ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

DEPARTMENT OF CHEMISTRY

SYNTHESIS OF IRON-III AND CHROMIUM-III BASED METAL-BETANIN COMPLEXES USING BETANIN AS A NATURAL LIGAND ISOLATED FROM BEETROOT

By:

Aklilu Gebeyehu

Advisor:

Dr. Mekonnen Abebayehu

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Approved by the Examining board	**Signature**
1. Dr. Mekonnen Abebayehu (Advisor)	_____
2. Dr. Estifanose Ele (Examiner)	_____
3. Dr. Merid Tessema (Examiner)	_____

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LIST OF ABBREVIATIONS

DOPA----------Dihydroxyphenylalanine

DMF----------Dimethyl formamide

FRAP----------Ferric reducing antioxidant power

NMR----------Nuclear Magnetic resonance

HPLC----------High Performance Liquid Chromatography

LDL----------Low–Density lipoprotein

NMR----------Nuclear Magnetic Radiation

ppm----------Parts per million

Rpm----------Revelations per minutes

TCA----------Trichloro acetic acid

TLC----------Thin layer chromatography

USDA----------United States Department of Agriculture

UV-Vis----------Ultra – Violet Visible spectroscopy

XRD----------X-ray diffraction

λ max---------- maximum wave length

SYNTHESIS OF IRON-III AND CHROMIUM-III METALS BASED BETANIN COMPLEXES ISOLATED FROM BEETROOT

ABSTRACT

The aim of this research work was to isolate betanin from beetroot (*Beta vulgaris L.),* investigate the antioxidant activity of betanin, synthesis of betanin-iron (III) and betanin-chromium (III) complexes and characterization.

The beetroot was collected from Meri local market, Addis Ababa. The essential compounds were extracted by solvent extraction and purified by liquid column chromatography. The result was determined by UV-Vis spectral studies. The analysis result indicates betanin is one of a beetroot component was observed at a peak of 538 nm wavelength. Structurally, betanin can potentially act as a bidentate ligand and can be used for complex formation. This study investigated the anti-oxidant activity of betanin, synthesis of betanin-iron (III) and betanin-chromium (III) complexes and characterization.

The ferric reducing power method was applied for anti-oxidant measuring of blue colour which is an indicator of higher antioxidant properties of betanin. The complex formation between Cr (III) and Fe (III) and betanin was carried out in distilled water. The resulting complexes were characterized by UV-Vis and XRD spectral studies. The UV-Vis spectral analysis results of iron (III)-betanin and chromium (III)-betanin complexes were at a wavelength of 358 nm and 458 nm respectively. This shows that betanin were inducing a blue-shifting due to the coordination or aggregation between iron (III) and chromium (III) to betanin. The XRD analysis results were also irregular base line. This indicates that the presence of amorphous phase within the sample.

Keywords: beetroot (beta vulgaris L), betanin, antioxidant,

CHAPTER ONE

1.1. INTRODUCTION

The *Beta vulgaris L.* is a medicinal and the taproot portion of the plant that belongs to the family Chenopodae (Singnarpi, and Cheng et al., 2017). The plant material is recognised in several cultivated varieties, the most well-known of which is the purple root vegetable also known as beetroot or garden beet. However, other cultivated strains include the leafy vegetables, chard spinach beets, and sugar beet. Beetroots have recently attracted great interest as potential therapeutic agents against a variety of diseases like those involving radical damage due to the presence of betalain compound. Betalain compounds and other natural antioxidants in beetroots have a wide range of activities such as antioxidative, antimicrobial, anti-mutagenic, and anti-inflammatory activities (Kong et al., 2003). They also show beneficial effects in age-associated disease such as cardiovascular diseases, some forms of cancer, and Alzheimer's diseases. Beetroot has been used as food (Tasneem et al., 2016) and as medicinal plant for the treatment of various ailments because of its lipotropic, antioxidant, and anti-tumour properties (Singnarpi et al., 2017). Beetroot has also been widely used industrially as food colorant due to its red color from the pigment betalain mainly from betanin, betanidin, and betaxanthin (Singh et al., 2017).

Betalains are hetrocyclic compounds and water-soluble nitrogen-containing pigments, found in high concentrations in red beet (*Beta vulgaris L.*). Betacyanins (red-violet pigments) and betaxanthins (yellow orange pigments) are the two sub classes betalains pigments. These pigments are classified as secondary metabolites. These classes of compounds show antimicrobial and antiviral effects and also can inhibit the cell proliferation of human tumor cells. It is reported that, betalain compounds mixtures can be used as a natural additive for various applications such as: for food, drugs and cosmetic products in the form of beet juice concentrate or beet powder (Dörnenburg & Knorr, 1996).

There are more than 50 known betalain colour principle, and the majority of analytical methods for beet colour focus on beet extraction and stability. Methanol solvents are generally used for extraction of plant materials. Other reported extraction procedures use

aqueous mixture of ethanol or methanol, and betanin may also be precipitated by a slight acidification with HCl (Delgado-Vargas et al., 2000).

Betalains are unstable and are degraded in the presence of light, oxygen, temperature, and enzymes. Therefore, focusing on betalain decomposition pathways and their stabilization methods meaningful to their potential applications in the pharmaceutical, cosmetic or food industry. Chromatographic separation methods are generally used for the isolation, purification, and quantification of crude betalain extracts (Eder, 2000).

Betanin (betanidin-5-O-β-glucoside), a red pigment compound belonging to one of the betalain classes is abundant in human diet and possesses potent antioxidant and metal ion complexes formation capacities and thus exerts various biological and biochemical effects including anti-inflammatory, and cardio protective activities. As an antioxidant, it protects various human cells, against oxidative damages. Betanin acts as a chemo preventive agent against oral carcinogenesis in vitro and in vivo. Besides, it is demonstrated that the coordination of metal like Cr (III) and Fe (III) ions with betanin as a bioactive ligand, can improve the pharmaceutical activity of the drugs themselves and reduce their toxicity effects.

The aim of this research work was to extract betanin from beetroot (*Beta vulgaris L.*), investigate the antioxidant activity of betanin, synthesis of the betanin-iron (III) and betanin-chromium (III) metal complexes and characterization.

CHAPTER TWO

2. LITRERATURE REVIEW

2.1. Historical Background

The beet plant is believed to have originated from the wild beet growing in prehistoric times in North Africa which then spread wild along the Mediterranean and European seashores extending quickly to British Isle, western Asian countries, India and as far east as China. In the beginning people ate beet leaves only and not roots. By the Roman era, it is believed that they were cultivated for their roots too. The ancient Romans appear to be one of the first civilizations to use beet roots as food. By the 16th century beet roots became popular throughout northern Europe.

Numerous cultivars have been selected and bred for several different characteristics. For example, the 'earthy' taste of some beet cultivars comes from the presence of the chemical compound geosmin. Investigators have not yet replied whether beets produce geosmin themselves, or whether it is produced by symbiotic soil microbes living in the plant (Lu et al., 2003). Nevertheless, breeding programs can produce cultivars with low geosmin levels yielding flavours more acceptable to shoppers (Nottingham, 2004). There are four main groups /forms of cultivated beets; leaf beet used as a leaf vegetable, fodder beet used as animal fodder, garden beet/table beet/or beetroot used as a root vegetable, and sugar beet grown for sugar.

The commercial value of beets escalated upon the discovery of its high sugar content in beetroot. The feasibility of commercial extraction of sugar from beetroot was demonstrated by the German chemist, Andreas Marggraf in 1747. This was quickly followed by setting up of the world's first commercial beet sugar factory in Poland in 1801. Before the war, Poland had 110 factories with an annual output of 1,000,000 tons of sugar. The US, the Russian Federation, France, Poland and Germany are the current leading commercial producers of beet for use in sugar manufacture and as a vegetable for general consumption (Ford-Lloyd 1995; Nottingham 2004).

The scientific name for red beet is Beta vulgaris L., family, *Chenopodiaceous, subspecies, vulgaris,* commonly known as garden beet and its five popular varieties are: 'Bonel',

'Detroit', 'Favorit', 'Nero' and 'Rubin'. Among from the variety 'Rubin' is considered the most appropriate one for food color making based on its red pigment content and composition (Gasztonyi et al. 2001).

The fleshy root petioles and leaf of Beta vulgaris are eaten. The most important form is the garden beet, but in many African countries spinach beet/Swiss chard/leaf vegetable is far more important (Oyen, 2004). The roots of the garden beet may be cooked or canned as a vegetable. They may also be used in soup. Garden beet juice is a common health food.

Beta vulgaris L roots contain significant amounts of vitamin C, while the leaves are excellent sources of vitamin A. they are also high in folate, soluble and insoluble dietary fibber and antioxidants. Betacyanin in beetroot may reason red urine and feces in some people who are incapable to break it down. This is termed beeturia (Eastwood and Nyhlin, 1995). Betanin, or beetroot red, a type of betalain obtained from the roots, are used industrially as red food colorants (such as to improve the colour of tomato paste, sauces, jams, sweets, etc.) (Oyen, 2004).

