmium and vitamin C on the level of threonine in *C. freundii*

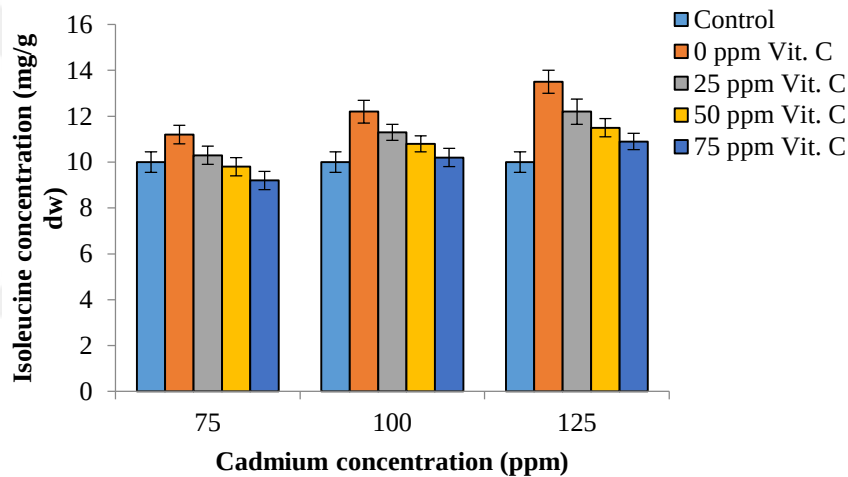


Figure 3.62. Effect of cadmium and vitamin C on the level of isoleucine in *C. freundii*

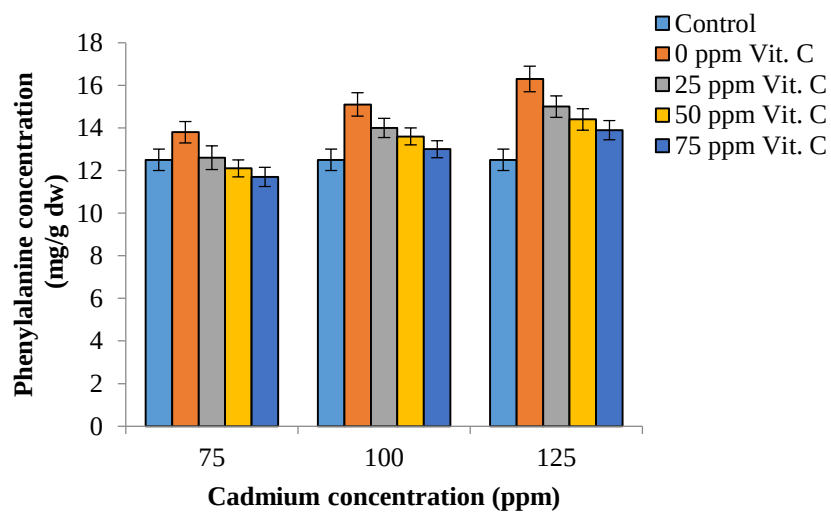


Figure 3.63. Effect of cadmium and vitamin C on the level of phenylalanine in *C. freundii*

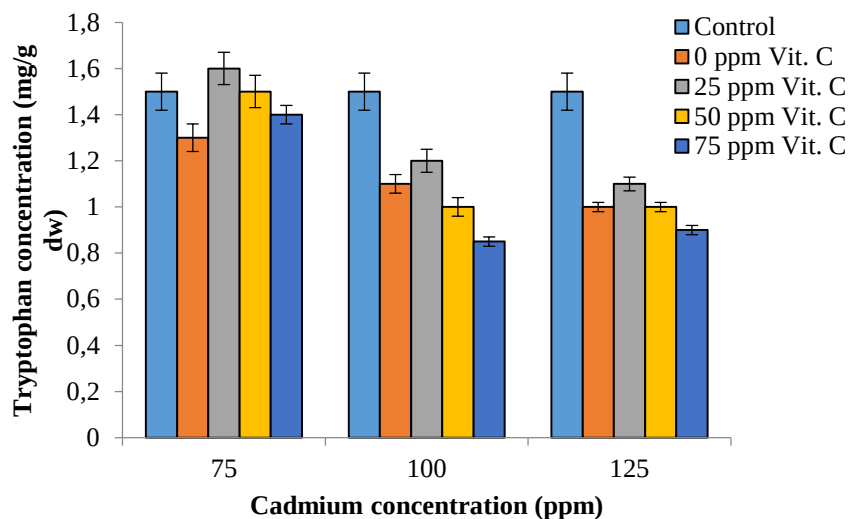


Figure 3.64. Effect of cadmium and vitamin C on the level of tryptophan in *C. freundii*

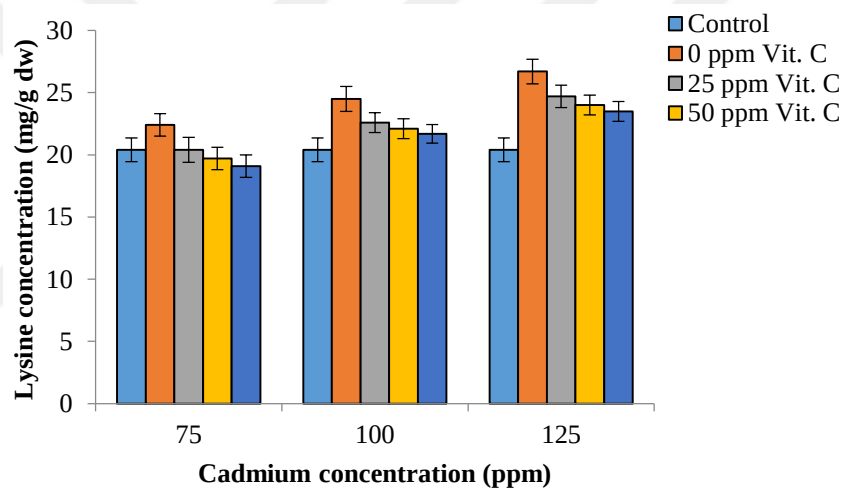


Figure 3.65. Effect of cadmium and vitamin C on the level of lysine in *C. freundii*

4. DISCUSSION

In this study, *C. freundii* was incubated (LB medium) at varying concentrations of cadmium and culture cell densities were examined by a VIS spectrophotometer. Changes in the level of stress biomarkers and the level of water and fat soluble vitamins were determined by high performance liquid chromatography (HPLC). The amino acids levels were determined also by HPLC. Total protein and enzyme activities were determined by UV-VIS spectrophotometer.

The bacteria were incubated at 0, 10, 20, 40, 60, 75, 100 and 125 ppm cadmium with the 0 ppm group serving as the control. No significant difference in the bacterial densities was observed in 10, 20 and 40 ppm groups compared with control group, while there was a significant decrease in the cell densities in cultures grown in the presence of 60, 75, 100 and 125 ppm cadmium ($p < 0.05$). There was a 40.5% and 45.5% decrease in the bacterial cell density at 100 and 125 ppm, respectively (Table 3.1 and Figure 3.2). Our result shows the ability of *C. freundii* to resist cadmium toxicity for up to 150 ppm. However, no growth was detected at 200 ppm cadmium (data not shown). Increasing the cadmium concentration significantly decreased the bacterial cell density (Table 3.1 and Figure 3.2). A previous study which is in line with our work showed the ability for *Citrobacter* sp to resist and accumulate cadmium but in higher concentration of cadmium showed a considerable inhibition of growth when compared with cadmium-free controls [159].

A significant decrease in the level of water and fat soluble vitamins was also observed. The highest effect of cadmium on the vitamins was seen at 100 and 125 ppm (Table 3.3 and 3.4). The bacterial cell density was also seen to be more affected at these cadmium concentrations (Table 3.1 and Figure 3.2).

A significant decrease in the amount of vitamin A was seen when cells were cultivated in the presence of 20, 40, 60, 75, 100 and 125 ppm cadmium ($p < 0.05$). However, no significant difference was observed in 10 ppm group (Table 3.3). Vitamin E (also known as alpha-tocopherol) is an antioxidant that protect the cell from oxidative stress and cell membrane damage [160]. A significant decrease in the amount of vitamin E was observed in 10, 20, 40, 60, 75, 100 and 125 ppm cadmium groups ($p < 0.05$) (Table 3.3).

Beta-carotene is also a very important antioxidant that protects the cell against oxidative damage [161]. A significant decrease in the amount of beta-carotene was determined in 10, 20, 40, 60, 75, 100 and 125 ppm cadmium groups ($p < 0.05$) (Table 3.3). Lycopene is a carotenoid important in beta-carotene synthesis. Another important function of lycopene is its ability to quench singlet oxygen which is a key role in antioxidation [162]. A significant decrease in lycopene levels was observed in 10, 20, 40, 60, 75, 100 and 125 ppm cadmium ($p < 0.05$) (Table 3.3). The levels of vitamin C and vitamin E at 125 ppm cadmium were found to significantly decrease. Vitamin C

decreased from 31.30 to 2.50 which corresponds to a 92.0% decrease. There was also a significant decrease of vitamin E (from 8.90 to 4.00, corresponding to a 55% decrease) (Table 3.3 and 3.4). We can relate the significant decrease in vitamin E, beta-carotene and lycopene with the bacterial ability to resist and tolerate the cadmium toxicity because these vitamins are very strong antioxidants that protect the cell against oxidative damage. Several studies have indicated the protective role of these vitamins against cadmium toxicity [163, 164].

