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A Comparative Analysis on the Antioxidant Activity and Anthocyanin content of Raphinus Sativus L and Beta vulgaris

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KEYWORDS

Raphinus sativus L, Beta vulgaris, Anthocyanin, Oxidative stress, Antioxidant.com parative analysis, HPLC.

ABSTRACT:

Introduction: **:-**Anthocyanins are natural pigments known for their antioxidant properties and potential health benefits. Antioxidants play a vital function to safeguard against oxidative stress (OS).This two vegetables having anthocyanin contents are reported to possess antioxidant properties.

Objectives: This study conducted a comparative analysis on the antioxidant properties and anthocyanin content, in two commonly consumed vegetables, Raphanus sativus L (red radish) and Beta vulgaris (beetroot), Anthocyanins were extracted by using a standardized (Std) method, and their profiles were determined using high-performance liquid chromatography (HPLC).

Methods: The antioxidant activities and anthocyanin contents of *Raphinus sativus L* and *Beta vulgaris* were evaluated by in vitro standard method by using spectroscopic Analysis. It was established what the antioxidant activities were by total antioxidant capacity, DPPH (1,1-diphenyl-2 picrylhydrazine) radical scavenging assay, hydroxyl radical scavenging assay, ferrous reducing antioxidant capacity and lipid peroxidation inhibition assay methods.

The pH-differential approach was utilized to ascertain the overall anthocyanin concentration. The process of maceration using ethanol acidified by concentrated HCl 0.5%, followed by liquid-liquid extraction using n-hexane, ethyl acetate, and water, allowed for the identification of anthocyanidin.

The purification of anthocyanin from water fraction was done with thin layer chromatography preparative using Acetonitrile : methanol : water: glacial acetic acid (2:7:1:0.1) as eluent.

Results: The results revealed significant differences in the anthocyanin content and composition between the two vegetables. Raphanus sativus L exhibited a higher total anthocyanin content compared to Beta vulgaris. And these two samples were identified by U.V Spectroscopy & quantified by HPLC. The anthocyanin profiles varied, with Raphanus sativus L, containing primarily cyanidin-based anthocyanins, while Beta vulgaris contained a mix of betacyanin and betaxanthins. Identified quantity of anthocyanin in Beetroot samples was from 71.362 $\pm$ 20 mg /Kg and for Red radish 146 $\pm$ 19 mg / 100 g.

Raphanus sativus L, showed the highest antioxidant activity followed by beta Vulgaris DPPH and hydroxyl radical scavenging activity, the Raphanus sativus L was the most effective one with IC_{50} 35.32 and 58.11 µg/mL, followed by Beta vulgaris with IC_{50} 39.76 and 115.15 µg/mL, respectively. The Raphanus sativus L had the most potent inhibitory activity against lipid peroxidation with IC_{50} 79.56 µg/mL. In addition, the reducing capacity on ferrous ion was in the following order: Raphanus sativus L > Beta vulgaris. The content of anthocyanin of Raphanus sativus L was found to be higher than Beta vulgaris.

Conclusions: The results indicate high correlation and regression (p-value <0 .001) between anthocyanin contents and antioxidant potentials of *Raphinus sativus L* and *Beta vulgaris*, hence the *Raphinus sativus L* and *Beta vulgaris* could serve as effective free radical inhibitor or scavenger which may be a good candidate for pharmaceutical natural products. However, further exploration is necessary for effective use in both modern and traditional system of medicines.

These will help to understand the diversity of anthocyanins in different vegetables and it will help in the future for their potential health-promoting effects.

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1. Introduction

The imbalance between the ability of cells to scavenge reactive oxygen species (ROS) and the amount of ROS produced by the cells is known as oxidative stress (OS). Numerous disorders, including cancer, diabetes, and heart disease, have been linked to OS as a possible etiology [1]. Numerous cellular constituents, such as lipids, proteins, and nucleic acids, including DNA, are harmed by ROS, which ultimately results in cellular death through necrosis or apoptosis [2]. The damage may spread more widely as a result of compromised antioxidant defense mechanisms within cells. An antioxidant defense mechanism exists in all biological systems, which guards against oxidative damage and fixes enzymes to eliminate harmed molecules. It is crucial to consume antioxidant-rich foods because the body's natural defense system may not always be effective. Eating fruits and vegetables is associated with a lower risk of a number of diseases, including cancer, cardiovascular disease, and stroke brought on by OS [3–4]. These health advantages are mostly attributed to the phytochemicals found in fruits and vegetables, which include anthocyanin, carotenoids, and vitamins E and C. Even though anthocyanins are frequently present in both edible and inedible herbs, cereals, fruits, vegetables, oils, spices, and other plant materials [5, 6], there is still a dearth of scientific data regarding the antioxidant qualities of endemic plants—plants that are unique to a given region and are only known to the local population—in these areas. As a result, evaluating these qualities is still a worthwhile and fascinating endeavor, especially when it comes to discovering fresh, promising natural antioxidant sources for functional foods and/or nutraceuticals [6, 7].

Anthocyanins are natural pigments found in plants, responsible for the vibrant red, purple, and blue colours seen in various fruits, vegetables, and flowers. These compounds belong to a class of flavonoids, which are secondary metabolites with numerous health benefits and ecological functions. The history of anthocyanins is a fascinating journey through centuries of human discovery and understanding of these colourful molecules.[8]

Throughout the 20th century, advancements in analytical techniques, such as chromatography and spectroscopy, enabled scientists to identify and characterize various anthocyanin compounds in different plant species. This led to the recognition of their diverse roles, from attracting pollinators to protecting plants from UV radiation.

The discovery of anthocyanins dates back to ancient civilizations. Early humans likely encountered these pigments in fruits and berries, appreciating their appealing colors. However, it wasn't until the 19th century that scientists began to unravel the chemistry behind anthocyanins. [9,11] .In 1841, the British chemist Sir John Herschel first coined the term "anthocyanin" to describe these compounds' blue or purple hues. Subsequent research by scientists like Robert Schunck and Arthur George Perkin in the late 19th century laid the groundwork for understanding anthocyanin structures.

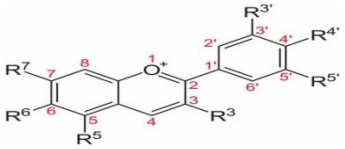

Fig1: Basic anthocyanin structure.

Anthocyanins have also garnered attention for their potential health benefits, with studies suggesting antioxidant properties that may contribute to human well-being. As research continues, anthocyanins remain a topic of great scientific interest, shedding light on their multifaceted roles in nature and their potential applications in human nutrition and medicine. This journey from ancient admiration to modern scientific exploration underscores the enduring fascination with anthocyanins and their colourful history [10].