The roots and leaves have medicinal uses (Grubben, 2004). The Romans used beetroot as a treatment of fevers and constipation, among other ailments. From the middle Ages, beetroot was used as a treatment for a variety of circumstances, particularly illnesses relating to digestion and the blood. Bartolommeo platina recommended taking beetroot with garlic to nullify the effects of 'garlic–breath ('Platina, 1475). During the middle of the 19th century, wine often was coloured with beetroot juice ('Nilsson et al., 1970).Other uses of beetroot, with large leaves, are also grown as ornamental plants. Ecologically, they provide food for many animals, including the larvae of a number of Lepidoptera species.

2.2. Scientific Classification of Beet root (Beta vulgaris)

Beetroot (beets) are classified, like all living organisms, in terms of class, order, family, genus and species. Beets are flowering plants and therefore within the class Dicotyeldae. Within this class, they are part of the order *caryophyllales.* Within this order, beets are part of the chenopodiaceae family. The *chenopodiaceous* or goosefoot family of plants also includes other edible species, including spinach (*spinaciaoleracea*), quinoa (*chenopodium quinoa*),

orache or orach (*Atriplexhortensis*) and Good King Henry (*chenopodium bonus-henricus*). Beets are in the genus Beta and the species *Beta Vulgaris L*.

Table-2.1*:* The scientific classification of *Beta-Vulgaris*

Kingdom	Plantae
Subkingdom	Tracheobionta
Super division	Spermatophyta
Division	Magnliophyta
Class	Magnoliopsida
Sub class	Caryophyllidae
Order	Caryophyllates
Family	Chenopodiaceae
Genus	Beta
Species	B.vulgaris

2.3 Nutritional Value of Beetroot

Beets are nutritionally diverse, low in calories, cholesterol-free, and fat free**.** The beetroot species (*Beta vulgaris L.*) is considered a good source of dietary fibre, minerals (potassium, sodium, iron, copper, magnesium, calcium, phosphorus and zinc), vitamins (retinol, ascorbic acid and B-complex), antioxidants, betalains and phenolic compounds, and possesses high nutritional value due to its high glucose content, in the form of sucrose (USDA, 2011). According to the data presented by the United States Department of Agriculture (USDA) for macronutrients, as follows.

Table 2.2: Nutritional value from 100 g of beetroot

Water	87.5 g
Energy	43 Kcal
Fat	0.17 g
Protein	1.61 g
Carbohydrates	9.56 g
Fiber	2.8 g

Potassium	325 mg
Sodium	78 mg
Phosphate	40 mg
Calcium	16 mg
Magnesium	23 mg
Iron	0.08 mg
Zink	0.35 mg
Vitamin C	4.9 mg
Vitamin B2	0.040 mg
Vitamin B6	0.067 mg
Vitamin A	361 U
Vitamin E	0.300 mg
Niacin	0.344 mg

Source (USDA, 2011)

2.4. Health Benefit of Beetroot (B.vulgaris L.)

Beetroots and beetroot juice have many health profits, particularly for heart health and exercise performance. Consumption of red beet which is rich source of antioxidants can contribute to protection from age related diseases. According to (Vinson, 1998, and Zitnanova et al. 2006), red beet is one of the most dominant vegetable with respect to antioxidant activity. Significant amount of Vitamin B1, B2, niacin, B6, B12 are found in beetroot, while the leaves are an excellent source of vitamin A. Consuming beetroot helps in curing many diseases such as anemia, blood pressure, cancer, dandruff, gastric ulcers, kidney ailments, liver toxicity or bile ailments like jaundice, hepatitis, food poisoning, diarrhoea or vomiting. In addition to their usefulness as colorants, betalains play an important role in human health because of their pharmacological activities such as antioxidant, anti-cancer, anti-lipid emic and antimicrobial.

2.5. Medicinal Benefits of Beetroot

Red beet products consumed regularly in the diet may provide protection against certain oxidative stress-related disorders in humans and also improve digestion and blood quality (Azeredo. 2009).

Beta vulgaris L., the beet root, has anti-cancer activity. The cancer chemo-preventive potential of the beet root is thought to be due to the betalains which are composed of two main groups: the red betacyanins and the yellow betaxanthins. Both are used as natural additives for food and are powerful antioxidant activity. It helps to preserve brain function with nitrates that improve blood flow and beet having the ability to increase the production of Glutathione naturally in body, that compound helps to prevent colon cancer. Due to its high fiber content, it prevents constipation and promotes regularity for healthy digestive tract (Tulp and Bohlin, 2004).

Under normal conditions, inflammation is noticed as a useful process, governing our innate answer to biological or physical stimuli such as trauma, infection and other pathogens that may cause the organism harm and disrupt homeostasis (Monteiro, et, al, 2010). In the short term, redness, swelling, pain and diminished function may be experienced at the site of inflammation; however, more concerning is the potential long-term implications if inflammation persists, and is unresolved (Yoon, et, al, 2005). Failure to eliminate the attacking element and restore normal immune function can cause chronic inflammation causing in long-term cell dysfunction. Chronic inflammation is often implicated in the onset and progression of several clinical disorders such as obesity, liver disease, cancer and heart disease (Monteiro, et, al, 2010).

Red beet product consumption provides a significant effect on nearly all organs of the digestive tract: pancreas, liver, colon. Red beetroot is well known as an “internal cleansing” substance and provides a mild laxative effect. Because of the extensive amount of literature on studies which have examined the physiological and nutritional effects of *Beta vulgaris* on both humans and animals, it is largely accepted that intestinal peristaltic improvement is related to dietary fiber. There are no differences between red beetroot and sugar beetroot effects. Beet fiber contains three main fractions: pectin, cellulose and arabinose polymers with a potential weight loss effect (Bobek, et al, 2000). It should be noted that laxative

effects are provided not only by fiber rich red beet products, but also by fiber free Beta vulgaris juice.

Red beetroot juice also diminishes blood cholesterol level. Beetroot containing polyphenols and dietary fiber stimulates intestinal excretion of cholesterol and cholesterol metabolites (Fugh-Berman, et al, 2004). The presence of both higher cellulose content and red beet fiber in the diet significantly reduced the incidence of precancerous lesions-aberrant crypt foci-in the colon (Tunnessen, et al, 1969).

Red beetroot compounds can stimulate intestinal iron absorption through several mechanisms. Ascorbic and other organic acids significantly enhance iron bioavailability and intestinal transport. The beetroot is rich in ascorbic and citric acids.

2.6. Color Chemistry of Beetroot

One major factor in the popularity of beets as a commercial crop is the abundance of natural red pigment that it provides. These pigments provide rich color; there is evidence to show that they may have health benefits, including anti-inflammatory and anti-cancer properties. These water-soluble, nitrogenous pigments are categorized as betalains, of which there are two main types: betacyanins and betaxanthins (Attokoran, 2011). The betacyanins appear red, while the betaxanthins contain a yellow color; it is the ratio of these components which decides the final hue (Neelwurne, 2013). In most beets, the ratio of betacyanins to betaxanthins is 3:1 (Goldman and Navazio, 2008). However, yellow cultivars exist that only contain betaxanthins. During the life of the beet, the amount of color present in the tuber increases; therefore, more mature beets are generally used for pigment extraction. One major issue in the color chemistry of the beet is the instability of these natural pigments. The individual types of pigments themselves display varying degrees of stability; betacyanins have been establish to be more stable than their betaxanthin complements, both at room temperature and during heating.

2.7. Betalains Classifications, Structures and Derivatives

Betalains are N-heterocyclic compounds which are classified into two sub-classes: betacyanins and betaxanthins based on their resonating double-bond structures. Betanin and indicaxanthin were the first betacyanin and betaxanthin being identified respectively. They are also the most dominant pigments amongst 78 betalaimic structures naturally occurring which have been reported recently (Herbach et al, 2006 and Stintzing et al., 2007).Betacyanins and betaxanthins are present within the structure of betalains precursor-betalamic acids (4-(2-oxoethylidene)-1,2,3,4-tetrahydro-pyridine-2,6-dicarboxylic acid). The conjugation of betalamic acid with variable proteinogenic /non-proteinogenic amino acids results in betaxanthins which display maximum absorption between λ 460 and 480nm. Vulgaxanthin I appeares as, one of the most abundant betaxanthins next to indicaxanthin (**Figure 2.1).**

