Purification and Stability Analysis of *S. cumini* Anthocyanins

A Thesis submitted in fulfillment of the requirement for the award of the degree of

Master Of Technology

In

Biotechnology

Submitted by

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Under the guidance of

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(July 2024)

CERTIFICATE

This is to certify that the thesis entitled **"Purification and stability analysis of *S. cumini* anthocyanins"**, submitted by **Ms. Arashdeep Kour (602204003)** in the partial fulfilment of the requirements of the award of the degree of Master of Technology in Biotechnology at Thapar Institute of Engineering and Technology (TIET), Deemed to be University, Patiala is a record of student's own work carried out under my supervision and guidance. This work has not been submitted in part or full to any other university or institute for the award of any other degree.

Date:

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Candidate's Declaration

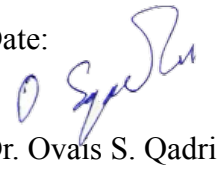
I hereby certify that the work, which is being presented in the thesis, entitled **"Purification and stability analysis of *S. cumini* anthocyanins"**, in partial fulfillment of the requirements for the award of the degree of Master of Technology and submitted to the institution, is an authentic record of my own work carried out during the period January 2024 to June 2024 under the supervision of Dr. Ovais Qadri Professor, Department of Food Technology, Thapar Institute of Engineering and Technology, Patiala, Punjab India. The matter presented in this thesis has not been submitted elsewhere for the award of any other degree or diploma from any institution.

Date:

Arashdeep Kour
Candidate

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

Date:



Dr. Ovais S. Qadri
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Date:

Arashdeep Kour

This thesis is dedicated to my Mother and Sister, Gurmeet Kour and Snehdeep Kour for their endless love, support, and encouragement.

Table of Contents

List of abbreviations

Acronym	Definition
A, Abs	Absorbance
ATPS	Aqueous two phase separation
TG	Triglycerides
PEG	Polyethylene glycol
ATPE	Aqueous two phase extraction
DW	Distilled water
TAC	Total Anthocyanin content
HPLC	High performance liquid chromatography
FRAP	Ferric reducing antioxidant power
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
OD	Optical density
H2O	Water
HPPLC	High Performance Preparative Liquid Chromatography
HSCCC	High speed counter current chromatography
MF	Micro filtration membrane
UF	Ultra filtration membrane
NA	Nutrient agar
NB	Nutrient broth
NF	Nano filtration membrane
ACNs	Anthocyanins
TMAC	Total Monomeric Anthocyanin Content
TPC	Total phenolic content
AOAC	Association of official agricultural chemists
+ve	Positive
-ve	Negative

List of symbols

Acronym	**Definition**
°C	Degree Celsius
cm	Centimetre
g	Gram
kg	Kilogram
L	Litre
%	Percentage
mg	Milligram
mg/g	Milligram per gram
min	Minute
ml	Millilitre
Mg	Microgram
nm	Nanometre
pH	Potential of Hydrogen
sec	Seconds
W	Watt

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Abstract

Syzygium cumini, commonly known as jambolana or Indian blackberry, is a fruit rich in anthocyanins with significant health-promoting properties. This study used conventional solvent extraction (using methanol as solvent) to recover anthocyanin from *Syzygium cumini* pulp. The TAC (Total anthocyanin content) was calculated and measured using the spectrophotometer which allowed rapid and accurate estimation of anthocyanin concentration. Additionally, the purification methods of *S. cumini* anthocyanins were investigated using two different methods: ATPS (Aqueous two-phase separation) and column chromatograph**y.** Regarding the Stability analysis, three parameters were taken into consideration; Light, pH and temperature. Each parameter was studied by subjecting purified samples to different incubation conditions and results were observed accordingly.

Chapter - 1
Introduction

A widely distributed tropical tree that originated in India and is now commonly found as an ornamental tree in various parts of Brazil is the *Syzygium cumini*; Syn.: *Eugenia jambolana* Lamarck, and *Eugenia cumini*. It belongs to Druce; Family: Myrtaceae. It is also known by other names such as jambolão, jamblon, jambul, jamelão, *S.cumini*, jamman, Indian black plum, or Java plum. A single large purple seed and a tart taste characterize the *S.cumini* fruit, which resembles a black olive. The fruit's extracts have been shown to have the potential to be used as a natural food colouring and antioxidant ingredient (de Carvalho et al., 2023).

Based on morphological and organoleptic characteristics, botanical studies have identified two primary morphotypes of *S.cumini* in the Indian subcontinent: the Kaatha Jamun, which is small and has an acidic taste, and the ras jaman, which is oblong, dark purple or bluish, with a pink, sweet fleshy pulp and small seeds. The trees have a wide canopy and can reach a height of 50 feet. The mature bark is scaly and darkish-brown, while the younger bark is pale brown. The leaves are smooth, glossy, leathery, fibrous, and elliptic to broadly oblong. The trees only flower once a year, and on the Indian subcontinent, that time usually falls in June or July. The tiny, sessile blooms have thin membrane petals, are white, and measure 7 to 12 mm. They are typically seen in groups of three and emerge from the incisions left by falling leaves.

Originally native only to the Indian subcontinent, *S.cumini* trees can now be found growing throughout Asia, Eastern and Southern Africa, Madagascar, and the warmer parts of the United States, including Florida. The mature bark is slightly darker brown, scaly, and occasionally peels off, whereas the younger bark is pale brown. The smooth, shiny, elliptic to broadly rectangular, leathery, and fibrous leaves. The trees only bear fruit and flowers once a year, in June and July, on the Indian subcontinent. The tiny, fragrant, sessile flowers have thin membrane petals, are white, and measure 7 to 12 mm. They typically appear from the scars left by fallen leaves and are generally organized in groups of three. Fruiting occurs from the flowering stage and takes about two months to finish. The fruits are found in clusters of four to twenty. When a bunch of *S.cumini* fruits ripens, they don't ripen all at once and fall off. Every fruit is 1/2 to 2 inches long, spherical, oblong, or ellipsoid, and has a big seed in the centre. When the fruits are young, they are green, but as they ripen, they become pale pink (Saldanha et al., 2018).

It is claimed that a variety of fruit-derived polyphenols, such as anthocyanins, influence different biological effects. A class of plant pigment ingredients known as anthocyanins is found in many common fruits, such as blueberries, black currants, cherries, and grapes. They have anti-inflammatory, antioxidant, and DNA-protective qualities and are linked to health advantages. Furthermore, it has been documented that consuming anthocyanins can lower human concentrations of triglycerides (TG), low-density lipoprotein (LDL), and total cholesterol (TC). Furthermore, various investigations involving human intervention have shown the protective effects of anthocyanin-rich products on DNA. Furthermore, anthocyanins enhance Nrf2 transcription and the activity of the antioxidant enzymes SOD, CAT, and glutathione peroxidase (GPx) by modulating the Nrf2-ARE signalling pathway. In a prior intervention trial, total DNA damage was found to decrease within 8 hours after consuming bilberry extract, which also affects the Nrf2/ARE-signaling pathway in individuals with a healthy gastrointestinal tract and ileostomies. This shows that the health benefits of anthocyanin-rich juice are caused by both anthocyanins and molecules that contain flavylium cations (Bakuradze et al., 2019). Thus, this study will focus on investigating efficient purification techniques and select the one that best isolates anthocyanins from the fruits of *S. cumini*.

Chapter - 2
Review of Literature

The term anthocyanin was coined by Marquart in 1835, derived from the Greek words ‘anthos’ and ‘kyanos’ for flower and blue, designating to the blue pigment of flowers (Markakis 2012). Members of the *Syzygieae* genus have been shown to have considerable economic potential due to their potential for usage as food and medicine. As a prospective raw material for the food and pharmaceutical industries, *S. cumini* is utilized for direct consumption and is a source of natural nutrients, bioactive chemicals, and antioxidants. Because of its exceptional strength and resistance, its wood is used to make charcoal and firewood. Jam, jelly, juice, ice cream, vinegar, wine, and pudding can all be made with the edible pulp. Its leaves can be used to extract essential oils for soap and perfume manufacture or fed to silkworms. Because they contribute to the production of premium honey, the nectar-rich flowers are beneficial for beekeeping. Furthermore, the peel and seeds are utilized as a natural remedy for diarrhoea, hyperglycemia, glycosuria, ulcers, bronchitis, and asthma in Unani, Chinese, and Ayurvedic therapy and claim that the plant's hypoglycemic, anti-inflammatory, anti-anaemic, antibacterial, antioxidant, antiallergic, hepatoprotective, hypolipidemic, and antipyretic qualities are present in all portions of the plant (do Nascimento-Silva et al., 2022).

The pulp and peel of *S. cumini* contain delphinidin, cyanidin, petunidin, peonidin, and malvidin. Delphinidin, which accounts for 37–48% of total anthocyanins during fruit ripening, is the most abundant anthocyanin. Petunidin (29–33%) and malvidin (19–27%) are the next most abundant anthocyanins, while cyanidin (3%) and peonidin (1-2%) are detected at lower levels. Research demonstrates the strong antioxidant properties of delphinidin, the phenolic compound present in higher concentrations in *S. cumini*. These actions include inhibiting the degradation of Nrf2 in HepG2 cells and increasing its nuclear translocation (Wu et al., 2020). Additionally, they prevent apoptosis caused by reactive oxygen species by significantly lowering apoptosis markers like c-caspase-3 and c-PAR.

Total phenolic components are significantly concentrated in the edible portion of *S. cumini*, ranging from 995 to 1117 mg GAE/100 g (d. w.). In terms of phenolic chemicals other than anthocyanins, the peel has four times as many as the pulp. Studies have revealed that the fruit's edible portion contains ellagitannins, gallotannins, flavonoids, and flavones. Gallic acid, galloyl glucose, hexahydroxydiphenoyl, and their derivatives were among the

compounds detected in higher concentrations (Nascimento-Silva et al., 2022). According to Faria et al. (2011), the edible portion of the fruit was noted for having high concentrations of all-trans-lutein and all-trans-β-carotene, which accounted for 43.7% and 25.4% of the pulp's total carotenoid content, respectively. Function of Bioactive Compounds and Health Advantages Anthocyanins have anti-inflammatory and antioxidant qualities. Polyphenols have anti-inflammatory and antioxidant qualities. Flavonoids' anti-inflammatory and antioxidant qualities Carotene benefits of antioxidants for eye health Ellagic Acid: An antioxidant with possible anti-cancer properties 19 Gallic Acid's antibacterial and antioxidant qualities Potential anticancer effects of tannins, an antioxidant Antioxidant and anti-inflammatory qualities of quercetin Potential anticancer effects of the antioxidant kaempferol Antioxidant and anti-inflammatory qualities of myricetin immunological support, antioxidant, and vitamin C benefits of vitamin A antioxidant for eye health Calcium muscle and bone health Calcium Heart health, blood pressure control, and iron energy generation and oxygen transportation.

