ADDIS ABABA UNIVERSITY
COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES
DEPARTMENT OF CHEMISTRY

EXTRACTION AND PURIFICATION OF BETANIN FROM RED BEETROOTS AND SYNTHESIS OF Co(II), Cu(II) AND Zn(II) BASED BETANIN METAL COMPLEXES

Thesis Submitted to the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemistry

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Table of Contents

List of Tables

List of Figures

List of Scheme

List of Abbreviations

DOPA	Dihydroxy phenyl alanine
DMF	Dimethyl formamide
EDTA	Ethylene di ammine tetra acetate
EFSA	European food safety authority
eV	electron Volt
FDA	Food and drug administration
FRAP	Ferric reducing antioxidant power
GRAS	Generally recognized as safe
HPCD	High pressure carbon- dioxide
HPCDAE	High Pressure Carbon – Dioxide Assisted Extraction
HPLC	High performance liquid chromatography
LC	Liquid chromatography
LDL	Low – Density lipoprotein
NaH_2PO_4	monoSodium Dihydrogen Phosphate
Na_2HPO_4	DiSodium monohydrogen Phosphate
$[Na_3Fe(CN)_6]$	Sodium ferricyanide
NMR	Nuclear magnetic resonance
PLE	Pressurized liquid extraction
POD	Peroxidases
PPOs	Polyphenyl Oxidase
rpm	Revelations' per minutes
TCA	Trichloro Acetic Acid
TLC	Tine Layer Chromatography
USDA	United States Department of Agriculture
UV-Vis	Ultra Violet Visible spectroscopy
XRD	X-ray diffraction

ABSTRACT

For this study, red beetroots were used as a source of betanin. Betanin extraction process was carried out by using methanol as a solvent. Column chromatography method was used to purify the extracted betanin and to isolate the purified betanin by slightly increasing the polarity of the solvent. The absorption spectra of the extracted and purified sample was efficiently monitored and recorded in the UV-Vis region (200–800 nm) by using Perkin Elmer, Lambda 950 UV/Vis/NIR scanning spectrometer with methanol reference. As observed from the experimental result, the maximum absorption spectra of pure betanin is around λ_{max} 540 nm is comparable result with absorption spectra data of pure betanin in different literatures. Ferric reducing power method was applied with slight modification to study the reducing capacity of betanin. Reducing power assay method to evaluate antioxidant ability was based on the reduction of Fe^{3+} to Fe^{2+} in which the yellow color of the test solution changes to various shades blue, which indicates the reducing capacity of the sample. Purified betanin were subjected to complexation with Co^{2+}, Cu^{2+} and Zn^{2+}. As observed from the experimental results, maximum absorption spectra of newly synthesized betanin-metal complexes showed blue shifting absorption spectra compared with absorption spectra of pure betanin. Finally, X-ray diffraction technique was used to draw conclusion about the structure and phase purity of the newly synthesized metal complexes. X-ray diffraction spectra results showed that the constituents of the resulting betanin-metal complex solids are arranged with no particular order indicating they are amorphous solids.

Key-words: Beetroot, Betanin, Stability, Super food, Medicinal plant, Oxygen, Temperature, Light and Antioxidant.

1. INTRODUCTION

Beetroot is the name used by the British and some other English speaking countries including Australia and the New Zealand for the vegetable that Americans in the USA call as the beet and also known as table beet, green beet, red beet dinner beet or golden beet **[1]**. Beetroots contain high amounts of biologically active substances including betalains and inorganic nitrate. The high concentration of betalains, which are water soluble pigments, is responsible for the intense red color of beetroots, especially betacyanins and betaxanthins. Besides the high amount of antioxidants, beetroot contains many other health benefiting compounds. Thus, beetroot is recognized as health promoting food due to the presence of essential components like soluble fiber, minerals (examples: calcium, magnesium, iron, potassium, phosphorus, sodium and zinc) and vitamins (examples: biotin, folic acid, niacin and vitamin B6) **[2]**.

In addition to health beneficial compounds, beetroots also contains significant quantities of oxalic acid, a strong metal ion chelator, interfering with iron and calcium metabolism and can lead to the formation of nephroliths. Effects of a commercially available beetroot juice on inflammation is strongly involved in the development and progression of several clinical conditions including coronary heart disease and cancer, beneficial effect of beetroot extract may relate to this anti inflammatory capacity **[3]**.

Betalains are natural pigments containing betalamic acid as the chromophores in their structure. Betalamic acid conjugates with cyclo-3,4-di hydroxy phenylalanine (DOPA) to produce the red–violet colored betacyanins, although, if the conjugation is with different amino acids or amines, yellow betaxanthins are produced. The structure of betacyanins consists of betanidin, a glycone that varies with substitutions of acyl groups and sugar moieties **[4]**.

Beetroots are used as sources of natural colorants in many fields of the food industry; however, their importance goes beyond their coloring ability, since many possible benefits for human health have been reported. These includes strong antioxidant and anti-inflammatory activities, inhibition of lipid per-oxidation, increased resistance to the oxidation of low density lipoproteins(LDL), hepato protective activity and chemo preventive effects [5,6].

Because, beetroot sugar concentration is low, it is not used for sugar production, and contrarily is grown for diverse food uses, in the forms of fresh vegetable, dehydrated or frozen product, or for food preparations, such as pickles or juices. The main sugar in beet root is sucrose with only small amounts of glucose and fructose **[7]**.

An increasing interest in more differentiated food products has been observed in consumers in the latest thousands of years with current yearly production of 227,158,114 tons **[8, 9]**. Despite their widespread consumptions and uses in commercial colorants, companies need to continually look for ways to innovate and to develop new or improved products. The main aim of this work was extraction and purification of betanin from beetroot and synthesis of Cobalt(II), Copper(II), and Zinc(II) based betanin metal complexes due to beet roots wide range of application in dairy and food product mainly as coloring agent and for production of value added functional food products.

2. LITERATURE REVIEW

2.1. Historical background

Ancient Babylonians were the first to use beetroots for various applications. Early Greeks and Romans used the root for its medicinal properties and the leaves as vegetables. In England, beet root juice or broth was recommended as an easily digested food for the aged, weak, or infirm. Even in mythology, Aphrodite is said to have eaten beets to retain her beauty. In folk magic, if woman and man eat from the same beet, they will fall in love. In Africa, beets are used as an antidote to cyanide poisoning **[10, 11]**.

From the middle ages, beetroot was used as a treatment for a variety of conditions, especially illnesses relating to digestion and the blood. During the middle of the 19^{th} century, wine often was colored with beetroot juice. Although the leaves have been eaten since before written history, the beetroot was generally used medicinally and did not become a popular food until French recognized their potential in the 1800's. Beet powder is used as a coloring agent for many foods **[11]**.

2.2. Nutritional value of beetroot

The nutritional value of beetroot juice is very high due to its high content of carbohydrate, float, fiber, iron, nitrate, manganese, potassium, vitamin C and in addition to that free fat, low in calories, inexpensive and beets are available throughout the year. Thus, beet root juice is not only blessed with a beautiful color but also packed with nutrients **[12]**. A detailed view of this parcel comes out to be like:

Vitamins: Beetroots are a good source of folic acid and vitamin C. It also contains small amounts of vitamins B1, B2, B3, and vitamin A in the form of beta-carotene.

Minerals: Beetroots are rich in calcium, magnesium, phosphorus, potassium, and sodium. It also contains smaller amounts of iron, zinc, copper, manganese, and selenium.

Amino acids: While raw beets are mostly water and carbohydrate, they also contain small amounts of all the amino acids (protein).

Calories: 5 cm beetroot contains 35 calories.

Antioxidants: Its carotenoids and flavonoids can help reduce the oxidation of low density lipoproteins (LDL) cholesterol which could lead to damaged artery walls and ultimately heart attacks and strokes.

Anti-carcinogenic color: The deep red color of beetroot comes from betacyanin. This prevents from colon cancer.

Silica: The rich stock of silica in it does perfect utilization of calcium in the body and is also required for healthy skin, hair, nails and bones.

2.3. Health benefits of beetroot

Besides their colorant proprieties, betalains have attracted much attention because of their bioactivities. These pigments are classified as antioxidants, i.e. compounds that stop or delay the oxidation processes. Consequently, they can be used in the treatment of inflammatory and cardiovascular diseases, cancer, asthma, arthritis, oxidative stress, intestinal inflammation, diabetes, and other diseases associated with aging. It is also useful to lower blood pressure and increased blood flow. It is helpful in tumor reduction, decreases the risk of obesity and overall mortality, diabetes, heart disease and promotes healthy hair, increase energy, and overall lower weight. Beetroot juice improves oxygenation to the brain, slowing the progression of dementia in older adults. Due to its high fiber content, it prevents constipation and promotes regularity for healthy digestive tract. 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 **[13]**.

Generally, beet roots have long been known for its amazing health benefits for almost every part of the body. Start adding beets to your juicing diet to enjoy all its heavenly goodness:

Anemia: The high content of iron in beets regenerates and reactivates the red blood cells and supplies fresh oxygen to the body. The copper content in beets helps make the iron more available to the body, a great blood builder.

Blood pressure: All its healing and medicinal values effectively normalize blood pressure, lowering high blood pressure or elevating low blood pressure.

Cancer: Betaine, an amino acid in beetroots, has significant anti cancer properties.

Constipation: Drinking beets juice regularly will help relieve chronic constipation.

Dandruff: Mix a little vinegar to a small cup of beets juice. Massage it into the scalp with your fingertips and leave on for about an hour, then rinse. Do this daily till dandruff clears up.

Detoxification: The choline from beetroot juice detoxifies (which remove toxic substance)**.**

Gastric ulcer: Mix honey with your beets juice and drink two or three times a week on an empty stomach for removing ulcer.

Kidney ailments: Coupled with carrot juice, the excellent cleansing virtues are exceptional for curing ailments**.**

Liver toxicity or bile: The cleansing virtues in beets juice is very healing for liver toxicity or bile ailments, like jaundice, hepatitis, food poisoning, diarrhoea or vomiting.

Skin disorders: The water in which beetroots and tops have been boiled is an excellent application for boils, skin inflammation and out breaks of pimples and pustules.

Tonic effects: Beetroot is a nutritious and suitable tonic for the entire digestive tract.

Increases sex drive: It contains high amounts of boron, which is directly related to the production of human sex hormones.

Lowers cholesterol: Beetroot have cholesterol lowering capacity, because, it contains soluble fiber.

2.4. The Chemistry of betalains

The term betalain originates from the Latin name of beet root (*Beta vulgaris*), from which betalains were first extracted. They are responsible not only for the bright coloration of fruits and flowers, but also of roots and leaves of plants. Betalains are water soluble vacuolar nitrogen containing pigments, which are synthesized from the amino acid tyrosine into two structural groups, namely betacyanins with color differences from purple to violet and betaxanthins with

color differences from yellow to orange. As showed in Figure 1 bellow, the highly aromatic structure in betanin is responsible for the deep violet color in betalain pigment **[4]**.