Consumption of foods high in polyphenolic components has been linked to a lower risk of degenerative and chronic illnesses in people. Researchers have demonstrated that polyphenols contribute significantly to antioxidant capacity in fruits and vegetables. Positive associations between eating foods high in polyphenols and a lower risk and death of chronic illnesses including cancer and heart disease have been reported by epidemiological research. Anthocyanins are one of the important phenolic compounds in fruits and vegetables in human diet [10].

The glycosidic forms of anthocyanidins that give flowers and fruits their blue, red, and purple hues are called anthocyanins. Fruits' primary anthocyanins are derived from cyanidin aglycon. Anthocyanins are extensively utilized in functional food formulations, pharmaceuticals, nutritional supplements, and other

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applications. Antioxidants (AO), anti-inflammatory (AI), and heart disease prevention are among the potential health advantages of anthocyanins.

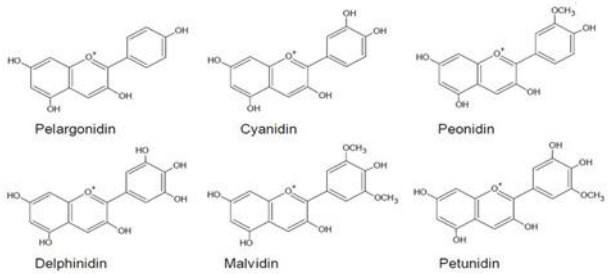

Fig 2: Different types of Anthocyanins

Radishes, often overlooked in the realm of vegetables, possess a unique charm that extends beyond their vibrant red hue. These crisp, peppery roots have earned a place in culinary traditions worldwide, offering a delightful addition to salads, garnishes, and even as a standalone snack. [12] Originating in Southeast Asia, radishes have traversed the globe, becoming a staple in various cuisines. Their journey through history mirrors the human penchant for exploring and cultivating diverse flavour. From ancient Egypt to Greece and Rome, radishes found favour among different civilizations, each adapting the root to suit their culinary preferences. Nutritionally, radishes are a powerhouse in a petite package. Low in calories and high in fibre, they contribute to digestive health while satisfying the palate with their distinctive crunch. Rich in vitamins C and B6, as well as essential minerals like potassium (K), radishes offer a nutritional boost to those seeking a health-conscious diet. [13]The composition of anthocyanins in red radish (Raphanus sativus L) is a subject of scientific interest and nutritional importance. Red radish is a widely consumed root vegetable known for its vibrant red or purple coloration, which is primarily attributed to the presence of anthocyanin pigments. Anthocyanins are a subclass of flavonoids, and they play a pivotal role in the pigmentation of various plant tissues, including fruits, flowers, and vegetables. Understanding the composition of anthocyanins in red radish involves a detailed analysis of the types and quantities of these pigments present in the vegetable. These compounds are water-soluble and belong to a diverse group of natural antioxidants. [14]

2. Objectives

In addition to their role in providing colour, anthocyanins have gained attention for their potential health benefits due to their antioxidant and anti-inflammatory properties. The anthocyanin composition in red radish can vary based on factors such as cultivar, growing conditions, and ripeness. Different anthocyanin molecules can be identified within red radish, each with its own unique chemical structure and potential biological activity. Analysing the specific anthocyanins in red radish not only contributes to our understanding of the plant's biochemistry but also holds implications for the vegetable's nutritional value and potential health-promoting properties. [14]

Rich in antioxidants, radishes contribute to overall health by combating oxidative stress. The presence of compounds like anthocyanins and vitamin C not only enhances the vegetable's vibrant hue but also augments its pharmacological value. These antioxidants play a pivotal role in neutralizing free radicals, thereby potentially mitigating inflammation and bolstering the body's defence mechanisms. [15]

Fig.3: Red radish

kingdom	Plantae-plant
Sub-kingdom	Tracheobionta-vascular plant
Super division	Spermatophyte-seed plant
Division	Magnoliophyta-flowering plant
Class	Magnoliopsida-Dicotyledons
Sub class	Dilleniidae
Order	Capparales
Family	Brassicaceae-Mustard family
Genus	*Raphinus* L
Species	*Raphinus sativus* L

Beetroot, scientifically known as Beta vulgaris, is a versatile vegetable celebrated for its vibrant colour, earthy flavour, and numerous health benefits. Rich in antioxidants, vitamins, and minerals, beetroot has earned its place not only in culinary delights but also as a nutritional powerhouse. One of the most striking features of beetroot is its deep red hue, attributed to the

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presence of betalins, a unique group of pigments with potent antioxidant properties. These antioxidants help combat oxidative stress in the body, contributing to overall well-being and potentially reducing the risk of chronic diseases.

Beyond its visual appeal, beetroot boasts a distinct earthy taste, often described as both sweet and savoury. This flavour profile lends itself well to a variety of culinary applications, from salads and soups to juices and pickles. The vegetable's versatility makes it a favourite among chefs, allowing for creative experimentation in the kitchen. [12,15]

Nutritionally, beetroot is a low-calorie vegetable that packs a nutritional punch. It is a good source of folate, manganese (Mn), potassium (K), and vitamin C. The nitrates found in beetroot have been linked to improved blood flow, potentially aiding cardiovascular (CV) health. Additionally, studies suggest that beetroot consumption may have positive effects on exercise performance due to its nitrate content.

In traditional medicine, beetroot has been utilized for its potential medicinal properties. From promoting liver health to supporting digestion, the vegetable has found a place in various holistic practices. However, it's crucial to note that while beetroot offers numerous health benefits, it should be consumed as part of a balanced diet, and individual dietary needs may vary. [15,16]

The composition of anthocyanins in beetroot (Beta vulgaris) is a subject of scientific interest and nutritional significance. Anthocyanins are water-soluble pigments responsible for the vibrant red and purple hues in various plant tissues, and beetroot is particularly known for its rich anthocyanin content, giving it its characteristic deep red color. Understanding the specific types and quantities of anthocyanins present in beetroot is essential for appreciating its potential health benefits, including antioxidant properties (AO), and for exploring its various applications in the food and pharmaceutical industries. This analysis can shed light on the distinct anthocyanin profile of beetroot and its potential contributions to human health and nutrition. [11,14]

Understanding the composition of anthocyanins in beetroot and red radish involves examining the quantities of these pigments present in each vegetable. Since these two plants belong to different species and may have distinct genetic backgrounds and environmental adaptations, their anthocyanin profiles are likely to vary. This comparative analysis provides insights into the diversity of anthocyanins within the plant kingdom and allows for meaningful comparisons between these commonly consumed root vegetables.