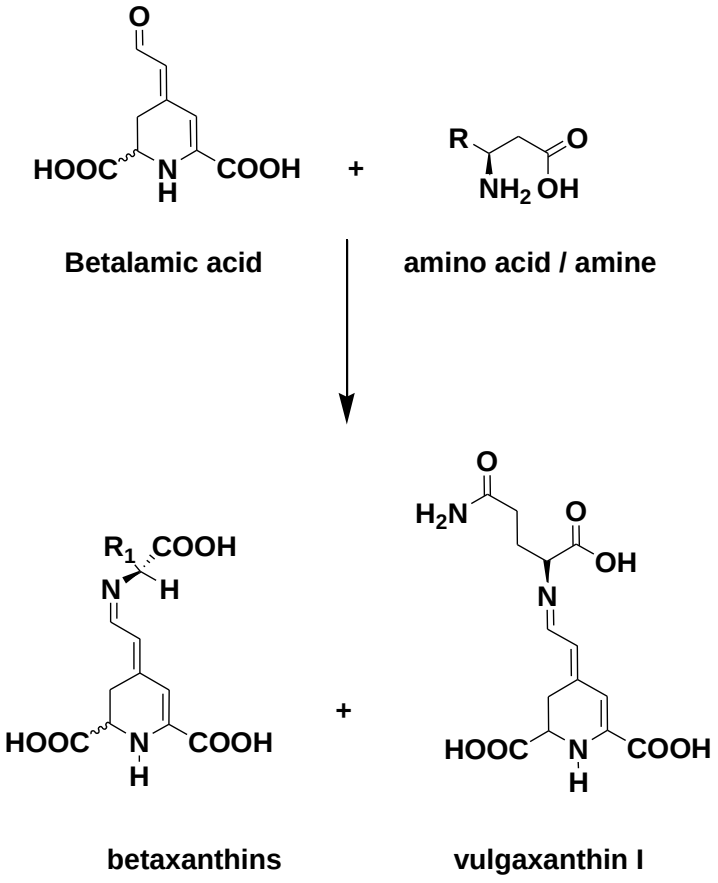


Figure 2.1: Betaxanthins formation and typical pigments

Betacyanins are generated by the condensation of betalamic acid with the aromatic system *cyclo-dopa* (cyclo-L-(3,4-dihydroxyphenylalanine)) to form the unifying aglycone betanidin, which reveals particular maximum absorbance at λ max 540 nm. Further characterizations of this aglycone with glycosylation and additional acylation create a great variety of derivatives **(Figure 2.2**).

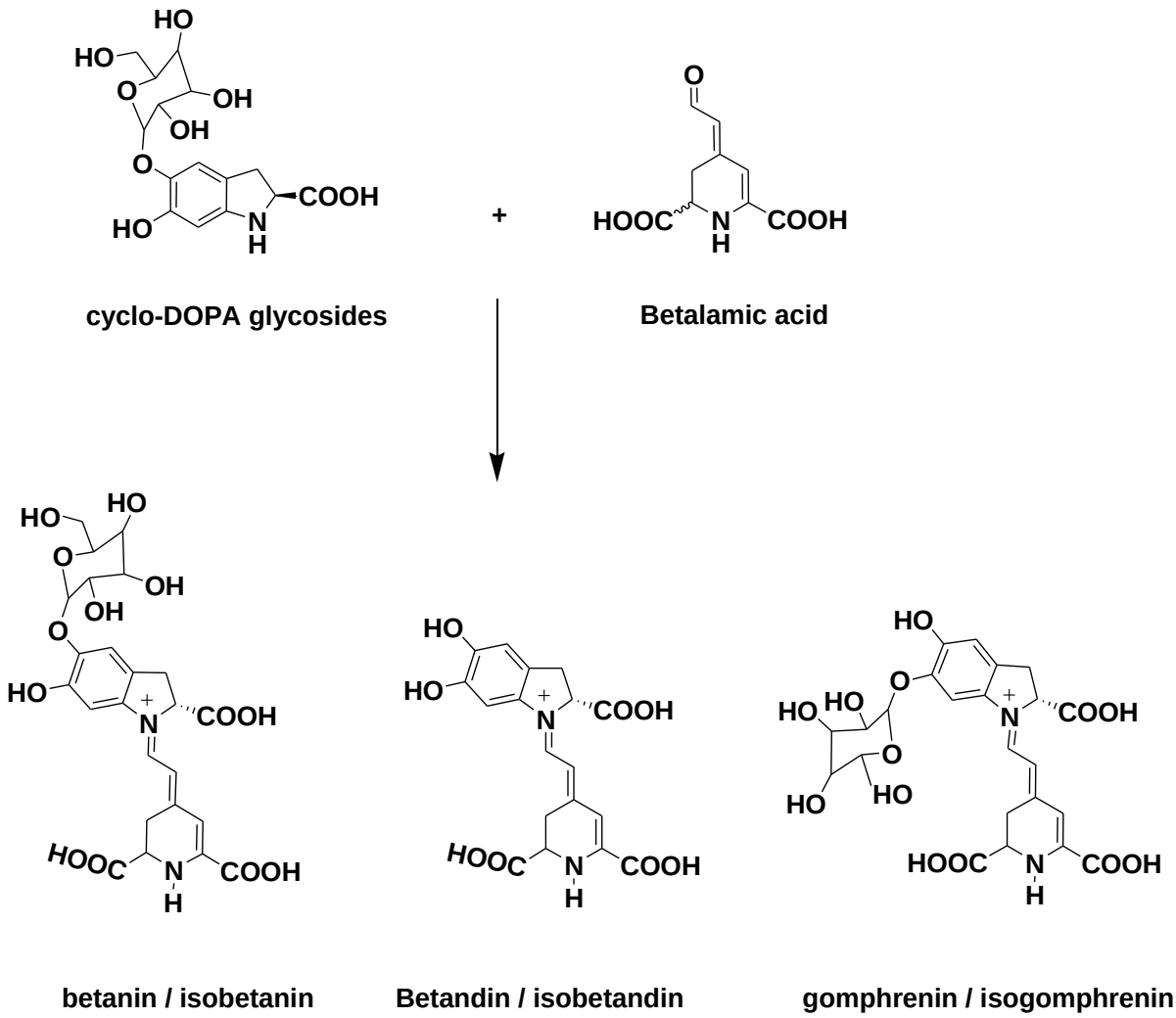


Figure 2.2: Typical structures of betacyanins

The typical glyosidic substituents are monosaccharaides namely glucoses, apiose and occasionally glucuronic acid, whereas the aliphatic acids or aromatic acid such as malonic, 3-hydroxy-3-methyl-glutaric, caffeic, p-coumaric, ferulicand sinapic acid are the principle acyl substituents (Stintzing et al., 2007 and Stintzing et al., 2008). Depending on the substitution position at C5 or C6, the corresponding structures are identified as betanin or gomphrenin (**Figure 2.2**).

The substituent structures cause variation in the chromic absorption of both betacyanins and betaxanthins. (Stintzing et al., 2008) summarized the effects of different structures on betalains maxima absorption; e.g. the 6-O-glycosylation and aromatic acylation led to the bathochromic shift λ of5 to 6 nm compared to the 5-O-and aliphatic substituents in betacyanin.

Generally, the glycosylation and acylation significantly change betacyanins bioactivity. The 6-O glycosylation exploits better bioactivities than its 5-O compartments, and acylation also enhances these abilities. On the other hand, the increased number of hydroxyl and imino groups improve radical scavenging properties of betaxanthins (Stintzing et al., 2004).

2.8. Method of Betalain Extraction

On a laboratory scale, betalains may be extracted by various methods, such as diffusion-extraction, solvent extraction, solid-liquid extraction (Lee & Wiley, 1981), reverse osmosis and ultrafiltration (Real & Cerezal, 1995).

Extraction procedures have employed aqueous alcoholic mixtures. However, betacyanins can be precipitated by a slight acidification with HCl (Delgado-Vargas, Jiménez and Paredes-López, 2000). Slight acidification of the extraction medium enhances betacyanin stability and avoids oxidation by polyphenol oxidases. The initial homogenate is clarified by either centrifugation or filtration but the crude extract may contain many potentially interfering substances hence additional purification steps are occasionally required such as column chromatography. (Azeredo et al, 2009) studied the effects of pH, solvent-to-sample ratio, and solvent temperature the efficiency of betanin extraction from beetroots. The most adequate extraction conditions were pH 3.0, solvent: sample ratio of 4:1, solvent temperature 40 ^{0}C.

The purification of crude betalain extracts is usually accomplished by liquid column chromatographic methods, thereby allowing separation and quantification of individual and total betalains (Eder, 2000).

2.9. Methods of Betalain Analysis

Spectrophotometric techniques have been used for betanin analysis. Betanin was identified by UV-Vis spectrophotometry in the range of 400 to 600 nm, and displayed a characteristic λ max at 538 nm.

2.10. Factors Affecting Chemical Stability of Betalains

Betalain degradation may occur by different mechanisms, which were detailed by (Herbach et al., 2006b) several factors, both intrinsic and extrinsic affect betalain stability.

Structure: glycosylated structures of betacyanins are more stable than aglycones, probably because of the higher oxidation–reduction potentials of the former (von Elbe & Attoe, 1985). Some studies have indicated increasing betacyanin stability resulting from esterification with aliphatic acids and aromatic acids.

Enzymes such as β-glycosidase, polyphenol oxidases and peroxidases, which if not properly inactivated by blanching may account for betalain degradation (Escribano et al., 2002). Betacyanins are more susceptible than betaxanthins to betalains degradation by peroxidases (Wasserman et al., 1984). The transformation of betalain glycosides into their respective aglycones produces a bathochromic shift of 4–6 nm. However, these aglycones are more labile and prone to further oxidation which results in red colour losses and subsequent browning.

