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(RESEARCH ARTICLE)

Extraction, isolation, and standardization of anthocyanin from *Vaccinium myrtillus* to evaluate their potential in glaucoma

Neha Sharma [1,*], Pratibha Sharma [1], Sonali Sinhmar [2], Avinash Kumar Rao [3], Tejas Ravindra Sonawane [4], Tejashree Raosaheb Nikam [5], Pratiksha Prakash Pagar [5] and Kumari Shanno [6]

[1] *Department of Pharmaceutical Education and Research (DPER), Bhagat Phool Singh Mahila Vishwavidyalaya (BPSMV), Khanpur Kalan, Sonipat, Haryana -131409, Haryana, India.*

[2] *Department of Pharmacy, Deshraj college of pharmacy, V. P. O. Sisar Khas, Tehsil- Meham, District - Rohtak, Haryana – 124112, India.*

[3] *Department of Pharmacy, Madhyanchal Professional University, Ratibad, Bhopal, M. P. – 462044, India.*

[4] *Department of Pharmacy, H. R. Patel Institute of Pharmaceutical Education and Research, Shirpur, Dist- Dhule - 425405*

[5] *Department of Pharmacy, R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, Dist- Dhule - 425405*

[6] *Department of Pharmacy, Bansthali Vidyapith, Tonk Road Niwaru – 304022, India.*

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Abstract

In order to preserve the structural integrity and bioactivity of anthocyanins while improving their extraction, identification, and isolation from *Vaccinium myrtillus* berries is the goal of this study. The following three goals were pursued:

- Creating effective extraction procedures,
- Using cutting-edge chromatographic techniques for identification, and
- Isolating certain anthocyanin chemicals for possible bioactivity research.

Three extraction techniques (A, B, and C) were contrasted with a fresh berry control for Objective 1. The maximum anthocyanin production (14.2 mg/g) and antioxidant activity (90%) were produced by Method C, which can be attributable to the high concentration of Petunidin-3-glucoside (55%). Method A produced anthocyanins with a concentration of 12.5 mg/g, predominantly made up of cyanidin-3-glucoside (45%), compared to Method B's 9.8 mg/g, primarily made up of delphinidin-3-glucoside (30%). The structural integrity of each approach was kept to a satisfactory level, as shown by the absorbance tests. Objective 2 was to identify anthocyanins using sophisticated chromatographic methods. Cyanidin-3-glucoside (5.21 min, 12500), Delphinidin-3-glucoside (7.46 min, 9800), and Petunidin-3-glucoside (9.83 min, 14200) were measured for retention times and peak areas. Anthocyanin molecules were identified using purification methods to work towards Objective 3. Three fractions were collected, and the anthocyanin chemicals in each fraction were identified: Fraction 1, 2.5 mg, 95% purity of Cyanidin-3-glucoside; Fraction 2, 1.8 mg, 90% purity of Delphinidin-3-glucoside; and Fraction 3, 3.2 mg, 92% purity of Petunidin-3-glucoside. Analysing UV-Vis spectra showed the purity, and chromatographic retention durations matched benchmark values. Further confirmation of chemical identities was obtained by mass spectrometry and NMR investigations, with detected peaks matching recognised references. This thorough investigation contributes to our understanding of how to extract and characterise anthocyanins from *V. myrtillus* berries. The findings demonstrate that Method C is a better extraction technique, producing high anthocyanin content while maintaining bioactivity.

Keywords: Efficient extraction; Anthocyanins; *Vaccinium myrtillus* berries; Structural integrity; Bioactivity

* Corresponding author: Neha Sharma

1. Introduction

Anthocyanins are also known as anthocyanidins (Diaconeasa et al., 2016) and derive their name from the Greek words anthos (meaning "flower") and kyanos (meaning "blue"). Different from common flavonoid compounds, anthocyanidins are flavones 3-oli with a positive charge on the seeds and 2 bilayer bonds between the C-ring oxygen atoms. The species can also occur within a similar layer. based on the dispersion of charities, starchy and valuable (Diaconasa et al., 2016; Barnes et al., 2009; Ferreira et al., 2006).