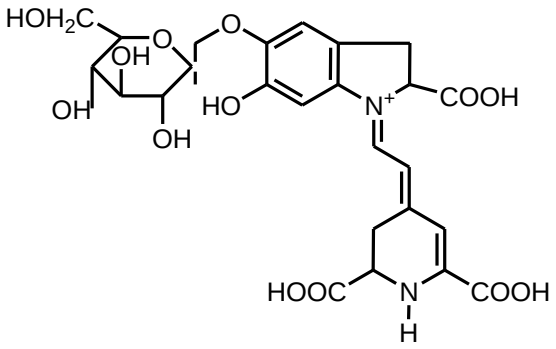
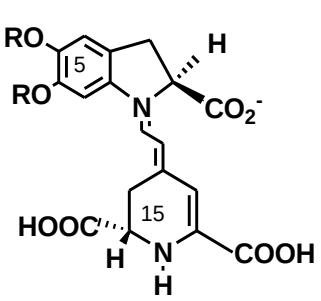
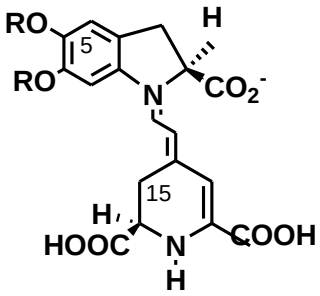


Figure 1: Structural formula of betanin

Betacyanins are derivatives of betanidin containing cyclo-3,4-dihydroxyphenylalanine residue. The simplest member of natural betacyanins is the non glycosylated betanidin or isobetanidin chromophores obtained by the condensation of cyclo di hydroxy phenylalanine with betalamic acid. The two molecules are differing only by the absolute configuration of their C-15 chiral center **[14]**.



Betanidin

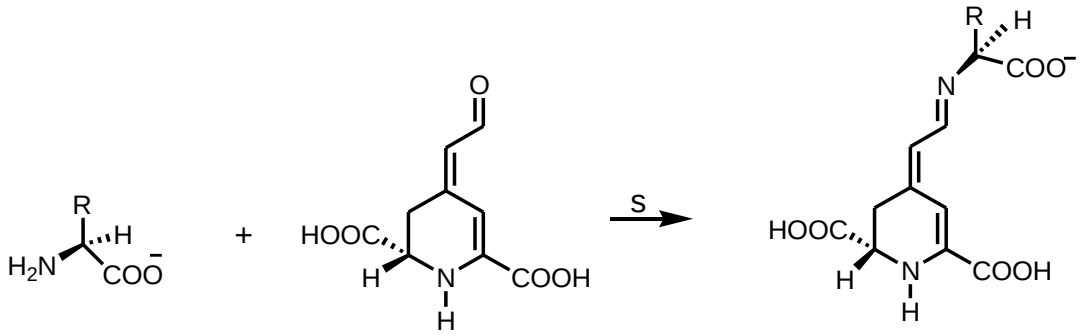


Isobetanidin

Figure 2: Structures of Betanidin and Isobetanidin

Betaxanthins are formed by the condensation of an amino acid or an amine with the aldehydes group of betalamic acid. This structure is responsible for the strong yellow or yellow-orange colors of betaxanthins and the maximum of absorbance between 470 nm – 486 nm. The yellow betaxanthins are immonium conjugates of betalamic acid with an amine or an amino acid.

Betaxanthins can also be easily synthesized none enzymatically by mixing betalamic acid and the desired amine at neutral or slightly acidic pH; this occurs spontaneously in the acidic plant vacuole **[15, 16].**



Amino acid Betalamic acid Betaxanthins

Where: S- refers to spontaneous aldimine formation.

Scheme 1: Betaxanthins synthesis

2.5. Sources of betalains

A. Natural sources of betalains

Betalains are a family of natural pigments found in most plants roots, fruits and flowers. They absorb visible radiation over the range of 476 nm – 600 nm with a maximum at 537 nm at pH=5. The few edible known sources of betalains are red and yellow beetroot, colored Swiss chard, grain or leafy amaranth and cactus fruits. Red beetroot is the major commercially exploited crop containing two major soluble pigments, betanin which is red in color and vulgaxanthine I which is yellow in color **[17]**.

B. Bio technological betalain production

Bio technological betalain production technology is another source of betalains. This production technology is helpful for easily controlling the quality and availability of betalain from external environmental factors such as temperature, light and oxygen that affects the stability of betalains. However, bio technological betalain production technology is unable to compete with the natural source for the production of betalains, i.e., beetroot, which is an abundant and

inexpensive crop which may produce up to 0.5 grams of betanin per kilo grams of roots. Generally, low productivity of bio technological betalain production system availability and the high cost of the process are some factors that impair its economic feasibility **[18]**.

2.6. Betalain extraction methodologies

Extraction is a frequently used technique to selectively transfer a compound of interest from one solvent to another. It is a process that selectively dissolves one or more of the mixture components in to appropriate solvent. Thus, extraction is based on solubility characteristics of the organic compound in the solvents being used for the extraction process. A compound can be separated from impurities in a solution by extracting the compound from the original solvent in second solvent. Recently, extraction techniques have been explored toward increasing efficiency of extraction process, reducing operating times and limiting the use of organic solvents, for the development of cheaper and greener analytical methodologies **[19, 20]**.

Extraction of bioactive compounds from various plant sources can be done by using various extraction procedures, conventional and non-conventional methods. Conventional techniques for the solvent extraction of bioactive compounds from natural matrices are based on the extraction power of different solvents coupled with the use of heat and agitation **[21, 22]**.

Betalains are mainly extracted through conventional extractions. Betalain containing materials are generally macerated or ground. Extraction of pigments is commonly performed with water, although, in some cases, the use of methanol or ethanol solutions (20–50 % v/v) is necessary to complete extraction. Nevertheless, the aqueous extractions promote better stability of these pigments. To enhance betacyanin stability and avoids possible oxidation by poly phenol oxidase activity, slight acidification of the extraction medium is recommended **[23]**.

In general, traditional extraction methods, used to obtain this type of compounds, have several drawbacks, such as long extraction time, evaporation of a huge amount of solvent, stability problems, batch-to-batch variations, low selectivity and relatively low yields of the extraction process. Therefore, there is growing demand for developing suitable extraction techniques that improves extraction process efficiency through enhanced mass transfer and which are more environmentally friendly **[19, 21]**.

Presently, extraction methodologies able to overcome the disadvantages mentioned above are being studied. These techniques are referred to as non-conventional extraction techniques. Among them, pressurized liquid extraction and high pressure Carbon dioxide-assisted extraction techniques could be used to obtain antioxidant pigment rich extracts from biological materials. These extraction techniques provide higher selectivity, shorter extraction times and do not use toxic organic solvents **[24].**

A. Pressurized Liquid Extraction (PLE)

Pressurized liquid extraction technique is one of the called green technologies. This technique is also known as pressurized solvent extraction, accelerated solvent extraction, enhanced solvent extraction and high pressure solvent extraction technique. Pressurized liquid extraction technique is suitable for a wide range of solutes, polar to non polar. The basic principle of pressurized liquid extraction technique relies on the combination of high pressure and high temperature to modify properties of solvents such density, diffusivity, viscosity and dielectric constant, allowing the selection of types of extracted compounds according to their polarity. The type of solvent, extraction time, temperature, particle size and water content of the sample are factors that affects this extraction process. Higher extraction temperature can promote higher analyte solubility and also decrease the viscosity and surface tension of solvents, allowing a better penetration of the solvent into the matrix **[19]**.

In pressurized liquid extraction process, pressure is another significant parameter which may influence compounds recovery. The main advantage of applying pressure during the extraction process is to keep the solvent in a liquid state at elevated temperatures, above the boiling point of the solvent. The use of elevated pressure and temperature reduce solvent surface tension, forcing the solvent within the matrix pore to contact the analyte and in this way making the analyte more available. Furthermore, depending on the location of the analyte within the matrix, high pressure could result in the disruption of the plant tissue, cellular wall, membrane and organelles, increasing its permeability and enhancing the mass transfer of the solvents into the matrix and the soluble constituents into the solvent used in extraction **[20, 22, 25]**.

B. High Pressure Carbon Dioxide-Assisted Extraction (HPCDAE)

High pressure Carbon dioxide-assisted extraction method is another important betanin extraction method that consists solvent extraction assisted with pressurized carbon dioxide. High pressure Carbon dioxide-assisted extraction technique combines the advantages of enhanced mass transfer rates that increases compounds diffusion from the vegetable matrix into the selected solvent extraction. Carbon dioxide is non-toxic, non-flammable, non-explosive and inexpensive agent. It is easily removed by depressurization and out gassing. In addition to the above points, this molecule is environmentally friendly and generally recognized as safe (GRAS) by United State Food and Drug Administration (FDA) and European Food Safety Authority (EFSA). Moreover, carbon dioxide has been described to ensure minimal alteration of the bioactive compounds and to preserve their functional properties **[25, 26]**.

Like many other natural pigments, betalains are extremely sensitive to oxidation especially that caused by peroxidases activity, which is one of the main causes of discoloration of this pigments. When betalains are effluxes from their cellular compartment, the vacuoles, come in contact with enzymes, mainly peroxidases (POD) and polyphenyl oxidase (PPOs), which quickly degrade the pigments. To avoid betalain enzymatic degradation, the enzyme can be effectively inactivated by a short heat treatment of the extract (70 ^{0}C, 2 minutes), although this may degrade some of the pigments and loss another nutritional components **[27, 28]**.

To overcome the disadvantages mentioned above, pressurized Carbon dioxide might be an alternative to inactivate not only enzymes but also pathogens. Pressure alone has no direct influence on enzyme at pressures below 20 MPa. The effect of pressurized Carbon dioxide is demonstrated to disrupt bacterial cells by the rapid release of gas pressure. Numerous studies have showed the efficacy of pressurized Carbon dioxide to inactivate microorganisms and enzymes in batch, semi continuous and continuous systems **[27, 28]**.

High pressure carbon dioxide besides microorganisms and enzymes inactivation might also strengthened extraction process by its superior abilities in disruption of the plant tissue, cell membrane modification, intracellular pH decrease, disordering of the intracellular electrolyte balance and removal of vital constituents from cells **[26]**.

2.7. Betalain analysis

Betalain analysis has been carried out by using Spectrophotometric analysis method and also chromatographic analysis techniques. Spectrophotometric analysis of betalain suggests that the external color of the beetroot depends on the relative concentration of betacyanins, red pigment with maximum absorbance at around 540 nm, and betaxanthins, yellow pigment with maximum absorbance at around 480 nm **[29].** Thus, Spectrophotometric analysis technique is the first methodology for betalain identification.

Chromatographic analysis methodology is another important betalain analysis methodology. Chromatography is a physical method of separation that distributes components to separate between two phases, stationary phase and mobile phase, moving in a definite direction. Structural modifications of betalains must be carried out considering at least high performance liquid chromatography (HPLC) separation and UV-visible spectroscopy, mass spectrometry and NMR spectroscopy for identification of individual compounds **[30]**.

2.8. Stability of betalains

Generally, betalains are stable between pH=3 and pH=7. Owing to their hydrophilic nature, betalains can be extracted using precooled water or aqueous methanol. Betalain containing plants also contains endogenous ascorbic acid which is important to protect the most labile compounds. Several authors recommended that the addition of ascorbic acid in the extraction medium since it leads to a slightly acidic pH, stabilizes betacyanins and avoids the formation of quinones by the effect of polyphenols oxidase. Isoascorbic acid was also reported to enhance betalains stability by oxygen removal. Moreover, chelating agents such as citric acid and Ethylene diamine tetra acetate (EDTA) were proven to be suitable for betalain stabilization, possibly by a partial neutralization of the electrophilic center of these pigments, through association around the positively charged amino nitrogen **[7, 31]**.

2.9. Microencapsulation techniques

Despite their coloring capacity and superior antioxidant activity, betalains have not been considered by the food industry as potential additives. This is in part due to their instability,

which prevents long term storage. The stability is an important aspect to consider for the use of these pigments as colorants in foods **[32]**.