The presence of cadmium significantly decreased the level of vitamin B₁ in *C. freundii* in the presence of 10, 20, 40, 60, 75, 100 and 125 ppm cadmium ($p < 0.05$) (Table 3.3 and Figure 3.8). The level of vitamin B₁ in *E.coli* was reported to be 7.4 µg/g of dry weight [165], while in *C. freundii* was reported to be 16.0 µg/g dw (Table 3.3). Our study showed a significant decrease in the level of Vitamin B₂ when cells were cultivated in the presence of 10, 20, 40, 60, 75, 100 and 125 ppm cadmium ($p < 0.05$) (Table 3.4). In a previous study, the level of vitamin B₂ in *E.coli* was reported to be 310.0 µg/g of dry weight [165] while in our study *C. freundii* showed a 12.0 µg/g dw in vitamin B₂ level (Table 3.4). In the presence of 10, 20, 40, 60, 75, 100 and 125 ppm cadmium there was a significant decrease in the amount of vitamin B₃ ($p < 0.05$) (Table 3.4). While *C. freundii* was found to have 262.0 µg/g dw vitamin B₃ (Table 3.4), the level of the same vitamin in *E.coli* was reported to be 450.0 µg/g of dry weight [165]. The level of vitamin B₅ was seen to decrease significantly when cells were cultivated in the presence of 10, 20, 40, 60, 75, 100 and 125 ppm cadmium ($p < 0.05$) (Table 3.4). In this study *C. freundii* was found to have a B₅ level of 170.0 µg/g dw (Table 3.4), while the level of the same vitamin in *E.coli* was reported to be 170.0 µg/g of dry weight [165]. The present study showed a significant decrease in the level of vitamin B₆ under 10, 20, 40, 60, 75, 100 and 125 ppm cadmium ($p < 0.05$) (Table 3.4). The amount of vitamin B₆ in *E. coli* was reported to be 6300.0 µg/g of dry weight [165] while in *C. freundii* was found to be 320.0 µg/g dw (Table 3.4). Vitamin B₉ (folate), in its active form as tetrahydrofolate, is a cofactor in several metabolic reactions, including DNA and amino acid synthesis [166]. The level of vitamin B₉ in this study significantly decrease when 10, 20, 40, 60, 75, 100 and 125 ppm groups were compared with control ($p < 0.05$) (Table 3.4). The amount of vitamin B₉ in *E. coli* was reported to be 1.10 µg/g of dry weight [165] while in *C. freundii* was found to be 3.60 µg/g dw (Table 3.4). Vitamin B₁₂ (cobalamin) is a cobalt-containing vitamin that in its active forms of methylcobalamin and adenosylcobalamin, catalyzes methionine synthesis. Together with vitamin B₆ and B₉, vitamin B₁₂ plays key functions in the formation of red blood cell and nucleic acid synthesis [167]. The present study indicates a significant decrease in the concentration of vitamin B₁₂ when 10, 20, 40, 60, 75, 100 and 125 ppm groups were compared with control ($p < 0.05$) (Table 3.4). The amount of vitamin B₁₂ in *K. pneumoniae* was reported to be 135.0 ng/g of dry weight [168] while in *C. freundii* was found to be 2.0 µg/g dw (Table 3.4). A similar study on *C. freundii* also shows that vitamin B₁₂ content to be 152.0 ng/g of dry weight [168]. Vitamin C has an anti-oxidative effect. It is an

electron donor and shows a reducing ability when found in the medium and therefore has the power to neutralize superoxide and hydroxyl radicals, thereby protecting the cell from oxidative damage [169]. In this study, a significant decrease in the amount of vitamin C was seen when 10, 20, 40, 60, 75, 100 and 125 ppm groups were compared with control ($p < 0.05$) (Table 3.4). Interestingly, our data showed that 20 ppm cadmium was able to decrease the initial concentration of vitamin C from 31.30 $\mu\text{g/g dw}$ to 14.73 $\mu\text{g/g dw}$ which is a 52.9% decrease (Table 3.4). We can correlate this with the antioxidant power of vitamin C which provides the bacteria ability to resist the cadmium toxicity at 20 ppm (Table 3.4).

Heavy metals such as cadmium cause metabolic, biological, and physiological modifications that are also expressed via protein inhibition [170]. From our findings, the bacterial total protein was found to be 23.77 ± 1.25 mg protein/g dw (control group) (Table 3.5). However, addition of 60, 75 and 100 ppm cadmium significantly reduced the total protein concentration to 18.12 ± 1.13 , 13.26 ± 0.92 and 8.99 ± 0.62 mg protein/g dw respectively ($p < 0.05$) (Table 3.5). Addition of 75 and 100 ppm cadmium showed a 44.2% and 62.2% reduction in total protein respectively. Addition of 25, 50 and 75 ppm vitamin C showed a significant increase in total protein concentration ($p < 0.05$) (Table 3.5).

One of the most important mechanism of metal tolerance in bacteria is the activity of the antioxidant system. Apart from the antioxidant system, various other mechanisms have been reported. The energy efflux of the metal, the deposition of the metal as insoluble salt, the modification of the permeability of the metal membrane, immobilization within the cell wall, the development of chelating agents and the biochemical transition of metal ions are some of these processes [171, 172]. Metals such as cadmium can generate physiological stress leading to the production of reactive oxygen species through hydrogen peroxide, superoxides and hydroxyl free radicals. In normal conditions, the level of these radicals remains low due to the presence and activities of protecting enzymes such as superoxide dismutase, catalase, peroxidase, glutathione reductase, glutathione peroxidase and lipoxygenase. Under metal stress conditions, the expression of these enzymes is believed to be increased in order to detoxify the reactive oxygen species [173]. In this study, the activities of all the five enzymes (SOD, CAT, POD, GSH-Px and GSH-Red) was seen to significantly increase after cadmium addition when compared with control ($p < 0.05$). (Table 3.5). These increases were seen in a concentration dependant manner. SOD is an antioxidant enzyme that function via conversion of highly toxic superoxide radical to oxygen and less toxic hydrogen peroxide. Its activity was reported to increase by cadmium [174]. In this study, the addition of 60, 75 and 100 ppm cadmium significantly increased the activity from 82.27 ± 4.89 to 120.40 ± 6.16 , 135.60 ± 6.84 and 150.0 ± 8.50 U/g dw respectively ($p < 0.05$) (Table 3.5). This shows a 46.2%, 64.8% and 82.3% increase in SOD activity in the presence of 60, 75 and 100 ppm cadmium respectively. CAT is another important antioxidant enzyme that participate in the

antioxidant enzymes system through detoxification of H_2O_2 to oxygen and water. In this study, the addition of 60, 75 and 100 ppm cadmium significantly increased the activity of CAT from 174.53 ± 11.20 to 223.99 ± 12.80 , 235.58 ± 13.10 and 250.12 ± 13.85 U/g dw respectively ($p < 0.05$) (Table 3.5). This shows a 28.3%, 34.9% and 43.3% increase in CAT activity in the presence of 60, 75 and 100 ppm cadmium respectively. Our result is in line with previous studies that also indicated an increase in CAT activity due to cadmium. In a similar study conducted by Banerjee et al, they reported an increase in CAT and SOD activities in the presence of cadmium in a concentration dependent manner [175].

GSH-Px is another vital enzyme that help to relieve stress via hydrogen peroxide removal in the presence of reduced glutathione. The present findings indicate a significant increase in GSH-Px activity with increase in cadmium concentration when compared with control. The addition of 60, 75 and 100 ppm cadmium significantly increased the GSH-Px activity from 22.89 ± 1.20 to 42.19 ± 2.05 , 75.66 ± 3.95 and 108.63 ± 5.29 U/g dw respectively ($p < 0.05$) (Table 3.5). This shows a 1.8 times, 3.3 times and 4.7 times increase in GSH-Px activity at 60, 75 and 100 ppm cadmium respectively when compared with control. This finding is also in line with previous work that showed an increase in GSH-Px activity in the presence of cadmium [171]. GSH-Red is the enzyme that catalyze the conversion of oxidized form of glutathione to its reduced form. This reaction is very important in maintenance of the glutathione level which is also important in oxidative stress conversion. In this study, the addition of cadmium significantly increase the activity of GSH-Red when compared with control. The addition of 60, 75 and 100 ppm cadmium significantly increased the activity of GSH-Red from 14.72 ± 0.98 to 26.47 ± 1.35 , 38.08 ± 1.82 and 54.50 ± 2.45 U/g dw respectively ($p < 0.05$) (Table 3.5). This result shows a 1.8 times, 2.6 times and 3.7 times increase in GSH-Red activity at 60, 75 and 100 ppm cadmium respectively when compared with control. This result is also in line with previous findings that indicate an increase in GSH-Red activity in the presence of cadmium [176]. In a similar work carried out by Sofia et.al on *Rhizobium leguminosarum*, the activities of both GSH-Red and GSH-Px where reported to increase in the presence of cadmium which is in line with the present work [177].

POD activity was also found to increase in this study after addition of varying concentration of cadmium. The addition of 60, 75 and 100 ppm cadmium significantly increased the activity of POD from 39.75 ± 1.92 to 81.93 ± 4.52 , 101.46 ± 5.06 and 122.22 ± 5.73 U/g dw respectively ($p < 0.05$) (Table 3.5). This result shows a 2.1 times, 2.6 times and 3.1 times increase in POD activity at 60, 75 and 100 ppm cadmium respectively when compared with control. This result is also in line with previous findings that indicate an increase in POD activity in the presence of cadmium [178]. A similar study carried out on two different bacterial species *Basillus subtilis* and *Pseudomonas putida* by Jin and Khalid, showed a significant increase in POD activity in the presence of heavy metals [179].

Many studies indicate an increase in stress biomarkers level due to cadmium toxicity [180]. Our result also shows an increase in the level of stress biomarkers which might be due to cadmium toxicity. Increasing the cadmium concentration significantly increase the level of MDA, 4-HNE and GSSG with the highest increase seen in 125 ppm cadmium (Table 3.6). MDA is one of the end products of arachidonic acid decomposition and also that of PUFA oxidation. For a very long time, MDA level has been used as lipid peroxidation maker in studies that are related to oxidative stress [181]. The present study indicates a significant increase in MDA level with increase in cadmium concentration in *C. freundii* when 10, 20, 40, 60, 75, 100 and 125 ppm groups were compared with control group ($p < 0.05$) (Table 3.6). A similar study conducted by Pandey et.al on *Ochrobactrum* sp, revealed a considerable increase in MDA level in the presence of cadmium which is in line with our work [171]. Another research conducted by Choudhary et.al on cyanobacteria *Spirulina platensi* also revealed a significant increase in MDA concentration under heavy metal stress condition [173].