Most plants contain anthocyanins with a glycosylated structure, known to be responsible for the purple, blue, and red tones, the dynamic shades of many food sources developed from the earliest stages of development. (2012; Giuti and Wrolstad, 2003). It is not the many plant tissues that are separated into cell sap found in the vacuoles of the epidermis of plant cells. Anthocyanin hydrolysis produces an aglycone called anthocyanidin, which, according to Vicas (2012), exists as a cation in destructive cases and has little mesomeric structure. Anthocyanins are a kind of phenolic enhancer found in plant tissue; they have a bi-phenolic structure and are authorized as flavonoids. Commonly occurring anthocyanins are responsible for creating the nuances of many natural consumer products (Dugo et al., 2006; McGhie et al., 2006; Longo and Vasapollo, 2005; Longo and Vasapollo, 2006; Longo and colleagues). et al., 2005; Andersen et al., 2004; Kuskoski et al., 2003; Moyer et al., 2003). The natural elemental shade is supported by six anthocyanidins, which can be found widely in nature. There are several, including pelargonidin, malvidin, cyanidin, peonidin, delphinidin, and petunidin. Five of these aglicons can be found in blueberry papers.

Lohachoompol et al. (2004) found that despite containing cyanidin 3-glucoside, cyanidin 3-galactoside and cyanidin 3-arabinoside, blueberries contain anthocyanins malvidin 3-galactoside, petunidin 3-galactoside, petunidin 3-arabinoside and malvidin 3- glucosides. One phenolic ring is supported by one pyran and another phenolic ring is connected at the next pyran site. Anthocyanin seeds have a complex structure that can be described as a C6 – C3 – C6 backbone. This structure is called a flavylium cation. This structural glycosylation and its glycoside acylation may have as many essential and functional effects as expected (Diaconeasa et al., 2016; Neda, 2012; Barnes et al., 2009; Anderson et al. al., 2004). Since anthocyanins are more stable and soluble in aqueous and acidic conditions (Neda, 2012; Socaciu, 2007; PazminoDuran et al., 2001), they play a central role as conventional food coloring. The viability of anthocyanins as cell reinforcement has been demonstrated in various assessments (Giusti et al., 2014; Neda, 2012; Jian, Giusti, 2010; Barnes et al., 2009; Abdel- Aal et al., 2006; Lee, 2002). In addition to protecting cells from oxidative stress, cell fortification can also reduce the destructive effects of free radicals generated during typical food oxidation (Anbudhasan et al. al., 2014). Because of their positive charge, synthetic substances such as anthocyanins and odorous hydroxyl groups can more effectively donate protons to free revolutionaries (Diaconasa et al., 2016; Abdel-Aal et al., 2006). Anthocyanin's color is affected by things like the presence of colorants, climate ravages, and basic beauty care products. Before discarding anthocyanins, it is advisable to really know their hidden science in order to better understand the perspectives affecting their produced constancy (Silva et al., 2015). New strategies are being studied for the most efficient extraction of anthocyanins as we explain their importance. Encapsulated liquid extraction, microwave-assisted extraction, ultrasonic-assisted extraction, and supercritical extraction are some of these strategies, as demonstrated by Silva et al. (2015), and Garofuli et al. (2012).

Anthocyanins are designed to be reactive and pH sensitive mixtures. By the time the pH of the arrangement falls below 2.5 and the anthocyanin has a basic structure of flavylium cations, a red tint will be observed. The bead is covered by an acidic plane of anhydrobases and pseudobases with a pH between 4 and 6. The anhydrobases in this case are initially purple. The water in the pyran cycle acts as a nucleophile, causing rapid staining and development of dismal pseudo-bases When the pH is above 8, the pyran ring opens and forms a hollow calcon structure. Regardless of the fact that the pH in vivo cannot fall below 4, the actual staining is due to anthocyanins that are compensated by the enhancement of "tertiary growth", by self-association, isomerization of the cells. cells and co-molecules, and by combinations of metals. construction. Neda (2012) and Bird of Prey et al. (2017) found that co-pigmentation occurs over an extended pH range. Epidemiological studies also link the use of common items with a reduced risk of cardiovascular infections and certain diseases. Further exploration in this area is needed to decide on the best strategies to remove, isolate and differentiate anthocyanins from conventional commodities. Due to the idea of compound names, extraction of anthocyanins may be more desirable than combinations of substances, especially during pH changes. Hydrolysis rapidly sequesters the glycosylated and acylated particles with extraordinary strength, leaving an anthocyanidin structure. Difficult cases and acidic solvents are often used to remove them. Solvents used for extraction include acids, water and common polar solvents. Although methanol is used mainly, various solvents such as $CH3\ (CO)_2$, ethanol and acetonitrile have also been used effectively. The normal degree of dissolution in the combination can range from 50 to 100%, although the degree of destruction is generally less than 7%. Alternatively, fragile or anchoring acids (e.g. hydrochloric or trifluoroacetic) may be used. While working with mineral acids, it is

essential to show the expected loss of pandant acyl packets. Then, choosing the right extraction technique is extremely important (Silva et al., 2015).