Table 1. Nutritive value of *Syzygium cumini*

Total sugars (%)	8.43-14.31
Thiamine (mg)	0.120
Magnesium (mg)	4-3
pH	4-5
Niacin (mg)	0.2-0.29
Reducing sugars (%)	5.7-9.85
Non reducing sugars (%)	8.35-8.58
Vitamin c (%)	10.70 – 29.52
Carbohydrates (%)	14.0
TSS (%) 9.0	9.0-18.6
Iron (mg)	0.8-1.2
Sodium (mg)	26.2-34.
Copper (mg)	0.23
Magnesium (mg)	4-3

Fats	0.15-0.27

Anthocyanins

The term anthocyanin was coined by Marquart in 1835, derived from the Greek words anthos and kyanos for flower and blue, designating to the blue pigment of flowers (Markakis, 2012). The anthocyanins are part of the very large and widespread group of plant constituents known collectively as flavonoids. Anthocyanins acylated with two or more aromatic acids are platyconin, cinerarin, gentiodelphin, and zebrinin. The color of anthocyanin-containing media depends on the structure and concentration of the pigment, pH, temperature, presence of copigments, metallic ions, enzymes, oxygen, ascorbic acid, sugars and their degradation products, sulfur dioxide, and other factors. Hydroxyl groups, methoxyl groups, sugars, and acylated sugars have a marked effect on the color intensity and stability of anthocyanins. Color intensification by copigmentation increases with increasing anthocyanin concentration and increasing ratio of copigment to anthocyanins. Self-association also occurs in concentrated "neutral" solutions and is affected by the type and concentration of anthocyanin. Increased anthocyanin concentration (s) in these and other plant tissues intensifies their color and may enhance color stability through intermolecular copigmentation and self-association. Intramolecular copigmentation is responsible for the color stability of anthocyanins containing two or more aromatic acyl groups (Mazza et al., 1993).

Anthocyanins are water-soluble pigments that occur in most vascular plants. Anthocyanin is a subgroup of large secondary plant metabolites called flavonoids. More than 5000 flavonoids have been identified. Flavonoids have two aromatic rings connected by a central C3 pyran ring. Common flavonoids are anthocyanins, aurones, chalcones, yellow flavanols, flavones, uncoloured flavanols, flavanones, dihydroflavonols, dihydrochalcones, leucoanthocyanidins, catechins, flavans, and isoflavonoids (Figure 1). Anthocyanins are modified by hydroxylation, methylation, glycosylation, and acylation. This adds versatility to the colors and stability of anthocyanins. As the number of hydroxyl groups in the B-ring increases, the color of the anthocyanin becomes bluer. Methylation, on the other hand, leads to a red shift in the color of anthocyanins. Methylation of the B-ring leads to a low susceptibility to oxidation and stabilization of the anthocyanins. Methyl-modified flavonoids are often found on the surfaces of leaves and flowers. Glycosylation of anthocyanins leads to a hypsochromic shift in the absorption maxima of the spectra and increases its stability for storage in the vacuoles.The glycosyl moieties of anthocyanins may be further modified by aromatic

(hydroxycinnamic or hydroxybenzoic acid) and/or aliphatic (malonic, acetic, or succinic acid) acyl moieties. While aliphatic acylation does not lead to a change in color, aromatic acylation leads to a shift towards blue. Acylation also increases the stability and solubility of anthocyanins.

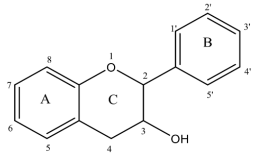
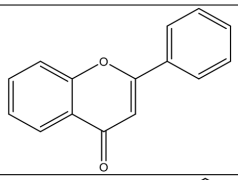
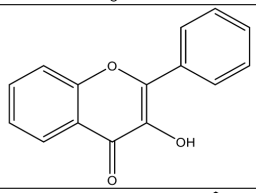
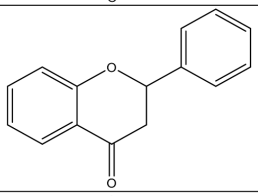
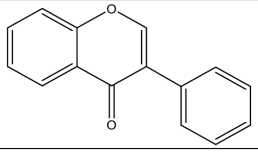
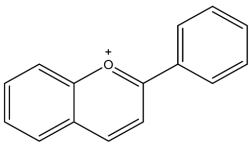
Class of Flavonoid	General Structure	Flavonoid	Substitution Pattern
Flavanol		Catechin	3,5,7,3′,4′-OH
Flavone		Chrysin Apigenin Luteolin	5,7-OH 5,7,4′-OH 5,7,3′,4′-OH
Flavonol		Kaempferol Quercetin Myricetin	5,7,4′-OH 5,7,3′,4′-OH 5,7,3′,4′,5′-OH
Flavanone		Naringenin Taxifolin Eriodictyol	5,7,4′-OH 3,5,7,3′,4′-OH 5,7,3′,4′-OH
Isoflavone		Genestein Daidzein	5,7,4′-OH 7,4′-OH
Anthocyanidin		Pelargonidin Cyanidin Delphinidin Peonidin Petunidin Malvidin	3,5,7,4′-OH 3,5,7,3′,4′-OH 3,5,7,3′,4′,5′-OH 3,5,7,4′-OH;3′-OMe 3,5,7,3′,4′-OH;5′-OMe 3,5,7,4′-OH; 3′,5′-OMe

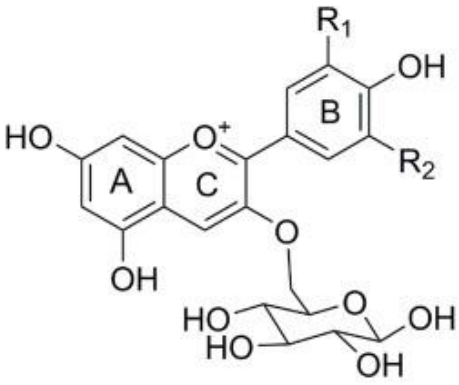
Figure 1. Classification of various flavonoids, their structures and substitution patterns. The ring system (A, B, and C) and numbering are shown for the flavanol structure only. Other structures have the same letters for rings and the same numbers for carbon atoms.

S. cumini pulp and seeds were analyzed for total anthocyanin content using the pH-differential method and expressed as cyanidin-3-glucoside equivalents. The S.cumini pulp powder contained 0.54% anthocyanins. Upon enrichment, the anthocyanin content in the pulp was not changed. After hydrolysis, the enriched pulp extract yielded 0.23% of anthocyanidins. *S.cumini* appears to be the only berry that contains the five anthocyanidins present in blueberry/bilberry, along with significant amounts of ellagic acid/ellagitannins. Because of the known anti-carcinogenicity potential of these five anthocyanidins in blueberry

and cyanidin together with ellagic acid/ellagitannins in black raspberry against estrogen-mediated breast cancer, this composition of anthocyanidins and ellagic acid/ellagitannins in *S. cumini* could potentially provide additive, if not synergistic, effects (Tanaka., 2008).

Structure and stability of anthocyanins

Anthocyanins, as the most common and widely distributed flavonoids, are water-soluble pigments in flowers, seeds, fruits, and leaves of many plants. Figure below shows the basic skeleton characteristics of anthocyanins C6-C3-C6, and 2-phenylbenzopyran is its basic structure. Generally, the properties of anthocyanins are extremely unstable and rarely exist alone under natural conditions, and they are often formed by glycosidic bonds with sugars such as glucose, galactose, rutose and rhamnose.



R_1=H	R_2=H	Pelargonidin (Pg)
R_1=OH	R_2=H	Cyanidin (Cy)
R_1=OH	R_2=OH	Delphinidin (Dp)
R_1=OCH_3	R_2=H	Peonidin (Pn)
R_1=OCH_3	R_2=OH	Petunidin (Pt)
R_1=OCH_3	R_2=OCH_3	Malvidin (Mv)

Figure 2. Basic structure of anthocyanins (Tan et al., 2022).

There are six common anthocyanins, namely, cyanidin, petunidin, petonidin, pelargonidin, delphenidin, and malvidin. The different types of anthocyanins are mainly due to the differences in the position of glycosidic bond binding, the type and number of sugars, the number of hydroxyl groups, the type and number of aromatic acids and so on. Anthocyanins contain unsaturated double bonds and easily oxidized groups, which lead to their extremely

unstable properties. The external environment can easily affect their stability. The main external factors affecting the degradation of anthocyanins are pH, temperature, and O2 concentration, and the secondary factors are enzymes, metal ions, ascorbic acid, and its degradation products . Moreover, the structure of anthocyanins, such as the number and position of hydroxyl groups, the degree of glycosylation, methylation and acylation, affects the stability of anthocyanins . Therefore, extraction factors (solid-to-liquid ratio, ultrasonic power, microwave power, extraction temperature, and extraction time) should be reasonably controlled to optimize the extraction rate of anthocyanins by using response surface analysis, orthogonal test, genetic algorithm, and genetic algorithm coupled neural network (Tan et al., 2022).

Anthocyanins as Bio colourant

Due to the dynamically growing natural, organic, and sustainable food markets, the demand for non-synthetic food colourants continues to increase. Anthocyanins have filled this gap for the last three decades. Anthocyanins have additional health benefits that give them a unique position among anthocyanins in this sector. Anthocyanins have been studied for their application as food colorants due to their vibrant colors (Cortez., 2017) Anthocyanins have also been used in various applications to render pink, red, purple, and blue colors. Because of their high reactivity and destabilizing interactions with other molecules in the media, the application of anthocyanins as food colorants has been limited. Co-pigmentation, complexation with various metal ions, and acylation with various organic acids have all been attempted to increase the stability of anthocyanins. Polyphenol oxidase is one of the enzymes that has been identified as the reason for the color destabilization of anthocyanins. The application of anthocyanin to produce various colors in a pH range of 2–8 was reported using a pelargonidin-based anthocyanin extracted and purified from the flowers of *Ipomea tricolor* (Hossain., 2016)

Anthocyanin Extraction using Solvent extraction method (SEM)

Solubility and the solvent extraction method (SEM) share a similar technical principle. Making a sensible choice when choosing organic solvents is crucial. Methanol, ethanol, acidified water, or acidified ethanols are the most often utilized solvents for the extraction of anthocyanins. An extensive analysis of prior research revealed that anthocyanins may be extracted by SEM with the following parameters: solid-to-liquid ratio of 1:15–1:30 g/mL; extraction period of 5 min–4.2 h; and extraction temperature of 34.7–52.03 °C. SEM provides

a few benefits, including straightforward implementation, basic equipment, and convenient operation.