The most commonly used application of betalain stabilization technique in the food industry is microencapsulation. Microencapsulation technique is described as a technique where bioactive compounds are encapsulated in a biopolymer. One of the objectives of microencapsulation is to increase the shelf life of the bioactive compound, protecting it from undesirable environmental conditions such as light, moisture and oxygen, thereby reducing its reactivity to its outside environment. This method is also used to change liquid solutions to powders, which are easier to handle **[33, 34]**.

One of the most widely used and accepted techniques of microencapsulation is spray drying, which is very economical, flexible, and yields good results. Rapid drying of the solutions leads to the formation of encapsulated form in which the colorant is surrounded by a wall material. Recent studies have described preparation of spray dried hydrophilic formulations containing betalains from red beet. However, the use of these hydrophilic forms to color food lipophilic matrices is very limited, due to solubility issues **[34]**.

2.10. Factors affecting betalains chemical stability

Stability of betanin is an important aspect to consider these bioactive compounds in foods, since, they are affected by several intrinsic and extrinsic factors such as light, oxygen, temperature and enzymatic activities **[31]**.

Concerning on the structural aspects of betalains, the condensation results of betalamic acid with amino compounds (betaxanthins) and the condensation results of 3,4-dihydroxyphenylananine with amino compounds (betacyanins), results in differing stability of pigment structures both at room temperature and on heating. For example, comparing stability of different betacyanins, glycosylated structures are more stable than aglycons, probably because of the higher oxidation-reduction potentials of the former molecule **[35, 36]**.

The uptake of dissolved oxygen by betanin was measured to understand the role oxygen in its degradation. Since lower oxygen level favors betanin pigment to be partially recovered after

degradation, storing betanin solutions under low oxygen levels results in decreased betanin pigment degradation compared with the storage of betanin under air atmosphere **[37, 38]**.

As reported by various authors, exposure to light has also a negative impact on pigment stability. In the presence of light, betalains oxidation is accelerated due to the excitation of electrons from **π** to **π*** orbital's with resultant increased molecular reactivity **[39].** Nevertheless, it has been reported that, in absence of oxygen, light induced degradation of betanin has minimal effect **[40].**

Temperature is the most important factor that plays role on the stability of naturally existing pigments. Most natural pigments depict color stability below 50 ^{0}C and were observed to begin degrading when exposed to temperatures exceeding 50 ^{0}C. If betanin is exposed to temperature exceeding 50 ^{0}C, rapid lose of its red color to brown takes place indicating that it is unstable to use it as food colorant **[41].**

Antioxidants are compounds that inhibits or slow down the oxidation of lipids and other molecules through the neutralization of free radicals **[42].** They are substances that may protect our cells against free radicals, which may play a role in brain disorder, cancer, heart disease and immune system decline and other diseases. The antioxidant activities of natural substances are based on their ability to donate hydrogen atoms. Some food antioxidants like ascorbic and isoascorbic acids have been described to enhance betalain stability. Addition of ascorbic acid or isoascorbic acid before thermal treatment of betanin resulted in higher effectiveness than their subsequent addition indicates that both acids do not only enhance pigment regeneration in red beet juice, but also prevent pigment degradation **[43].**

3. OBJECTIVE OF THE STUDY

3.1. General objective

The general objective of this study was extraction and purification of betanin from red beetroot and synthesis of Cobalt (II), Copper (II) and Zinc (II) based betanin-metal complexes due to beetroots wide range of application in dairy such as drinking yogurt and food products mainly as coloring agent and for production of value added functional food products.

3.2. Specific objectives

The specific objectives of this study includes:

Extraction and purification of betanin from red beetroots,
To determine the antioxidant properties and absorption spectra of pure betanin pigment and
To synthesize Cobalt (II), Copper (II) and Zinc (II) based betanin metal complexes.

4. EXPERIMENTAL

4.1. General description

Materials and methods

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 coated on Aluminum foil. X-ray diffraction measuring instrument was used to draw conclusion about the structure and phase purity of the newly synthesized metal complexes.

Chemicals

Methanol, Ethanol, water, Hexane, Ethyl acetate , Di methyl formamide (DMF), Ascorbic acid, Silica gel and selected heavy metal nitrate and chloride salts such as $Co(NO_3)_2.6H_2O$, $CuCl_2.2H_2O$ and $ZnCl_2$ are obtained from Adiss Ababa University laboratory equipments and chemicals store room.

4.2. Sample collection and preparation

Fresh red beetroot were purchased from a local market, Adiss Ababa Ethiopia, washed, peeled and chopped up into small pieces and ground by grinder in order to increase contact area of the sample. For the laboratory experiments, raw juice was prepared directly from the diced beet (700 grams) with the aid of 2.0 L of methanol.

(*a*) (b*)* (c)

Figure 3: (a), (b) and (c) fresh, sliced and ground red beetroot, respectively.

4.3. Sample extraction and purification

The chopped up red beetroot sample was soaked overnight with methanol as a solvent. This extraction process was carried out twice at room temperature in dark. The solution, which contains most of the red pigment is then subjected to suction-filter to remove suspended particles and the solvent is removed using rotary evaporator. After extraction process was completed, the extracted juice was stored in 250 ml round bottom flask in dark.

Figure 4: Methanol extract of the red beetroot juice.

The extracted and collected sample juices were subjected to purification by liquid column chromatography method. Silica gel was used as the stationary phase in a glass column and packed under methanol. The elution was performed with methanol as the mobile phase. Yellow pigments and other troublesome contaminants are thereby removed.

Figure 5: purifying methanol extract betanin by using liquid column chromatographic technique.

After complete elution, fractions containing betanin were collected and the solution was concentrated under temperature of 50 ^{0}C using the rotary evaporator and 35.54 grams of red extract was obtained.

Figure 6: Rotary evaporator for concentration.

This 35.54 grams extract was dissolved in methanol and applied to silica gel column chromatography which was packed with methanol (100%), the column was eluted using methanol and total of 5 fractions were collected.

Figure 7: Fractions of methanol extract betanin collected for TLC test.

Out of those 5 fractions collected, by increasing polarity using ethyl acetate : hexane (7:3) solvent system, 4 fractions show the same profile on TLC, when mixed and the solvent was removed by rotary evaporator and 1.74 grams of pure compound was obtained. Red spot was formed on the TLC for this pure compound. Again when polarity of the solvent system ethyl acetate : hexane increase to (8:2), all fractions show the same profile on TLC with the red color of the pure compound.

Figure 8: TLC analysis for methanol extract betanin.

4.4. Analysis of purified sample

4.4.1. Ferric reducing antioxidant power (FRAP) analysis

Ferric ions reducing power was measured according to the method of Oyaizu **[44]**, with a little modification. In this assay of reducing activity was based on the reduction of Fe^{3+} (ferricyanide complex) to Fe^{2+} (ferrous) form in the presence of reductants (antioxidants) in the tested samples. Fe^{2+} was then monitored by measuring the formation of blue color which indicates the reduction of ferric to ferrous. This was approved by measuring absorbance of the sample with different concentration at 700 nm wave lengths.

Reagents and sample solution preparation

0.2 M Sodium phosphate buffer solution (pH = 6.6), which consists of mixture of monobasic di hydrogen phosphate (NaH_2PO_4) and dibasic mono hydrogen phosphate (Na_2HPO_4) was prepared as the following: 28.4 grams of Na_2HPO_4 was dissolved in 100 ml distilled water to give 2.0 M solution and 24.0 grams of NaH_2PO_4 was dissolved in 100 ml distilled water to give 2.0 M solution. The two solutions mixed up 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 has been checked using pH meter at room temperature. The combined stock solution (2.0 M) was diluted to 1.0 liter with distilled water and obtained 2.0 M. 1% sodium ferricyanide was prepared by dissolving 1.0 grams of sodium ferricyanide in 100 ml of distilled water. Trichloro acetic acid (TCA) was prepared by dissolving 10 grams of TCA in 100 ml. 0.1 % $FeCl_3$ was prepared by dissolving 0.05 grams of $FeCl_3$ in 50 ml. 0.5 mg/ml of standards ascorbic acid was prepared by dissolving 0.005 grams of standards in 10 ml of methanol (dilute stepwise until the absorbance is corrected to the range of sodium in order case it was 0.12, 0.06, 0.03 and 0.01) and the calibration curve was drawn from these concentrations.

The reducing power of extracted betanin from red beetroot with different concentrations can be determined according to the method Oyaizu (1986). 0.05 ml of betanin was dissolved in 50 ml of methanol. From the solution prepared methanolic of betanin was taken and mixed with 2.5 ml of 0.20 M sodium phosphate buffer of pH=6.6 and 2.5 ml of 1% sodium ferricyanide [$Na_3Fe(CN)_6$]. After shaking well using vortex shaker, mixture was incubated in water bath at

50 ^{0}C for 20 minutes. Then, 10% TCA of 2.5 ml was added to the mixture and centrifuged at 200 rpm for 10 minutes. 2.5 ml of the solution mixture was taken and mixed with 2.5 ml of distilled water and 0.5 ml of 1 % $FeCl_3$. The absorbance of the mixture was measured at 700 nm against ascorbic acid as the control. The concentration of betanin complex was calculated by stating equivalent to regression equation of ascorbic acid calibration curve **[45, 46]**.

4.4.2. Spectro photometric analysis

UV-Vis spectrometer is used to measure the absorbance rate in visible light spectrum. The betanin concentration was determined by assuming a molar absorption coefficient (ε) of 6.5 x 10^4 L $mol^{-1}cm^{-1}$ at 536 nm. Detailed procedure on UV-Vis measurement can be obtained in Gomesh et al., 2014 **[47].** The photon energy absorbed in to the sample is calculated by using a general equation 1.

$$\text{Energy } (E) = \frac{hc}{\lambda} \quad \text{- Equation (1)}$$

Where *h*- is the Planck's constant, *λ*- is the wave length and *c*- is speed of light in vacuums. The numerical values of the symbols are; h= 6.63 × 10^{-34} Js and c = 3.0 × 10^{8} m/s.

5. RESULTS AND DISCUSSION

5.1. Results of betanin antioxidant property study

The results of the present investigation show the extracted and purified betanin provided antioxidant activity.

Sodium ferricyanide + ferric chloride $\xrightarrow{\text{antioxidant}}$ Sodium ferricyanide + ferrous chloride

$Na_3Fe(CN)_6 + FeCl_3 \xrightarrow{\text{antioxidant}} Na(Fe^{3+}[Fe^{2+}(CN)_6]) - \text{Complex}$

The reducing power assay was associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicates that they were electron donors and can reduce the oxidized intermediates. Presence of the reducers causes the conversion of the ferricyanide to the ferrous form. The reducing power assay increase with increase in concentration. Reducing power assay method to evaluate antioxidant ability was based on the reduction of Fe^{3+} to Fe^{2+} that is observed at 700 nm in which the yellow color of the test solution changes to various shades blue, depending on the reducing power of the sample. Increasing absorbance to 700 nm indicates an increase in reductive ability.

The concentration of complexes was calculated from ascorbic acid calibration curve. Ascorbic acid was used as the positive reference standard. All assays were run in triplicates and averaged. The reducing ability of the sample betanin was directly proportional to the absorbance reading i.e. a higher reducing power was indicated by a greater absorbance. As shown in Table 1, the standard curve was prepared with the help of different diluted concentrations (0.01 ppm, 0.03 ppm, 0.06 ppm and 0.12 ppm) of ascorbic acid and the relation is linear.