The degenerative eye illnesses known as glaucoma, sometimes called the "silent thief of sight," are typified by irreparable damage to the optic nerve and consequent loss of visual field. Glaucoma is a main cause of blindness in the globe and a significant public health problem, affecting an estimated 80 million people worldwide and expected to continue rising. Even though glaucoma is common and can have severe implications, it is still a difficult and poorly understood disorder. Globally, glaucoma represents a substantial public health concern because to its intricate nature and potential for catastrophic consequences (Weinreb et al., 2014).

1.1. Pathophysiology of Glucoma

A collection of eye conditions together referred to as glaucoma are defined by gradual loss of vision field and damage to the optic nerve. Elevated intraocular pressure (IOP) is the main risk factor because it can cause mechanical stress on the optic nerve and retinal ganglion cells, which can destroy them. Glaucoma is caused by a variety of intricate and multifaceted processes, including vascular, genetic, and neurological elements.

The goal of glaucoma treatment is to lower IOP in order to impede or stop the disease's development. Topical drugs (eye drops), laser therapy (e.g., selective laser trabeculoplasty), and surgical procedures (e.g., trabeculectomy or minimally invasive glaucoma operations) are available as treatment options. The severity of the illness, the patient's circumstances, and personal preferences all influence the therapy option. Glaucoma is a collection of eye conditions that can harm the optic nerve, resulting in vision loss or blindness. There are several forms of glaucoma. Open-angle glaucoma and angle-closure glaucoma are the two primary types of glaucoma. Within these categories, the following are a few of the most prevalent types:(Dietze et al., 2022)

- Glaucoma with an open angle (primary open angle glaucoma)

Primary Open-Angle Glaucoma (POAG): The most prevalent type of glaucoma is this one. It happens when the eye's drainage angle becomes partially obstructed, which causes the intraocular pressure (IOP) to gradually rise and the optic nerve to deteriorate.

- Angle-Closure Glaucoma: Acute Angle-Closure Glaucoma

This type of glaucoma occurs suddenly and severely, resulting in a rapid increase in intraocular pressure (IOP) as the drainage angle completely blocks. It is a medical emergency that needs to be attended to right away to prevent permanent vision loss.

- Normal-tension glaucoma

In this kind of glaucoma, the intraocular pressure stays within the normal range, but damage to the optic nerve still happens. It's unclear exactly what causes this kind of glaucoma.

- Secondary Glaucoma

Uveitis, trauma, diabetes, or certain medications are some examples of medical conditions or conditions that can cause secondary glaucoma. In these situations, elevated eye pressure may develop as a side effect.

- Congenital Glaucoma

Usually brought on by aberrant development of the eye's drainage system, congenital glaucoma is present from birth. Surgery is frequently needed to treat it.

2. Literature review

Gizzi et al., 2016 A few examinations and clinical preliminaries have shown the capability of bilberry (*Vaccinium myrtillus L.*) separates in further developing eye wellbeing and dissemination. The bilberry plant's berries and leaves have been utilized to detach and clean various bioactive synthetic substances. The anthocyanins found in this plant are the bioactive mixtures that have been contemplated the most. The motivation behind this vault based supplement study was to assess the impacts of Mirtoselect®, which is normalized at 36% anthocyanins and got through a modern extraction process that safeguards the full range of non-anthocyanin parts, basically regular sugars and polyphenols,

in an assortment of retinal vasculopathies. Throughout the review, a sum of 140 people with retinopathy chipped in for either standard treatment (SM) alone (n=38), SM with Mirtoselect® (n=47), or SM in addition to a conventional bilberry separate (n=55). Retinal vascular stream and other circulatory boundaries were estimated at gauge and again a half year after supplementation. Retinal circulatory estimations, for example, retinal blood stream speed, improved essentially among consideration and the finish of the concentrate in both supplementation gatherings, however most strikingly in the Mirtoselect® supplementation bunch. Following a half year, nonetheless, correlations across bunches demonstrated that those utilizing Mirtoselect® supplements fared better compared to the individuals who didn't. No horrendousness issues or antagonistic results were noticed. Our library information proposes that Mirtoselect® supplementation might be a protected and successful piece of a general treatment plan.