Glutathione happen to be a valuable antioxidant with the ability to protect damage caused by ROS such as free radicals, peroxides, lipid peroxides and heavy metals to important cellular component of the bacteria [182]. Therefore, keeping an optimum ratio of the reduced and oxidized forms of glutathione (GSH: GSSG ratio) is very vital for the cell survival [183]. In the present work., the addition of cadmium significantly decrease the amount of reduced glutathione (GSH) when 20, 40, 60, 75, 100 and 125 ppm groups were compared with control group ($p < 0.05$). However, no significant difference when 10 ppm group was compared with control (Table 3.6). The level of GSH was seen to significantly decrease as we increase the cadmium concentration which leads to a decrease in the GSH: GSSG ratio which is an evidence of increased ROS in the bacteria due to cadmium toxicity (Table 3.6). The level of oxidized glutathione was seen to significantly increase when 20, 40, 60, 75, 100 and 125 ppm groups were compared with control group ($p < 0.05$). However, no significant difference was observed when 10 ppm group was compared with control (Table 3.6). The ratio of reduced to oxidized glutathione (GSH: GSSG ratio) was found to reduce (Table 3.6). The decreases in this ratio explain the evidence of cadmium in having the ability to induce stress in the bacteria. The decrease in the amount of GSH is an evidence of metal toxicity tolerance by the bacteria. This is in line with a research conducted on *Streptococcus pneumonia* by Adam et.al where they showed the importance of GSH in defence against oxidative stress and metals toxicity such as cadmium, copper and zinc [184]. Another similar work carried out on *Rhizobium leguminosarum* showed a significant decrease in the amount GSH and a significant increase in GSSG and also a decrease in GSH/GSSG ration in the presence of cadmium [177]. This work is also in line with our findings. These data are consistent with the role of GSH as a radical scavenger, being oxidized by ROS and producing GSSG. 4-Hydroxyneonal is another product of lipid peroxidation which is more toxic than MDA [185]. In this study, the

level of 4-HNE was seen to significantly increase when 20, 40, 60, 75, 100 and 125 ppm groups were compared with control group ($p < 0.05$). However, no significant difference was observed when 10 ppm group was compared with control (Table 3.6). At 125 ppm cadmium, there is 76.2% decrease in GSH level, 4.0 fold increases in GSSG level, 5.7 fold increases in MDA and a 10.2 fold increase in 4-HNE level.

Addition of vitamin C to the bacterial LB significantly increased the concentration of the bacteria (Table 3.2). This result is in line with many studies that report the effect of vitamin C in antagonizing cadmium toxicity [186]. There is a significant increase in the bacterial concentration even in the absence of cadmium from 4.37 ± 0.04 to 4.64 ± 0.01 , 4.77 ± 0.03 and 4.82 ± 0.02 g dw/L after addition of 25, 50 and 75 ppm vitamin C respectively ($p < 0.05$) (Table 3.2). At 75 ppm the bacterial concentration was 3.55 ± 0.05 g dw/L which significantly increased to 3.88 ± 0.02 , 4.10 ± 0.03 and 4.30 ± 0.06 g dw/L after addition of 25, 50 and 75 ppm Vitamin C respectively ($p < 0.05$) (Table 3.2). The initial concentration of the bacteria was 4.37 ± 0.04 g dw/ L (control group). At 75 ppm cadmium, the concentration decreased to 3.55 ± 0.05 g dw/L which is 18.76% decrease. However, addition of 75 ppm vitamin C to the 75 ppm cadmium group increased the concentration from 3.55 ± 0.05 to 4.30 ± 0.06 g dw/L which almost reverse the cadmium effect by taking back the concentration close to the initial one 4.37 ± 0.04 g dw/L. So also, addition of 25 ppm vitamin C to 100 ppm cadmium group shows a significant difference 2.60 ± 0.08 to 2.70 ± 0.06 g dw/L respectively and further addition of 50 and 75 ppm significantly increased the concentration to 2.80 ± 0.01 and 3.04 ± 0.09 g dw/L respectively ($p < 0.05$) (Table 3.2). The initial level of the bacteria was 2.38 ± 0.13 g dw/L at 125 ppm cadmium which significantly increased to 2.65 ± 0.01 , 2.75 ± 0.01 and 3.00 ± 0.01 g dw/L after addition of 25, 50 and 75 ppm vitamin C respectively ($p < 0.05$) (Table 3.2).

Furthermore, the levels of both fat and water soluble vitamins were seen to significantly increased after addition of 25, 50 and 75 ppm vitamin C to the 75, 100 and 125 ppm cadmium groups ($p < 0.05$) (Tables 3.3 and 3.4). Interestingly, the initial level of B₉ was 3.60 ± 0.20 µg/g dw (control group), after addition of 75 ppm cadmium, the level drop to 2.00 ± 0.08 µg/g dw which is a significant decrease ($p < 0.05$). Addition of 75 ppm vitamin C to this group increased the concentration to 3.60 ± 0.20 µg/g dw which is the same as that of the control group (Table 3.4 and Figure 3.14).

Addition of 25, 50 and 75 ppm vitamin C showed a significant increase in total protein concentration and a significant decrease in the activities of the enzymes ($p < 0.05$) (Table 3.5). This further explain the ability of vitamin C to relieve stress. The initial total protein concentration was 23.77 ± 1.25 mg protein/g dw (control group). After addition of 60 ppm cadmium, the concentration drops to 18.12 ± 1.13 mg protein/g dw ($p < 0.05$). Addition of 75 ppm vitamin C, increase the concentration to 22.20 ± 1.24 mg protein/g dw ($p < 0.05$) which is close to the initial total protein

concentration (Table 3.5 and Figure 3.16). The activity of CAT was seen to statistically increase from 174.53 ± 11.10 U/g dw to 223.99 ± 12.80 U/g dw ($p < 0.05$) after addition of 60 ppm cadmium. Addition of 75 ppm vitamin C to this group drop the activity to 172.33 ± 10.75 U/g dw ($p < 0.05$) which is almost the same with that of the control group (Table 3.5 and Figure 3.21).

Vitamin C was reported to have the ability to lower the MDA concentration in cadmium-induced stress [186]. Our result shows a significant decrease in MDA concentration in *C. freundii* after addition of vitamin C to 75, 100 and 125 ppm groups ($p < 0.05$) (Table 3.6). In its ability to ameliorate oxidative stress induced by cadmium, vitamin C increase the level of GSH and decrease the level of GSSG thereby improving the activity of glutathione reductase enzyme and increasing the reduced to oxidized glutathione (GSH:GSSG) ratio [187]. In our study. addition of 25, 50 and 75 ppm vitamin C to 0 ppm cadmium group (control) showed a significant increase in GSH level (Table 3.6). However, all other groups shows a significant increase in GSH ($p < 0.05$) (Table 3.6). A significant decrease in GSSG ($p < 0.05$) (Table 3.6) was observed and also an increase in GSH:GSSG ratio when 25, 50 and 75 ppm vitamin C was added to 75, 100 and 125 ppm groups (Table 3.6). The amount of 4-HNE in our study also significantly decrease after addition of 25, 50 and 75 ppm vitamin C to both control group, 75, 100 and 125 ppm groups ($p < 0.05$) (Table 3.6).

Selenium was reported to have a protective role against cadmium toxicity in bacteria [188]. We also added selenium in the bacterial LB which significantly increase the concentration of the bacteria in the presence of cadmium (Table 3.7), indicating the effect of selenium in ameliorating cadmium toxicity as previously reported by several researchers [189]. In this study. addition of 1.0 ppm selenium to control group (0 ppm group) shows no sinificant difference in the bacterial concentration 4.37 ± 0.04 to 4.50 ± 0.06 g dw/L, but addition of 3.0 ppm selenium significantly incese the bacterial concentration from 4.37 ± 0.04 to 4.58 ± 0.01 g dw/L ($p < 0.05$) (Table 3.7). Addition of 1.0 ppm and 3.0 ppm selenium to 75 ppm cadmium group significantly increase the bacterial concentration from 3.55 ± 0.05 to 3.75 ± 0.02 and 4.02 ± 0.09 g dw/L respectively ($p < 0.05$) (Table 3.7). Further more. addition of 1.0 ppm and 3.0 ppm selenium to 100 ppm cadmium group significantly increase the bacterial concentration from 2.60 ± 0.08 to 2.68 ± 0.05 and 2.90 ± 0.01 g dw/L respectively ($p < 0.05$) (Table 3.7). Statistically, no significant difference was observed after additon of 1.0 ppm selenium to 125 ppm cadmium group (Table 3.7). However, additon of 3.0 ppm selenium to the 125 ppm cadmium group significantly increase the initial concentration of 2.38 ± 0.01 to 2.74 ± 0.06 g dw/L ($p < 0.05$) (Table 3.7).

Additionally, the levels of both fat and water soluble vitamins were seen to significantly increased after addition of 1.0 and 3.0 ppm selenium to the 0, 75, 100 and 125 ppm cadmium groups ($p < 0.05$) (Tables 3.8 and 3.9).

Having the ability to antagonize the toxic effect of oxidative damage induced by cadmium, selenium was found to decrease the level of stress biomarkers via prevention of lipid peroxidation

[189, 190]. Addition of 1.0 and 3.0 ppm selenium to 0, 75, 100 and 125 ppm cadmium groups significantly decreased the level of 4-HNE ($p < 0.05$) (table 3.10). Addition of 1.0 and 3.0 ppm selenium to 0, 75, 100 and 125 ppm cadmium groups significantly decreased the level of MDA ($p < 0.05$) (Table 3.10). However, no significant difference in the level of GSH after addition of 1.0 ppm selenium to 0 ppm group, but a significant increase was observed after adding 3.0 ppm selenium ($p < 0.05$). Also, addition of 1.0 and 3.0 ppm selenium to 75, 100 and 125 ppm groups showed a significant increase in GSH level ($p < 0.05$) (Table 3.10). No significant difference in the level of GSSG after addition of 1.0 ppm selenium to 0 ppm group, but a significant decrease was observed after adding 3.0 ppm selenium ($p < 0.05$). Also, addition of 1.0 and 3.0 ppm selenium to 75, 100 and 125 ppm groups showed a significant decrease in GSSG level ($p < 0.05$) (Table 3.10). A significant increase in the GSH: GSSG ratio was also observed after addition of 1.0 and 3.0 ppm selenium to 0, 75, 100 and 125 ppm groups ($p < 0.05$) (Table 3.10).