Fig.4: Beetroot

kingdom	Plantae-plant
Division	Magnoliophyta-flowering plant
Class	Magnoliopsida
Sub class	Caryophillanae
Order	Caryphyllales
Family	Chenopodiaceae
Genus	*Beta*
Species	*Vulgaris*

Such a study can shed light on the influence of genetic and environmental factors on the production of anthocyanins in these vegetables and may have implications for their nutritional value and potential health benefits. By comparing the anthocyanin composition in beetroot and red radish, we can uncover the chemical diversity within these vegetables, contributing to our broader understanding of plant biochemistry (BC) and the potential advantages of including them in our diets. [13].

3. Methods

Plant identification and Material Collection:

Beetroot and Red radish were collected from local village of Mysuru, Karnataka, India and were identified by an expert taxonomist at the Department of Botany, University of Mysore,Mysuru. A voucher specimen (SVCP/C/2023) was deposited to the herbarium in the Department of Botany, University of Mysore. After being cleaned separately with clean water to get rid of any dirt, the plant components were shade-dried for a few days and occasionally sun-dried. After being dried and processed into a coarse powder using a grinding machine, the materials were kept for later usage at room temperature.

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Extract preparation

DAY 1: Firstly, the process begins with the careful selection of fresh beetroot and red radish. Subsequently, they are trimmed and sorted to eliminate any damaged or overripe radish and beetroot then they are rinse with distilled water to remove dirt substances and impurities, and wiped with a cotton cloth to remove the water. [17]

Beetroot and red radish are weighed in analytical balance and it is found to be 569.21gms and 539.48gms respectively. The vegetables are peeled and cut into small pieces, and then it is kept in the hot air oven for drying process. Before keeping into hot airoven the instrument has to be cleaned and hot air oven set at a constant temperature of 50ºC, a batch of beetroot weighing 569.21gms and a batch of red radishes weighing 539.48gms underwent a drying process that spanned a total of 14.5 hours. This drying procedure was carefully monitored over three days, with the initial conditions documented on day 1. At the end of the first 5 hours, the red radishes final weight is 308.23 grams and beetroot final weight is 341.18gms. This weight loss was indicative of the moisture removal from the beetroot & radish, a crucial step in the production of dry beetroot & radish powder.

DAY 2: Continuing the drying process for 7 hours, the weight of red radish further decreased to 112.79 grams, and beetroot to 133.25 grams

DAY 3: On the third day, after a total drying span of 14.5 hours, the final weights were observed. The red radish weighed 24.58 grams, and the beetroot weighed 40.23 grams. [18,19,20]

Following the completion of the drying process, the dried red radish and beetroot were ground using a mixer. The resulting mixture was then sieved to obtain a fine powder. This process transforms the dehydrated vegetables into a convenient and versatile powdered form. These drying and processing steps not only preserve the nutritional content of red radish and beetroot but also enhance their shelf life, allowing for easy storage and utilization in various culinary applications. The powder sample used for water extraction by hot percolation process but the boiling temperature below 40° C and analysed.

List of Chemicals

Chemicals used in this research were:-1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide, catechin (CA), ferrous ammonium sulphate, butylated hydroxytoluene (BHT), ascorbic acid (AA), trichloro acetic acid (TCA), sodium phosphate, ammonium molybdate, tannic acid,DMSO, EDTA, acetyl acetone and FeCl3 were purchased from Sigma Chemical Co. (St. Louis, MO, USA); potassium acetate, phosphate buffer, thiobarbituric acid were purchased from Sigma-Aldrich, USA,acetonitrile (Merck), alumunium chloride,aquadest, cellulose TLC plates (Merck), ethanol 96%, filter paper, formic acid (Brataco), hydrochloric acid 96% (Merck), methanol, potassium chloride, sodium chloride, Formic acid, ammonium acetate, glacial acetic acid and distilled water

Table 3: List of Instruments and Equipment

Sl.no	Instruments/equipment	Mode/maker
1.	Hot air oven	Bio gene laboratory, India
2.	Grinder	Kaps engineers, India
3.	Sieve	Manikaran scientific works, India
4.	Bunsen burner	Jay brass Industries, India
5.	Sonicater	Oscar Ultrasonic, India
6.	Electronic analytical balance	Mettler Toledo, India
7.	Whatman filter paper no.1	Spectrum two
8.	U.V Spectroscopy	Beckman coulter DU-730
9.	HPLC	Waters Alliance e2695 HPLC system with Waters 2489 UV/Vis Detector

Analysis of Anthocyanin Compounds:

Natural bioactive substances called anthocyanins have been linked to a lower risk of cancer and cardiovascular disease when consumed in food.(Anna Rafaela Cavalcante Braga *et al*., 2018)

Acetone was used to remove the anthocyanins from liquid nitrogen-powdered epidermal tissue of red radish (Raphanus sativus L.), which were then separated using chloroform and purified using C-18 resin.Based on pH-differential analysis, the monomeric anthocyanin concentration was found to be 154 ± 13 mg/100g of epidermal tissue.(pelargonidin-glucoside basis).

Beet root,Betacyanins is responsible for the red-violet color of fruits and vegetables (Sri Priatni *et al*., 2015). 1 ml of extract was added in 1 ml of NaOH then heat it for 5 min at 100^0 C bluish green color indicates the presence of anthocyanin and formation of yellow colour indicates the presence of betacyanin.

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Determination of Total Anthocyanin Content:

Beet root

The betalain content was analyzed as described by Ravichandran et al. [21]. Red beetroot contains natural pigments called betalains, which primarily consist of red-violet betacyanins or yellow-orange betaxanthins. However, betalains exhibit poor stability during processing and storage due to their high sensitivity to heat, pH, light, and oxygen.

After dissolving one gram of the material in ten milliliters of 20% ethanol and mixing for ten seconds, the homogenate was centrifuged at 6000× g for ten minutes. To ensure that the most betalains could be extracted, the centrifugation process was repeated twice. The amount of betalains was measured using the supernatant. Using a UV-Vis spectrophotometer, the extracts' betaxanthin and betacyanin contents were measured spectrophotometrically at 538 and 480 nm. [22]. The betalain content (BC) was calculated according to Equation (1):

$$BC=\frac{A\cdot\ Df\cdot M_W\cdot V\cdot L\cdot W_d,\ mg/g}{d\varepsilon} \quad ...(1)$$

where Wd is the sample weight in grams, L is the cuvette's path length (1 cm), Vd is the solution volume in milliliters, Df is the dilution factor, and A is the absorbance. The measurements of MW = 550 g/mol and ε = 60,000 L/(mol cm) were used for the quantification of betacyanins; corresponding values for betaxanthins were MW = 308 g/mol and ε = 48,000 L/(mol cm).Five milliliters of two distinct buffers (0.025 M potassium chloride, pH = 1.0, and 0.4 M sodium acetate, pH = 4.5) were used to dilute a volume of three milliliters of extract.At both λ = 510 and 700 nm, absorbance (A) was measured after a 30-minute incubation period at room temperature. Every extract was examined three times. The molecular weight of 449 Da and the molar absorptivity coefficient (ε) values of 26,900 $M^{-1}cm^{-1}$ were utilized to calculate total anthocyanins as cyanidin glucoside equivalents, or CGE. [23].