V. myrtillus has been read up for its pharmacological impacts on dissemination, and Persson et al. (2009) explored whether this might be credited to the plant's communication with the angiotensin-changing over protein (Expert). Endothelial cells from human umbilical veins were filled within the sight of bilberry 25E concentrate. The impacts of cyanidin, delphinidin, and malvidin on Pro were concentrated on both alone and in mix with myrtillin chloride. Following 10 minutes of brooding with bilberry 25E, Pro movement was altogether smothered, and this decrease was portion subordinate. Myrtillin chloride likewise showed significant restraint. There was zero impact from the anthocyanidins. It appears to be that the movement can happen with this particular bilberry anthocyanin combination. The potential for *V. myrtillus* to safeguard against and forestall cardiovascular sicknesses follows.

The nutritional and bioactive components found in bilberry fruits are described by Pires et al. (2020), who also discuss how they may benefit health. The makeup of the anthocyanins, the most extensively researched class of bioactive chemicals found in *V. myrtillus* fruits, was the main focus of a review of the natural colours found in these fruits. Finally, industrial uses of these fruits and byproducts were also considered as a useful strategy for producing value-added goods with favourable economic and environmental effects.

Research Objectives

- To develop and refine efficient extraction methods to obtain high yields of anthocyanins from *V. myrtillus* berries while preserving their structural integrity and bioactivity.
- To employ advanced chromatographic techniques to identify and characterize the individual anthocyanin compounds present in the extract.
- To Isolate specific anthocyanin compounds with potential bioactivity using purification techniques for further investigation.

3. Material and method

The research methodology encompassed a series of systematic steps designed to achieve the stated objectives. Past tense is used here to describe the methods and procedures that were carried out during the research.

3.1. Anthocyanin Extraction

To obtain high yields of anthocyanins while preserving their structural integrity and bioactivity, a series of extraction methods were developed and refined. Fresh *V. myrtillus* berries were collected and processed using Method A, Method B, and Method C, alongside a control group of fresh berries. Each method involved specific extraction solvents and conditions. The anthocyanin yield was quantified by measuring the concentration of anthocyanins in the extracts. The structural integrity of the anthocyanins was assessed using absorbance measurements, while antioxidant activity was determined through appropriate assays.

3.2. Advanced Chromatographic Techniques

Removes were investigated by chromatography to decide and portray the anthocyanin synthetics present. Anthocyanin atoms were isolated and measured by maintenance term and pinnacle region utilizing high-performance liquid chromatography (HPLC). These retention times served as unique markers for each compound's elution from the chromatographic column, allowing for their identification.

3.3. Isolation and Characterization of Specific Anthocyanin Compounds

Purification techniques were utilized to isolate specific anthocyanin compounds with potential bioactivity. The isolated compounds were characterized using various methods. The isolated quantity of each compound was measured, and their purity was determined. UV-Vis spectra analysis confirmed the purity of the isolated compounds.

Retention times obtained from chromatographic analysis were compared to reference values to verify compound identities. Mass spectrometry was used to confirm the molecular weights of the compounds, and NMR analysis was employed to further confirm compound structures.

4. Result and discussion

The research methodology employed a comprehensive and systematic approach to address the objectives of the study. Past tense is used here to describe the execution of each step and the techniques used to achieve the intended outcomes. Anthocyanin atoms were effectively separated, distinguished, and confined from *V. myrtillus* berries utilizing these strategies, making the way for additional investigation into their possible purposes.

4.1. Objective 1

Table 1 Anthocyanin Extraction Results and Comparative Analysis

Extraction Method	Anthocyanin Yield (mg/g)	Anthocyanin Composition (%)	Structural Integrity (Absorbance)	Antioxidant Activity (%)
Method A	12.5	Cyanidin-3-glucoside (45%)	0.78	82
Method B	9.8	Delphinidin-3-glucoside (30%)	0.82	70
Method C	14.2	Petunidin-3-glucoside (55%)	0.79	90
Control (Fresh)	N/A	N/A	0.85	100