Anthocyanin Purification through Chromatography

Anthocyanins are extracted alongside various contaminants, such as soluble sugar, protein, and organic acid, during the extraction process. The presence of an excessive amount of contaminants will greatly affect the physiological activities, stability, and overall quality of anthocyanins. Hence, the process of isolating and refining the raw extract is a crucial step in acquiring anthocyanins that possess exceptional stability, robust physiological effects, and superior quality. Currently, the primary techniques used for purifying anthocyanins include column chromatography, membrane separation, high-speed counter-current chromatography (HSCCC), and high-efficiency preparative liquid chromatography.

The column chromatography method is widely used for the isolation and purification of anthocyanins. The approach is based on the disparity in distribution coefficients of anthocyanins between solid and mobile phases, enabling more effective separation of anthocyanins from contaminants. The packed column typically comprises macroporous resins, sephadex-100, and polyamide resins. Macroporous resin is a high polymer adsorbent that has a porous structure and lacks ion exchange groups. Furthermore, macroporous resin possesses the benefits of rapid adsorption rate, substantial adsorption capacity, economical production cost, and recyclability. Consequently, this technique has emerged as a very efficient purification approach, extensively employed in the isolation and purification of bioactive compounds from plants. Chen et al. (2016) conducted a comparative analysis of the adsorption and desorption characteristics of five macroporous resins on anthocyanins extracted from mulberry. The study revealed that XDA-7HP macroporous resin had superior adsorption and desorption capabilities compared to the other four macroporous resins. The purity of anthocyanins obtained after elution with a 40% ethanol solution was 8.5-fold more than that of the crude extract. Wang et al. (2014) employed macroporous resin to segregate and refine the unrefined extract of anthocyanins derived from blueberry. According to the findings, the concentration of anthocyanins rose from 7.5% to 32.0% following the process of purification using macroporous resin. In their study, Xue et al. (2019) initially employed macroporous resin to dynamically adsorb the crude extract of anthocyanins from blueberries. Subsequently, they performed dynamic desorption using a pH of 3 and a 60% ethanol aqueous solution, resulting in the collection of the desorbed solution. The initial anthocyanins

content was measured at 4.58%. Subsequently, the anthocyanins were isolated from the analytical solution using Sephadex LH-20. Finally, the anthocyanins content in the resulting powder was found to be 90.96%. Column chromatography is the predominant technique used to separate and purify anthocyanins. Nevertheless, the limited quantity of preparation hinders the industrial-scale purification of anthocyanins using this approach.

The membrane separation method utilizes both manufactured and natural synthetic membranes to effectively separate and purify material. The separation principle relies on the utilization of distinct molecular weights to effectively segregate contaminants from the desired constituents. Currently, the membranes utilized for separation and crude extraction mostly consist of micro-filtration membranes (MF), ultra-filtration membranes (UF), and nanofiltration membranes (NF). Membrane separation technology is a purely physical method that does not include chemical reactions. It has several advantages, including gentle treatment, no phase changes during separation, resilience to acids and alkalis, and minimal energy consumption. Consequently, this technology is extensively employed in the domains of biology, medicine, and the treatment of food and water. Woo et al. (2008) employed membrane separation technique to extract anthocyanins from cranberry pulp wastes. Following the processes of ultrafiltration, infiltration, and concentration, the resultant powder exhibited a concentration of 0.11% anthocyanins. In their study, He et al. (2017) isolated anthocyanins from black rice. Initially, a membrane with a relative molecular weight of 3 kDa was employed to selectively capture and eliminate proteins and polysaccharides. The anthocyanins were absorbed onto DM 301 resin, then washed out using an ethanol solution containing 85% ethanol. Finally, they were captured using a 200 Da membrane to get purified anthocyanins with a purity of 95.93%. When employing membrane separation, it is worth considering enhancing the pretreatment technique of the raw materials. Various purification techniques were employed, including membrane separation, resin adsorption, gel separation, and membrane separation. The selection of a suitable and effective method for the separation and preparation of anthocyanins was based on the experiment's feasibility.

The method used is High-speed counter-current chromatography (HSCCC). High-speed counter-current chromatography (HSCCC) is a widely employed technique for extracting bioactive chemicals from natural plant resources. It is a continuous liquid-liquid separation method. HSCCC can prevent the irreversible attachment of samples to a solid support, hence enhancing the capacity to load samples and enabling the quick and efficient synthesis of

bioactive chemicals on a wide scale, as opposed to standard column chromatography. Furthermore, the presence of contaminants in the desired fraction will be significantly diminished, leading to a substantial enhancement in the efficiency of separating active chemicals by HSCCC. The research group effectively extracted and refined two highly pure anthocyanin monomers (cyanidin-3-glucoside and delphinidin-3-glucoside) from blueberry and cranberry using HSCCC. The purity of these monomers exceeded 94%. Furthermore, we successfully extracted and refined seven prominent types of anthocyanins from the leftover raspberry wine using HSCCC. In addition, Xiao et al. (2010) extracted anthocyanins from Perilla frutescens using HSCCC. The analysis revealed that the malonylshisonin and shisonin had purities of 96.7% and 97.5% respectively. This indicates that HSCCC is a rapid and effective method for obtaining pure anthocyanin monomers from natural sources. Liang et al. (2014) extracted cyanidin-3-glucoside from blue honeysuckle fruits using HSCCC. According to the findings, every 100 mg injection of crude extract produced 22.8 mg of cyanidin-3-glucoside with a purity level of 98.1%. The identification of a suitable extraction solvent system remains a pressing issue that needs to be addressed in HSCCC technology. Hence, additional investigation is required for the advancement of the technology.

A procedure called High Performance Preparative Liquid Chromatography (HPPLC) is used. The principle of high performance preparative liquid chromatography (HPPLC) relies on the distinct physicochemical properties of each component in the crude extract of natural products. These components are distributed to varying degrees in two immiscible phases and move at different speeds within the two phases. As a result, they are eluted from the column, leading to improved separation efficiency. When employing HPPLC for the separation and purification of the crude extract, the primary elements taken into account are the purity, yield, operational components, and production cycle of the desired chemical. HPPLC has several benefits, including effective separation and purification, precise detection accuracy, versatile applications, and automated continuous separation. Consequently, it is extensively employed in the extraction of valuable substances with a high economic worth, such as organic acids, bioactive alkaloids, and flavonoids. Nevertheless, this method requires a significant investment in equipment and only a minimal amount of preparation. As a result, it is currently incapable of being implemented on a broad scale in industrial settings. Ultimately, the pretreatment of samples can enhance the extraction efficiency and purity of anthocyanins, while also saving time, minimizing the need for organic solvents, and preserving the environment. Based on the production needs, choosing suitable and practical equipment, and

utilizing purification technology in combination can facilitate the efficient separation and purification of anthocyanins with a high level of purity (J et al., 2022).

During the extraction process, several pollutants such as soluble sugar, protein, and organic acid are extracted simultaneously with the anthocyanins. The excessive presence of contaminants will have a significant impact on the physiological actions, stability, and quality of Anthocyanins as a final product. Therefore, in order to obtain Anthocyanins with excellent physiological activity, high stability, and superior quality, it is necessary to separate and purify the crude extract. The main techniques currently employed for anthocyanin purification are high-speed counter-current chromatography (HSCCC), two-phase separation, membrane separation, column chromatography, and high-efficiency preparative liquid chromatography (Tan et al., 2022).

The extract (40 mL) was purified by successively partitioning it three times with 40 mL of ethyl acetate (EtOAc). The aqueous phase obtained was further reduced to a volume of 20 mL to eliminate any remaining ethyl acetate (EtOAc) residues. It was then applied onto a column packed with Amberlite XAD-7HP resin (50 × 1.0 cm) with a bed volume (BV) of 40 mL. The resin selectively adsorbed anthocyanins and flavonols, whereas sugars, organic acids, and other water-soluble chemicals were eliminated by washing the column with 1 L of deionized water acidified with HCl (pH 2.0) at a flow rate of 1 BV/h. The elution of anthocyanins was conducted by employing a solution consisting of 40% ethanol in water, which was acidified with HCl to achieve a pH of 2.0. The elution process was carried out at a flow rate of 1 bed volume per hour. The fractions were gathered in 10-mL tubes and each tube was examined using a double-beam UV–vis spectrophotometer.The target eluate was gathered and concentrated at temperatures below 40 °C, and the resulting solution was subjected to freeze-drying. The anthocyanin powder, which had been freeze-dried and weighed 45.59 ± 3.61 mg, was diluted in 10 mL of deionized water. This solution was then placed onto a YMC*Gel ODS-AQ-HG chromatographic column with dimensions of 50 × 1.7 cm and a bed volume of 80 mL. The column was washed with 500 mL of a solution containing 20% ethanol and water, which was acidified with HCl to have a pH of 2.0. The elution process was carried out at a rate of 0.5 bed volumes per hour. Monomeric anthocyanins were separated and collected based on their easily distinguishable color bands. The collected anthocyanins were then freeze-dried to obtain a powdered form. Subsequently, the individual monomers were precisely measured in terms of weight, and their purities were

determined by calculating the percentage of their peak area in the total chromatogram area using HPLC-PDA chromatogram (200–600 nm) (Zhao et al., 2017).

The blueberries from the Lake Saint-Jean region in Quebec, Canada have a total anthocyanin level of 290 mg/100 g. Additionally, twenty derivatives of anthocyanin were found. The primary anthocyanins present are the monoglycoside isomers of malvidin, peonidin, cyanidin, and delphinidin, with relative quantities of 163, 93, 73, and 91 mg of Cyanin equivalent per gram of extract. To achieve a high level of purity, it was necessary to perform two consecutive solid phase extractions using hydrophobic silica gel (DSC-C18) and cationic exchange resin (DSC-SCX), followed by fractionation by preparative HPLC, to isolate anthocyanin molecules. Delphinidin-3-galactoside exhibits the largest capacity to absorb oxygen radicals, measuring at 13.062 ± 2.729 μmol TE/μmol. On the other hand, malvidin-3-glucoside has the lowest capacity, measuring at 0.851 ± 0.032 μmol TE/μmol.The reference is from (Chorfa et al., 2016).