Importantly, addition of both vitamin C and selenium shows a significant decrease in the level of stress biomarkers MDA, 4-HNE and increase the GSH: GSSG ratio (Tables 3.6 and 3.10). This further explains the positive effect of both vitamin C and selenium in antagonizing cadmium toxicity.

Amino acids are highly important molecules for all living cells. They are the building blocks of protein and intermediates in metabolic pathways. They also play a pivotal role in metabolism, regulating gene expression, immunity, signal transduction, anti-oxidative responses, growth and cell survival [191]. Table 3.11 shows the amino acid composition of *C. freundii* obtained in this study by the use of HPLC. As seen from the results, the amino acid with the highest concentration is glutamic acid (38.70 ± 1.50 mg/g dw) and the amino acid with the lowest concentration is tryptophan (1.50 ± 0.08 mg/g dw). The order from highest to lowest concentration is Glutamic acid > Aspartic acid > Alanine > Leucine > Threonine > Lysine > Serine > Asparagine > Phenylalanine > Proline > Isoleucine > Glycine > Tyrosine > Valine > Glutamine > Arginine > Histidine > Methionine > Cysteine > Tryptophan. Our findings indicated the amount of glutamic acid, glycine, serine, histidine, valine, aspartic acid, proline, tyrosine, alanine, methionine and phenylalanine to be 38.70 ± 1.50 , 9.60 ± 0.40 , 19.60 ± 0.90 , 5.00 ± 0.25 , 8.40 ± 0.40 , 30.00 ± 1.20 , 12.00 ± 0.50 , 9.50 ± 0.40 , 27.60 ± 0.90 , 4.95 ± 0.20 and 12.50 ± 0.50 mg/g dw in *C. freundii* respectively (tables 3.14) while that of *E.coli* was reported to be 35.00 ± 3.02 , 94.00 ± 4.00 , 1.10 ± 0.01 , 3.00 ± 1.40 , 1.80 ± 1.30 , 51.00 ± 3.70 , 1.60 ± 0.01 , 13.00 ± 0.50 , 4.30 ± 0.40 and 0.40 ± 0.02 nmoles/mg dry cell mass respectively [192].

Moreover, in this study, addition of cadmium in *C. freundii* culture medium shows a contrasting pattern in the concentration of the amino acids in the bacteria (Tables 3.12). When compared with control, addition of 75, 100 and 125 ppm cadmium significantly increase the level of leucine (Figure 3.46), asparagine (Figure 3.48), glycine (Figure 3.49), histidine (Figure 3.52),

arginine (Figure 3.53), valine (Figure 3.56), aspartic acid (Figure 3.55), proline (Figure 3.56), tyrosine (Figure 3.57), alanine (Figure 3.58), cysteine (Figure 3.60), threonine (Figure 3.61) isoleucine (Figure 3.62), phenylalanine (Figure 3.63) and lysine (Figure 3.65) ($p < 0.05$), while glutamic acid (Figure 3.47), serine (Figure 3.50), glutamine (Figure 3.51), methionine (Figure 3.59) and tryptophan (Figure 3.64) shows a significant decrease when compared with control ($p < 0.05$).

These irregular changes that were observed in the concentrations of the amino acids are an evidence of cadmium toxicity resistance and tolerance by the bacteria. This is in line with many studies conducted on different plant and bacterial species [193, 194]. Cadmium toxicity affects a number of metabolic processes. Bacteria and plants can accumulate specific amino acids in the presence of cadmium, in which doing so is highly beneficial for the plant and bacteria to develop cadmium tolerance [193-195]. In vitro studies carried out on *Lactobacillus plantanum* revealed a significant up-regulation of hydrophobic amino acids [196]. A chorismate mutase (aro A) is a protein that was reported to be up-regulated during cadmium exposure in which it triggers the biosynthesis of phenylalanine and tyrosine which in turn increases the hydrophobicity of the bacterial cell surface to prevent cadmium-induced cell damage [197]. The above findings are also in line with our result which shows a significant increase in hydrophobic amino acids (with the exception of methionine and tryptophan) with increase in cadmium concentration (Tables 3.12). In another recently published research, histidine-rich peptides were reported to confer a protective role against cadmium stress in *E.coli* [198]. We might also relate these findings with the significant increase in histidine concentration with increasing cadmium concentration as shown in table 3.12. The peptide glutathione is an important antioxidant which exists in reduced and oxidized forms and consists of glutamine, cysteine and glycine. Exposure to cadmium leads to an increase in ROS generation which the cells respond to that via the use of enzymes and molecules like glutathione to protect the cell against oxidative damage. So collectively this leads to an increase in expression and activities of glutathione synthase, glutathione peroxidase and glutathione reductase [199]. In view of this, we can also relate the irregular and contrasting pattern of amino acid levels observed in this study (Table 3.12).

Moreover, our results indicated that the addition of varying concentrations of vitamin C to *C. freundii* culture medium showed a change in the pattern of amino acid levels. Vitamin C is a strong antioxidant and as such it relieves the toxicity of cadmium thereby causing a change in the concentrations of the amino acids. In the case of methionine, addition of 75, 100 and 125 ppm cadmium shows a significant decrease in the concentration (Table 3.12, Figure 3.59) ($p < 0.05$). Addition of 25 and 50 ppm vitamin C to the 75, 100 and 125 ppm cadmium groups, shows a significant increase in the concentration of methionine (Table 3.12, Figure 3.59) ($p < 0.05$), but further addition of 75 ppm vitamin C shows no difference statistically when compared with control (Table 3.12, Figure 3.59).

5. CONCLUSIONS

Due to increased addition of heavy metals to the environment, pollution has become a serious problem and of great concern to the world. Heavy metals such as cadmium are not readily absorbed or captured by microorganisms and as such become a great danger to aquatic and environmental system. Isolating bacteria capable of bioaccumulating these heavy metals has become a focus of research for researchers around the globe. *C. freundii* was previously reported to have the ability to bioaccumulate metals such as copper [200]. The present study shows the ability of *C. freundii* to grow under cadmium stress. In this work, increased levels of MDA and 4-HNE suggested the production of free radicals in the cadmium stressed bacteria, while increased activities of SOD, CAT, GSH-Px, GSH-Red and POD indicated the occurrence of a scavenging process. *C. freundii* show resistance or tolerance which indicates its potential to be useful in bioremediation of cadmium. This study also further indicates the effect of vitamin C and selenium in inhibiting cadmium toxicity in living cells which is in line with previous studies.

6. RECOMMENDATION

The present study shows the ability of *C. freundii* to grow under cadmium stress. Future studies on the same bacteria using different heavy metals such as lead and chromium can provide more understanding of this bacterium's ability to resist heavy metal stress. Also, future studies should consider determination of protein expression fluctuations by the use of SDS-PAGE. Same study plan can also be employed on different species of the *Citrobacter* genus such as *C. koseri* and *C. amalonaticus*. Effects of other antioxidant vitamins can be investigated in heavy metal applications. Similar work can also be applied to other bacteria in order to determine their heavy metal tolerance capability. Intracellular and extracellular oscillations can be determined by looking at the parameters in the nutrient medium.

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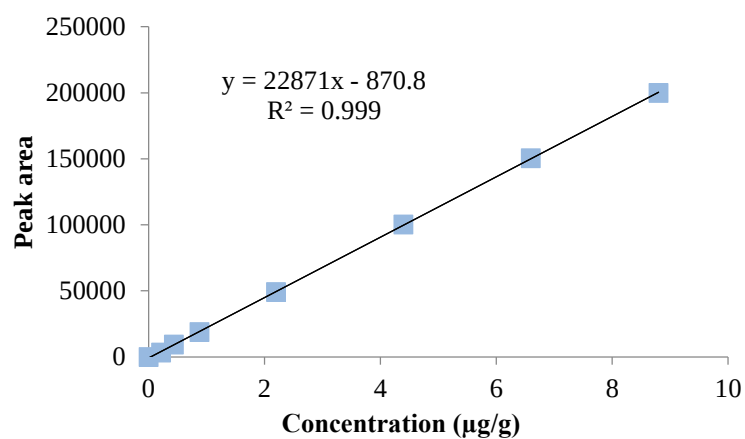
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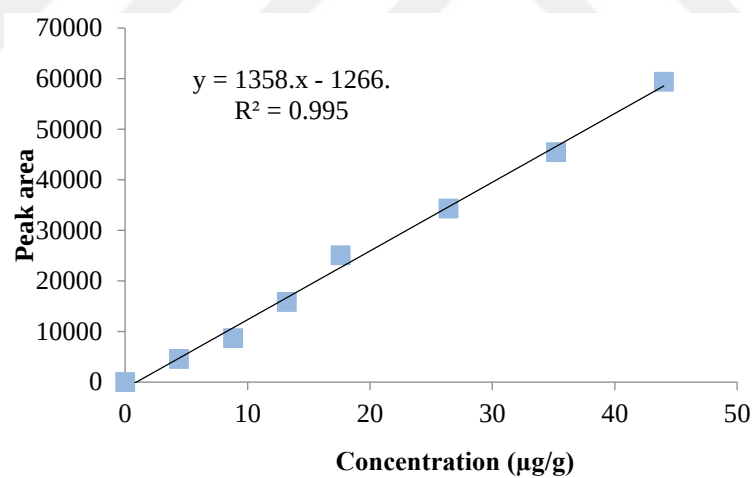
APPENDICES

APPENDIX- 1:

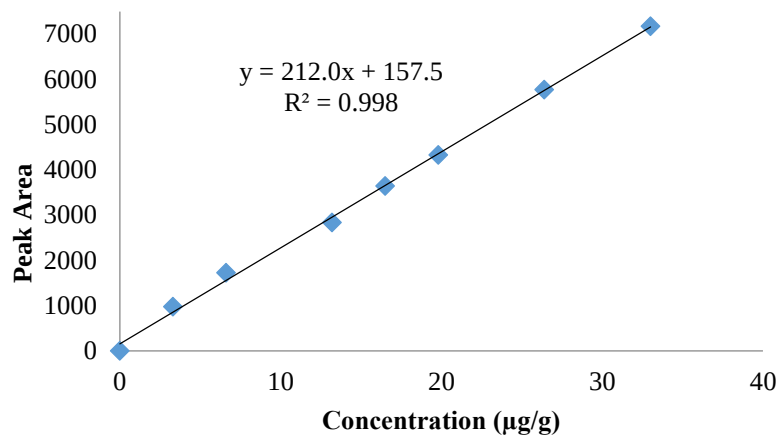
FigureA 1. *C.freundii* calibration curve