The results were calculated similarly to [24] as follows:

$$Asp = (A_{510} - A_{700})\ pH1.0 - (A_{510} - A_{700})\ pH\ 4.5$$

The content of total anthocyanins (TA) were calculated as follows:

$$TA = (Asp \times M \times D_f \times 1000) / (\varepsilon \times \lambda \times m) \quad .....(2)$$

where D_f is the dilution factor, λ is the cuvette optical path length (1 cm) and m is the weight of the sample in grams. The total anthocyanin content was expressed as mg CGE/g dw.

Acetone was used to remove the anthocyanins from liquid nitrogen-powdered epidermal tissue of red radish (Raphanus sativus L.), which were then separated using chloroform and purified using C-18 resin.Based on pH-differential analysis, the monomeric anthocyanin concentration was found to be 154 ± 13 mg/100g of epidermal tissue.(pelargonidin-glucoside basis).

Identification of isolates and quantify

(By chromatographic technique)

Thin layer chromatography:-

Preparative Thin Layer Chromatography:

For preparative TLC to separate anthocyanin components from other compounds, water extract was used. Since the anthocyanin fraction is polar, the water extract will have the highest concentration of anthocyanin.When performing preparative thin layer chromatography using cellulose plates, the TLC findings serve as the standard.Beet root The analyses were carried out by the thin layer chromatography (TLC) using the RP-18F254 splates.During the development , the following mobile phase was used: Acetonitrile : methanol : water: glacial acetic acid (2:7:1:0.1).

High Performance liquid chromatography:-

Acetone was used to remove the anthocyanins from liquid nitrogen-powdered epidermal tissue of red radish (Raphanus sativus L.), which were then separated using chloroform and purified using C-18 resin.Based on pH-differential analysis, the monomeric anthocyanin concentration was found to be 154 ± 13 mg/100g of epidermal tissue.(pelargonidin-glucoside basis).

Radishes processed thermally demonstrated the pigments' heat stability. Using spectrum studies, mass spectroscopy, and HPLC, four main pigments were identified as pelargonidin-3-sophoroside-5-glucoside derivatives. Ferulic or p-coumaric acid, along with malonic acid, were used to acylate the two main pigments. All that was needed to acylate the other two pigments was ferulic or p-coumaric acid. Pigment resistance to acid hydrolysis was enhanced by acylation.

Purity Isolate Test:

Beet root (B.R) :- Two-way TLC was used to test the isolate's purity utilizing two different kinds of mobile phase. The first one is methanol: water: formic acid (1.6:0.4: 0.1) and the second is Acetonitrile : methanol

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: water: glacial acetic acid (2:7:1:0.1).The two-way findings demonstrate the purity of the chemical produced, with a pink spot appearing at Rf 0.42 with the first mobile phase. One pink spot appears at Rf 0.81 after eluting with a second mobile phase and turning 90º to the right.

Red radish (RR)

Acetone was used to remove the anthocyanins from liquid nitrogen-powdered epidermal tissue of red radish (Raphanus sativus L.), which were then separated using chloroform and purified using C-18 resin.Based on pH-differential analysis, the monomeric anthocyanin concentration was found to be 154 ± 13 mg/100g of epidermal tissue.(pelargonidin-glucoside basis).

Radishes processed thermally demonstrated the pigments' heat stability. Using spectrum studies, mass spectroscopy, and HPLC, four main pigments were identified as pelargonidin-3-sophoroside-5-glucoside derivatives. Ferulic or p-coumaric acid, along with malonic acid, were used to acylate the two main pigments. All that was needed to acylate the other two pigments was ferulic or p-coumaric acid. Pigment resistance to acid hydrolysis was enhanced by acylation.

a)

V SPECTROSCOPY:

UV spectroscopy, or ultraviolet-visible spectroscopy, is a dominant analytical method used in chemistry and biochemistry to study the electronic transitions of molecules. This method relies on the absorption of ultraviolet or visible light by atoms or molecules, providing valuable information about their structure and concentration. The basic principle of UV spectroscopy involves the interaction of light with electrons in a molecule. When a molecule absorbs UV or visible light, the energy is absorbed by electrons, causing them to transition from a lower energy state to a higher energy state. The wavelength of light absorbed corresponds to the energy difference between these states, and this information can be used to identify functional groups and characterize chemical compounds.

The U.V-visible spectrum of red radish and beetroot extracts were recorded using a U.V spectrometer (Beckman coulter DU-730) at 2nm resolution to ensure the presence of anthocyanin using ascorbic as a reducing agent. Samples were scanned in the 200-600nm range, with a scanning speed of 300nm/min at 1cm optical path and at room temperature. Deionized water was used as a blank sample.

Weigh 1mg of red radish and beetroot powders respectively and dissolve them in two different beakers containing mild hot distilled water (100 ml). Use glass rod for homogenous mixing of the samples. Then the sample is kept aside for 5 min, after that the solution is filtered using Whattmann filter. From this filtrate 2ml is separated and subjected to U.V spectrometer (Beckman coulter DU-730). To identify the presence of anthocyanin in sample. [16,18]

b)

PLC ANALYSIS

Anthocyanins can be quantitatively as well as qualitatively analyzed using high-performance liquid chromatography. The idea can be used to quantitative analysis since it is based on the observation that the concentration of anthocyanins is proportional to the peak area of anthocyanins in the chromatogram. Currently, a lot of fruit, wine, juice, and flower quantitative analyses of total and single anthocyanins are conducted using HPLC.[13]

Preparation of mobile phase:

Solvent **U**A: Add 10 ml of formic acid solution (chromatographic grade) to 990 ml of ultrapure water, shaking it evenly, and then sonicate to expel air bubbles.

Solvent B: Acetonitrile solution (chromatographic grade) sonicate to expel air bubbles.

Standard and sample preparation

Solutions were prepared by dissolving 1mg of each powdered sample in distilled water in a volumetric flask, filling it up to the mark. Use a stirring rod to assist the dissolving substance in water and complete dissolution for the homogenous solution. The solution was then filtered in an amber vial using a 0.22µm regenerated cellulose syringe filter. 1N Ascorbic acid(2ml) was added as a stabilizer to enhance the stability of the solutions.