$Na[Fe(III)(CN)_6]_3 + Ph\text{-}OH \rightarrow Na_X(Fen^{3+}[Fe^{2+}(CN)_6]_3) + Ph\text{-}O^{\bullet} + H^+$

Yellowish color color of betanin the formation blue color

Table 1: The average absorbance's of Ascorbic acid at 700 nm**.**

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

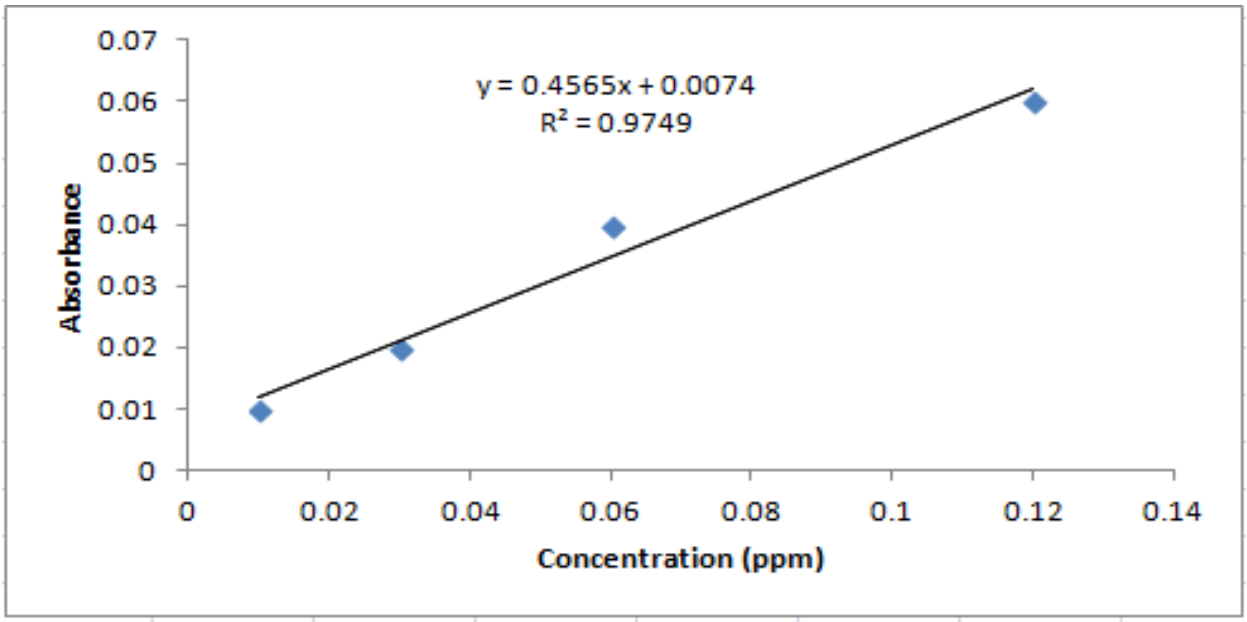


Figure 9: Calibration curve constructed from ascorbic acid.

The regression equation obtained for ascorbic acid was y = 0.4565x + 0.0074 (R^2= 0.9749), Here, y = absorbance obtained at 700 nm and X = concentration of ascorbic acid used. The reduction ability or antioxidant activity of extract betanin was stated in terms of ppm ascorbic acid equivalents as calculated from the standard curve found for ascorbic acid. The concentration of complex $Na_X (Fe_n^{3+} [Fe^{2+} (CN)_6]_3)$ of betanin was calculated from regression of ascorbic acid whose absorbance of equivalents taken as reference at 700 nm **[48]**.

In this study, the reducing capacity of betanin was performed using Fe^{3+} to Fe^{2+} reduction assay in which the yellow color changes to blue color depending on concentration of antioxidants in the samples was calculated based on ascorbic acid standard calibration curve as reference.

Table 2: Average absorbance of betanin complex Na_X (Fe^{3+} [Fe^{2+} $(CN)_6]_3$) blue sample prepared.

Species	Average absorbance			Concentration calculated from standard calibration carve(ppm)		
Betanin –complex	A-1	A-2	A-3	C-1	C-2	C-3
	0.10467	0.085	0.10467	0.213	0.170	0.123

A-1 = average absorbance of originally prepared complex Na_X (Fe_n^{3+} [Fe^{2+} $(CN)_6]_3$) blue color sample prepared used concentration before dilution,

A-2 = average absorbance of first dilution and

A-3 = average absorbance of second dilution.

C-1, C-2 and C-3 concentration calculated from standard calibration curve concentration (ppm) versus absorbance.

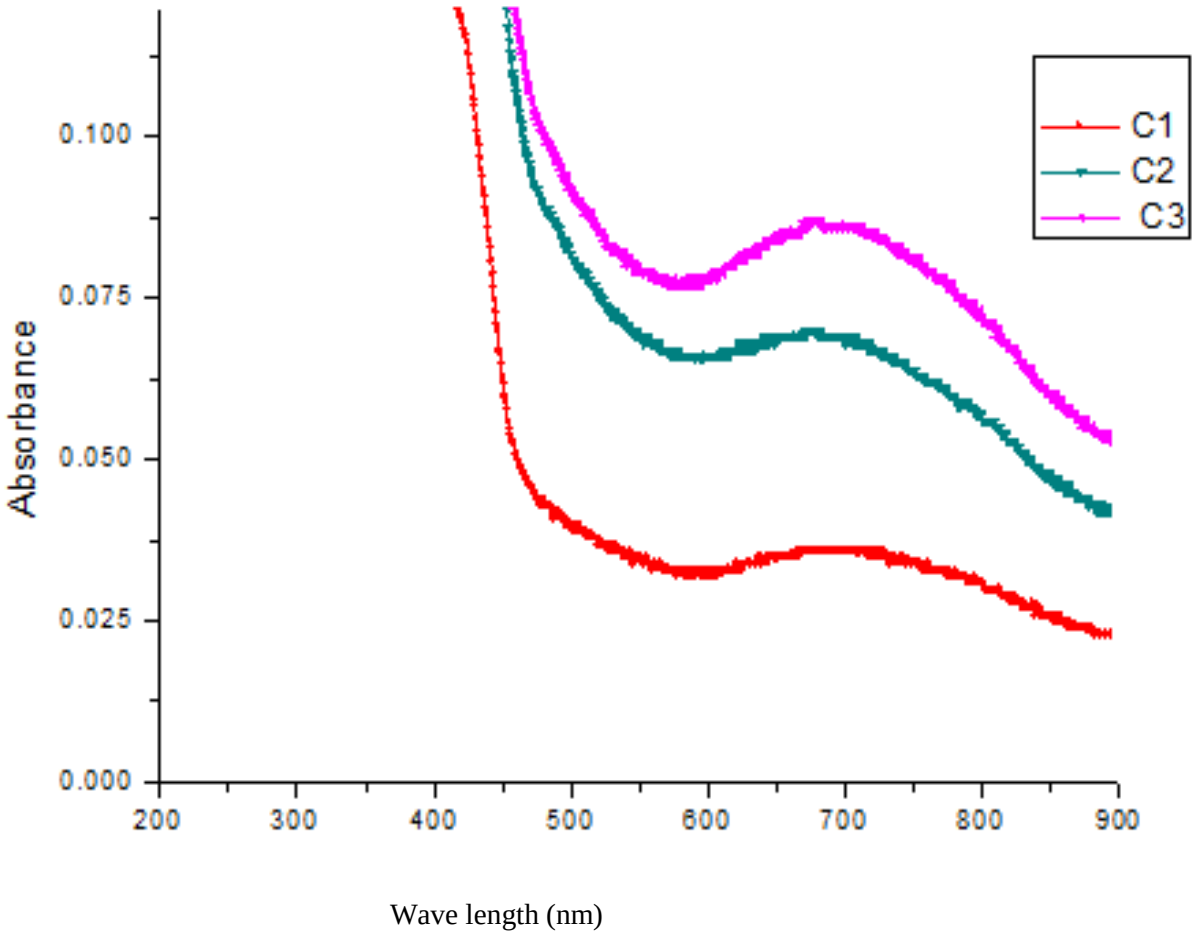
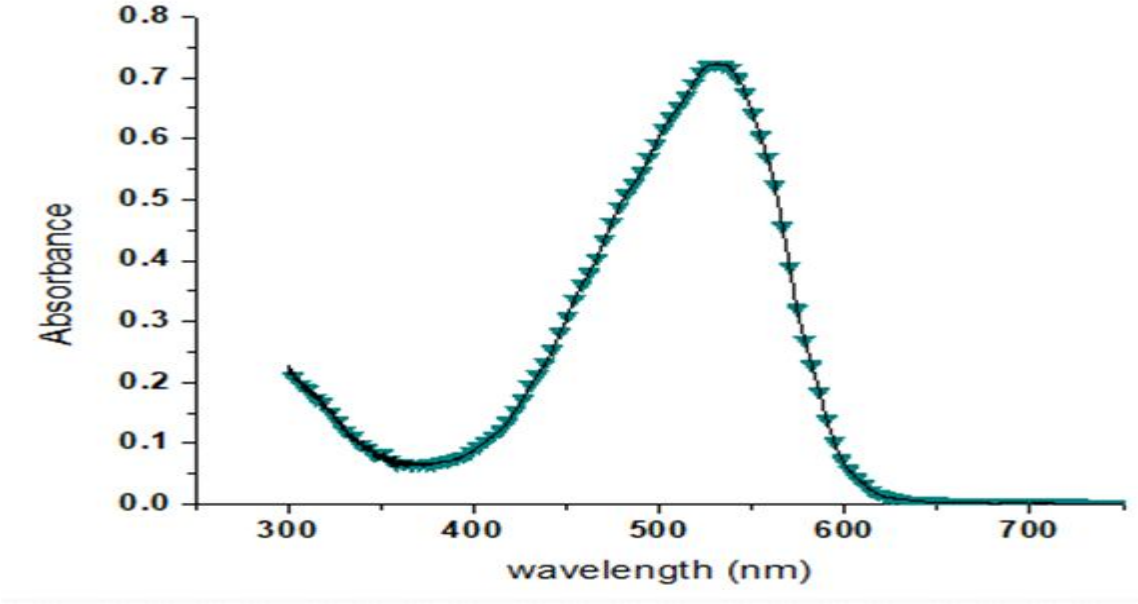


Figure 10: Absorbance of Betanin complex with different concentrations at 700 nm.