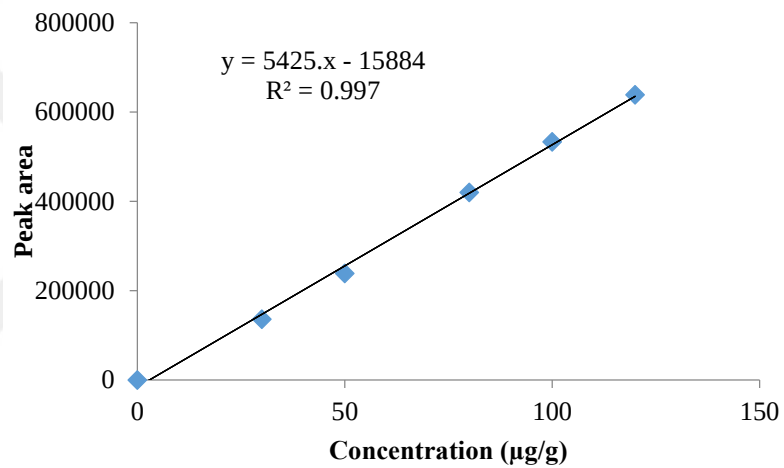
FigureA 2. The correct working graph for Vitamin A



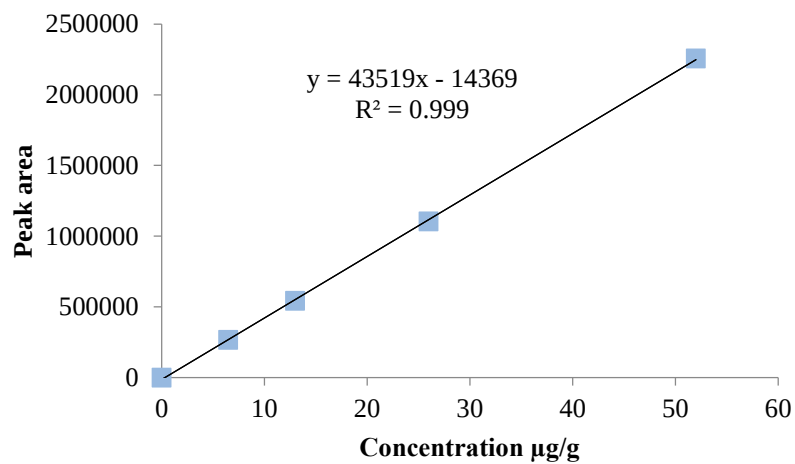
FigureA 3. The correct working graph for Vitamin E



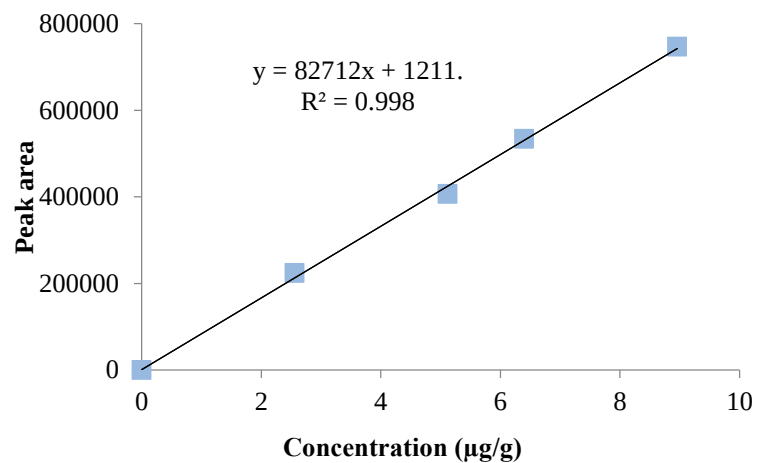
FigureA 4. The correct working graph for Beta-carotene



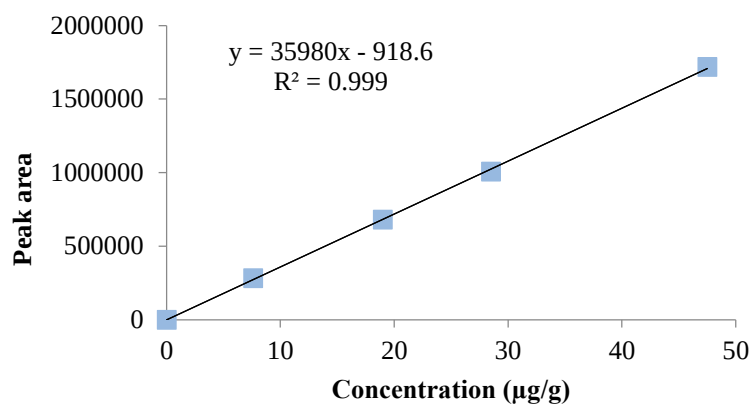
FigureA 5. The correct working graph for Lycopene



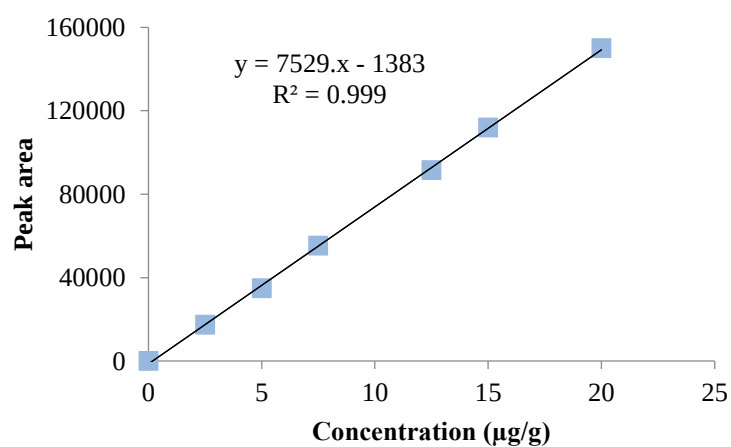
FigureA 6. The correct working graph for Vitamin B₁ (Thiamine)



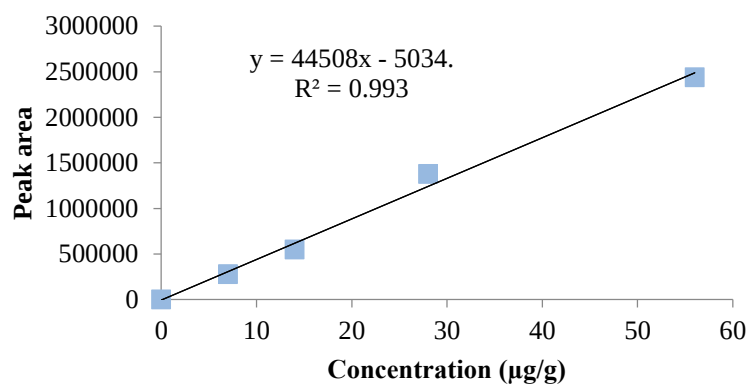
FigureA 7. The correct working graph for Vitamin B₂ (Riboflavine)



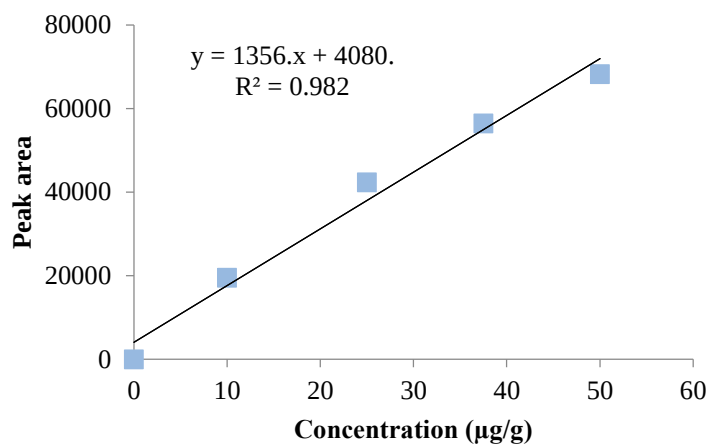
FigureA 8. The correct working graph for Vitamin B₃ (Niacine)



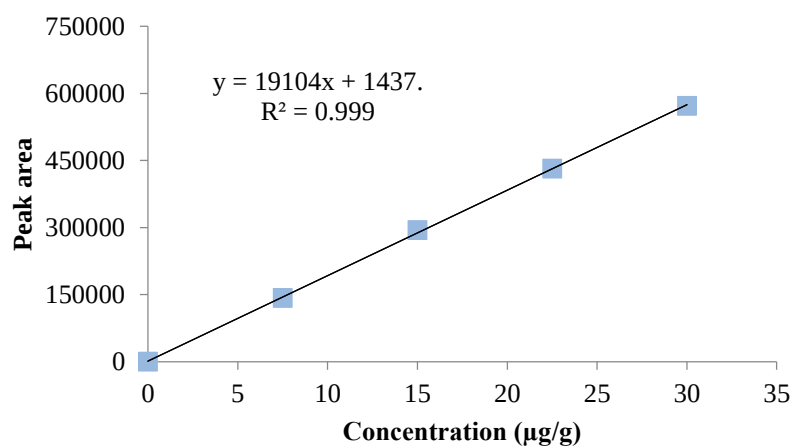
FigureA 9. The correct working graph for Vitamin B₅ (Pantothenic acid)



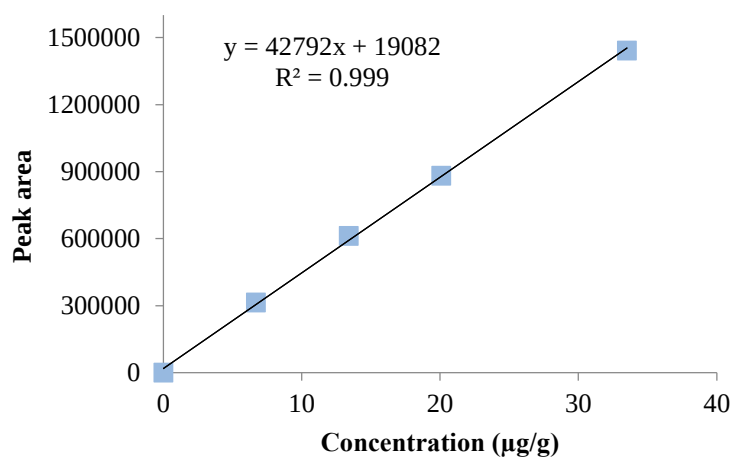
FigureA 10. The correct working graph for Vitamin B₆ (Pyridoxine)