The anthocyanin standard solution and sample solution were prepared by dissolving 1mg of standard powder that was previously procured from INPLA Pvt Ltd, Mysore Karnataka in distilled water in a volumetric flask, and stirring rod was used to assist uniform mixing and were filtered in an amber vial using a 0.22µm regenerated cellulose syringe filter. 1N Ascorbic

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acid(2ml) was added as a stabilizer to enhance the stability of the solutions. [14]

Instrument parameter setting

Selecting the C-18 column and connecting it to the instrument (waters E2659) is the first step. Connect the mobile phase pipeline and detection system, and turn on the HPLC workstation, which includes the chromatograph and computer software. Configure the injection volume and flow rate, start the full wavelength scan mode, set the monitoring wavelength to 280 and 520 nm, set the pressure alarm value and column temperature. [13,15]

Adjust the volume ratio between phase A and phase B to elute anthocyanins after setting the gradient elution method and ensuring that both phases have a 100% total volume. Phase B's percentage is first set to a lower figure, usually between 5 and 6%. The mobile phase will not elute the anthocyanins at this time.

Subsequently, increase the phase B volume ratio gradually until it reaches 95–100%. As the two mobile phases shift, the anthocyanins will gradually elute out. Ultimately, B's volume ratio is brought back to what it was prior to the tested sample's initial run, and the balance is kept for a while. A program's duration shouldn't be excessively long or short.

Otherwise, the peak shape won't be distinguishable if the time is too long or too short. The typical program duration is 20 to 70 minutes, however the precise duration can be changed based on the sample's characteristics and the mobile phase. The particular circumstance is as follows: 0–3 minutes: 6–6% B; 3–6 minutes: 6–13% B; 6–26 minutes: 13–30% B; 26–28 minutes: 30–95% B; 28–30 minutes: 95–95% B; 30-32 minutes: 95–6% B; 32–35 minutes: 6–6%.[16].

Determination of total antioxidant capacity:-

Total antioxidant capacity (TAC) of samples was determined by the method reported by Prieto et al. [25] with some modifications. Three milliliters of reaction mixture comprising 0.6 M sulfuric acid, 28 mM sodium phosphate, and 1% ammonium molybdate was combined with half a milliliter of samples or standard at varying concentrations and put into test tubes.To finish the process, the test tubes were incubated at 95°C for ten minutes.

After cooling at room temperature, the absorbance was measured at 695 nm using a spectrophotometer against blank. Catechin was used as standard.Three milliliters of reaction mixture and the appropriate volume of the same solvent used for the samples/standard made up a typical blank solution. The solution was incubated at 95°C for ten minutes, and the absorbance at 695 nm was measured.

Ferrous reducing antioxidant capacity assay:-

The ferrous reducing antioxidant capacity (FRAC) of samples was evaluated by the method of Oyaizu [26].

The test tubes were filled with 0.25 ml of the standard solution at various concentrations, 0.625 ml of potassium buffer (0.2 M), and 0.625 ml of potassium ferricyanide (1%), or [K3Fe (CN)6] solution. To finish the reaction, the reaction mixture was incubated at 50°C for 20 minutes.

The test tubes were then filled with 0.625 ml of a 10% trichloroacetic acid (TCA) solution. For ten minutes, the entire mixture was centrifuged at 3000 rpm.

Subsequently, 1.8 ml of the supernatant was taken out of the test tubes and combined with 0.36 ml of 0.1% ferric chloride ($FeCl_3$) solution and 1.8 ml of distilled water.

A spectrophotometer was used to test the solution's absorbance at 700 nm in comparison to a blank. The same solution mixture without extracts or standard was used as a typical blank solution. It was incubated under the same conditions, and its absorbance was measured at 700 nm. A higher reducing capability was demonstrated by the reaction mixture's increased absorbance.

DPPH radical scavenging assay:-

Free radical scavenging ability of the extracts was tested by DPPH radical scavenging assay (DRSA) as described by Choi et al. [27] and Desmarchelier et al. [28].

2.4 ml of a 0.1 mM DPPH in methanol solution and 1.6 ml of an extract in water at several concentrations were combined. After carefully vortexing the reaction mixture, it was kept at room temperature for 30 minutes in the dark. Using spectrophotometry, the mixture's absorbance was calculated at 517 nm. BHT served as the reference.

Percentage DPPH radical scavenging activity (% DRSA) was calculated by the following equation,

$$(\%DRSA) = \{(Ao - A_1)/Ao\} x100$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the extractives/standard.

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Then % of inhibition was plotted against concentration, and from the graph IC_{50} was calculated.

Hydroxyl radical scavenging activity:-

Hydroxyl radical scavenging activity (HRSA) of the extractives was determined by the method of Klein et al. [29] with a slight modification.

Test tubes were filled with 0.5 ml of extractives/standard at various concentrations. The test tubes were filled with 1 milliliter of Fe-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 milliliters of 0.018% EDTA solution, 1 milliliter of 0.85% DMSO solution, and 0.5 milliliters of 22% ascorbic acid. The test tubes were placed in the water bath with their caps securely on and heated to 85°C for 15 minutes. Following incubation, the test tubes were unscrewed, and each test tube received an instant addition of 0.5 ml of ice-cold TCA (17.5%).

All the tubes were filled with 3 ml of the Nash reagent (7.5 gm of ammonium acetate, 300 µl glacial acetic acid, and 200 µl acetyl acetone combined to make 100 ml), which was then incubated at room temperature for 15 minutes. At 412 nm wave length, absorbance was measured in a UV spectrophotometer.

Percentage hydroxyl radical scavenging (% HRSA) activity was calculated using the following equation,

$$\%HRSA=\{(Ao–A_1)/Ao\}x100$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the extractives/standard.

Then % of inhibition was plotted against concentration, and from the graph IC_{50} was calculated.

Lipid peroxidation inhibition assay:-

The lipid peroxidation inhibition assay (LPI) was determined according to the method described by Liu et al. [30] with a slight modification.

To create liposomes, excised rat liver was homogenized in buffer and centrifuged. To make the final volume of 1 ml, 0.5 ml of supernatant, 100 µl 10 mM FeSO4, 100 µl 0.1 mM AA, and 0.3 ml of extractives or standard at varying concentrations were combined. For twenty minutes, the reaction mixture was incubated at 37°C.