Several study showing the factors affecting the stability of betalain such as; pH (Jackman & Smith, 1996, Schwartz & von Elbe, 1983 and Mabryet al., 1967), Oxygen (Attoe & von Elbe, 1985, Attoe & von Elbe, 1985 and Drunkler et al., 2006), impaired by light exposure (Von Elbe et al., 1974 and Cai et al., 1998 and Jackman & Smith, 1996). Temperature is the most important factor on betalain stability during processing and storage (Garcı´a Barrera et al., 1998, Drda´k & Vallova´, 1990 and Schwartz & von Elbe, 1983). Betanin with different decarboxylation levels were identified together with their corresponding neo-derivatives as heating degradation products of betacyanins from red beetroot juice (Wybraniec & Mizrahi, 2005, Huang & von Elbe, 1985).

2.11. Biological Properties of Betalains

The antioxidant activities of betalains is well documented in several works and using diverse methodologies, e.g. scavenging of the 2, 2-azino-bis-(3-etyhyl-benzthiazoline-6-sulfonic acid) (ABTS. +) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals (Polturak, 2018).

Betalains, particularly red-violet gomphrenin type betacyanins and yellow betaxanthins, have strong antioxidant activity when compared to the traditional standards (ascorbic acid, rutin and catechin). There is an effect of the chemical structure of betalains on the antioxidant activity: the ability for scavenging free radicals increased with the number of hydroxyl groups and imino groups and decreased with more glycosylation of aglycones in the molecules. The capacity for scavenging other free radicals, such as peroxyl (ROO.) free radicals or nitric oxide (NO.), by some betacyanins was also dependent on the number of hydroxyls. For example, betanin was less effective than betandin (Tesoriere et al., 2009).

The capacity for scavenging ABTS.+ of betalamic acid was better when related to that of trolox. The same pigment was also able to reduce Fe^{3+} to Fe^{2+}. These events were attributed to the extended conjugated system of betalamic acid. PH values above 5.5 increased the activity of betalamic acid (Herrero et al., 2012). The significance of the PH on the activity was also informed for betanin, the main pigment of red beet and other betalains (Herrero et al., 2010).

The oxidation mechanism of betanin and some of its derivatives (2-decarboxybetanin, 17-decarboxybetanin, 2, 17-bidecarboxybetanin and neobetanin) in the presence of ABTS and the intermediates that are generated during the reactions (Wybraniec et al., 2013).

CHAPTER THREE

3. Objective of the Study

3.1. General Objective:

The general objective of this study was to extract betanin from beetroot and by using this as a natural ligand, to synthesize complexes of betanin to Fe^{3+} and Cr^{3+}.

3.2. Specific Objectives:

The specific objectives of this study were:

- to extract and purify betanin from beetroots.
- to determine the antioxidant properties and absorption spectra of betanin and
- to synthesize betanin-metal complexes

CHAPTER FOUR

4. EXPERIMENTAL

4.1. General

The UV-Vis spectra were taken on Perkin Elmer, Lambda 950 UV/Vis/NIR scanning spectrometer (200-800 nm). Analytical tin layer chromatograms were run on a readymade 0.2 mm thick layer of Merck silica gel 60 F_{254} coated on Aluminum foil. Compound on TLC were detected using eye protected UV- instrument.

The X-ray diffraction measurements were obtained using a Philips X' pert-Pro System powder diffractometer with Bragg-Brentano geometry in continuous mode with a scanning speed of $1/2^{o}$/min. A *Cu Kα* radiation tube with line focus was operated at 40 kV and 30 mA. The X-ray powder diffraction patterns were obtained in the range of $15\text{-}90^{o}$, in steps of 0.02^{o}.

4.2. Material and Method

4.2.1. Material

Samples of fresh red beet roots (*Beta vulgaris L.*) were purchased from a local market Meri, Addis Ababa. The whole root was washed with cold water and sliced with knife into small pieces for preparation to the extraction procedure.

(a) *(b)*

Figure 4.1: (a) fresh and (b) sliced red beetroot respectively.

The chemicals used for this experiment were: water, ethyl acetate, DMF, ascorbic acid, Silica gel, $FeCl_3.6H_2O$, $CrCl_3.6H_2O$, ferrous chloride, n-hexane, ethanol, and methanol. All chemicals were obtained from Addis Ababa University, Department of Chemistry chemicals store.

4.3. Methods

4.3.1. Extraction of Sample

Solvent extraction method was used to extract betanin from red beetroot, using methanol as the extracting solvent. A 700 g sliced beetroot samples were put into a conical flask for the laboratory experiments. Methanol was added into the conical flask just enough to cover all the samples and soaked for 2 days.

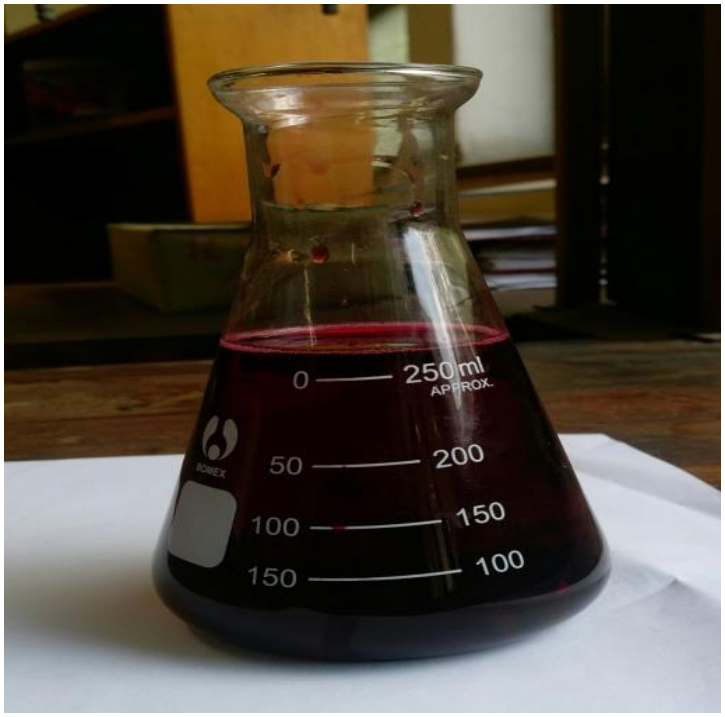


Figure 4.2: Methanol extracts of the red beetroot juice

This extraction process was carried out at room temperature in the dark. The sample solution is then subjected to suction filtration to remove suspended particles and concentrated by using a rotary evaporator at 40 ^{0}C to remove the solvent**.** The result presented in **Table 5.1.**

Figure 4.3: Concentrating betanin pigment by using rotary evaporator at 40 ^{0}c

Figure 4.4: Adsorption of the crude extract with silica gel

4.3.2. Purification of the Extract

After extraction process was completed, the extracted samples were collected and purified by liquid column chromatography by adsorbing the extract on silica gel as shown in **Figure 4.4**. Silica gel was used as the stationary phases in glass column. The elution was performed with methanol and used as the mobile phase. Red pigments were isolated from Yellow pigments and other contaminants.

Figure 4.5: purifying methanol extract betanin by using liquid Colum chromatographic technique

Figure 4.6: Fractions of methanol extract of beetroot pigments collected after column chromatography

Six fractions were collected from methanol extract of beetroot. As shown in **Figure 4.6**, from the total of six fractions, four of them were put together based on their similar TLC profile using ethyl acetate: n-hexane (7:3) solvent system then after concentrated using a rotary evaporator. These were further purified by liquid column chromatography for UV Vis, and NMR analyses. The final yield was 1.74 g.

4.4. Analysis of the Isolated Purified Betanin

4.4.1. Spectrophotometric Analysis of Betanin

The wavelength of UV-Vis spectrophotometry (Perkin Elmer, Lambda 950 UV/Vis/NIR) was set to 200-800 nm. Distilled water in a quartz cuvette was used to blank the spectrophotometer. Then, the absorbance of the methanol extract of the beetroot in the visible light was measured. The result is shown in **Figure 5.1.**

4.4.2. Ferric Reducing Antioxidant Power (FRAP) Analysis

Ferric ions reducing power was measured according to the method of Oyaizu **(**Oyaizu, 1986**)** with a little modification. In this assay of reducing activity was based on the reduction of Fe^{3+} (ferricyanide complex) to ferrous complex form in the presence of an antioxidants in the tested sample solutions. Fe^{2+} was then monitored by measuring the absorbance of blue color with different concentration at 700 nm (Oyaizu, 1986).

Reagents and Sample Solution Preparation

200 mM Sodium phosphate buffer solution (pH = 6.6), which consists of a mixture of monobasic dihydrogen phosphate (NaH_2PO_4) and dibasic monohydrogen phosphate (Na_2HPO_4) was prepared as follow: 28.4 g of Na_2HPO_4 was dissolved in 100 mL distilled water and 24.0 g of NaH_2PO_4 was dissolved in 100 mL distilled water. The two solutions were combined in the ratio of 38.1 mL of Na_2HPO_4 and 61.9 mL of NaH_2PO_4. The pH of the prepared solution was checked using PH meter at room temperature. The combined stock solution was diluted to 1.0 liter of distilled water and 200 mM Sodium phosphate buffer solution was prepared. A 1% sodium ferricyanide was prepared by dissolving 1.0 g of sodium ferricyanide in 100 mL of distilled water. TCA was prepared by dissolving 10.0 g of TCA in 100 mL distilled water. A 0.1% $FeCl_3$ was prepared by dissolving 0.05 g of $FeCl_3$ in 50 mL. 0.5 mg/mL of standard ascorbic acid was prepared by dissolving 0.05 g of standards in 10.0 mL of methanol.