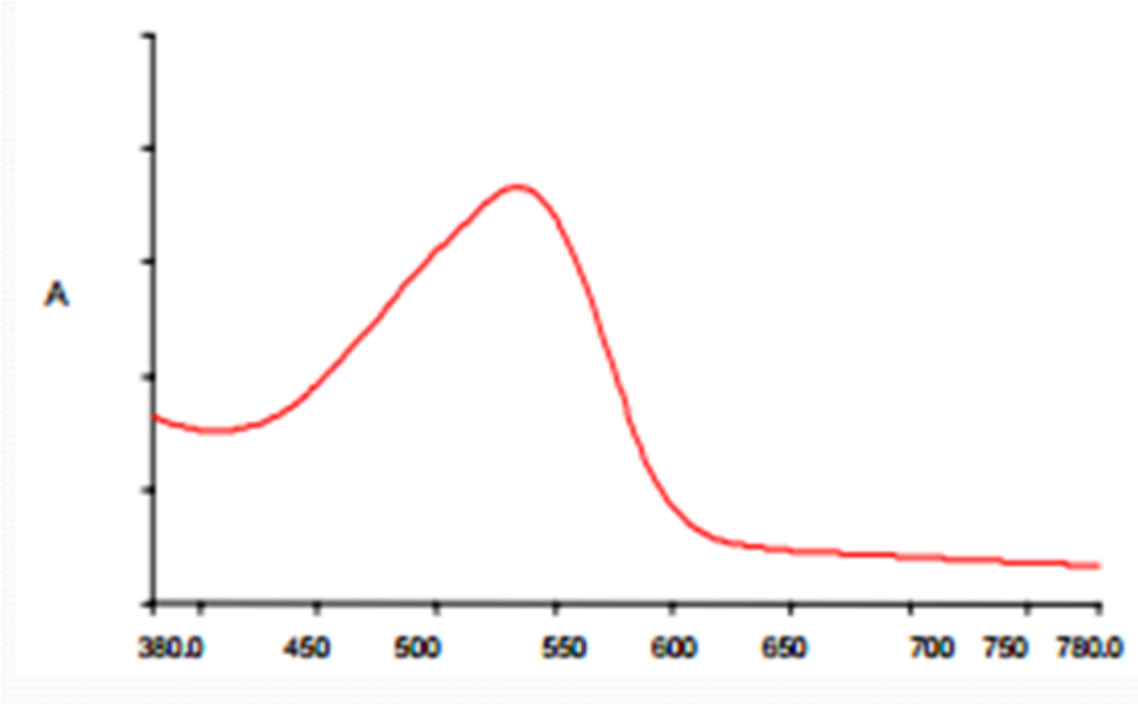
As indicated in Table 2 and Figure 10 above, the presence of reducers (antioxidants) causes the reduction of the Fe^{3+} (ferricyanide complex) to the Fe^{2+} (ferrous) form. Ferric ions were reduced to ferrous ions in the presence of an antioxidant which formed a blue colored ferrous.

5.2. Absorption spectra result of betanin

Absorption spectra of the sample was efficiently monitored and recorded in the UV–Vis region (200–800 nm) by using Perkin Elmer, Lambda 950 UV/Vis/NIR scanning spectrometer with methanol reference. As shown in Figure 11 A, experimentally obtained maximum absorption wave length for extracted betanin around λ_{max} 540 nm indicates the characteristic property of absorption chromophores centers for betanin is comparable with absorption spectra data for pure betanin in different literatures as indicated in Figure 11 B.



(A)



(B)

Figure11:(a) and (b) shows experimentally obtained absorption spectra of Betanin with methanol reference and absorption spectra of Betanin obtained from literature, respectively.

5.3. Synthesis of betanin-metal Complexes

In most food colorant applications, an estimated quantity of less than 50 mg betanin per kilo gram could produce the desired color. In order to increase its commercial applications, betalain need to resist or slow down these changes through stabilization techniques. Among the various ways of stabilizing betalains; complex formation, co-pigmentation and encapsulation are promising techniques. In this study, complex formation of betanin with three selected heavy metal cations, Co^{2+}, $Cu^{2+}$and Zn^{2+} was synthesized and studied.

Experimental setting

Procedures

1, 1.65 grams (0.12M) of betanin ($C_{24}H_{26}N_2O_{13}$) sample was taken from total of 1.74 grams of crude betanin sample.

2, 0.04M of each selected metal salts, i.e., 0.17grams of $CuCl_2.2H_2O$, 0.29 grams of $Co(NO_3)_2.6H_2O$ and 0.14 grams of $ZnCl_2$ salts were taken to conduct the experiment in the ratio of 3:1 betanin to metal salts.

3, 80 % (v/v) of organic solvent methanol was used to dissolve the mixture of betanin heavy metal salts in separated beaker and the mixture was heated overnight at 40 ^{0}C. The resulting betanin-metal salt solutions with different colors were obtained as shown in Figure 13. These solutions were kept in dark for 24 hours.

4, After 24 hours, solutions containing betanin-metal complexes were concentrated with suction filter paper.

(a) (b)

Figure12: (a) and (b) indicates color of betanin and color of betanin after complexation with metal cations Zn^{2+}, Co^{2+} and Cu^{2+}, respectively.

5, Total of 15 mL (5 mL for each) organic solvent DMF was used to dissolve new compounds in order to measure absorbance spectra of each compounds.

(a) (b) (c)

Figure 13: (a), (b) and (c) shows betanin-Cu^{2+} complex, betanin-Zn^{2+} complex and betanin-Co^{2+} complex solutions dissolved in DMF, respectively.

6, The UV-Vis spectra (absorbance) of each betanin- metal complexes on a film of regular thickness (~ 10 μm) were measured using a diffuse reflectance set up on a Perkin Elmer, Lambda 950 UV/Vis/NIR scanning spectrometer(200-800 nm) with DMF reference.

5.3.1. Results of metal complexes absorption spectra study

Absorbance spectra of new compounds were efficiently monitored by registration of UV-Vis spectra of the resulting products. The UV-Vis spectra (absorbance) of betanin- metal complex on a film of regular thickness (~ 10 μm) was measured using a diffuse reflectance set up on a Perkin Elmer, Lambda 950 UV/Vis/NIR scanning spectrometer(200-800 nm) with DMF reference. Selected qualitative data, based on arising of new absorption bands, testifying on account of formation of betanin - metal complexes, are presented in Table 3.

Table 3: Absorption maxima data of betanin- metal complexes in DMF solvent.

Organic solvent used	Absorption maxima λ_{max} (nm) data of betanin – metal complexes in DMF solvent observed from the experiment		
	Co^{2+}	Cu^{2+}	Zn^{2+}
DMF	385	380	405

Comparison between spectra of complexes resulted by three metal cations (Co^{2+}, Cu^{2+} and Zn^{2+}) added separately to DMF organic solvent was presented in Figure 14.

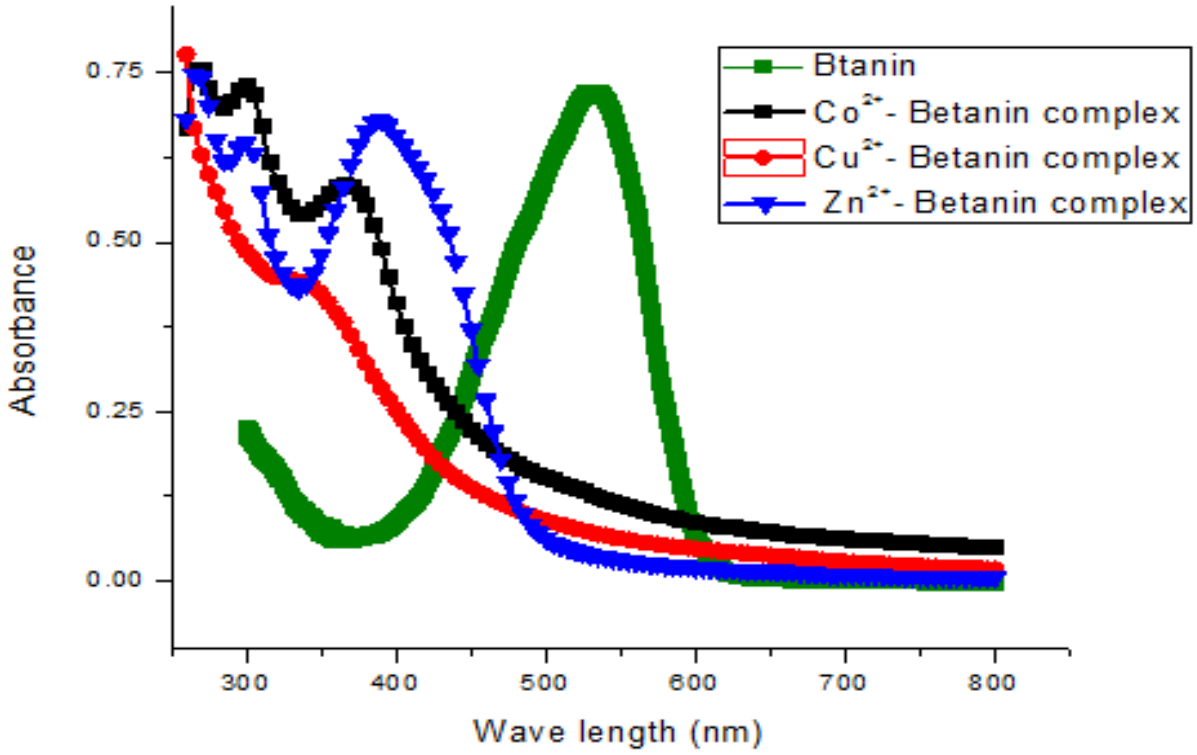


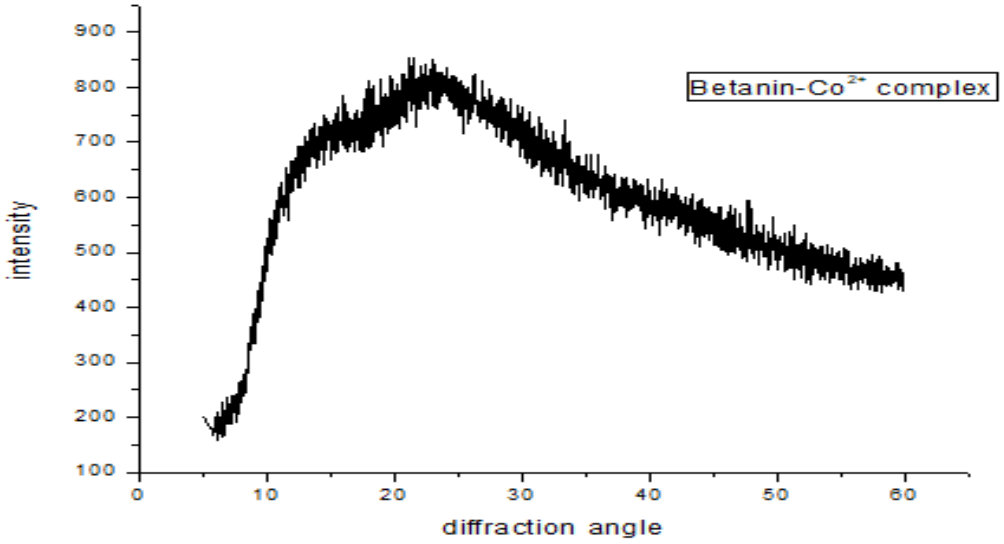
Figure14: Absorption Spectra of betanin-Cu^{2+}, betanin-Co^{2+} and betanin-Zn^{2+} metal complexes in MDF solvent and betanin, respectively.

As indicated in Table 3 and Figure 14 above, for all metal cations, the most remarkable changes in the betanin-metal complex spectra with DMF reference was observed bellow 500 nm. The fastest changes in the spectra were observed for Cu^{2+} ion, which causes rapid formation of a complex, because the hypsochromic (blue) shifts of absorption maxima, λmax, for the resulting reaction mixtures to about 380 nm 420 nm was generated.