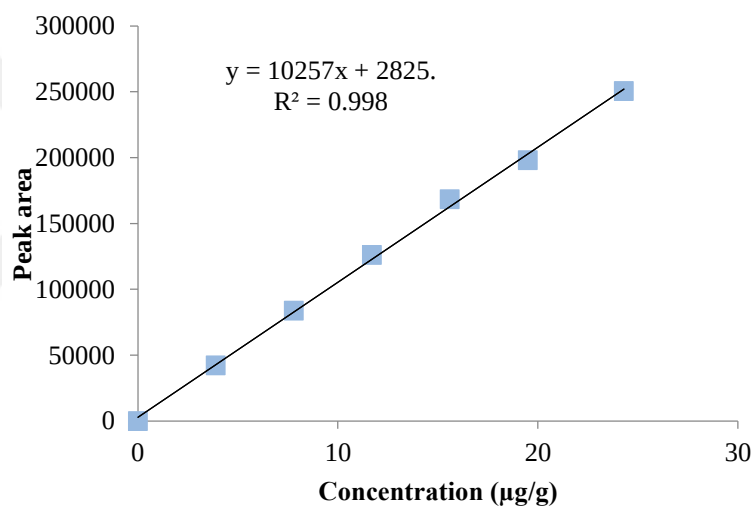
FigureA 11. The correct working graph for Vitamin B₉ (Folic acid)



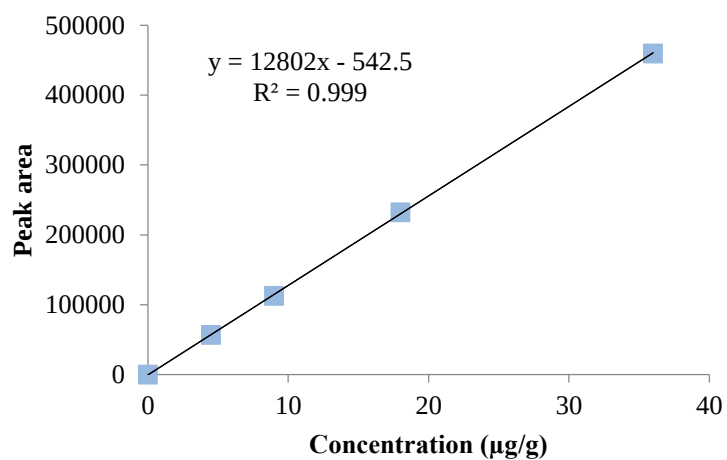
FigureA 12. The correct working graph for Vitamin B₁₂ (Cyanocobalamin)



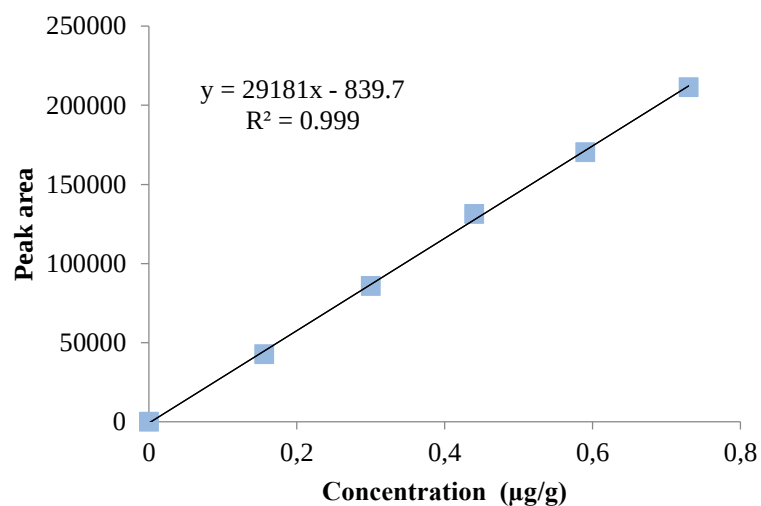
FigureA 13. The correct working graph for Vitamin C



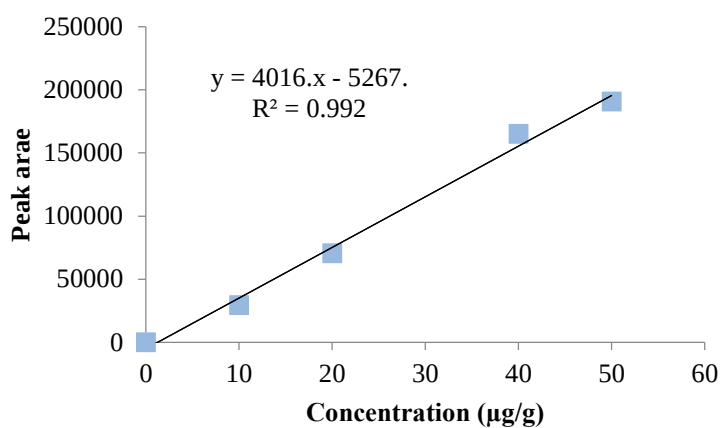
FigureA 14. The correct working graph for GSH



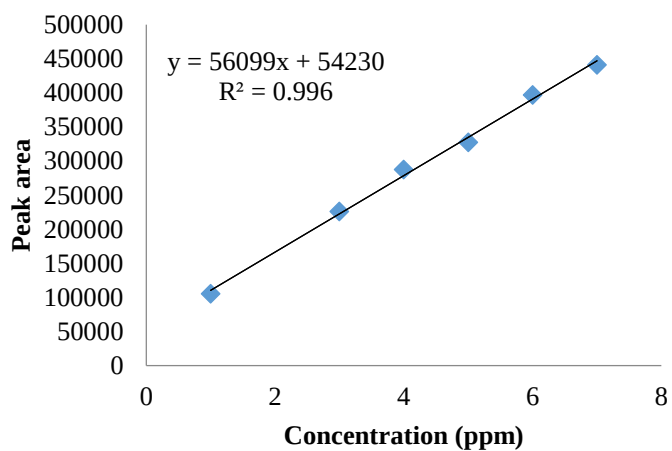
FigureA 15. The correct working graph for GSSG



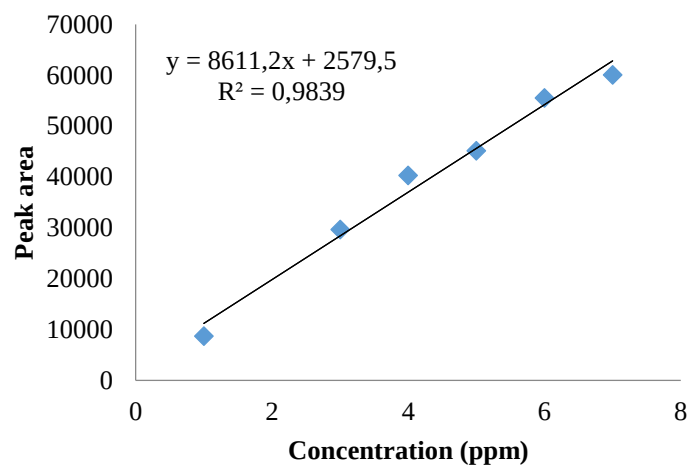
FigureA 16. The correct working graph for MDA



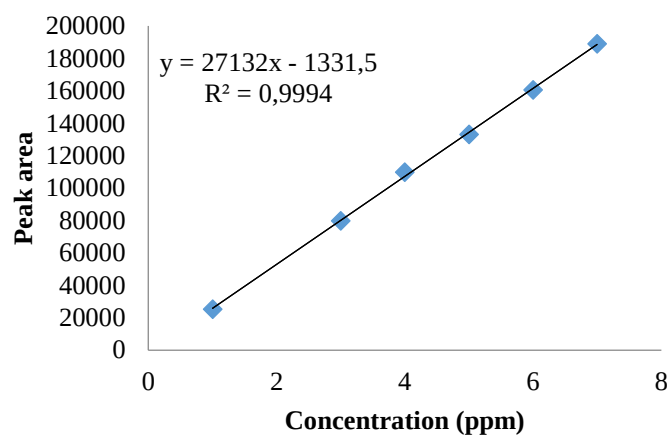
FigureA 17. The correct working graph for 4-HNE



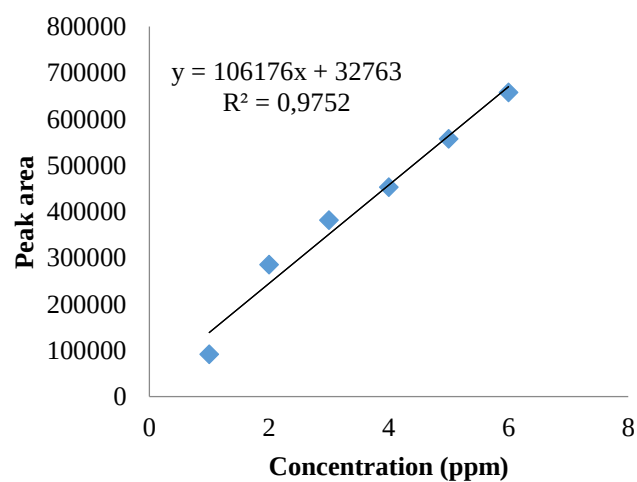
FigureA 18. The correct working graph for Aspartic acid