Immediately upon heating, 1.5 ml of (1%) TBA and 1 ml of (28%) TCA were added. After 15 minutes of heating at 100°C, the reaction mixture was finally cooled at room temperature. The absorbance was measured at 532 nm after cooling.Percentage inhibition of lipid peroxidation (% LPI) was calculated by the following equation,

$$\%LPI =\{(Ao–A_1)/Ao\}x100$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the extractives/standard.

Then % of inhibition was plotted against concentration, and from the graph IC_{50} was calculated.

Statistical analysis:-

Every analysis was performed three times. The data was displayed as mean ± SD. The F- and t-tests (p-value <0.001) were utilized to assess significant associations between experimental parameters through regression analysis and correlation. R program for free, version 2.15.1 (http://www.r-project. org/) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations.

4. Results

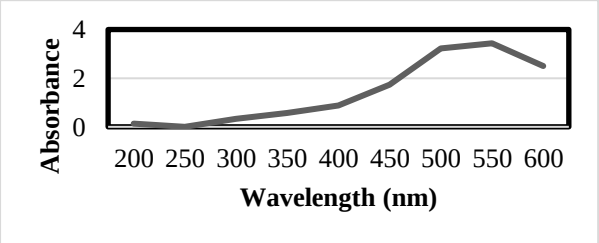

Fig 5: U.V chromatogram of red radish

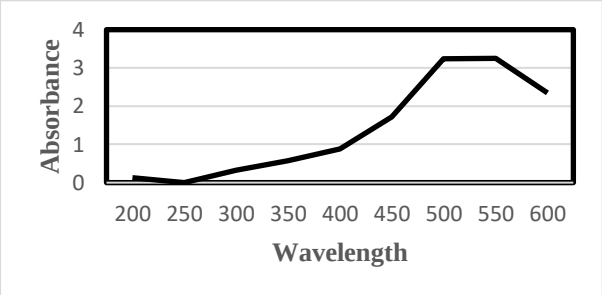

Fig 6: U.V chromatogram of Beetroot

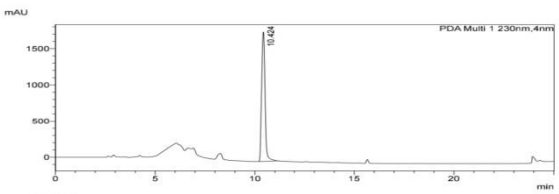

Fig 7: HPLC chromatogram of standard

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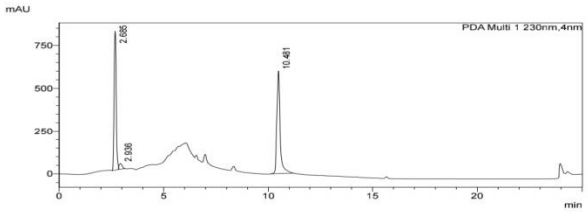

Fig 8: HPLC chromatogram of Beetroot

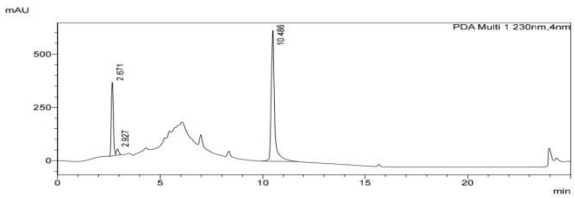

Fig 9: HPLC chromatogram of red radish

Fig.5 and Fig.6 U.V chromatogram for red radish and Beetroot by which identified the presence of anthocyanin compare with standard anthocyanin U.V chromatogram.

The UV-Vis spectra recorded in the range of 200-700nm show characteristic maxima of absorbance for the left and the right band for both,the red beet juice(540nm) and betanin (540nm).

Fig.7 reports the HPLC chromatogram of standard anthocyanin compound that was procured from INPLA Pvt. Ltd. As we can see peak at retention time 10.424 with area of 100%. In fig 8 we can see HPLC chromatogram of beetroot extract, anthocyanin peak is absorved at retention time 10.481 in contrast to standard(fig7) the area of the peak of anthocyanin is 51.923%. In fig 9 we can see HPLC chromatogram of red radish extract, peak indicating anthocyanin have a area of 75.566% with retention time 10.486

Beet Root-In the course of the analysis obtained an surprising result, namely HORIZONTAL separation of the mixture of red pigments instead of the awaited verticals eparation. The R_F coefficients for the left-hand-side and the right-hand-side bands were determined for both the red beet juice (0.79 and 0.78respectively) and betanin (0.81 and 0.80 respectively).

Acetone was used to remove the anthocyanins from liquid nitrogen-powdered epidermal tissue of red radish (Raphanus sativus L.), which were then separated using chloroform and purified using C-18 resin. Based on the peragonidin-glucoside basis, the monomeric anthocyanin concentration was 154 ± 13 mg/100g of epidermal tissue, as evaluated by pH-differential. Radishes processed thermally demonstrated the pigments' heat stability. Using spectrum studies, mass spectroscopy, and HPLC, four main pigments were identified as pelargonidin-3-sophoroside-5-glucoside derivatives. Ferulic or p-coumaric acid, along with malonic acid, were used to acylate the two main pigments. All that was needed to acylate the other two pigments was ferulic or p-coumaric acid. Pigment resistance to acid hydrolysis was enhanced by acylation.

Determination of TAC and FRAC

The TAC and FRAC of water extractives of RR and BR were shown in Table 1. Both Water extracts of RR and BR showed considerable antioxidant activity compared to CA (standard). At the concentration of 100 µg/mL, the absorbance of water extract of RR,BR and (+)-catechin were 0.721, 0.522 and 1.781, respectively; while at 400 µg/mL, the absorbance of water extracts of RR, BR and (+)-catechin were 2.156, 1.670 and 3.775. The extractives were found to increase the total antioxidant activity with the increasing concentration of the extracts (Table 1).

The water extracts of roots of RR and BR plant showed reducing activity, although less than that of ascorbic acid, a reference antioxidant, the extractives increased the reducing activity with the increased concentration of the extracts. At 100 µg/mL,the absorbance of water extracts of RR, BR and ascorbic acid were 0.598, 0.712 and 2.32 respectively, while at 400 µg/mL, the absorbance of water extracts of RR, BR and AA were 2.323, 2.249 and 3.17, respectively. A higher absorbance indicates a higher reducing power. These results demonstrated that the water extracts of RR and BR had considerable iron reducing capacity

DPPH radical scavenging activity Figure 1A shows the dose–response curve of DPPH radical scavenging activity of the water extracts of RR and BR, compared with BHT. It was observed that the extract of RR had higher activity than that of the other extractives. At a concentration of 100 µg/mL, the scavenging activity of the RR and BR reached 93.27 and 81.74%, while at the same concentration, that of the BHT was 96.128%. The IC_{50} of water extracts of RR and BR were 35.32 and 39.76 µg/ml, respectively. The IC_{50} of BHT (standard) was 8.3 µg/mL (Figure 10A).