The reducing power of an extracted betanin from red beetroot with different concentrations determined according to the method (Oyaizu (1986). 0.05 mL of betanin was dissolved in 50.0 mL of methanol. Added 2.5 mL of 200 mM or 0.20 M sodium phosphate buffer solution of pH=6.6 and 2.5 mL of 1% sodium ferricyanide [$Na_3Fe(CN)_6$]. Shake well and the mixture was incubated in water bath at 50^0C for 20 minutes. Then, a 2.5 mL of TCA was added to the mixture and centrifuged at 200 rpm for 10 minutes. 2.50 mL of the mixture was taken and mixed with 2.5 mL of distilled water and 0.50 mL of 1% $FeCl_3$. The absorbance of the mixture was measured at 700 nm. The concentration of betanin complex was calculated from the regression equation of ascorbic acid calibration curve **(**Huang et al., 2005, Maruthamuthu et al., 2016 and Oyaizu, 1986**)**.

4.5. Synthesis of Iron-III and Chromium-III-betanin Complexes

Steps to synthesis of iron-III and chromium-III-betanin complexes

Step 1: A 1.650 g of betanin ($C_{24}H_{26}N_2O_{13}$) sample was taken from total of 1.74 g of purified betanin sample and dissolved in a 20 mL of distilled water.

Step 2: 0.270 g of $FeCl_3.6H_2O$ and 0.266 g of $CrCl_3.6H_2O$ was put into a separate reaction flask to conduct the experiment.

Step 3: 10 mL of betanin solution was added to each reaction flask. The solution was stirred overnight at 40 ^{0}C, precipitate was formed. The resulting precipitate was centrifuged and the supernatant solution was decanted, washed with distilled water and dried in vacuum (**Figure 4.7).**

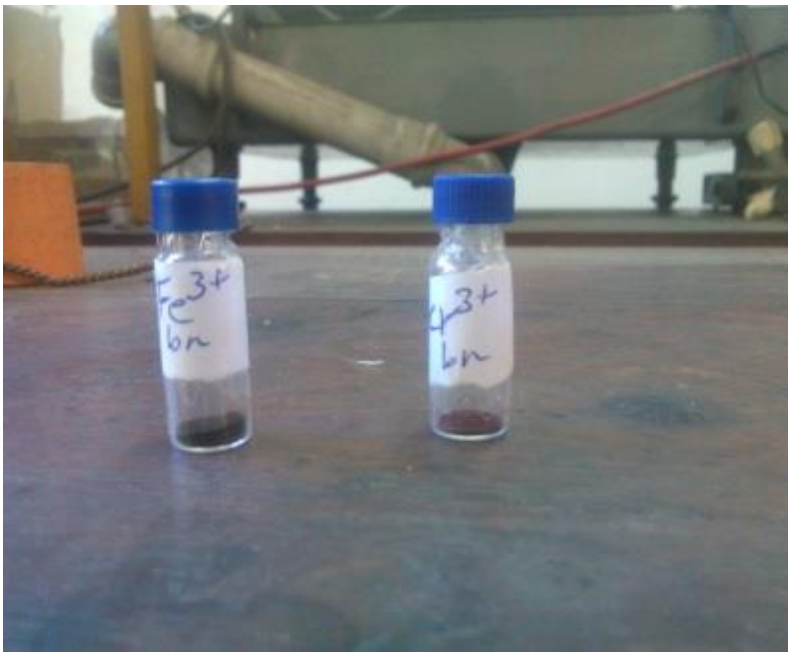


Figure 4.7: The solid product of the complexion of iron-III and chromium-III with betanin

4.6. Analysis of Iron (III)-betanin and Chromium (III)-betanin Complexes

4.6.1. Spectrophotometric Analysis of the Complexes

Sample preparation

5 mL of DMF was added into a vial containing the solid product of iron-betanin and chromium-betanin complexes and heated to completely dissolve (**Figure 4.8).**

(a) Fe^{3+}- betanin complex (b) Cr^{3+}- betanin complex

Figure 4.8: Complex formation of betanin with (a) Fe^{3+} and (b) Cr^{3+}after dissolved in DMF for UV-Vis measurement

4.6.2. XRD Analysis of Fe (III)-betanin and Cr (III)-betanin Complexes

The powder of Fe (III)-betanin and Cr (III)-betanin complexes were recorded in the range of 15-90^{o}, in steps of 0.02^{o} using a Philips X' pert-Pro System powder diffractometer with Bragg-Brentano geometry in continuous mode with a scanning speed of 1/2^{o}/min. The result is presented in **Figure 5.6.**

CHAPTER FIVE

5. RESULTS AND DISCUSSION

5.1. Yields of Extraction of the Root Part of the Beetroot Plant Material

Table 5.1: extraction of the root part of the beetroot plant material

Sample	**Solvent**	**Crude Extract Yield**	**Texture/color**
Beetroot (700 g)	methanol	34.35 g, (4.9%)	Viscous solid/red

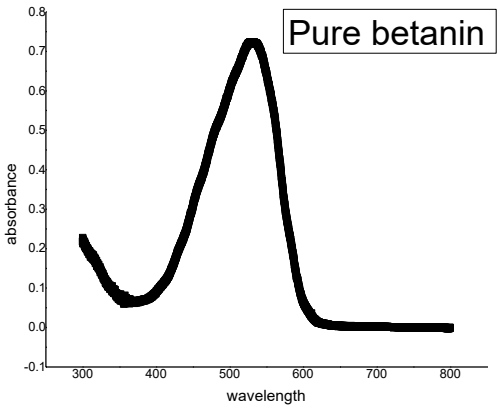
The root part of a beetroot was extracted with methanol and a viscous red product was obtained. The viscous red product was then subjected to column chromatography for purification. Six fractions were collected. From the total of six fractions four of them were put together based on their similar TLC profile using ethyl acetate: n-hexane (7:3) solvent system. Again subjected to column chromatography for further purification. Finally, 1.74 g sample was obtained. This study has resulted in the isolation and characterization of betanin from beetroot. The sample was finally submitted for full NMR analysis for structural elucidation.

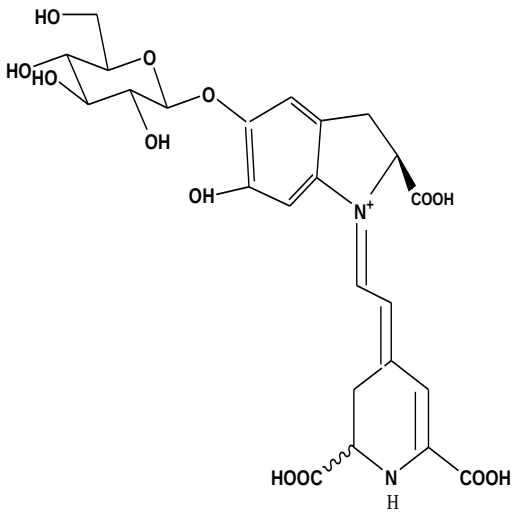
Betanin was extracted from red beet root plants (*Beta vulgaris L*.) and the yields were 1.74 g. The percentage yield for the extraction was 0.25% which was calculated from the mass of betanin obtained divided by the mass of the sliced beetroot used in the extraction multiplied by 100 i.e. (Y= (mass of extract/mass of raw material) x100)). These results were lower than literature's result, but, in the expected range. In general, the result obtained in this study is almost comparable to that of literatures reported by different authors (Schwartz & von Elbe, 1983).

5.2. Characterization of Betanin with the UV-Vis Spectrophotometry

The UV-Vis spectra of beetroot extracts in distilled water are depicted in **Figure 5.1.** A strong absorption band was observed at 538 nm in the visible range.

The presence of an absorption peak at 538 nm wavelength confirms that the isolated compound is betanin which is comparable to the literature data. A peak that appears is given by the conjugated double bonds contained in betanin structure.





Betanin structure

Figure 5.1: UV-Vis spectrum of betanin at a wavelength of 538 nm

5.3. Ferric Reducing Antioxidant Power (FRAP) Analysis

The results of the present investigation show the solvent extraction of betanin showed antioxidant activity.

$$Na\,[Fe\,(III)\,(CN)_6]_3 \;+\; Ph\text{-}OH \;\longrightarrow\; Na_X\,(Fe_n^{3+}\,[Fe^{2+}\,(CN)_6]_3) \;+\; Ph\text{-}O^{\cdot} + H^+$$

(Yellowish color) (Color of betanin) (Blue color)

The reducing power assay was associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they were electron donors and can reduce the oxidized intermediates. The presence of the reducers (antioxidants) causes the conversion of the ferricyanide to the ferrous form. The reducing power assay increase with increase in concentration and betanin exhibit higher reducing activity. Reducing power assay method to evaluate antioxidant ability was based on the reduction of Fe^{3+} to Fe^{2+} of the test solution.