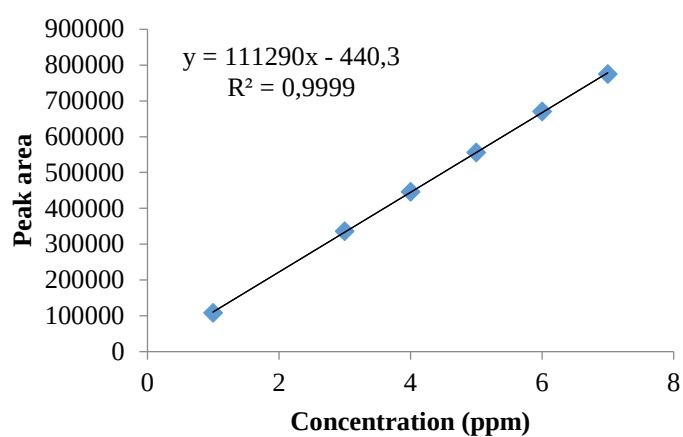
FigureA 19. The correct working graph for Glutamic acid



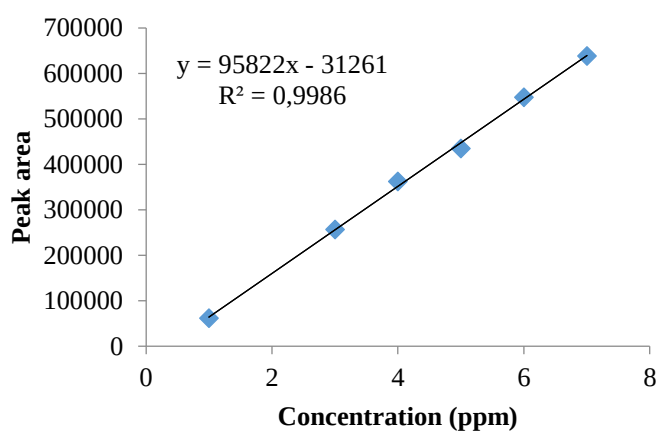
FigureA 20. The correct working graph for Asparagine



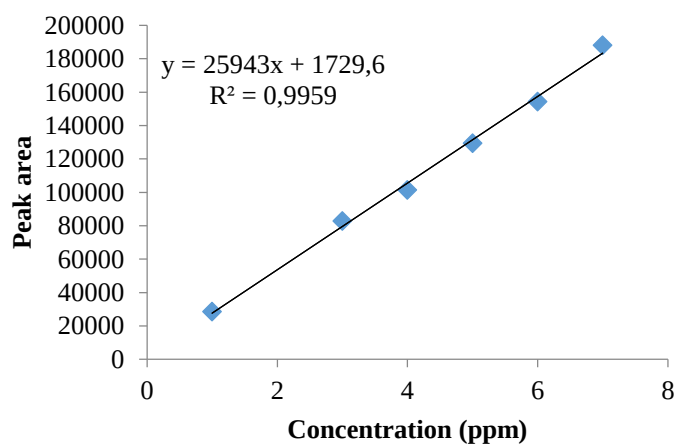
FigureA 21. The correct working graph for Serine



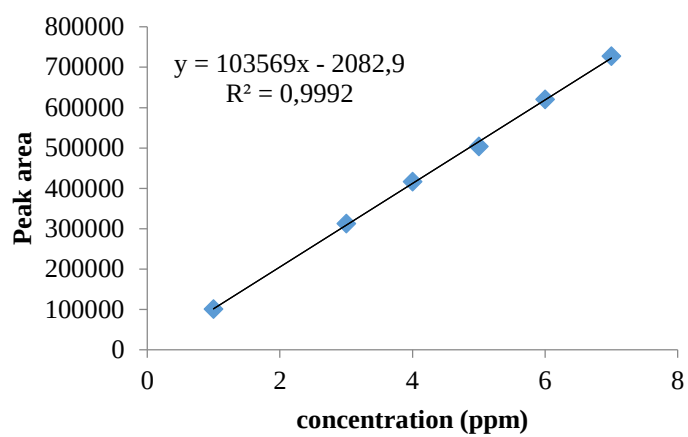
FigureA 22. The correct working graph for Glycine



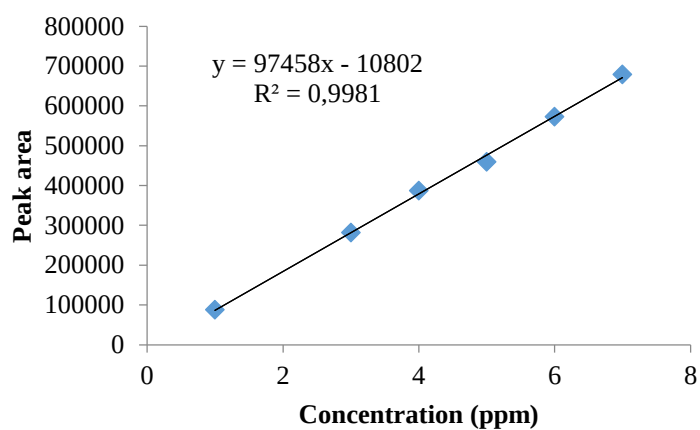
FigureA 23. The correct working graph for Glutamine



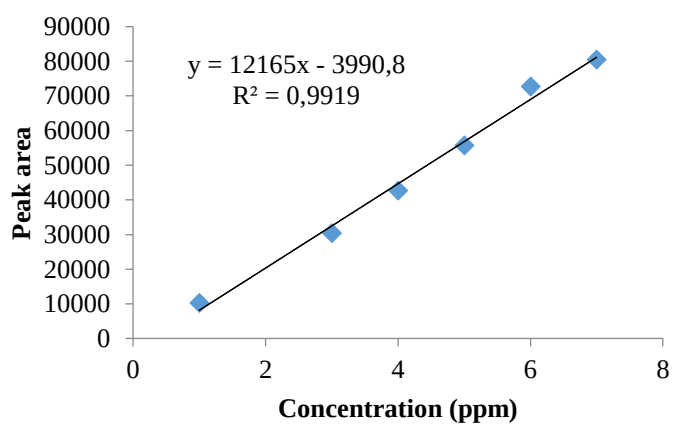
FigureA 24. The correct working graph for Histidine



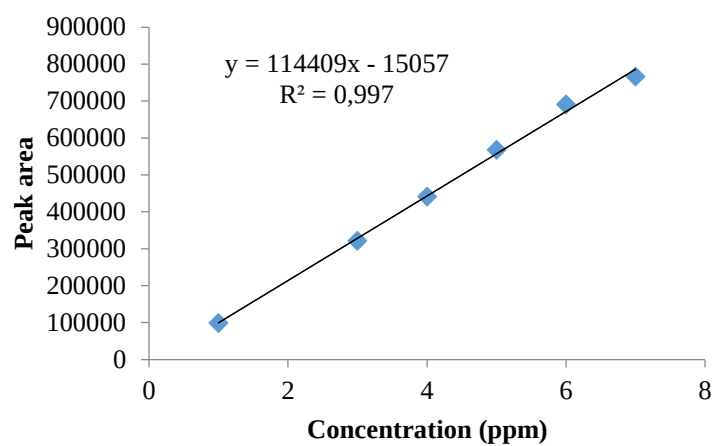
FigureA 25. The correct working graph for Arginine



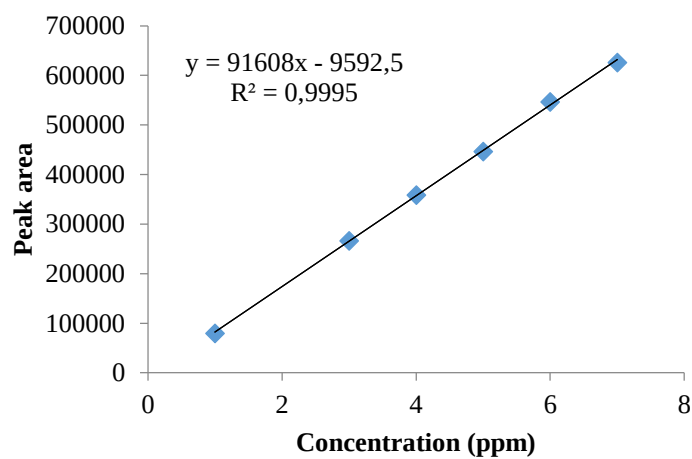
FigureA 26. The correct working graph for Threonine



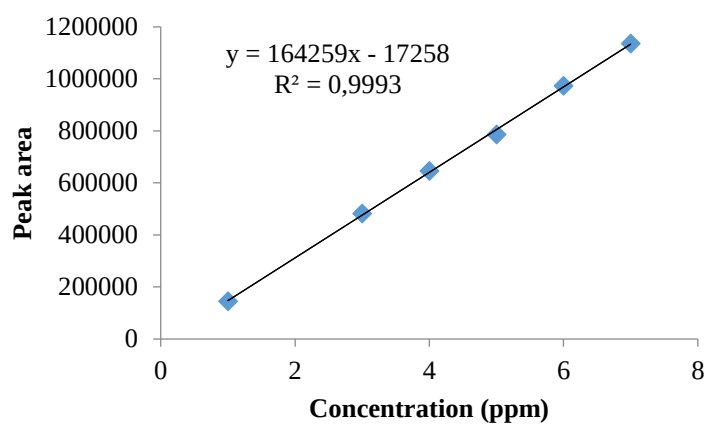
FigureA 27. The correct working graph for Alanine



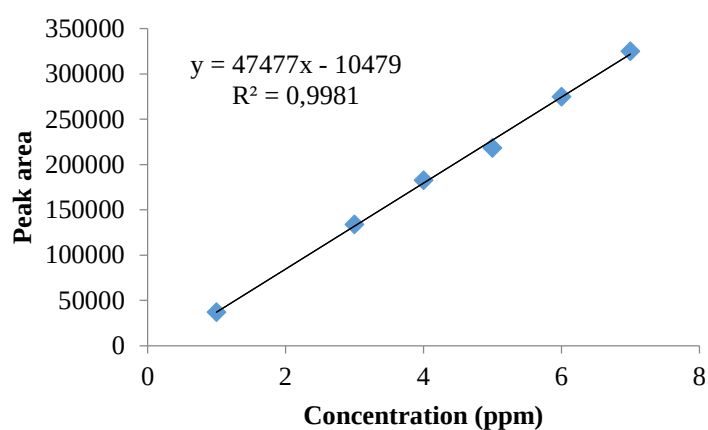
FigureA 28. The correct working graph for Proline