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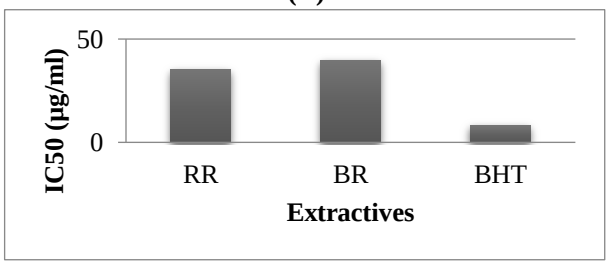
Table 4 Absorbance of TAC and FRAC of RR and BR at different concentration

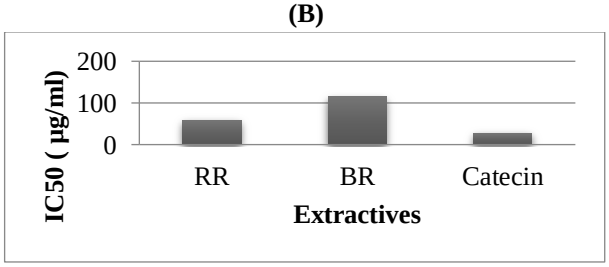
Extractives	TAC		FRAC	
	At 100 µg/ml	At 400 µg/ml	At 100 µg/ml	At 400 µg/ml
RR	0.721 ± 0.022	2.156 ± 0.023	0.598 ± 0.031	2.323 ± 0.033
BR	0.522 ± 0.015	1.670 ± 0.032	0.712 ± 0.017	2.249 ± 0.012
AA			2.32 ± 0.026	3.17 ± 0.031
CA	1.781 ± 0.027	3.775 ± 0.014	-------	-------

Hydroxyl radical scavenging activity:- The hydroxyl radical scavenging activity of the water extracts of the RR and BR dose–response curve, compared with CA. It was observed that extract of the RR had higher activity than that of the other extractives. At a concentration of 200 µg/mL, the scavenging activity of the RR and BR reached 82.32 and 73.87%, while at the same concentration, that of the CA was 81.85%. The hydroxyl radical scavenging activity of RR was closely resembled to that of CA. The IC_{50} of water extracts of RR and BR were 58.11 and 115.15 µg/mL, respectively. The IC_{50} of CA (standard) was 27.62 µg/mL (Figure 10B).

(A)



(B)



(C)

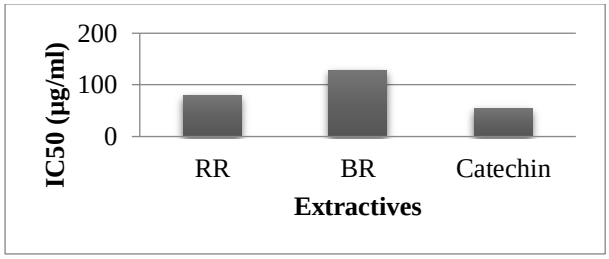


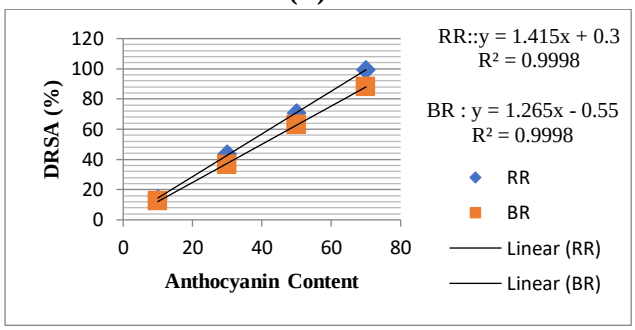
FIg 10 Determination of IC_{50} of water extractives of RR and BR : (A) DPPH Assay (B) Hydroxyl radical scavenging assay and (C) Lipid peroxidation inhibition assay. Data expressed as mean± SD (n=3, P<0.05) for all tested dosages

Table 5 Anthocyanin content of the water extracts of RR and BR

Natural water soluble pigment	RR	BR
Anthocyanins	146 ± 19 mg / 100 g	71.362 ± 20 mg /Kg

Lipid peroxidation inhibition assay:-The lipid peroxidation inhibition activity of the water extracts of RR and BR was compared with CA. The water extract of RR had higher activity than that of the other extractives. At a concentration of 200 µg/mL, the scavenging activity of the RR and BR reached 61.27 and 42.51%, while at the same concentration, that of the catechin was 63.47%. The IC_{50} of water extracts of RR and BR were 79.56 and 127.72 µg/mL, respectively. The IC_{50} of catechin (standard) was 55.21 µg/mL (Figure 10C).

(A)



(B)

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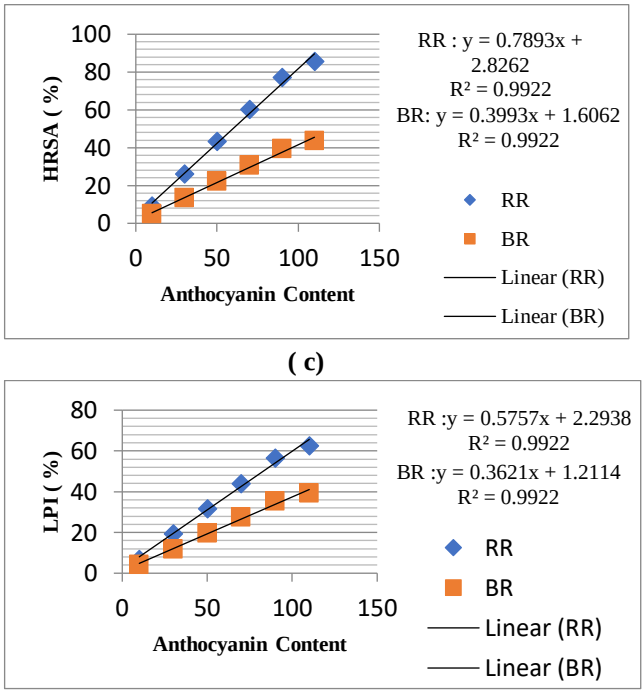

Fig 11 Relationship of total Anthocyanin contents with

(A) DRSA % (B) HRSA % (C) LPI %.Data expressed as mean ± SD (n=3, p<.001).

Total Anthocyanidin contents

The total anthocyanin in the RR and BR water extract is displayed in Table 5. Figure 11 displays the correlation between the extractives' total anthocyanin contents and the percentage LPI and free radical (DPPH and OH) scavenging efficiency.