The components of E. uniflora that were separated from Diaion HP-20SS using a mixture of water and methanol were further purified using reversed-phase column chromatography. This process resulted in the isolation of cyanidin-3-glucoside and delphinidin-3-glucoside.The Diaion HP-20SS resin offers an efficient means of eliminating sugars and ascorbic acid from aqueous plant extracts, hence serving as an initial purification step for the polyphenolic antioxidants. The technique successfully identified the anthocyanin cyanidin-3-glucoside from the semi-purified aqueous fractions of Surinam cherry, star apple, and jaboticaba. Additionally, delphinidin-3-glucoside was identified from Surinam cherry (Einbond et al., 2004).

The tobacco leaf was extracted using petroleum ether in a water bath at a temperature of 50 °C, with reflux for 2 hours. The resulting mixture was then filtered, and the powdered leaf was subjected to another extraction using petroleum ether. The extracts obtained from this process were combined and concentrated to obtain a thick residue. The remaining substance was converted into soap and then placed into a column filled with silica gel for separation. The chemical was separated using a binary solvent mixture of petroleum ether–acetone (90:10, v/v) and the resulting solution was collected in fractions of 5 ml each. Preliminary identification was conducted using thin-layer chromatography (TLC). The fractions primarily consisting of solanesol were combined and dehydrated using rotary evaporation. The solenosol yield from tobacco leaf was 0.38% of its dry weight, with a purity of 83.04%.(Tang et al., 2007).

This work aimed to investigate the process of isolating and purifying anthocyanins from blood oranges using column chromatography. Subsequently, the identification of the anthocyanins present in blood oranges was conducted. A comparison was made between the static adsorption and desorption, as well as the dynamic adsorption and desorption, of 12 different types of resins. The findings demonstrated that NKA-9 macroporous resin was the most effective for extracting blood orange anthocyanins, and the ideal solvent for elution was a mixture of 50% ethanol and citric acid (pH 2.5). The Toyopearl TSK HW-40S column was used to separate and purify the anthocyanin extracts obtained from blood orange. The optimal separation of the Toyopearl TSK HW-40S column was achieved by using a mobile phase consisting of 35% methanol and 2% formic acid, with a flow rate of 0.6 mL min−1. Three different types of anthocyanins were isolated from blood oranges. Subsequently, the anthocyanins present in blood orange were determined using HPLC-ESI/MS analysis. The analysis revealed that cyanidin-3-glucoside (35.2%) and cyaniding-3-(6″-malonyl) glucoside (42.9%) were the primary anthocyanins found in blood orange. In addition, the compounds cyanidin-3-(3″-malonyl) glucoside, cyanidin 3-(6″-dioxalyl) glucoside, and cyanidin-3-glucoside adduct:4-vinylcatechol were found in blood orange. The utilization of NKA-9 macroporous resin in conjunction with Toyopearl TSK HW-40S column chromatography proved to be a successful technique for the extraction and purification of anthocyanins from blood orange. Additionally, the application of -ESI/MS analysis was found to be a convenient, rapid, and efficient method for the identification of these anthocyanins.The reference is from (Cao et al.,2010).

To monitor the development of anthocyanin-derived pigments and contribute to the investigation of their chromatic qualities, stability, and relative impact on the color of red wines, a technique for separating the coloring material was established. The approach relied on the unique reactivity of various pigment families towards bisulfite (hydrogen sulfite). The wine, which had been acidified and bleached using NaHSO3, was loaded into a Toyopearl® HW-40(s) gel column and subjected to elution with ethanol. Two fractions with distinct pigment contents were gathered and examined using liquid chromatography diode array detection-mass spectrometry. The compounds in each fraction were identified based on their UV-visible and MSn mass spectra. The first fraction consisted mainly of pyranoanthocyanins, whereas the second fraction primarily included anthocyanins and condensation products of anthocyanin-flavanol. A diverse array of novel pigments were identified, including those that have not been previously documented in red wines, to the best of our knowledge. Each family

of chemicals exhibited distinct MS2 and MS3 fragmentation patterns, which can be utilized to identify undiscovered pigments in different wines (Alcalde-Eon et al., 2004).

The separation of wine anthocyanins was achieved by directly injecting them into a low-pressure chromatographic column. The gradient method was adapted to incorporate water as the eluent. The methodology was employed to examine five distinct wine varieties produced from Vitis vinifera and Vitis rotundifolia grapes. The gradient profile reliably separated the pigments in both Vitis vinifera and Vitis rotundifolia wines into four different fractions. The chromatographic profiles of the wines revealed variations in the levels of mono- and diglucoside anthocyanins depending on the aging process. The inclusion of acetaldehyde had varying effects on anthocyanins, resulting in distinct chromatographic patterns compared to untreated samples. The reference for this information is (Johnston et al., 1996.)

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Anthocyanin Purification using ATPS Method

The worldwide wine business produces almost 8.5 million tons of trash each year, which contains numerous valuable biomolecules. The current investigation centres on the retrieval of anthocyanins from grape pomace via solid/liquid extraction employing aqueous solutions of the ionic liquid 1-ethyl-3-methylimidzaolium acetate [(C2mim]OAc) and further refinement in aqueous two-phase systems (ATPS) with supplementary salts. The ideal parameters for solid/liquid extraction were determined using response surface methods and resulted in a maximum anthocyanin yield of 3.58 mg g−1. The most effective purification factor (PF = 16.2 fold) during ATPS synthesis was achieved in the lower phase, which is rich in salt. This was accomplished by employing K2CO3 (29.41 wt%) and [C2mim]OAc (29.28

wt%) at a temperature of 35 °C and atmospheric pressure. The partition process is characterized by being exothermic, spontaneous, and controlled by enthalpy forces.

Anthocyanins in grape juice (GA) were extracted and first purified by aqueous two-phase extraction (ATPE). To determine the ideal extraction circumstances, the effects of several parameters were examined, including the kind and concentration of salt, the amount of alcohol and grape juice, the extraction period, the pH and temperature of the system, and the system's temperature. These were as follows: 1 mL of grape juice, 298.15 K in temperature, 1 hour, no pH adjustment, and concentrations of sodium dihydrogen phosphate (NaH_2PO_4) and ethanol (28% and 25%, respectively). The sugar-removing rate was 75.08%, and under ideal circumstances, the relative and absolute recovery of GA in the top phase reached 99.35% and 99.26% in a single step, respectively (Wu et al., 2014).

Table 2. Purification techniques employed for anthocyanins from different source

Fruit sample	**Source**	**Purification technique**	**Observations/ results**	**Limitations**	**References**
1.Blood oranges (*Citrus sinensis)*	Squeezed fruit juice	Column chromatography, Toyopearl (TSK HW-40S) column	NKA-9 macroporous resin was optimum for isolation of blood orange anthocyanin.	Less yield, equipment constraints, other means of identification also required.	CAO et al. (2010)
2.Red Cabbage *(Brassica oleracea var. capitata f.rubra*)	Leaves	Adsorption (6 adsorbents used)	Amberlite XAD7HP showed the highest adsorption capacity.	Stays focused on red cabbage only. Adsorbent specificity. Solvent dependence.	Chandra sekhar et al. (2012)
3.Purple fleshed potato *(Solanum tuberosum)*	Raw tubers	Adsorption	XAD-7HP polymeric adsorbent is an efficient method.	There are no details about adsorbent material or its selectivity. Large-scale extraction is not discussed.	Heinonen et al. (2016)
4.Roselle (*Hibiscus sabdariff)*	Roselle powder	Comparison of the adsorption property of six macroporous resins on anthocyanins from Roselle.	On Comparison of the adsorption property of six macroporous resins on anthocyanins from Roselle DM21 gave the best result.	Less yield. No large scale production. Lack of economic analysis (cost of resins, solvents & overall process).	Yang et al. (2022)

The anthocyanin partitioning in the crude extract of *S. cumini* fruit was studied in aqueous two-phase systems (ATPS) comprising of Na_2SO_4/$(NH_4)_2SO_4$ and isopropanol. Compared to $(NH_4)_2SO_4$, Na_2SO_4 triggered ATPS formation more successfully. In the $(NH_4)_2SO_4$ ATPS, a temperature increase promoted phase separation, whereas in the Na_2SO_4 ATPS, the converse was true. Tie-line length and slope values increase with increasing temperature or overall mixture concentration. Anthocyanins shown a preference for partitioning to the top phase in all systems and settings. In the top phase of the $(NH_4)_2SO_4$ ATPS, the partition coefficient and theoretical recovery yield ranged from 1.14–1.77 and 53.31–63.87 percent, respectively, while in the Na_2SO_4 ATPS, they varied from 1.94–21.50 and 65.90–95.55 percent (Caldeira et al., 2022).

Anthocyanin Stability

Anthocyanins are sensitive to light, pH, and temperature. In these circumstances, anthocyanins' color changes to yellow or colorless breakdown products. A number of mechanisms have been suggested to guarantee anthocyanin stability. The A or B ring of anthocyanin molecules may be the site of molecular breakdown and metabolism. A great deal of research has also been done on anthocyanin metabolism.It was found that in simulated gastric digesting processes, the stability of anthocyanins was enhanced by the replacement of the B-ring's hydroxyl groups with methoxy groups. Under these circumstances, vanillic acid, syringic acid, and protocatechuic acid were the primary breakdown products (Yang, 2018).

The loss of color in anthocyanins has been linked to the probability that they are hydrated. Numerous mechanisms have been investigated for color stabilization, such as metal ion complexation, intramolecular sandwich-type stacking of the anthocyanin nucleus and acyl groups, self-association, and the complex formation of anthocyanins with flavonoid co-pigments (co-pigmentation) (Hayashi., 1996).

Various anthocyanin sources were employed to ascertain the impact of thermal treatment and varying degrees of acidity. There are four main kinds of anthocyanins: the chalcone pseudobase form, quinoidal bases, carbinol pseudobase, and flavylium cation. At pH 2 or lower, the flavylium form is predominant, but at higher pH values, the colorless chalcone pseudobase takes over. Chalcone production can result from exposure to increased temperatures and pH levels in addition to other factors (McDougall et al., 2007).