The presence of a reducing agent causes the conversion of ferricyanide complex to the ferrous form due to the formation of Perl's Prussian blue (Na_X (Fe_n^{3+} [Fe^{2+} (CN) $_6]_3$)). The concentration of the complex increases the absorbance is also increase indicates an increase in reductive ability of the compounds. The concentration of a complex was calculated from Ascorbic acid calibration curve. Ascorbic acid was used as the positive standard reference. All assays were run in triplicates and averaged. The reducing ability of samples was directly proportional to the absorbance reading. A standard curve was prepared with the help of different diluted concentrations (0.02, 0.03, 0.06, and 0.12) of ascorbic acid and their absorbance, the relation is linear.

Table 5.2: Average absorbance of ascorbic acid at 700nm

Sample number	Concentration of ascorbic acid (ppm)	Average absorbance 700nm
1	0.02	0.01
2	0.03	0.02
3	0.06	0.04
4	0.12	0.06

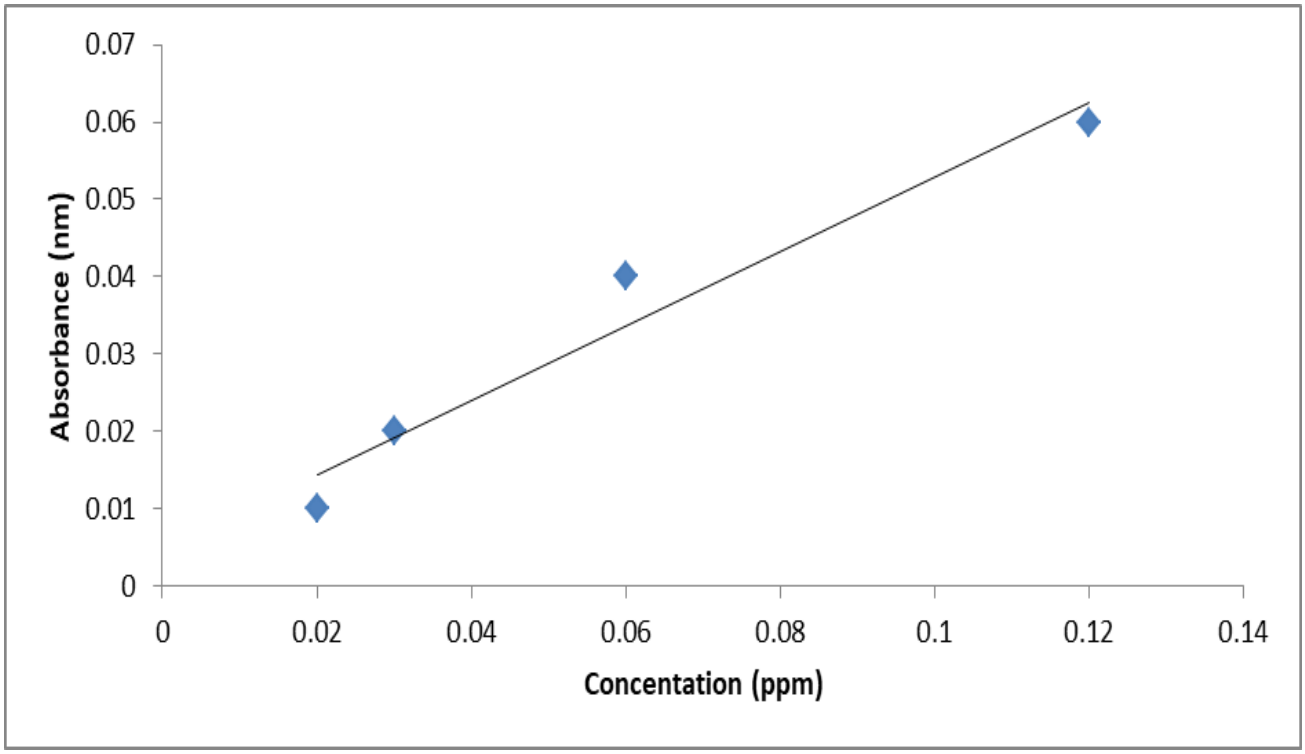


Figure 5.2: Linear regression relationship between concentration (mg/mL) of ascorbic acid and its absorbance at 700 nm

The regression equation obtained for ascorbic acid was, [Y=0.48148X+0.00481(R^2=0.97714)] Here, y = absorbance obtained at 700 nm and X = concentration of ascorbic acid used. The reductive ability or antioxidant activity of the betanin extract was stated in terms of mg/mL. The concentration of complex of betanin (Na_X (Fe_n^{3+} [Fe^{2+} $(CN)_6]_3$)) was calculated from regression equation of ascorbic acid whose absorbance of equivalents taken as a reference at 700 nm.

The capacity of a compound to reduce Fe^{3+} (ferricyanide complex) to the Fe^{2+} (ferrous) form, is as an indicator of its potential antioxidant activity (Prieto P.et al, 1999). In this assay, the yellow color of the $FeCl_3$/Na_3Fe $(CN)_6$ changes to blue, depending on the reducing power of the test solution. Obtaining the greater absorbance of blue color solution at 700 nm indicates betanin have a greater reducing power (Rice-Evans et.al. 1997).

Table 5.3: Average absorbance of a blue sample betanin complex

Species	**Average absorbance of betanin**			**Concentration calculated from standard calibration curve(ppm)**		
Betanin-complex	A-1	A-2	A-3	C-1	C-2	C-3
	0.06	0.08	0.10	0.12	0.16	0.20

Where: A-1 = average absorbance, before dilution, A-2= average absorbance of first diluted and A-3= average absorbance of second diluted. C-1, C-2 and C-3 concentration calculated from regression equation of ascorbic acid

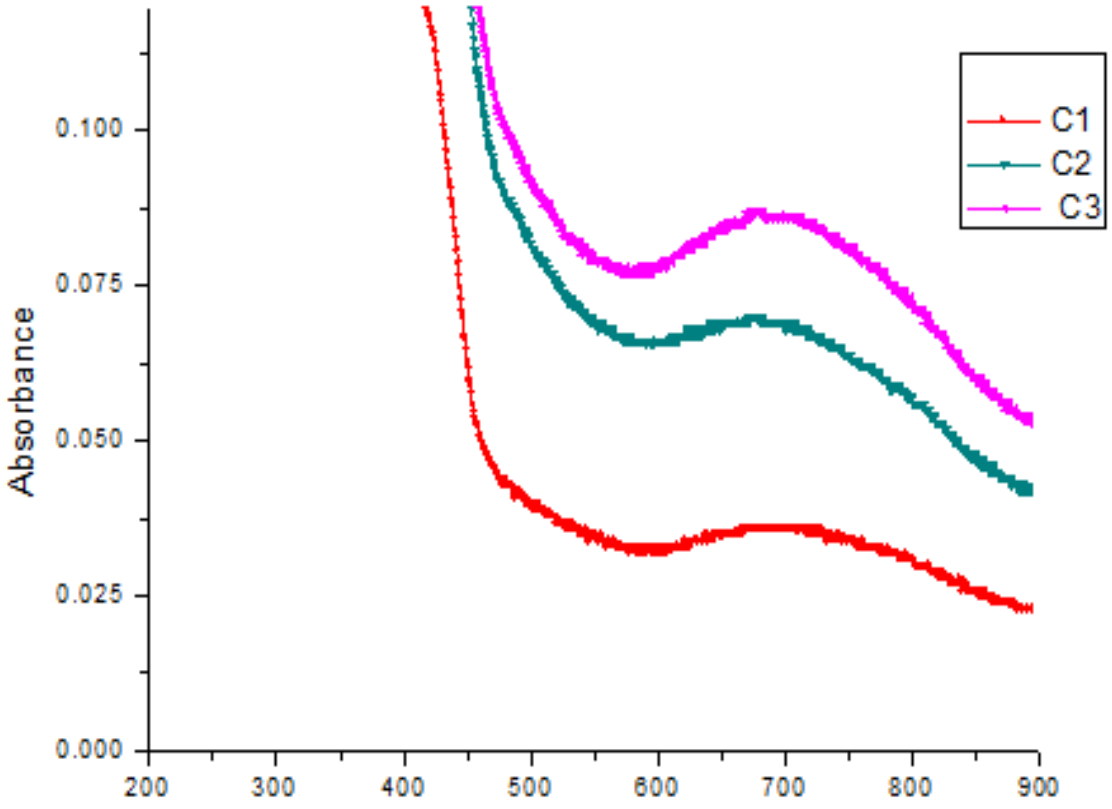


Figure 5.3: Absorbance of betanin complex with different concentrations at 700nm

As indicated in **Table 5.3** and **Figure 5.3** above, when the betanin complex concentration was increase the absorbance also Increases indicates the betanin were a good reductive ability (antioxidant).