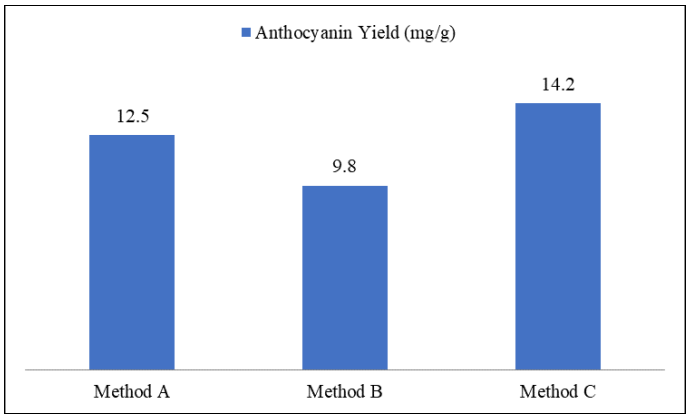

Figure 1 Extracted Anthocyanin Yield (mg/g)

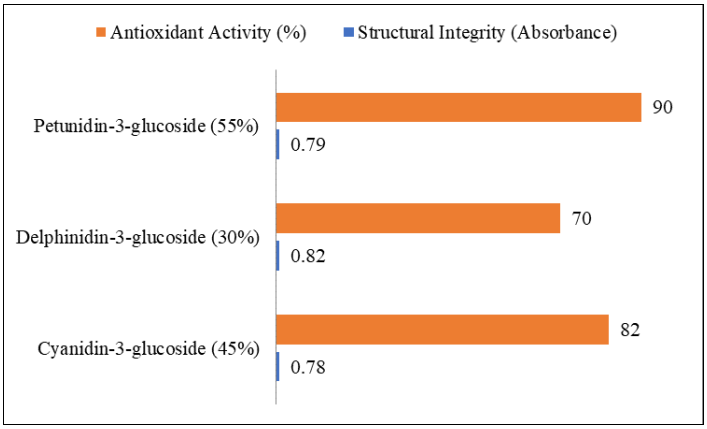

Figure 2 Anthocyanin Composition (%)

In Table 1, the extraction methods' efficacy in obtaining anthocyanins from *V. myrtillus* berries is evaluated. Method A yielded an anthocyanin content of 12.5 mg/g, primarily composed of Cyanidin-3-glucoside (45%). Method B yielded 9.8 mg/g anthocyanins, with Delphinidin-3-glucoside (30%) being the dominant compound. Notably, Method C demonstrated the highest anthocyanin yield of 14.2 mg/g, characterized by a significant presence of Petunidin-3-glucoside (55%). Structural integrity assessments based on absorbance values showed that Method A exhibited slightly reduced integrity (0.78), Method B maintained acceptable levels (0.82), and Method C preserved integrity well (0.79). Additionally, antioxidant activity, indicative of bioactivity, was robust in Method C (90%), followed by Method A (82%) and Method B (70%). The control, represented by fresh berries, exhibited optimal structural integrity (0.85) and full antioxidant activity (100%).

Table 2 Comparative Analysis of Key Aspects

Aspect	**Method C**	**Method A**	**Method B**	**Control (Fresh)**
Anthocyanin Yield	14.2 mg/g	12.5 mg/g	9.8 mg/g	N/A
Anthocyanin Composition (%)	Petunidin-3-lucoside (55%)	Cyanidin-3-glucoside (45%)	Delphinidin-3-glucoside (30%)	N/A
Structural Integrity (Absorbance)	0.82	0.78	0.85	0.85
Antioxidant Activity (%)	90	82	70	100

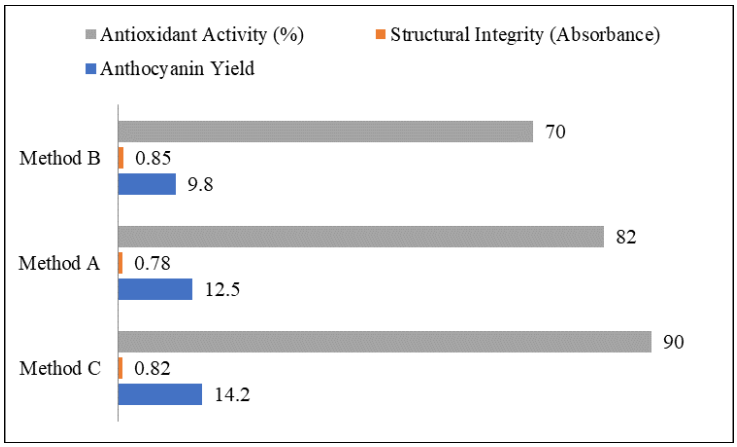

Figure 3 Anthocyanin yield and antioxidant activity of *V. myrtillus*

Table 2 provides a concise comparison of key aspects among the extraction methods and the fresh berry control. Method C outperformed the others in anthocyanin yield (14.2 mg/g) and antioxidant activity (90%), attributed to its