An increase in pH and/or temperature led to an increase in anthocyanin degradation, as predicted. The kind of anthocyanin source, pH, and heating duration all had a substantial

($P < 0.05$) impact on the anthocyanins' degradation, according to statistical analysis. Following treatment at 70 °C, the anthocyanins in red cabbage, black carrot, and grape skin were destroyed by 11.82, 9.36, and 2.57%, respectively, in pH 3 buffer systems. It was shown that at higher temperatures, especially when the samples were heated to 90 °C, the anthocyanins broke down more quickly. The variations in the anthocyanin profiles of grape skin, red cabbage, and black carrot may be the cause of these findings.

The anthocyanin stabilities of red cabbage were found to be higher at temperatures of 70, 80, and 90 °C compared to those of grape skin and black carrot.Similar research was conducted in 2004 by Cevallos-Casals and Cisneros-Zevallos, who found that acylated anthocyanin sources were more stable than non-acylated ones when examining the heat and pH stability of red sweet potato, purple corn, and commercial purple carrot colorants. Red cabbage's distinct anthocyanin makeup gave its anthocyanins the highest thermal stability. Specifically, the composition of acylated anthocyanins in red cabbage anthocyanins contributes to their best thermal stability (Cevallos-Casals and Cisneros-Zevallos 2004; Dyrby et al. 2001; Pliszka et al. 2009). The main aglycone of red cabbage is cyanidin, which is found as cyanidin-3-sophoroside-5-glucoside and cyanidin 3,5-diglucoside and is acylated with sinapic, malonic, ferulic, and p-coumaric acids (Walkowiak-Tomczak & Czapski 2007). Conversely, most anthocyanin sources, such the monosides found in grape skin anthocyanins, are less acylated and exhibit light resilience to heat, light, and pH changes (Hong and Wrolstad 1990). Higher hydration, light, and pH stability are displayed by the anthocyanin pigment in black carrots that have been acylated with p-coumaric, ferulic, p-hydroxybenzoic, and sinapic acids (Kammerer et al. 2004).According to (Dai et al.,2009), anthocyanin structures and pH are responsible for the colour stability of anthocyanins.

The heat stability of red cabbage, blackcurrant, elderberry, and black grape colorants was found by Dyrby et al. (2001) to be red cabbage > blackcurrant > grape skin > elderberry at pH 3 and 80 °C. At pH ranges between 0.9 and 4, (Cevallos-Casals and Cisneros-Zevallos 2004) discovered that the order of stability of some anthocyanin sources was red sweet potato > purple carrot > purple corn > red grape. These findings suggest that stability is influenced by anthocyanin compositions and that acylated anthocyanins exhibit greater resistance than their non-acylated counterparts.

Table 3. Stability Purification techniques employed for anthocyanins

Fruit sample	Source	Stability analysis technique	Observations/ results	Limitations	References
L. rutheni cum Murr	Fruit	The combination of purified anthocyanins with Whey Protein Isolate (WPI) to assess its impact on thermal stability.	WPI can enhance the thermal stability of anthocyanins, making them more resilient to temperature-induced degradation. Purification process effectively removed impurities that might contribute to thermal instability.	Less detailed analysis of the thermal degradation profile.	Liu et al. (2020)
Prunus nepalen sis L.	Freeze dried fruit	pH from 3.0 to 7.0	pH from 3.0 to 6.0 entailed a decreasing trend in the colour intensity. Increase in pH to 7.0 showed increase in the colour intensity.	Potential Variability in Plant Material. Lack of Detailed Composition Analysis	Swer & Chauhan (2019)

The impact of pH on anthocyanin stability: The pHs (0, 1.5, and 3) are another element that influences the stability of anthocyanin. Our findings demonstrate that anthocyanin in samples is more severely destroyed as pH rises. Salts of flevelylium are stable only in extremely acidic environments. At higher pH values, these salts lose their proton and change into the unstable pigment quinoidal base. They then instantly combine with water to produce the colorless molecule known as chromenol.Every experiment conducted by (Maccarone et al.,1985) to examine the stability of five distinct Berberis species with a pH of 0 to 1.5 was carried out over 84 days at a fixed temperature of 25 EC. B. integerrima pH = 3 67%, 68%, and 94% B. vulgaris 44.87% 64% 33% 44.87% 10.27% 27% 81.69% B. khorasanica B. orthobotrys 68.11% 88.94% 28.26% The degree of anthocyanin degradation between pH=1.5 and 3 is significantly higher than that between pH = 0 and pH=1.5, as the results demonstrate.According to Morris et al. (1986), the juice sector may face difficulties due to high pH grapes during harvest in warm climates. Increased pH in grapes can lead to color fading and reduce product stability. The combination of color pigments in strawberry jam and packaged strawberries at 37.7 C over time was investigated by (Angela and Little 1977). Her findings for pH values of 2, 3, and less than 1 demonstrated that an increase in pH causes an increase in the degradation of anthocyanin pigments. The impact of temperature on

anthocyanin degradation: Another component that contributes to the instability of the anthocyanin molecular structure is temperature; as temperature rises, anthocyanin degradation increases. These studies support the findings we found. The impact of light, either present or absent, on anthocyanin stability Another element that influences anthocyanin stability is light. Graphs 10–13 show how light speeds up the anthocyanin degradation process in the four species of Berberis. Every sample had a pH of 2, and they were all maintained at 25 °C. Five different times over the 84-day experiment, the data was rearranged. This study showed the anthocyanin destruction percentage for each of the four Berberis species in the presence or absence of light.

The impact of light on the stability of anthocyanin in grape juice was also examined by Palamidis and Markakis (1975), who demonstrated that the pigments' breakdown is accelerated when exposed to light. According to their experiments, nearly 30% of the pigments in the juice samples containing anthocyanin were destroyed after being left in the dark for 135 days at 20 C. However, when the same samples were left in the same conditions for the same amount of time in the presence of light, more than 50% of the pigments were destroyed (Laleh et al.,2006).

Objectives

1. Separation of Anthocyanin from *S. cumini* pulp
2. Purification of Anthocyanin extract from *S.cumini*
3. To study various parameters affecting the stability of *S.cumini* anthocyanins

Chapter - 3

Materials and Methods

3.1 Sample preparation of *S. cumini* fruit

Matured *S. cumini* fruits were purchased from the local market in Patiala, Punjab. The drying and extraction of the sample was performed in the Thapar Institute of Engineering and Technology. The fruit was first washed thoroughly under tap water to remove all the dust and dirt. Further blanching of *S. cumini* fruit was done in which the fruits were immersed briefly in boiling water followed by their rinsing in cold water to stop any enzymatic activity and remove any surface bacteria.

Figure 3: Sample prepration

Pulping was done to the blanched fruits. The pulp was manually cleaned of the seeds, and it was then dried for an entire night at 50°C in a hot air oven. The pulped fruits were grinded after being dried into a fine powder. The grounded *S. cumini* fruit pulp was packed and kept in aluminium packs in a freezer refrigerator at -18°C for further use.

3.2 *S. cumini* anthocyanin extraction

The extraction of anthocyanins from the pulp was done using a solvent. 5g of *S. cumini* pulp powder was taken for 100 ml of methanol (solvent). To improve the process of anthocyanin extraction, assist breakdown of the fruit's cells open and draw out anthocyanins from it. Ultrasonication was employed as a pre-treatment. The remaining extraction was finished when kept in an incubator shaker for 2 hrs at 37°C at 120 rpm. Further, the filtration was done and the extract was kept in the refrigerator for further use.

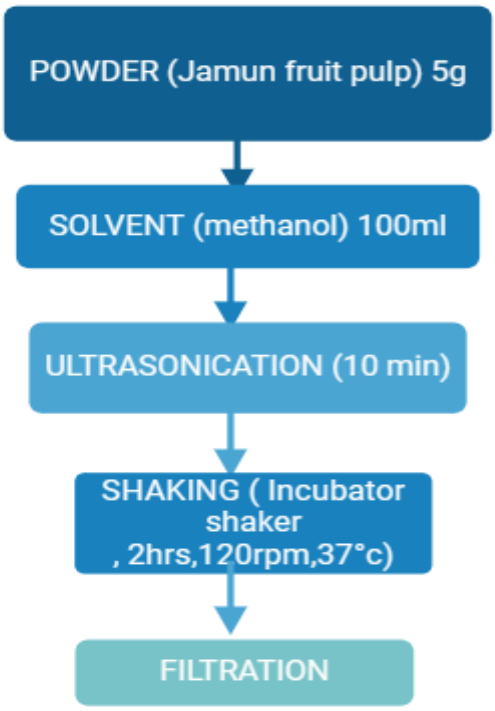

Figure 4. Solvent Extraction of *S. cumini* anthocyanins

3.3 *S. cumini* anthocyanin purification

During the extraction process, soluble sugar, protein, and organic acid are among the many contaminants that are extracted concurrently with the anthocyanins. Excessive contaminants will negatively affect anthocyanins' physiological activity, stability, and quality as a finished product. Therefore, to get anthocyanins with great physiological activity, high stability, and high quality, the separation and purification of the crude extract is a crucial step. These days, high-speed counter-current chromatography (HSCCC), membrane separation, column chromatography, and high-efficiency preparative liquid chromatography are the primary techniques used to purify anthocyanins.

Purification using ATPS (Aqueous two-phase system)

PEG 4000 (14.8g) & Magnesium sulfate (10.3g) were taken 74.9g of crude extract was added to make up 100% weight on a w/w basis. The solution was kept for 1 hour in a magnetic stirrer to mix thoroughly.The mixture was kept for phase separation. The supernatant was collected and volume was noted. Multistage ATPE was performed to remove sugars from the PEG-rich top phase. The distilled water was added to the supernatant to make up the volume to 100ml. The mixture was again kept in magnetic stirrer for 30 minutes. After 30 minutes the mixture was kept for phase separation. The supernatant was collected, its volume was noted and again kept for magnetic stirring for 30 minutes. The mixture was kept overnight for phase separation . Next day the clear supernatant was collected and volume was noted.To separate PEG from the mixture, chloroform was added to it to make up the volume to 100 ml. It was kept in a magnetic stirrer for 30 minutes and the mixture was allowed to settle for phase separation.A pinkish-coloured supernatant was collected containing Anthocyanins. Finally, Anthocyanins determination test was performed and OD was checked at 530 &700 nm.(Jampani et al., 2015).