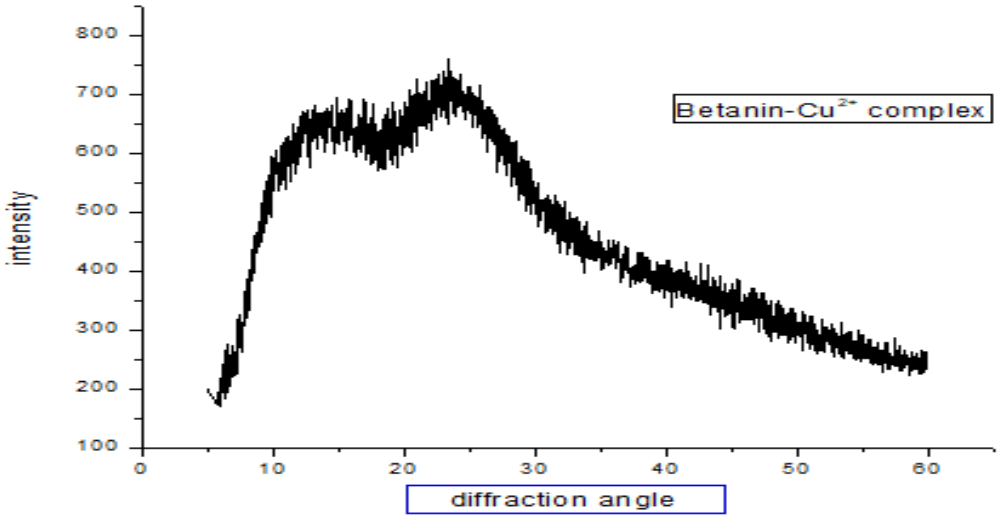
As indicated from Figure above, a broad band absorption spectra is observed for betanin -Zn (II) complex and its absorption standing band lies between wave lengths 450 nm to 500 nm. The absorption spectra band for betanin – Cu (II) complex is narrower than absorption spectra band of both betanin – Co (II) and betanin – Zn (II) complexes. Generally, as observed from the experimental results, the maximum absorption spectra for newly synthesized betanin-metal complexes showed blue shifting absorption spectra compared with absorption spectra of pure betanin.

5.3.2. XRD results of metal complexes

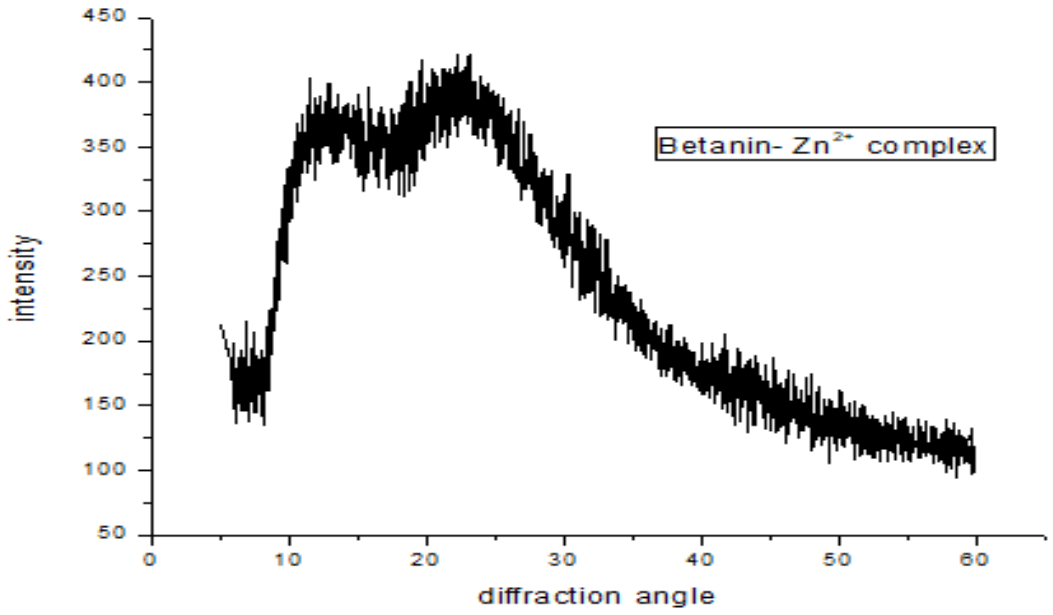
X-ray diffraction is a technique used to characterize the crystallinity and phase purity of the newly synthesized betanin – metal complexes. Thus, the X-ray diffraction of newly synthesized betanin –metal complexes was efficiently monitored and recorded by using X-ray diffraction measuring instrument.



(a)



(b)



(c)

Figure 15: (a), (b) and (c) shows X-ray diffraction spectra for betanin-Co $^{2+}$, betanin-Cu $^{2+}$ and betanin-Zn $^{2+}$ complexes, respectively.

As indicated in Figure 15 above, the X-ray diffraction spectra results shows that the constituents of the resulting betanin-metal complex solids are not arranged in a regular repeating three-dimensional structure, but, they are arranged with no particular order indicating that, the three newly synthesized betanin-metal complexes are amorphous solids.

6. CONCLUSION

From this study, we observed that red beetroot consisted essential compound betanin having potential anti-oxidant activity. In this assay, the presence of reducers (antioxidants) betanin causes the reduction of the Fe^{3+} (ferricyanide) complex to the ferrous form. Ferric ions were reduced to ferrous ions in the presence of antioxidant which formed a blue colored ferrous. Measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentrations with higher absorbance which is the indicator of higher antioxidants of betanin. The antioxidant activity of betanin was also found to be higher than the reference ascorbic acid absorbance value. In addition to ferric reducing antioxidant power analysis method, spectro photometric analysis and spectra deconvolution method was used for analysis of red beetroot extract betanin. The experimental result showed that, maximum absorption wavelength obtained for pure betanin around λ_{max} 540 nm indicates the characteristic absorption property for chromophores centers of betanin that confirms with absorption spectra for pure betanin data on different literatures.

This study was also concerned with synthesis of Cobalt (II), Copper(II) and Zinc (II) based betanin-metal complexes by using betanin as ligands. As observed from the experimental results, the maximum absorption spectra of newly synthesized betanin-metal complexes showed blue shifting absorption spectra compared with absorption spectra of pure betanin. Finally, X-ray diffraction (XRD) technique is used to draw conclusion about the structure and phase purity of the newly synthesized betanin-metal complexes. As indicated from the experimental results, X-ray diffraction spectra results showed that the constituents of resulting betanin-metal complex solids are not arranged in a regular repeating three-dimensional structure, but, they are arranged with no particular order indicating that the three newly synthesized betanin-metal complexes are amorphous solids.

7. RECOMMENDATION

Future studies will be required knowing the difference between diffraction patterns of betanin-metal complexes of heavy metals with oxidation states II and oxidation states III that provides information about their structural differences.

8. REFERENCES

[1]. Yashwant Kumar., **2015**. Beetroot: A Super Food. *International Journal of Engineering Studies and Technical approach*, 01(3).

[2]. Azeredo, H.M., **2009**. Betalains: properties, sources, applications, and stability–a review. *International journal of food science and technology*, *44*(12), pp.2365-2376.

[3]. Wadsworth, J.I., Velupillai, L. and Verma, L.R., **1990**. Microwave-vacuum drying of parboiled rice. *Transactions of the ASAE*, *33*(1), pp.199-210.

[4]. Strack, D., Vogt, T. and Schliemann, W., **2003**. Recent advances in betalain research. *Phytochemistry*, *62*(3), pp.247-269.

[5]. Georgiev, V.G., Weber, J., Kneschke, E.M., Denev, P.N., Bley, T. and Pavlov, A.I., **2010**. Antioxidant activity and phenolic content of betalain extracts from intact plants and hairy root cultures of the red beetroot Beta vulgaris cv. Detroit dark red. *Plant foods for human nutrition*, *65*(2), pp.105-111.

[6]. Tesoriere, L., Butera, D., D'arpa, D., Di Gaudio, F., Allegra, M., Gentile, C. and Livrea, M.A., **2003**. Increased resistance to oxidation of betalain-enriched human low density lipoproteins. *Free radical research*, *37*(6), pp.689-696.

[7]. Kanner, J., Harel, S. and Granit, R., **2001**. Betalains a new class of dietary cationized antioxidants. *Journal of Agricultural and Food chemistry*, *49*(11), pp.5178-5185.

[8]. Neelwarne, B., **2013**. Red Beet Biotechnology: Food and Pharmaceutical Applications. *Springer: New York*, pp. 435-74.

[9]. Stolzenbach, S., Bredie, W.L. and Byrne, D.V., **2013**. Consumer concepts in new product development of local foods: Traditional versus novel honeys. *Food Research International*, *52*(1), pp.144-152.

[10]. Grubben, G.J.H. & Denton, O.A., **2004**. Plant Resources of Tropical Africa 2: *Vegetables. PROTA Foundation, Wageningen; Backhuys, Leiden; CTA, Wageningen*.

[11]. Hopf, Maria; Zohary, Daniel., **2000**. Domestication of plants in the old world: the origin and spread of cultivated plants in West Asia, Europe, and the Nile Valley. Oxford [Oxfordshire]: *Oxford University Press*, pp. 200.

[12]. Boswell, V.R., **1967**. Growing Table Beets. *USDA Leaflet*, pp.360.

[13]. Kapadiaay't, G.I., Tokuda, H., Konoshima C, T. and Nishjnod, H., **1996**. Chemoprevention of lung and skin cancer by Beta vulgaris (beet) root extract. *Cancer letters*, *100*, p.211v214.

[14]. Carmen Socaciu., **2008**. Food colorants: chemical and functional properties. *Washington, DC*: *Taylor and Francis*, pp.169.

[15]. Escribano, J., Gandía-Herrero, F., Caballero, N. and Pedreño, M.A., **2002**. Sub cellular localization and isoenzyme pattern of peroxidases and polyphenols oxidase in beet root (Beta vulgaris L.). *Journal of Agricultural and Food Chemistry*, *50*(21), pp.6123-6129.

[16]. Schliemann, W., Kobayashi, N. and Strack, D., **1999**. The decisive step in betaxanthins biosynthesis is a spontaneous reaction1. *Plant Physiology*, *119*(4), pp.1217-1232.

[17]. Stintzing, F.C., Schieber, A. and Carle, R., **2002**. Identification of betalains from yellow beet (Beta vulgaris L.) and cactus pear [Opuntia ficus-indica (L.) Mill.] by high-performance liquid chromatography− electro spray ionization mass spectrometry. *Journal of Agricultural and Food Chemistry*, *50*(8), pp.2302-2307.

[18]. Gasztonyi, M.N., Daood, H., Hajos, M.T. and Biacs, P., **2001**. Comparison of red beet (Beta vulgaris var conditiva) varieties on the basis of their pigment components. *Journal of the Science of Food and Agriculture*, *81*(9), pp.932-933.

[19]. Herrero, M., Castro-Puyana, M., Mendiola, J.A. and Ibanez, E., **2013**. Compressed fluids for the extraction of bioactive compounds. *TrAC Trends in Analytical Chemistry*, *43*, pp.67-83.

[20]. Huang, H.W., Hsu, C.P., Yang, B.B. and Wang, C.Y., **2013**. Advances in the extraction of natural ingredients by high pressure extraction technology. *Trends in Food Science and Technology*, *33*(1), pp.54-62.

[21]. Azmir, J., Zaidul, I.S.M., Rahman, M.M., Sharif, K.M., Mohamed, A., Sahena, F., Jahurul, M.H.A., Ghafoor, K., Norulaini, N.A.N. and Omar, A.K.M., **2013**. Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of food engineering*, *117*(4), pp.426-436.

[22]. Sun, H., Ge, X., Lv, Y. and Wang, A., **2012**. Application of accelerated solvent extraction in the analysis of organic contaminants, bioactive and nutritional compounds in food and feed. *Journal of Chromatography A*, *1237*, pp.1-23.

[23]. Delgado-Vargas, F., Jiménez, A.R. and Paredes-Lopez, O., **2000**. Natural pigments: carotenoids, anthocyanins, and betalains—characteristics, biosynthesis, processing, and stability. *Critical reviews in food science and nutrition*, *40*(3), pp.173-289.