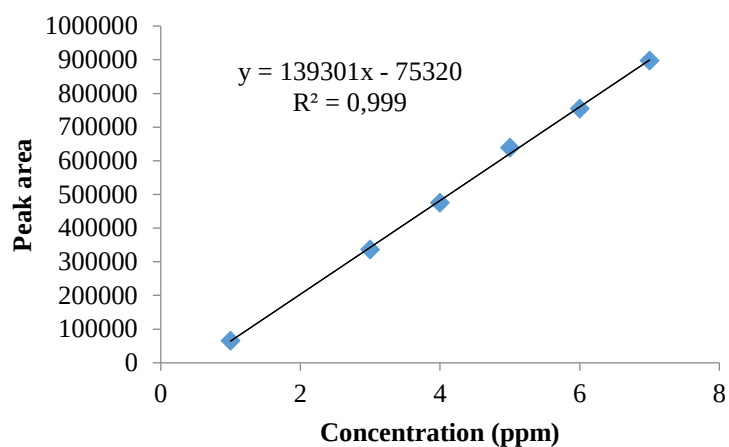
FigureA 29. The correct working graph for Tyrosine



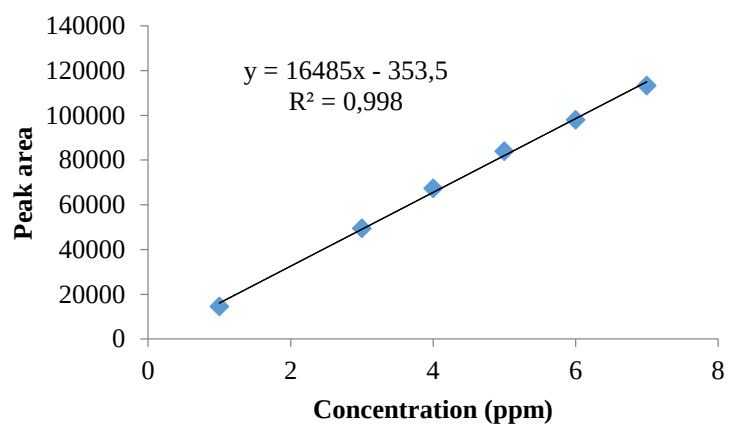
FigureA 30. The correct working graph for Valine



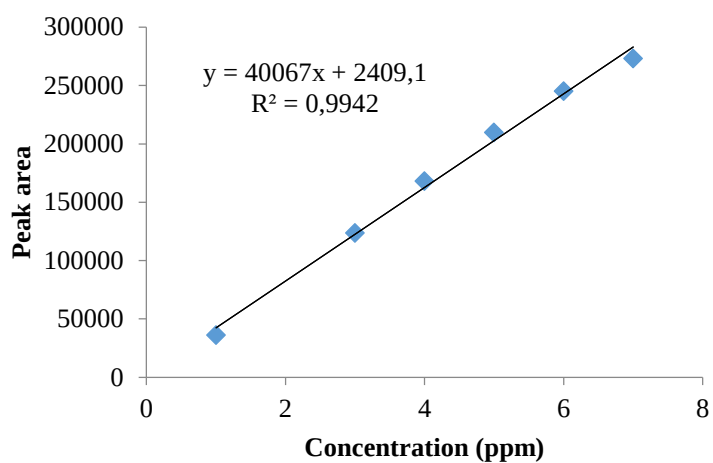
FigureA 31. The correct working graph for Methionine



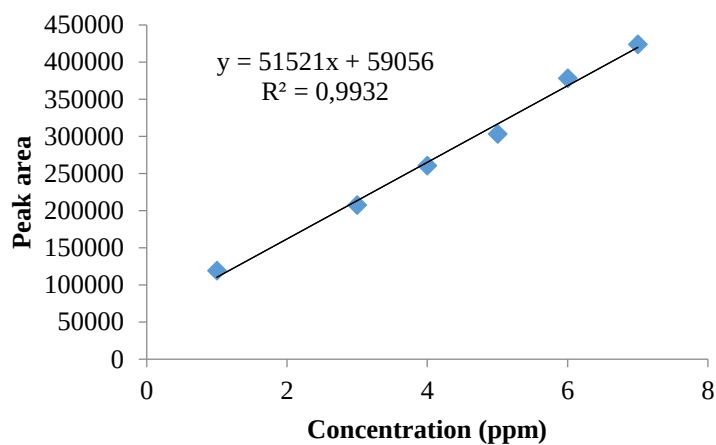
FigureA 32. The correct working graph for Cysteine



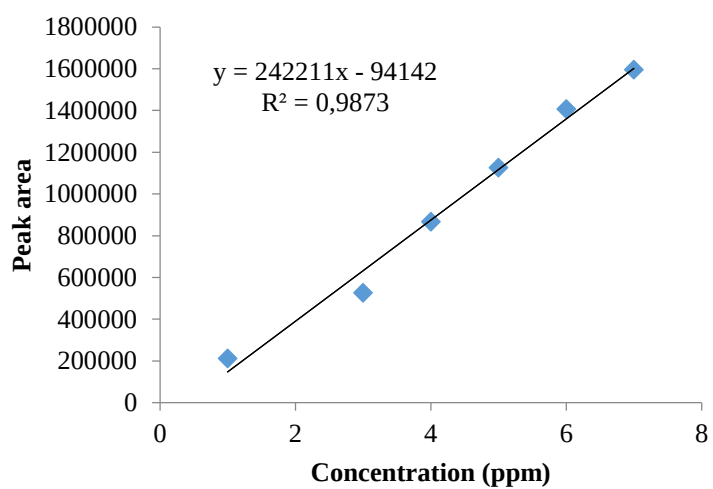
FigureA 33. The correct working graph for Leucine



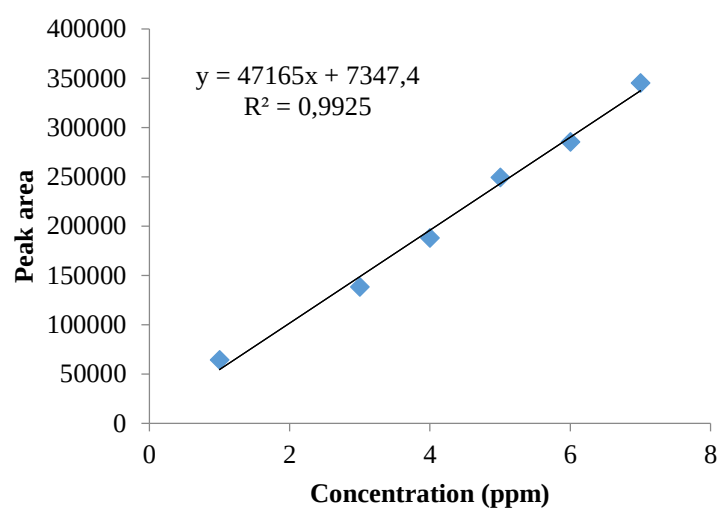
FigureA 34. The correct working graph for Isoleucine



FigureA 35. The correct working graph for Phenylalanine



FigureA 36. The correct working graph for Tryptophan



FigureA 37. The correct working graph for Lysine

CURRICULUM VITAE

Muhammad Salihu IBRAHIM

PERSONAL INFORMATIONS

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RESEARCH EXPERIENCES

- ✓ Good knowledge of bacterial growth by using autoclave, shaking incubator and spectrophotometer.
- ✓ Good sample preparation for vitamins analysis using HPLC

WORK EXPERIENCE

- Student Industrial Work Experience Scheme (SIWES) at Murtala Muhammad Specialist Hospital. Kano Nigeria 2009.
- 6 months at Aminu Kano teaching Hospital (AKTH) Kano Nigeria 2010.
- 6 months training at Princes Heya Biotechnology Center king Abdallah University hospital Jordan 2013.
- Lecturer at Kano state polytechnic (school of technology) since 2015 to date.

ACADEMIC ACTIVITIES

PUBLISHED ARTICLES

1. **IBRAHIM MS.** Ibrahim YI. Mukhtar ZG. Karatas F (2017) Amount of Vitamin A. Vitamin E. Vitamin C. Malondialdehyde. Glutathione. Ghrelin. Beta-Carotene. Lycopene in Fruits of Hawthorn. Midland(Crataegus laesssvigata). J Hum Nutr Food Sci 5(3): 1112.

2. Mukhtar, Z. G., **IBRAHİM. M. S.**, Ibrahim, Y. I., Karatas, F. (2019). Amounts of vitamin A, vitamin E, vitamin C, β -carotene, lycopene, ghrelin, glutathione, and MDA in fruits of Diospyros kaki L. GIDA (2019) 44(4) 585-592 doi: 10.15237/gida.GD19033
3. Bakar, B., Çakmak, M., **IBRAHİM. M.S.**, Ozer, D., Karatas, F., Saydam, S (2020). Investigation of Amounts of Vitamins, Lycopene, and Elements in the Fruits of Opuntia ficus-indica Subjected to Different Pretreatments. Biol Trace Elem Res <https://doi.org/10.1007/s12011-020-02050-w>
4. ÇAKMAK, M., BAKAR, B., **IBRAHİM. M.S.**, Dursun, Ö.Z.E.R., KARATAŞ, F. and SAYDAM, S.. (2020). Effect of Freezing and Drying Methods on Some Biochemical Properties of Prickly Fig (Opuntia ficus-indica) Fruit. *Yüzüncü Yıl Üniversitesi Tarım Bilimleri Dergisi*. 30(3). pp.535-543.

ACADEMIC CONFERENCE ATTENDED

- 7th Cairo international biomedical engineering conference (CIBEC 2014) Egypt.
- 1st annual International Conference Northwest University (November 2015) Nigeria.
- International Conference on Physical Chemistry and Functional Materials 19-21. June 2018. Firat University, Elazığ-Turkey (2018).
- 3rd International Eurasian conference on Biological and Chemical Science (Eurasian biochem 2020) Ankara Turkey.

CONFERENCE PAPERS

- Determination of heavy metals in some plant and soil samples from Sharada district of Kano State.
- Evidence of oxidative stress in experimental rats exposed to graded dosages of Transfluthrin based paper Rambo insecticide.
- Investigation of changes in growth and amount of water soluble vitamins in *Citrobacter freundii* under cadmium stress

PERSONAL MEMBERSHIP

- Member, Nigerian institute of food science and technology (NIFST)
- Member, Nigerian society for biochemistry and molecular biology (NSBMB)