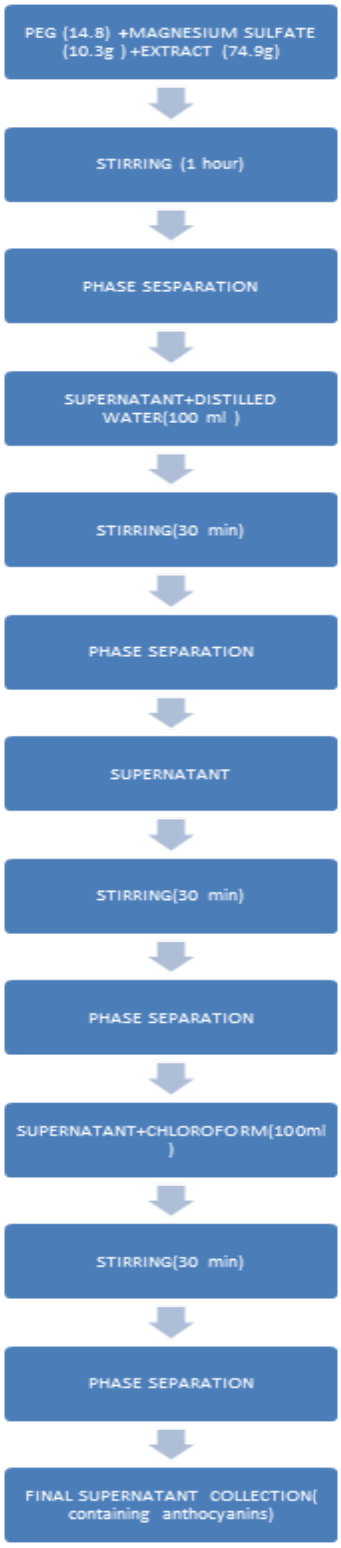

Figure 5. Purification of *S. cumini* anthocyanins by ATPS method

Purification using column chromatography

Firstly 100 ml of *S. cumini* fruit pulp extract was taken and kept in hot air oven for 1 hour at 50°C until it got concentrated to 20 ml. The concentrates were stored in the refrigerator at

-18°C in air-tight bottles until the column was prepared. For the preparation of a Silica gel column, 20g of silica gel was taken as the stationary phase and three trials were done replacing the mobile phase each time and keeping the stationary phase constant (silica gel). Acetone, 2% Formic acid acidified methanol and Ethanol: Distilled water: HCl (80:20:1 v/v/v) were the different mobile phases used in column chromatography. During each column a coloured liquid was eluted which was the purified form of anthocyanins. Finally, Total anthocyanins determination test was performed and OD was checked at 530 & 700 nm in all the 3 cases respectively (Chen et al., 2017).

3.4 *S. cumini* anthocyanin stability analysis

3.4.1 Thermal stability

The extract prepared was adjusted to pH 3.5. Then the extract was transferred into air-tight bottles and subjected to heating at temperatures 50 °C, 70°C and 90 °C for 5 complete hours in the hot water bath. The samples with different temperatures were examined after being cooled to room temperature and the absorbance was read immediately at 530 and 700 nm for anthocyanin determination test periodically at an interval of 1 hour. Analyses were performed in triplicates (Swer et al., 2019).

3.4.2 pH stability

The extract prepared was divided into 3 different categories based on their pHs. Three equal volumes of extracts were taken and the pH to 3, 5 and 7 for each category respectively. The percentage of pigment retention was calculated daily for 10 days to study the effect of pH on the degradation of anthocyanins concerning time and the absorbance of each sample**.** The samples with different pHs were examined and the absorbance was read immediately at 530 and 700 nm for anthocyanin determination test periodically at an interval of 1 day. Analyses were performed in triplicates (Swer et al., 2019).

3.4.3 Light stability

The extract prepared was transferred into 3 different categories and pH was adjusted to 3.5. Three equal volumes of extracts were stored in different light conditions like shade, sunlight and amber respectively. The percentage of pigment retention was calculated on daily basis for 10 days to study the effect of pH on the degradation of anthocyanins concerning time and the absorbance of each sample. The samples with different pHs were examined and the absorbance was read immediately at 530 and 700 nm for anthocyanin determination test

periodically at an interval of 1 day. Analyses were performed in triplicates (Swer et al., 2019).

3.5 Determination of total anthocyanins from *S. cumini*

The pH differential approach, based on absorbance measurements at pH 1.0 and 4.5 and structural changes in chemical forms of anthocyanin, will be used to determine the total anthocyanin concentration. The crude extracts will be diluted individually using 0.4 M sodium acetate buffer (pH = 4.5) and 0.025 M hydrochloric acid–potassium chloride buffer (pH = 1). The buffers diluted each sample so that the absorbance reading ranged from 0.2 to 1.4. A UV-Vis spectrophotometer measured the mixture's absorbance at 700 nm and 530 nm. The following equation represents the total anthocyanin content given as cyanidin-3-glucoside equivalents.

$$\text{Anthocyanin pigment (mg/L)} = \frac{A \times MW \times DF \times V \times 1000}{a \times l \times m}$$

where A is the absorbance, MW is the molecular weight of cyanidin-3-glucosode (449.2 g/mol), DF is the dilution factor, V is the solvent volume (mL), a is the molar absorptivity (26,900 $L.mol^{-1}.cm^{-1}$), and l is the cell path length (1 cm) (Le et al., 2019).

Chapter - 4

Results and Discussion

4.1 Solvent-extraction of anthocyanins from the pulp of *S. cumini* fruit

The anthocyanin extraction was performed using conventional solvent extraction, where methanol was used as the solvent. The crude anthocyanin extract was analysed for its total anthocyanin content, to establish an experimental design for the purification process. The total anthocyanin content obtained for the *S. cumini* fruit pulp extracts was 81.20 mg C3G/ 100 g. A study reported similar findings of total anthocyanins from the crude extract of *S. cumini* (Kaur et al., 2024).

4.2 Purification (ATPS)

Purification of Anthocyanins was done using Aqueous two-phase system in which a mixture of salt (magnesium sulfate), crude extract and PEG was taken. After a series of stirring and supernatant collection steps, multistage ATPE was performed in order to remove sugars from PEG rich phase. Chloroform was finally added to the solution to remove PEG from it. A clear pink coloured liquid was obtained as shown in Figure 6 attributing to the purified anthocyanins from the *S. cumini* fruit pulp. The crude extract exhibited total anthocyanin content of 455.04 mg C3G/ 100 g whereas after purification it decreased to 303.41 mg C3G/ 100 g. The decrease in the anthocyanin content may be due to the removal of impurities from the *S. cumini* fruit pulp extract. However, we experimented with other techniques of purification and sticked with column chromatography, discussed in the next section.

Vietnamese herb Peristrophe bivalvis (L.) Merr. (Acanthaceae) is high in anthocyanin, a naturally occurring colouring agent. Nevertheless, contaminated chemicals are frequently present in conjunction with the popular extraction techniques for this color. As a result, the aqueous two-phase system (ATPS), a green technique for removing and purifying anthocyanin from leaf extract, was the main focus of this investigation. The acquired results showed that an ATPS consisting of ammonium sulfate and ethanol at concentrations of 25% and 32.5%, respectively, could remove 80% of polluted nitrogen compounds from leaves while recovering 80% of the anthocyanin. Additionally, compared to the crude extract, the pigment's shelf life in the refined extract was much longer (Le et al., 2022).

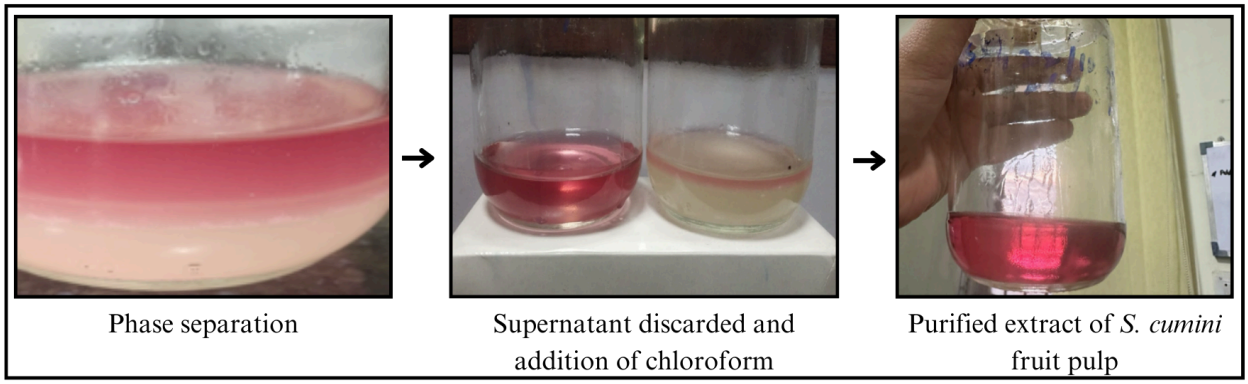

Figure 6. Various stages of purification of *S. cumini* extracts by ATPS

The anthocyanin partitioning in the crude extract of *S. cumini* fruit was studied in aqueous two-phase systems (ATPS) comprising of Na_2SO_4/$(NH_4)_2SO_4$ and isopropanol. Compared to $(NH_4)_2SO_4$, Na_2SO_4 triggered ATPS formation more successfully. In the $(NH_4)_2SO_4$ ATPS, a temperature increase promoted phase separation, whereas in the Na_2SO_4 ATPS, the converse was true. Tie-line length and slope values increase with increasing temperature or overall mixture concentration. Anthocyanins showed a preference for partitioning to the top phase in all systems and settings. In the top phase of the $(NH_4)_2SO_4$ ATPS, the partition coefficient and theoretical recovery yield ranged from 1.14–1.77 and 53.31–63.87 percent, respectively, while in the Na_2SO_4 ATPS, they varied from 1.94–21.50 and 65.90–95.5 percent (Caldeiraetal., 2022).

4.3 Purification using Column Chromatography

The extraction of anthocyanins from *S.cumini* was done in the best possible conditions using preliminary trials. The trials included a set of procedures starting from usage of methanol as solvent, followed by 10 minutes of ultrasonication and incubation at 37°C and 120 rpm for 2 hours in an incubator shaker. The extract prepared was kept in a hot air oven for 1 hour at 50° C to concentrate it and get it ready for the upcoming purification process. Further, the TAC was determined before and after purification.