[24]. Borges, M.E., Tejera, R.L., Diaz, L., Esparza, P. and Ibáñez, E., **2012**. Natural dyes extraction from cochineal (Dactylopius coccus). New extraction methods. *Food Chemistry*, *132*(4), pp.1855-1860.

[25]. Joana Gil-Chávez, G., Villa, J.A., Fernando Ayala-Zavala, J., Basilio Heredia, J., Sepulveda, D., Yahia, E.M. and Gonzalez-Aguilar, G.A., **2013**. Technologies for extraction and production of bioactive compounds to be used as nutraceuticals and food ingredients: an overview. *Comprehensive Reviews in Food Science and Food Safety*, *12*(1), pp.5-23.

[26]. Hu, W., Zhou, L., Xu, Z., Zhang, Y. and Liao, X., **2013**. Enzyme inactivation in food processing using high pressure carbon dioxide technology. *Critical reviews in food science and nutrition*, *53*(2), pp.145-161.

[27]. Liu, X., Gao, Y., Peng, X., Yang, B., Xu, H. and Zhao, J., **2008**. Inactivation of peroxidases and poly phenol oxidase in red beet (Beta vulgaris L.) extract with high pressure carbon dioxide. *Innovative food science and emerging technologies*, *9*(1), pp.24-31.

[28]. Liu, X., Gao, Y., Xu, H., Hao, Q., Liu, G. and Wang, Q., **2010**. Inactivation of peroxidases and poly phenol oxidase in red beet (Beta vulgaris L.) extract with continuous high pressure carbon dioxide. *Food chemistry*, *119*(1), pp.108-113.

[29]. Schliemann. W et al., **1996**. Betacyanin from plants and cell cultures of Phytolacca Americana. Phytochemistry, 40(4), pp.1039–1046.

[30]. Stintzing,F.C.,Conrad,J.,Klaiber,I.,Beifuss, U. and Carle, R., **2004**. Structural investigations on betacyanin pigments by LC NMR and 2D NMR spectroscopy. *Phytochemistry*, *65*(4), pp.415-422.

[31]. Herbach, K.M., Stintzing, F.C. and Carle, R., **2006 b**. Betalain stability and degradation—structural and chromatic aspects. *Journal of food science*, *71*(4), pp.R41-R50.

[32]. Gandía-Herrero, F. and García-Carmona, F., **2013**. Biosynthesis of betalains: yellow and violet plant pigments. *Trends in plant science*, *18*(6), pp.334-343.

[33]. Ravichandran, K., Palaniraj, R., Saw, N.M.M.T., Gabr, A.M., Ahmed, A.R., Knorr, D. and Smetanska, I., **2014**. Effects of different encapsulation agents and drying process on stability of betalains extract. *Journal of food science and technology*, *51*(9), pp.2216-2221.

[34]. Saénz, C., Tapia, S., Chávez, J. and Robert, P., **2009**. Microencapsulation by spray drying of bioactive compounds from cactus pear (Opuntia ficus-indica). *Food chemistry*, *114*(2), pp.616-622.

[35]. Sapers, G.M. and Hornstein, J.S., **1979**. Varietal differences in colorant properties and stability of red beet pigments. *Journal of Food Science*, *44*(4), pp.1245-1248.

[36]. Von Elbe, J.H. and Attoe, E.L., **1985**. Oxygen involvement in betanine degradation—Measurement of active oxygen species and oxidation reduction potentials. *Food chemistry*, *16*(1), pp.49-67.

[37]. Attoe, E.L. and Von Elbe, J.H., **1985**. Oxygen involvement in betanine degradation: effect of antioxidants. *Journal of Food Science*, *50*(1), pp.106-110.

[38]. Von Elbe, J.H., Maing, I.Y. and Amundson, C.H., **1974**. Color stability of betanin. Journal of Food Science, 39(2), pp.334-337.

[39]. Jackman, R.L. and Smith, J.L., **1996**. Natural food colorants. *Anthocyanins and Betalains (Springer, New York)*, pp.244-309.

[40]. Von Elbe, J.H., Schwartz, S.J. and Hilden brand, B.E., **1981**. Loss and regeneration of betacyanin pigments during processing of red beets. *Journal of Food Science*, *46*(6), pp.1713-1715.

[41]. Herbach, K.M., Stintzing, F.C. and Carle, R., **2006**. Stability and color changes of thermally treated betanin, phyllocactin, and hylocerenin solutions. *Journal of agricultural and food chemistry*, *54*(2), pp.390-398.

[42]. Zheng, W. and Wang, S.Y., **2001**. Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food chemistry*, *49*(11), pp.5165-5170.

[43]. Han, D., KIM, S.J., KIM, S.H. and KIM, D.M., **1998**. Repeated regeneration of degraded red beet juice pigments in the presence of antioxidants. *Journal of Food Science*, *63*(1), pp.69-72.

[44]. Oyaizu M., **1986**. Studies on product of browning reaction prepared from glucose amine. *J Nutr*, 44, pp. 307-15.

[45]. Huang, D., Ou, B. and Prior, R.L., **2005**. The chemistry behind antioxidant capacity assays. *Journal of agricultural and food chemistry*, *53*(6), pp.1841-1856.

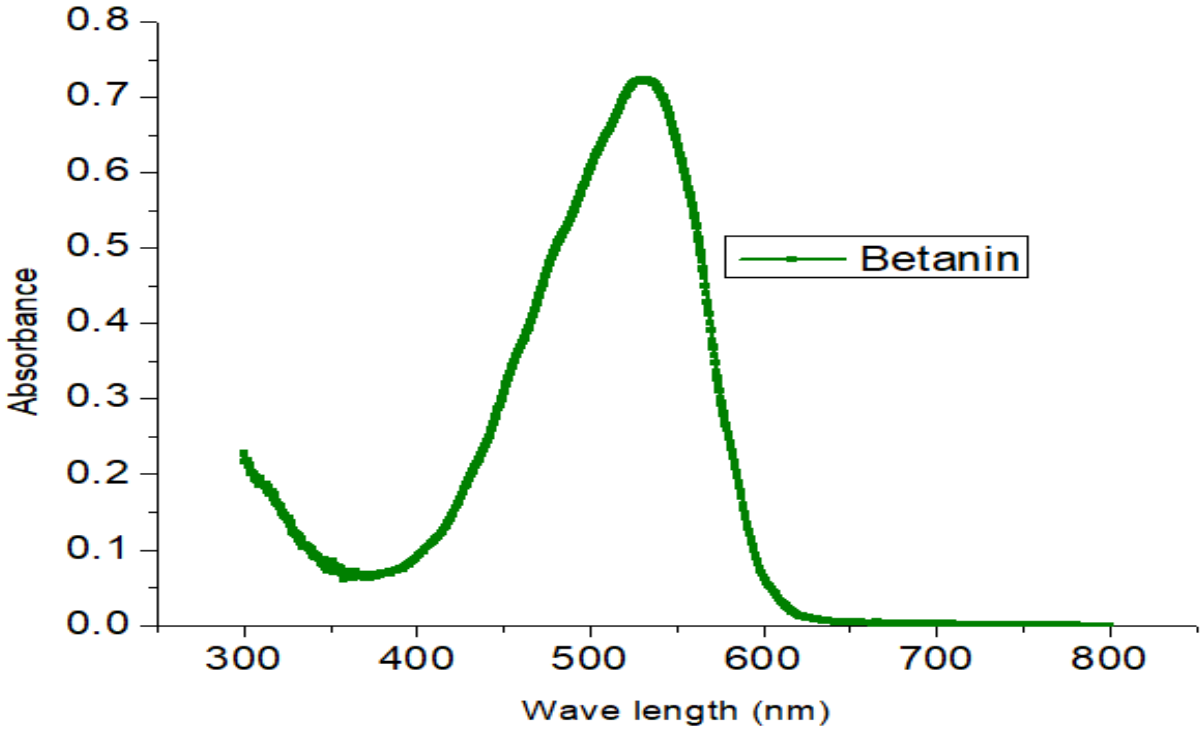
[46]. Maruthamuthu Vijayalakshmi and Kandansamy Ruckmani., **2016**. Ferric reducing antioxidant power assay in plant extract. *Bangladesh journal of pharmacol*, 11, pp. 570-572.

[47]. Leow, W.Z., Irwan, Y.M., Irwanto, M., Gomesh, N. and Safwati, I., **2014**. PIC 18F4550 controlled solar panel cooling system using DC hybrid. *Journal of Scientific Research and Reports*, pp.2801-2816.

[48]. Rice-Evans, C.A., Sampson, J., Bramley, P.M. and Holloway, D.E., **1997**. Why do we expect carotenoids to be antioxidants in vivo?. *Free radical research*, *26*(4), pp.381-398.