5.4. Results of betanin-metal complexes absorption spectra study

(a)Pure betanin (b) Fe^{3+}-betanin complex (c) Cr^{3+}-betanin complex

Figure 5.4: Betanin before complexation (a), betanin after complexation with Fe^{3+} (b) and Cr^{3} (c)

The absorption spectra of the ligands (betanin), iron-betanin and chromium-betanin complexes were measured using a Perkin Elmer, Lambda 950 UV/Vis/NIR scanning spectrometer in DMF (N, N-dimethylformamide) solvent in range of 200-800 nm. The spectra of the ligand and its complexes illustrate in **Table 5.4.**

Table 5.4: The wave length of the pure betanin and metal of betanin

Samples	**Absorption band measured in nm**
Betanin	538
Fe^{3+}-betanin complex	360
Cr^{3+}-betanin complex	480

The spectra of betanin (ligand) showed one band at 538 nm due to the intra molecular charge transfer (ICT) transition of the conjugated bond of the ligand (betanin). For iron (III)-betanin and chromium (III)-betanin complexes, the bands appeared at 360 nm and 480 nm respectively. From the absorption spectra of the complexes shown in **Figure 5.5**, note that main characteristic absorption peaks corresponding to the ligand (betanin) induce blue-

shifting due to the aggregation of the metal complexes. The aggregation of the iron (III)-betanin complexes is more favourable compared to that of chromium (III)-betanin complex.

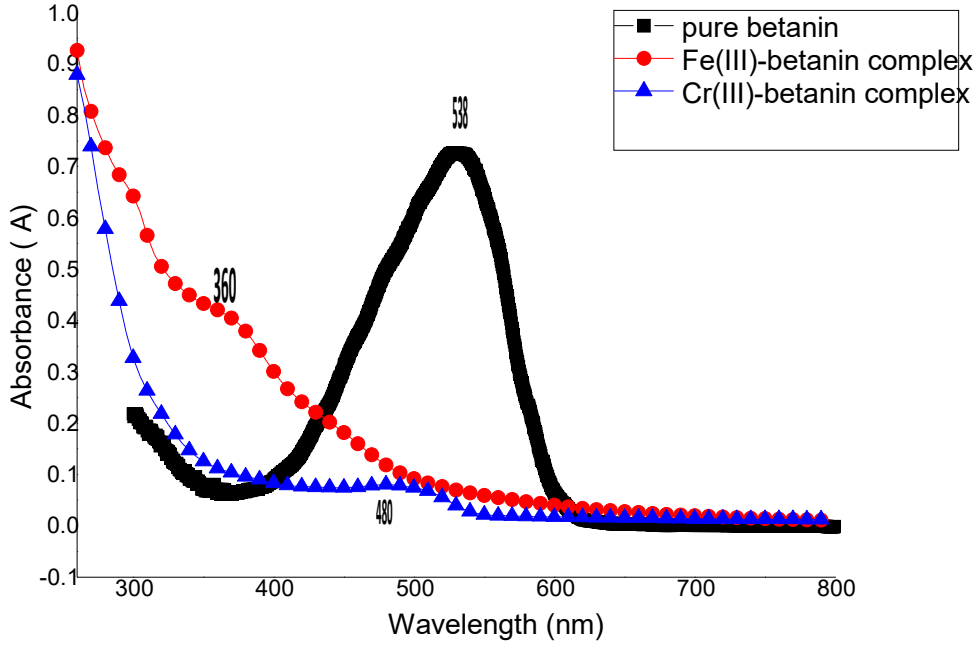
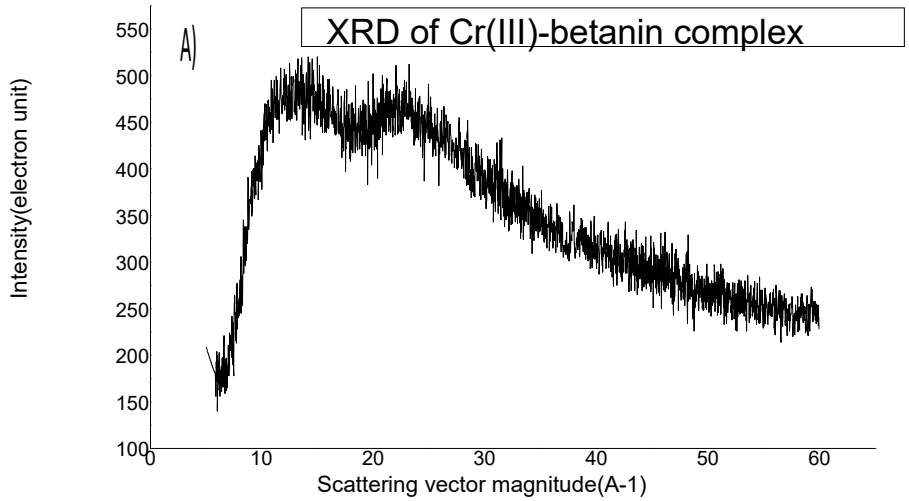


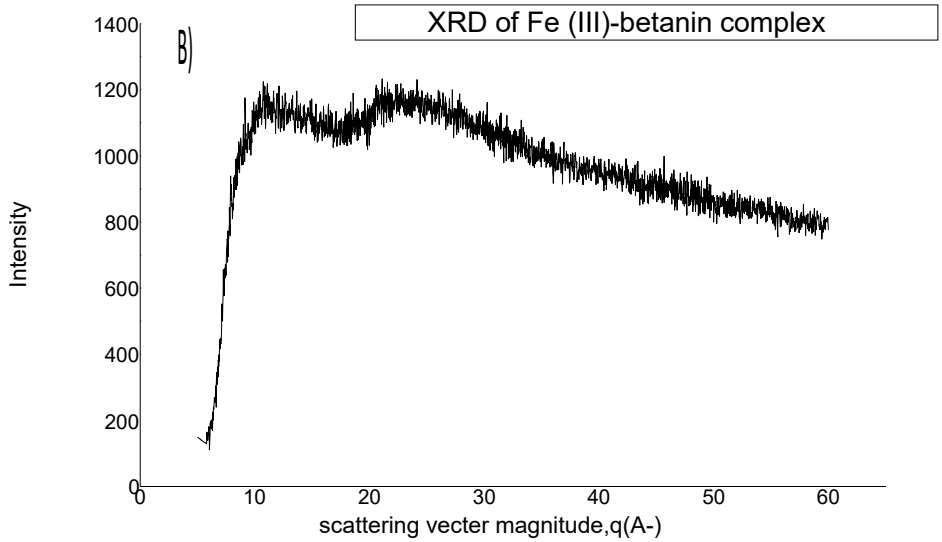
Figure 5.5: The absorption bands of the pure betanin, chromium-betanin complex, and iron-betanin complex

5.5. Results of Betanin-metal Complexes XRD Spectra Study

One of the most important differences between XRD patterns of amorphous to that of crystalline material is the base line of the obtained spectra. The presence of amorphous phase within the sample may result in irregular base line with noise. On the other hand, we can presume that highly crystalline materials possess smooth base line with sharp peaks. **Figure 5.6** depicts the XRD data for both the Cr (III)-betanin complex and Fe (III)-betanin complex. The result shows irregular base line indicating that both complexes are highly amorphous solid.



A) Cr (III)-betanin complex



B) Fe (III)-betanin complex

Figure 5.6: XRD result of (a) Cr (III)-betanin and (b) Fe (III)-betanin complexes

CHAPTER SIX

6. CONCLUSION

In this study, the compound betanin, which is one of the essential constituent of beetroot, is successfully isolated in good yield from methanol extract (0.25% w/w). The absorption peak at 538 nm wavelength confirms that the compound is betanin which is exactly comparable to the literature data.

The electron scavenging properties of the compound was investigated through Ferric Reducing Antioxidant Power (FRAP) Analysis. The result of the reducing power assay increases with increase in concentration indicating that betanin exhibit higher reducing activity (antioxidant activity).

By benefiting the structural feature of betanin, we have successfully synthesis iron (III)-betanin and chromium (III)-betanin complexes. The resulting metal-betanin complexes are characterized by UV-Vis and XRD spectral studies. Compared to pure betanin, absorption spectral of the complexes induce blue-shifting due to the aggregation (coordination) of the metal-betanin complexes. The formation (aggregation) of Iron (III)-betanin complex is more favourable when compared to that of Chromium (III)-betanin complex. The XRD analysis results of both complexes have irregular base line indicating that the complexes are an amorphous solid.

Future studies should be done to fully characterise and investigate the application of the resulting betanin-metal complexes.