Correlation and regression of LPI with DRSA and HRSA

The correlation and regression (p-value < 0.001) between LPI and DRSA and HRSA are shown in Figure 12. For all extraction, significant associations (p-value < 0.001) were found (Figure 12).

5. Discussion

The identification of anthocyanins present in water extract of Red radish and Beet root identified by TLC,UV and HPLC, again quantified by HPLC method amount of anthocyanin in red radish and beetroot is calculated by dividing peak area by its respective response factor. After drying the anthocyanin present in Red radish is found to be 32.7377% and anthocyanin in Beetroot is found to be 30.6476% respectively.

Total antioxidant property and ferrous reducing Antioxidant property

The antioxidant potentials of water extracts of RR and BP were estimated from their ability to reduce the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The reducing ability of the extractives was in the range of 1.670±0.032 to 2.156± 0.023 µg/ml green phosphate/Mo (V) (Table 4). Antioxidant activity increased proportionally with the increase of Anthocyanins content.

The iron reducing capacity of the water extractives of RR and BR were estimated from their ability to reduce the Fe3+-ferricyanide complex to the ferrous form by donating an electron. The reducing ability of the extractives was in the range of 2.249± 0.012 to 2.323± 0.033 µg/ml Fe (II)/g (Table 4). In this study, ferrous reducing antioxidant capacity was increased with the increase of Anthocyanins contents. Our results are consistent with the result published previously [31].

DPPH radical scavenging activity

Antioxidants are hypothesized to have an influence on DPPH because of their capacity to donate hydrogen [32]. Engaging in radical scavenging activities is crucial to halting the harmful effects of free radicals on a variety of illnesses, including cancer. The recognized method by which antioxidants work to prevent lipid peroxidation is called DPPH free radical scavenging. Because the analysis takes only a short while, this method has been widely utilized to predict antioxidant activity.

Comparing the aqueous extract of RR with normal BHT, our findings showed that they exhibited comparable free radical scavenging ability (Figure 10A). The findings demonstrated the extractives' capacity to donate protons, suggesting that they may be used as main antioxidants as well as scavengers or inhibitors of free radicals. The work done sufficiently shows that the extractives' anthocyanin contents and their antioxidant qualities are correlated. Thus, this could be used as a supplement to medical care.[31]

Hydroxyl radical scavenging activity

Because hydroxyl radicals directly interact with DNA, free radicals have the ability to mutate cells, which is why they are crucial in the development of cancer [33]. Biochemical reactions have the ability to produce

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hydroxyl radicals. Superoxide dismutase transforms superoxide radical into hydrogen peroxide, which when combined with divalent metal ions like iron and copper can result in the production of extremely reactive hydroxyl radicals.

When compared to catechin, a typical antioxidant, the results showed that the aqueous extract of RR and BR exhibited significant hydroxyl radical scavenging activity (Figure 10B). Additionally, by preventing hydroxyl radical interaction with DNA, the extract may have anticancer properties. Lipid peroxidation prevention may be directly related to the extracts' capacity to squelch hydroxyl radicals.

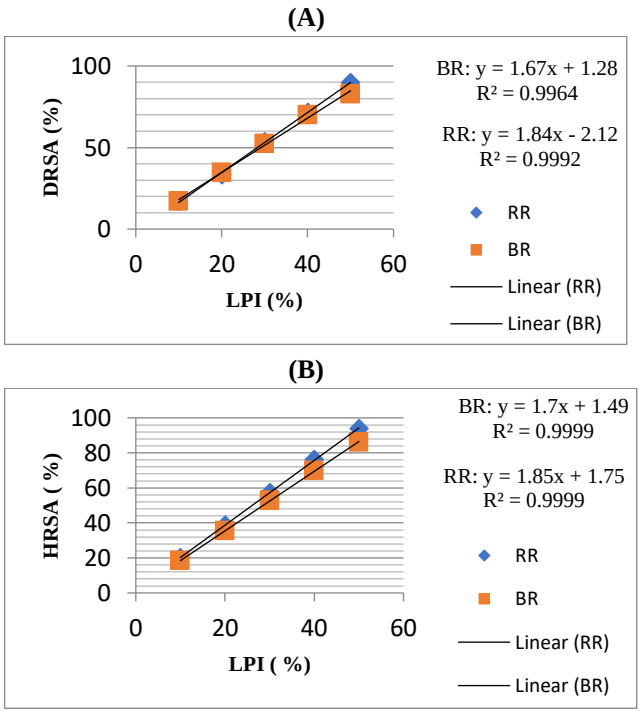

Fig 12 Relationship of % LPI with (A) % DRSA and (B) % HRSA. Data expressed as mean ± SD (n=3, p< .001).

Lipid peroxidation inhibition assay

Lipid peroxidation, a chain reaction that produces damage to lipid molecules, particularly polyunsaturated fatty acids, is how reactive oxygen species (ROS) cause damage to membranes [34]. A second radical is produced by the first reaction, and this radical can then react with another macromolecule to start a chain reaction that causes irregularities in cell function. In this work, ascorbic acid and ferric ions were used to cause lipid peroxidation in rat liver homogenates. It was discovered that RR has a stronger lipid peroxidation inhibition ability than other extractives (Figure 10C). These findings suggested that RR extracts might be investigated for the treatment of liver illness.[35,36]

Total Anthocyanins contents

Figure 11 illustrates the considerable and strong positive connection (p-value <.001) between the extractives' total anthocyanin concentrations and the percentage of LPI and free radical (DPPH and OH) scavenging efficiency. These findings point to the potential critical function that the extracts' anthocyanin components may play in inhibiting lipid peroxidation and neutralizing free radicals.

Correlation and regression of LPI with DRSA and HRSA Significant correlations (p-value < 0.001) were observed for all the extractives for all dosages (Figure 12). This infers that the extractives differentially inhibit lipid peroxidation by virtue of their varying degrees of free radical quenching potential.

6. Conclusion

The Red radish and Beet root have been used to treat a variety of diseases in India as folk medicine. Little is known about the antioxidant activities of Red radish and Beet root. In this research identified then quantified about anthocyanin present in water extracts of Red radish and Beet root as a major active constituent, then studied about antioxidant activities of both and compare, Our results clearly showed that the water extract of RR had strong hydroxyl and DPPH radical scavenging activities as compare to BR. The reducing capacity of RR on ferrous ion was higher than that of water extractive of BR. In addition, the potent antioxidative activity of Red radish and Beet root might result from its high contents of anthocyanins compounds. Hence, the water extract of Red radish and Beet root could be used as a health-care food supplement and in the pharmaceutical industry.

Conflict Of Interest

The authors declare no conflict of interest.

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