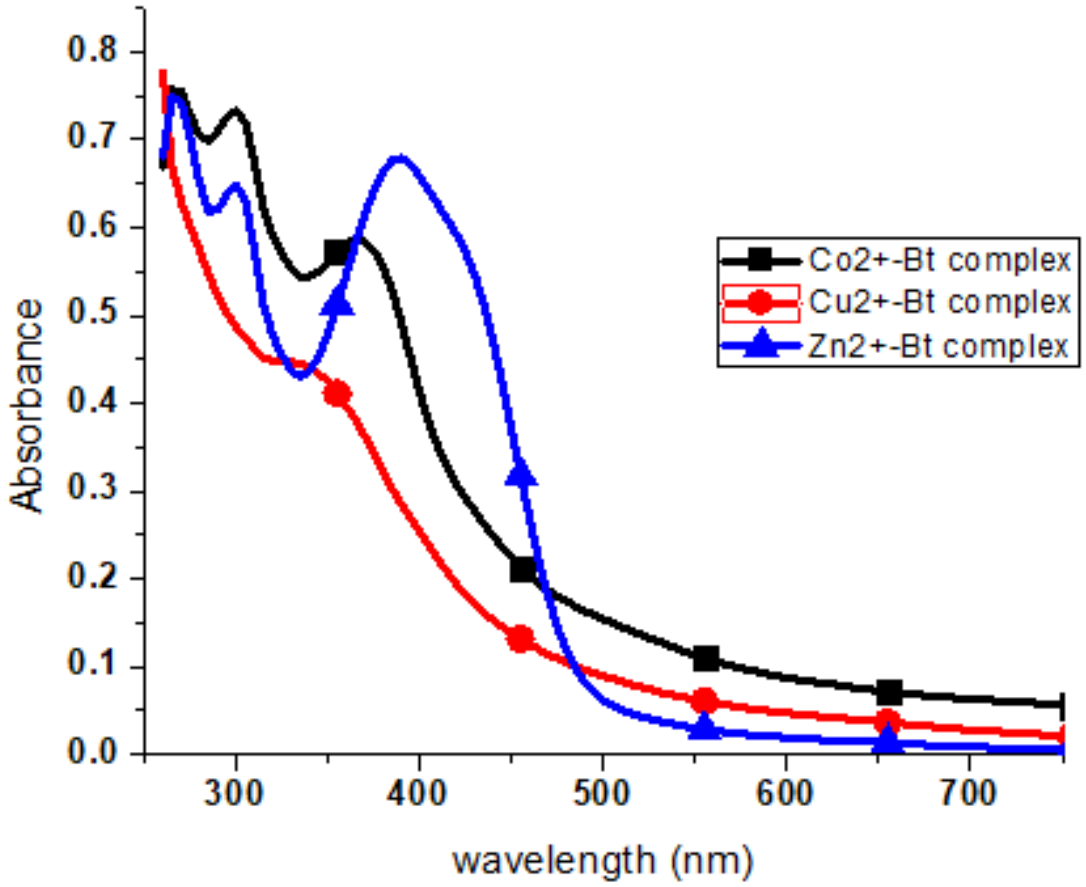
9. APPENDIXES

Appendix 1



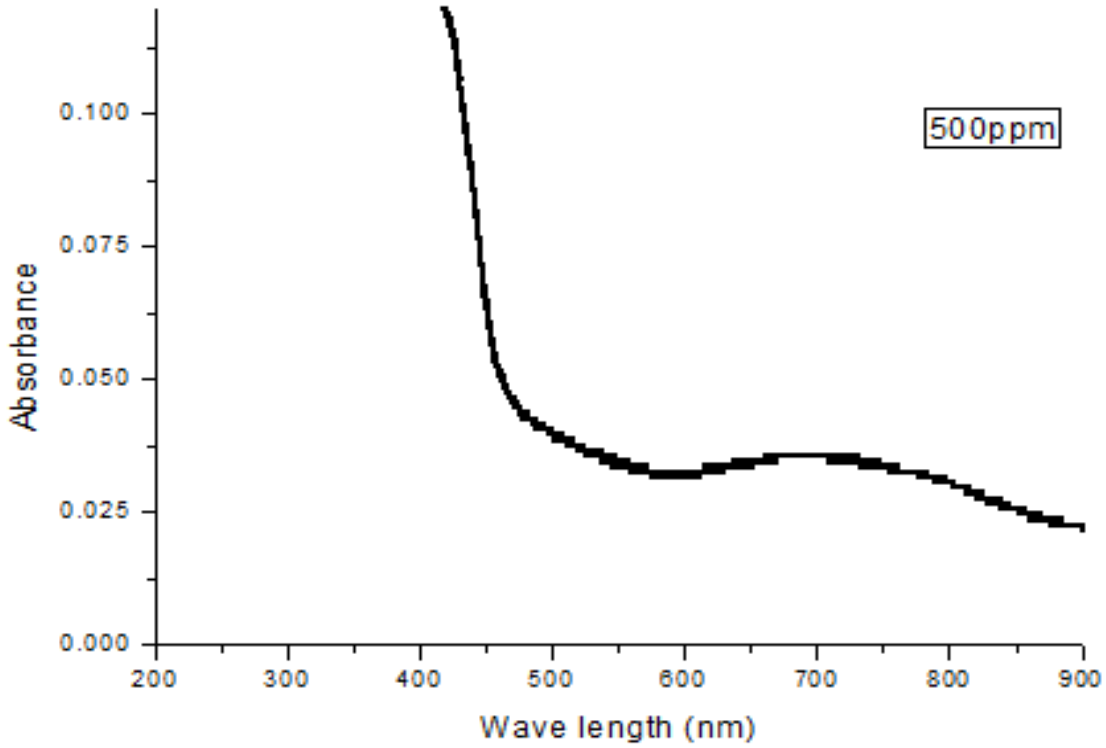
Absorption spectra of pure betanin pigment with methanol reference.

Appendix 2



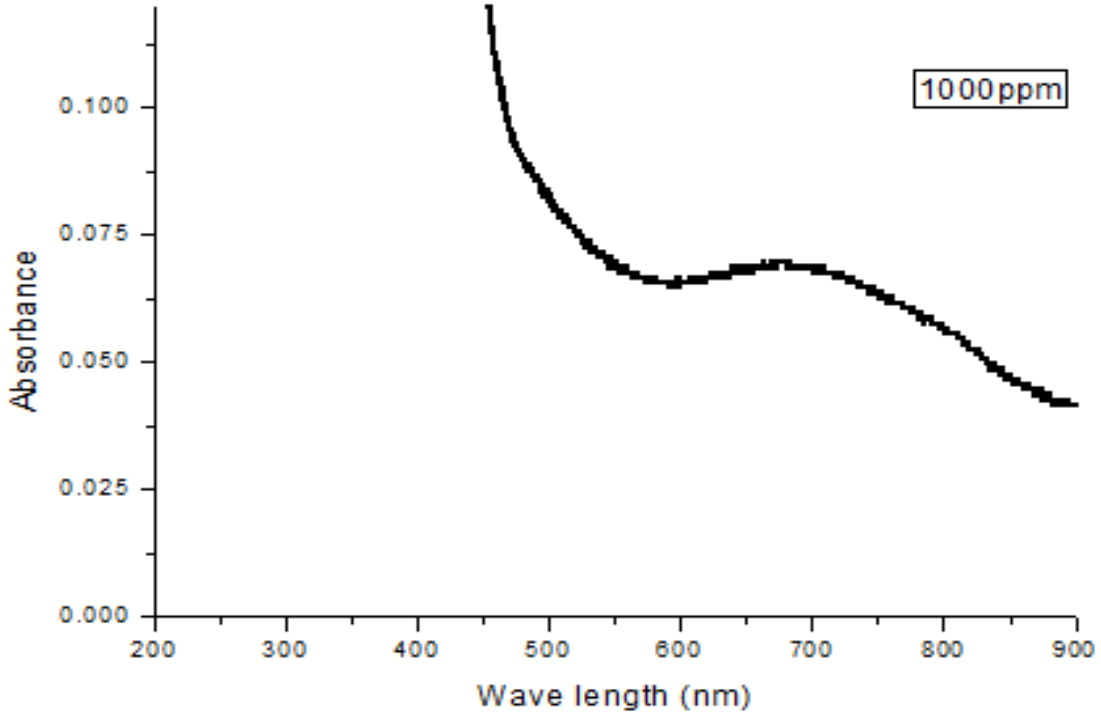
Absorption Spectra of betanin-Cu^{2+}, betanin-Co^{2+} and betanin-Zn^{2+} metal complexes in MDF solvent respectively.

Appendix 3



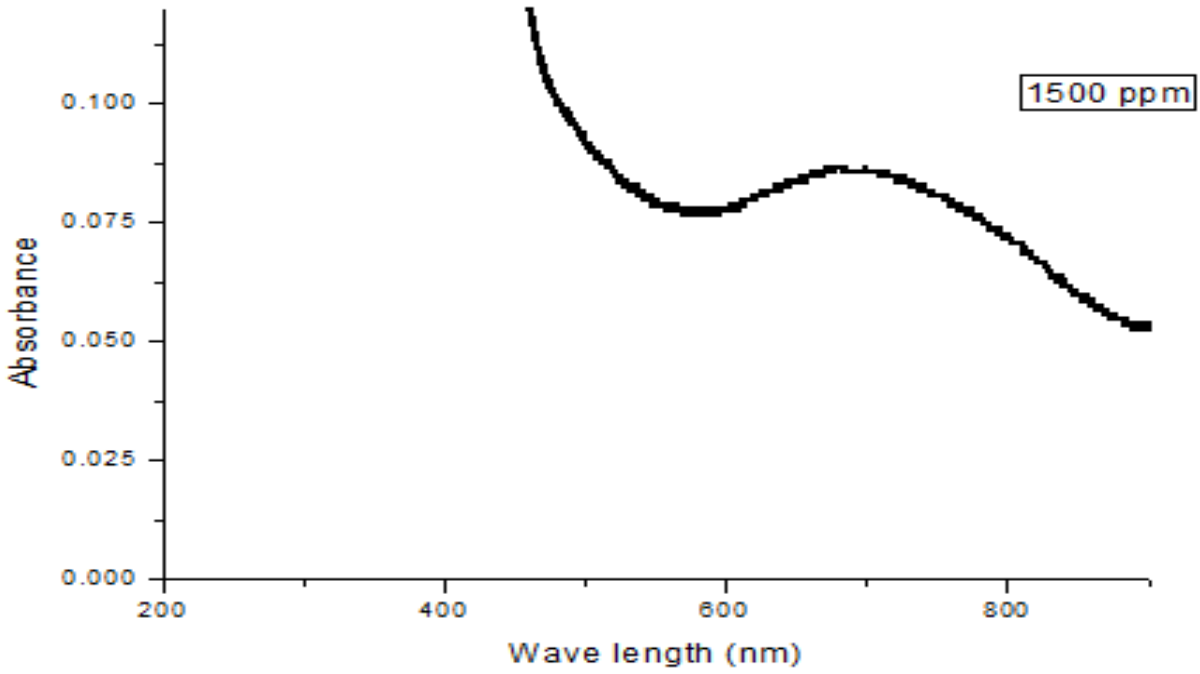
Absorbance spectra of betanin complex with concentration of 500ppm at 700nm.

Appendix 4



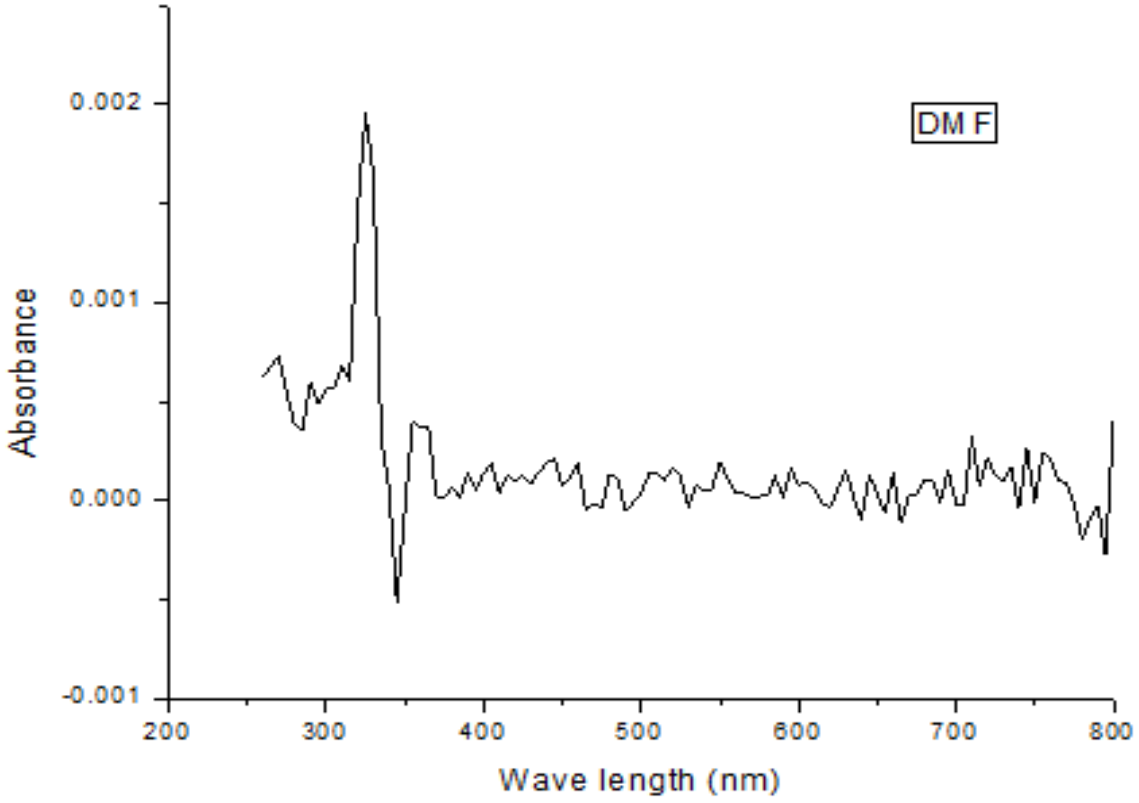
Absorbance spectra of betanin complex with concentration of 1000 ppm at 700nm.

Appendix 5



Absorbance spectra of betanin complex with concentration of 1500ppm at 700nm.

Appendix 6



Absorbance spectra of DMF solvent as reference for UV-Vis spectra study of betanin-metal complex.