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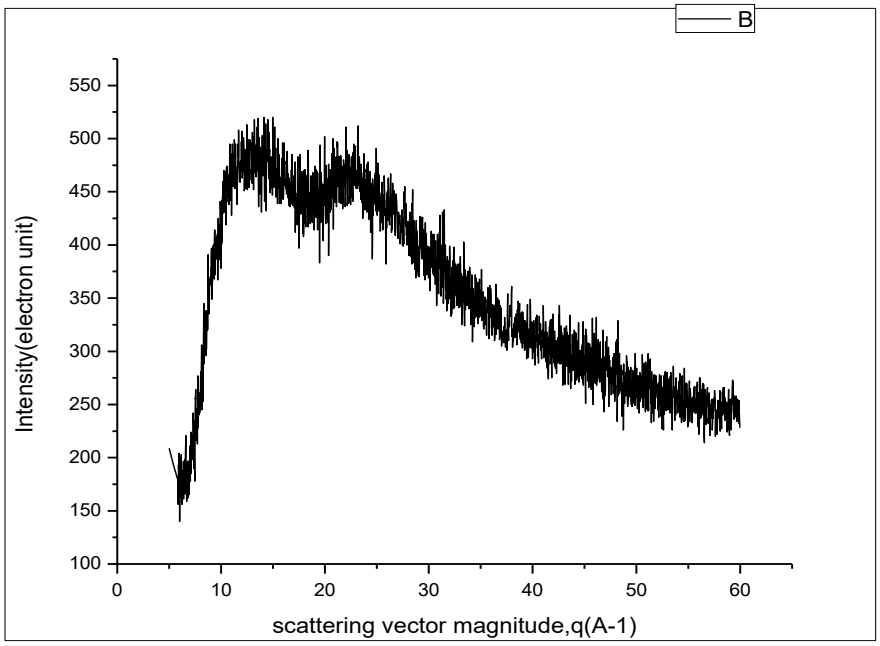
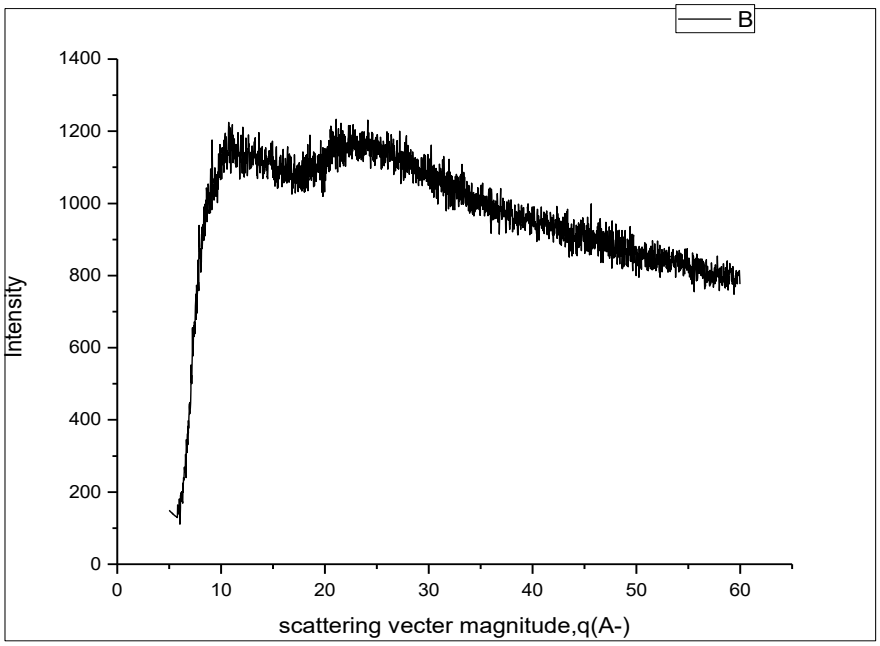
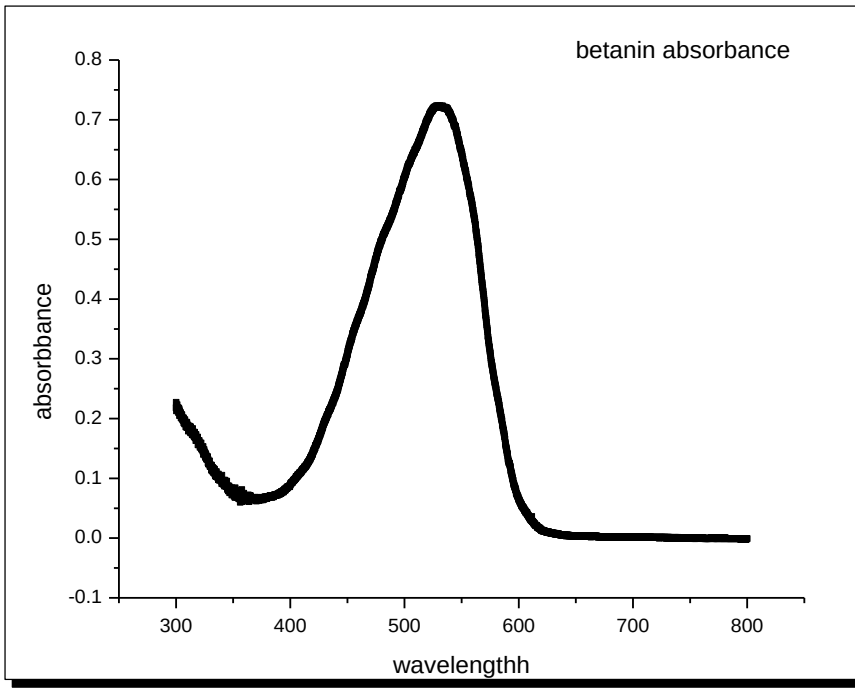
APPENDICES



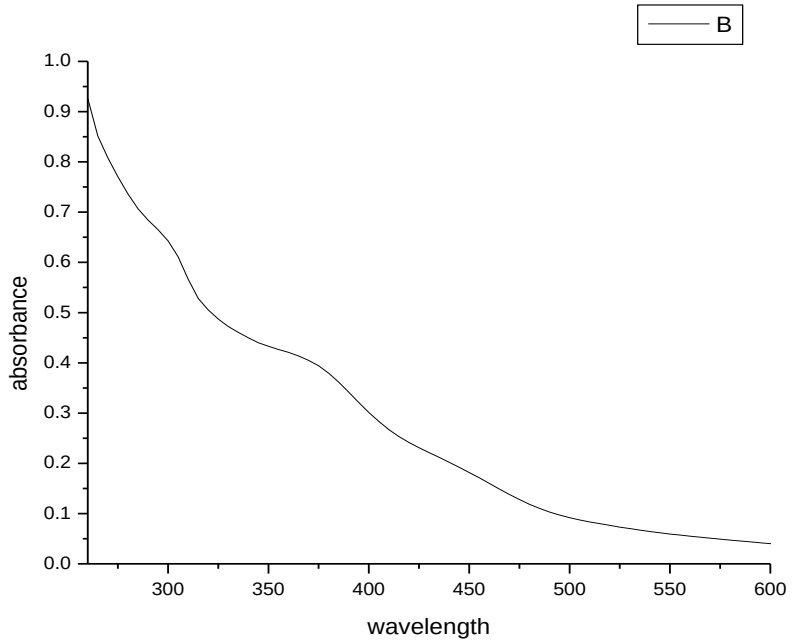
APPENDIX 1: XRD results of Cr (III)-betanin complex



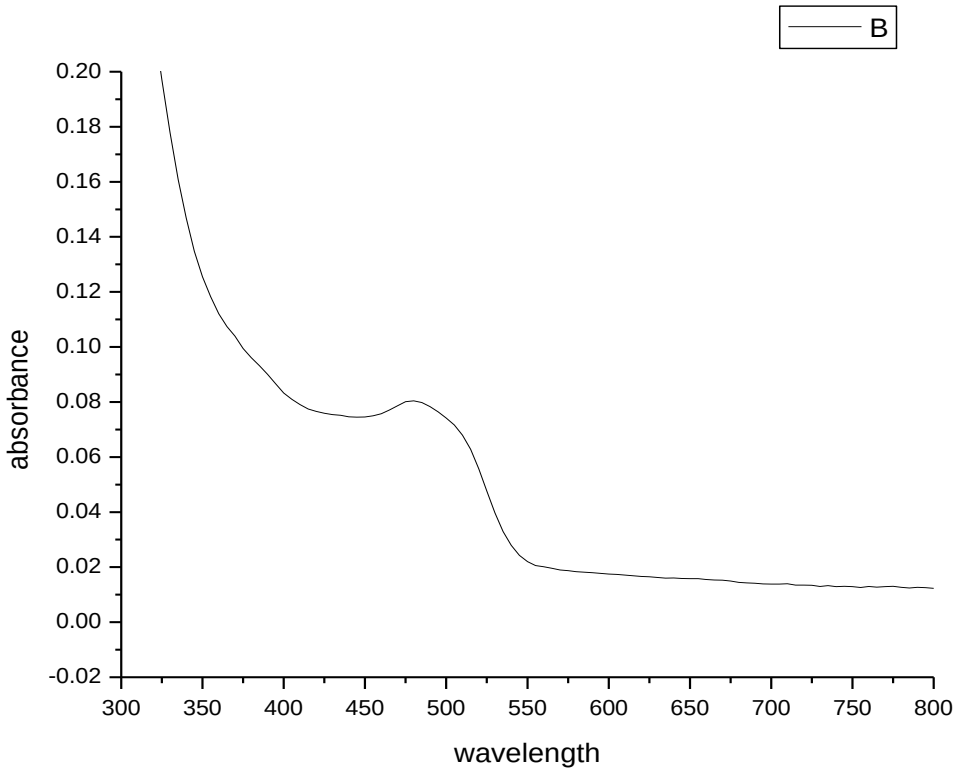
APPENDIX 2: XRD results of Fe (III)-betanin complex



APPENDIX 3: Absorption spectra of isolated betanin



APPENDIX 4: Absorption spectra of Fe (III)-betanin complex



APPENDIXE 5: Absorption Spectra of Cr (III)-betanin complex