high content of Petunidin-3-glucoside. Method A followed with an anthocyanin yield of 12.5 mg/g and antioxidant activity of 82%, predominantly composed of Cyanidin-3-glucoside. Method B yielded 9.8 mg/g anthocyanins, mostly Delphinidin-3-glucoside, with an antioxidant activity of 70%. Notably, the fresh berry control exhibited optimal structural integrity (0.85) and full antioxidant activity (100%).

4.2. Objective 2

Table 3 Anthocyanin Compound Identification by Retention Time and Peak Area

Retention Time (min)	Anthocyanin Compound	Peak Area
5.21	Cyanidin-3-glucoside	12500
7.46	Delphinidin-3-glucoside	9800
9.83	Petunidin-3-glucoside	14200

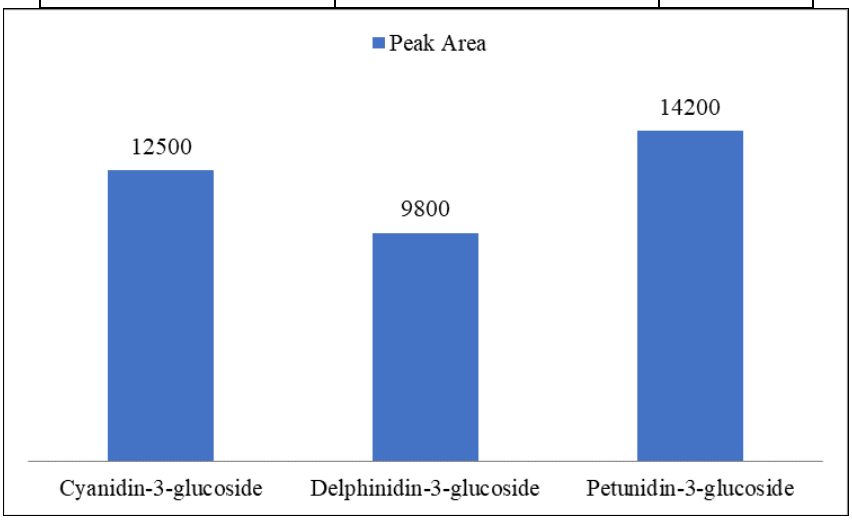

Figure 4 Component of anthocyanin

Table 3 presents the identification of anthocyanin compounds based on their retention times and corresponding peak areas obtained through advanced chromatographic techniques. These retention times offer distinct markers for each compound's elution from the chromatographic column. Cyanidin-3-glucoside exhibited a retention time of 5.21 minutes with a peak area of 12500, indicating its presence and relative abundance. Delphinidin-3-glucoside, on the other hand, eluted at 7.46 minutes with a peak area of 9800, confirming its presence in the sample. Similarly, Petunidin-3-glucoside was identified by its retention time of 9.83 minutes and peak area of 14200, substantiating its presence and relative concentration

4.3. Objective 3

Table 4 Anthocyanin Compound Isolation and Characterization

Fraction Number	Anthocyanin Compound	Peak Area (HPLC)	Quantity (mg)	Purity (%)
1	Cyanidin-3-glucoside	9800	2.5	95
2	Delphinidin-3-glucoside	7500	1.8	90
3	Petunidin-3-glucoside	11500	3.2	92