The purification of anthocyanins was done using 3 different solvents (Acetone, Ethanol: HCl: DW (80:20:1) and 2% Formic acid acidified methanol as mobile phases. Out of three mobile phases analysed, 2% Formic acid acidified methanol turned out to give the best results for anthocyanins. The primary criterion for the determination of the best mobile phase was TAC.

Figure 7. Purification of *S. cumini* anthocyanin extracts using Silica-Gel Column Chromatography

Several polar solvents, including acetone, methanol and ethanol were used as elution solvents because the phenolic acids and flavonoids are highly polar compounds. The highest extraction yields of target compounds were obtained when methanol was used as an elution solvent. It demonstrated that methanol should be suitable for desorbing phenolic acids and flavonoids from S-SIL. Therefore, methanol was finally selected as elution solvent (Wang et al., 2014). As shown in the graph (Figure 8) there is a variation in total anthocyanins of the concentrated and purified form of anthocyanin extracts from the fruit pulp of *S. cumini*.

There was an evident increase in total anthocyanin content when 2% Formic acid acidified methanol (374.22 mg C3G/ 100g (concentrated form) to 476.97 mg C3G/ 100g (purified form)) was used as a mobile phase, followed by the Ethanol: HCl: DW. However, in case of Acetone as a mobile phase, anthocyanin concentration after purification decreased.

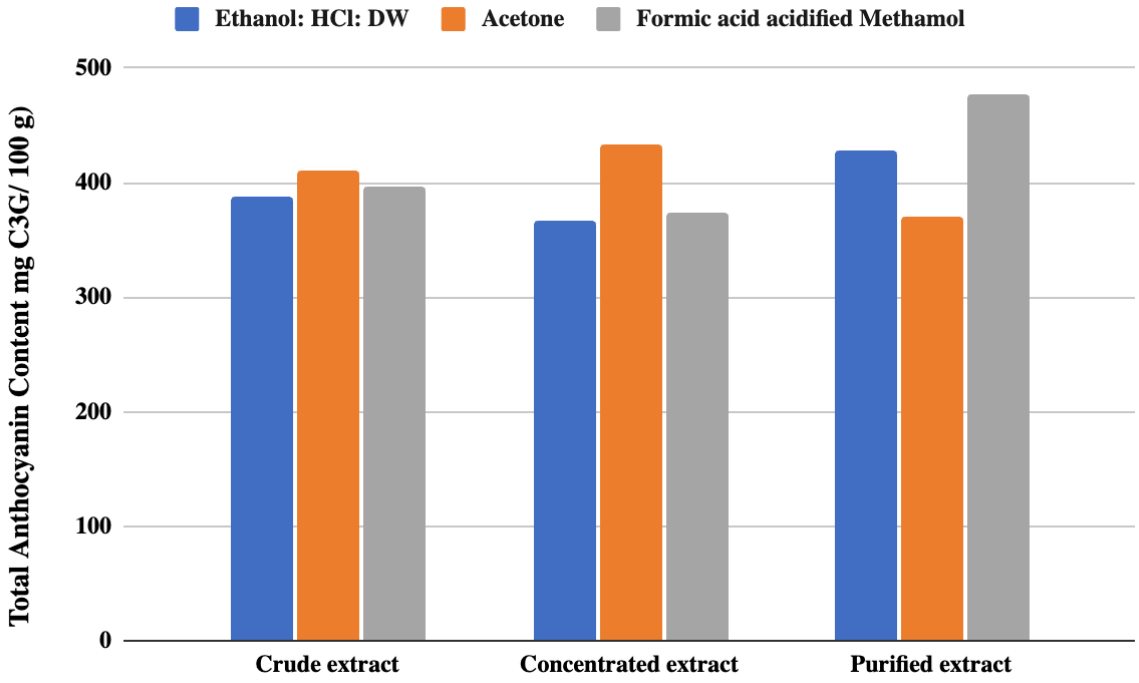

Figure 8. Variation in total anthocyanin content of *S. cumini* extracts before and after purification.

Similarly, in case of Ethanol: HCl: DW as mobile phase, it was observed that the concentration of *S. cumini* anthocyanins increased from 367.54 mg C3G/ 100g (concentrated form) to 428.15 mg C3G/ 100g (purified form). In contrast, in case of Acetone as mobile phase, it was observed that the concentration of *S. cumini* anthocyanins decreased from 433.67 mg C3G/ 100g (concentrated form) to 370.69 mg C3G/ 100g (purified form).

4.4 Stability of *S. cumini* anthocyanins

Stability analysis was done by considering various parameters such as Light, pH and Temperature.

4.4.1 Effect of light on the stability of anthocyanins

There is an impact of light on anthocyanin stability. The aromatic acyl group of anthocyanins absorbs the light energy which breaks the conjugated double bond causing photodegradation (Han et al., 2024). UV light photons are absorbed by organic molecules and affect conjugated bonds, such as aromatic rings, double rings, and compounds (e.g., disulfide bonds), which may reduce anthocyanin content Long-term exposure to UV-C light degrades anthocyanins and increases the total color difference (Koutchma et al., 2009). Fuleki et al.,1968 mentioned that red–blue pigments such as anthocianines in cranberry juice could be affected by UV

light. Anthocyanins are the colorful pigments in cranberries. The major anthocyanins in fresh cranberries and cranberry products are cyanidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-galactoside and peonidin-3-arabinoside Fuleki et al.,1968).

Three light conditions were maintained; shade, amber and 6 hours sunlight exposure. Out of all three a visible colour change (Figure 9) was observed in the sunlight-exposed sample which showed decreased anthocyanin content (Figure 10) on determining the anthocyanin content while as other two did not showed a visible change in colour as well as the anthocyanin content.This was because anthocyanins can be UV light affected in their double bonds and lead to degradation to render decolorization (Francis., 1975). While as in shade and amber storage conditions no exposure to UV light was there due to which there was no significant change observed except the one kept under sunlight exposure.

Figure 9. Effect on color of *S. cumini* anthocyanin extracts on sunlight exposure

UV light can cause degradation in the color of organic molecules of fruit products. UV energy is absorbed by double bonds in organic molecules, and these energized double bonds can react with oxygen, rendering single bonds. Accordingly, free hydroxyls can react with each end of the double bonds, at which point color may start to pale (Nassau et al.,1998).

A study analyzed the effects of light intensity on the anthocyanin stability of blueberry peels and found that anthocyanins were stable without the presence of light in indoor, but decreased when exposed in indoor or outdoor with increase of light intensity (Peng et al., 2016). A similar effect was observed by Liu et al. when light accelerated the breakdown of blueberry anthocyanins (Liu et al., 2018). In this sense, it was also reported that blueberry anthocyanins were less resistant when exposed to outdoor natural light, with a retention rate

below 65% after 10 days of light exposure, while indoors avoiding light, the retention rate was higher than 80% (Xu et al., 2019).

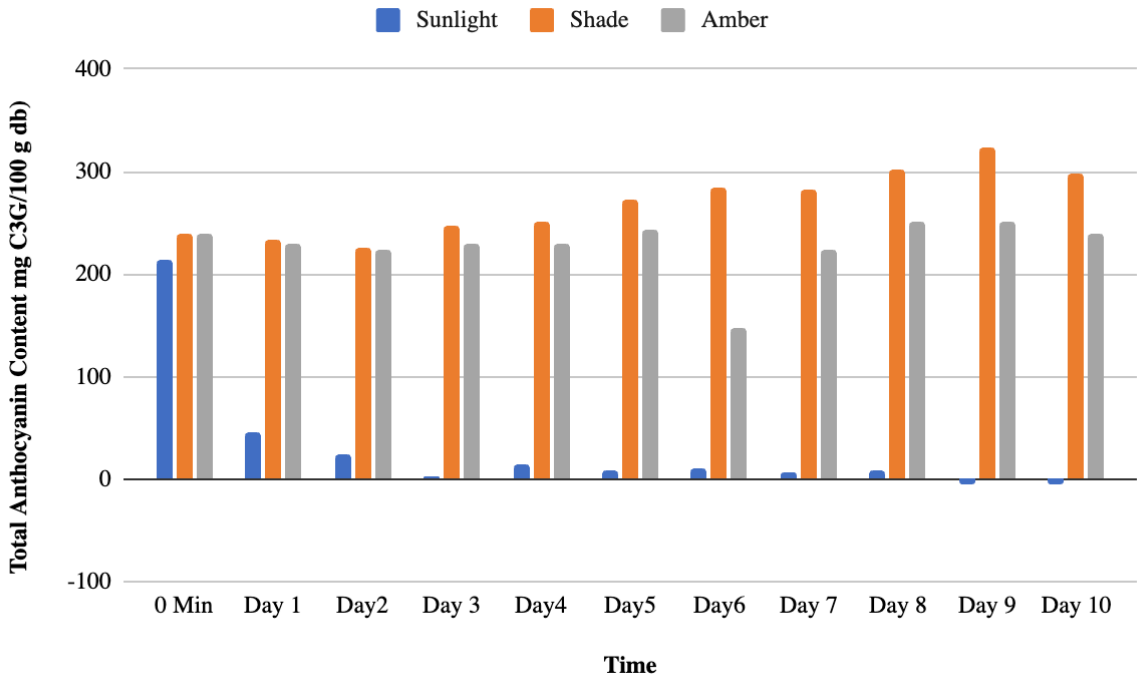

Figure 10. Effect of light exposure (sunlight, amber and shade) on stability of *S. cumini* anthocyanins

4.4.2 Effect of pH on stability of *S. cumini* anthocyanins

According to research, anthocyanins exhibit a reddish color in acidic environments (Khoo, Azlan, Tang, & Lim, 2017), however they tend to break down in alkaline environments, which impacts the stability of ring B, which is a light yellowish color. An increase in pH causes the anthocyanins to be degraded and look colorless due to the synthesis of chalcone and carbinol pseudobase (Castaneda-Ovando et al.,2009).

Figure 11. Effect of varying pH visible through color changes showing a) 3 pH, b) 5 pH, c) 7 pH.

In pH analysis, three pHs 3, 5 and 7 were selected for stability analysis of anthocyanins. A regular change in colour (Figure 11) was seen in all however anthocyanin content (Figure 12) did not show much prominent change in all the samples with different pH used.

Increasing pH cause greater destruction of anthocyanin in samples. Flavylium salts are stable only in highly acidic conditions. These salts loose the proton in higher pH and transform into quinoidal base, which is an unstable pigment, and immediately bond to water and form colourless compound called chromenol (Laleh et al., 2006).

Anthocyanin Structure and pH Dependent Extraction Characteristics from Blueberries (Vaccinium et al., 2021). It is in an acidic environment (pH = 1) that anthocyanins are found in the form of the red flavylium cation, which is highly soluble in water and gives rise to the red and purple colors. There is an abundance of quinoidal blue species at pH values between 2 and 4, and at pH values between 5 and 6, colorless substances called chalcones and carbinol pseudobases are visible . Ultimately, the anthocyanins will break down in accordance with the substituent groups at a pH greater than 7. Anthocyanin Structure and pH Dependent Extraction Characteristics from Blueberries (Vaccinium et al., 2021).

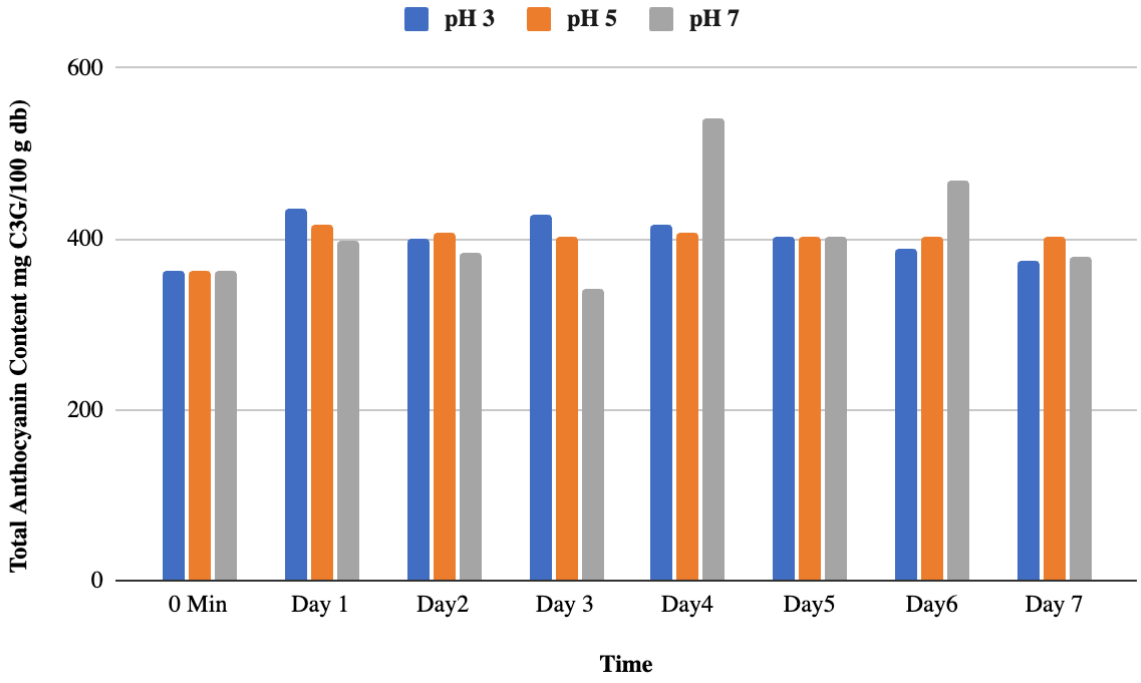

Figure 12. Effect of varying pH (3, 5 and 7) on stability of total anthocyanins in *S. cumini*

Ring B substituents and the presence of additional hydroxyl or methoxyl groups are responsible for the stability of anthocyanins, which in neutral environments decrease

aglycone stability. On the other hand, although in a neutral environment, aglycones are not stable, monoglycosides and diglycoside derivatives are more stable under these conditions, because sugar molecules will avoid the degradation of unstable intermediates into phenol and aldehyde acid molecules. In short, the stability of anthocyanins increases with increasing methylation and decreases with an increasing number of hydroxyl groups in the B ring of anthocyanidins (Fleschhut et al., 2006).

4.4.3 Effect of temperature on stability of anthocyanins (Thermal stability)

Temperature also is another factor, which has a role in destabilising the anthocyanin molecular structure; with increase in temperature we see a greater degree in anthocyanin destruction. It results in decay and discolouration. At high temperatures, thymol turns into pseudo-alkali glucoside, which opens up to generate chalcone glycoside. Alpha-dione, an isomer of chalcone, and chalcone glycoside are formed by further removal. The final products of this process include phenolic acid, aldehydes, cyanidin-3-glucoside, and geranium-3-glucoside (Connor et al., 2014).

We suggest that the speedy destruction of anthocyanin in higher temperatures could be due to hydrolyzation of 3-Glycoside structure, which has a protective effect in unstable anthocyanin. The other suggestion is that the hydrolyzation of the pyrilium ring resulted in production of chalkons, which are responsible for brown colour developed in food containing anthocyanin (Laleh et al., 2006).

Due to processing under high temperature for certain periods of time, foods can undergo color changes, the amount of anthocyanins but also their antioxidant capacity. Due to heat processing, anthocyanins can undergo a multitude of mechanisms such as glycosylation, nucleophilic attack of water, cleavage and polymerization that will cause the loss of this pigment and their degradation. Therefore, the temperature is another factor that affects the stability of the molecular structure of anthocyanins, so with increasing temperature the degradation of these compounds occurs (Laleh et al., 2005). Kirca in 2003 reported the degradation of anthocyanins in blood orange juice occur faster with increasing storage temperature (Kırca et al., 2003).

Three temperatures were chosen 50°C, 70°C and 90°C. Out of all three, a prominent and visible decrease in anthocyanin content was seen in the case of temperature set to 90°C due to the degradation of anthocyanin as a result of high temperature (Figure 13).

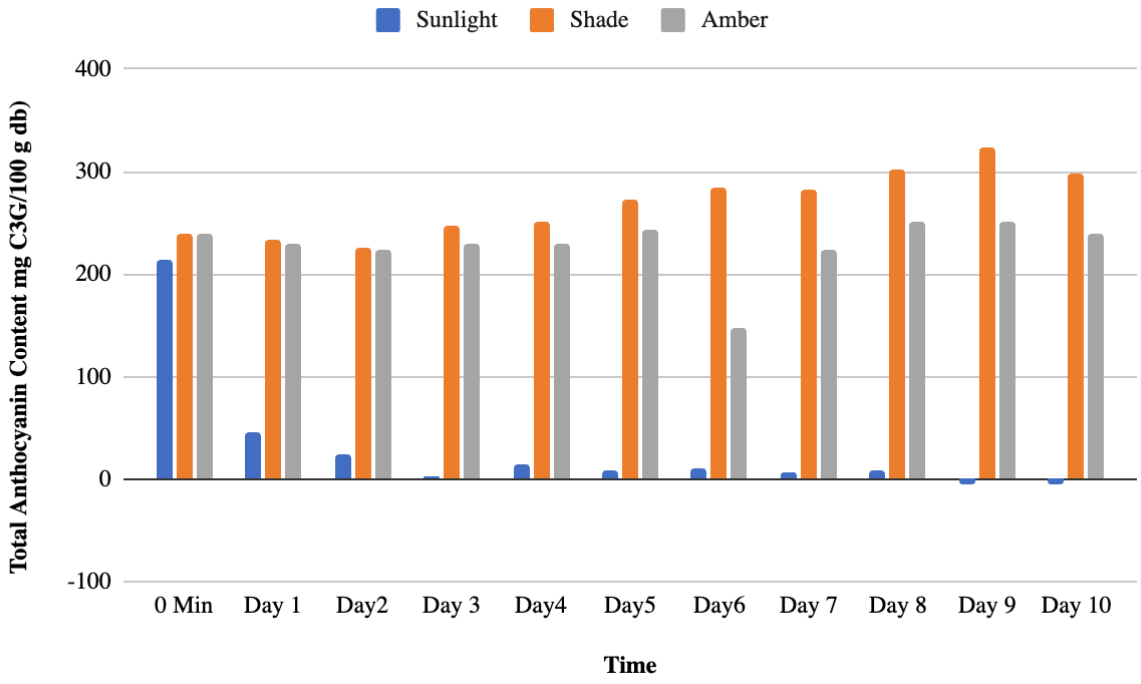

Figure 13. Effect of different temperatures (50°C, 70°C, 90°C) on stability of total anthocyanins in *S. cumini*

Sun et al. (2011) reported that the thermal degradation of anthocyanins occurs in three ways: the hydrolysis of the glycosidic bond, which forms a highly unstable aglycone anthocyanidin and proceeds through an intermediate α-diketone pathway to form aldehydes and benzoic acid derivatives. Another route involves the hydrolytic opening of the pyrrolic ring and subsequent hydrolysis of the glycosidic bond to generate the pseudo-base carbinol. The third form is the route in which the pseudo-carbinol is transformed into chalcone, and later, into glycosides of coumaric acid, which are both colorless (Sun et al., 2011). Samples placed at 80, 90, 100, 110, and 115 °C had a 50% loss of anthocyanins at the time intervals of 180, 115, 40, 15, and 10 min, respectively (Buckow et al., 2010).

Chapter - 5
Conclusion

Due to their abundant supply, beneficial physiological effects, and deep color, anthocyanins are widely employed in food, medicine, cosmetics, and other industries. The extraction and purification processes for anthocyanins are maturing in step with the ongoing advancement of research. The properties of the sample substrate and the extraction parameters (pH value, solvent, temperature, duration, etc.) are the primary factors influencing the extraction of anthocyanins. Anthocyanins are effective natural coloring agents that have a range of biological properties, such as hypoglycemic, anti-inflammatory, anti-cancer, and antioxidant properties. Anthocyanins' low stability and rapid disintegration restrict their use in a variety of industries, including food, cosmetics, and medicine. The relationship between anthocyanins and proteins has drawn more attention in recent years.. Numerous studies have suggested that the interaction between proteins and anthocyanins may improve the proteins' digestibility, foaming and emulsifying qualities, and stability as well as give them additional nutritional value. It is imperative that we integrate various analytical techniques to thoroughly examine the possible mechanisms of interaction between proteins and anthocyanins and to broaden the areas in which they can be used in the future.

Chapter - 6

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