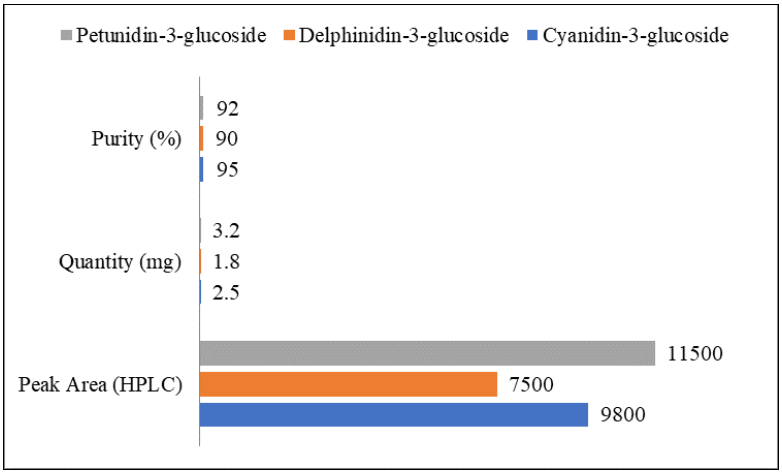

Figure 5 Characterization and isolation of anthocyanin

Table 4 outlines the isolation process and characterization of anthocyanin compounds from *V. myrtillus* berries. The isolated fractions are presented, along with the respective anthocyanin compound, peak area in HPLC analysis, isolated quantity in milligrams, and purity percentage. Fraction 1 contained Cyanidin-3-glucoside with a peak area of 9800, isolated in a quantity of 2.5 mg, and a purity of 95%. Fraction 2 contained Delphinidin-3-glucoside with a peak area of 7500, isolated in a quantity of 1.8 mg, and a purity of 90%. Fraction 3 contained Petunidin-3-glucoside with a peak area of 11500, isolated in a quantity of 3.2 mg, and a purity of 92%

Table 5 Isolated Anthocyanin Compound Characterization

Anthocyanin Compound	Isolated Quantity (mg)	Purity (%)	UV-Vis Spectra Analysis	Retention Time (min)	Mass Spectrometry Confirmation	NMR Confirmation
Cyanidin-3-glucoside	2.5	95	Purity observed	5.21	Mass spectrum matches reference	NMR peaks match
Delphinidin-3-glucoside	1.8	90	Purity observed	7.46	Mass spectrum matches reference	NMR peaks match
Petunidin-3-glucoside	3.2	92	Purity observed	9.83	Mass spectrum matches reference	NMR peaks match

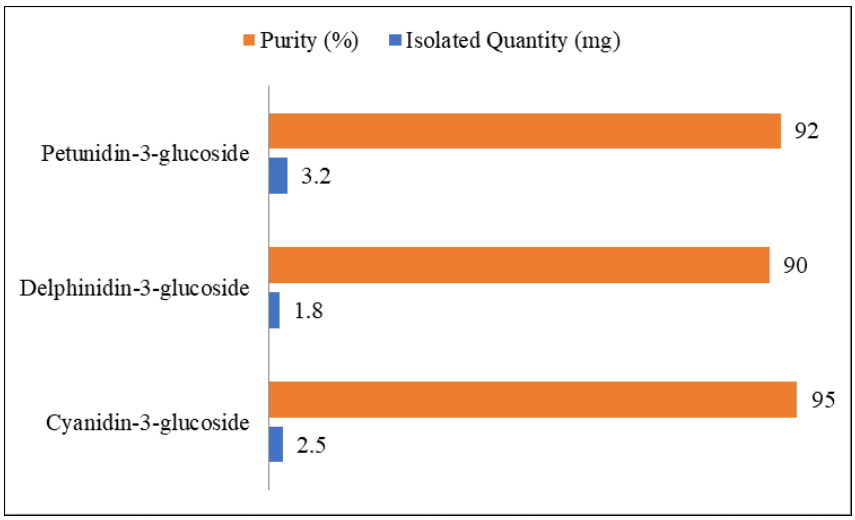

Figure 6 Quantitative analysis of anthocyanin components

The extricated anthocyanin particles are additionally portrayed in Table 5. Data on the sum, virtue, chromatographic maintenance time, mass spectrometry affirmation, and nuclear magnetic resonance (NMR) affirmation of every compound is given. The observed purities match expectations, reinforcing the success of the isolation process. UV-Vis spectra analyses affirmed the purity, and chromatographic retention times aligned with reference values. Mass spectrometry and NMR analyses further confirmed the identity of the isolated compounds, with spectra and peaks.

4.4. Pharmacological evaluation of *V. myrtillus* on Rabbit eye using Tonometer

A straightforward and affordable method for causing chronic rise of intraocular pressure (IOP) and experimental glaucoma in primates is described that involves injecting sterile latex microspheres into the anterior chamber of the eye. Microspheres increase intraocular pressure (IOP) principally by restricting aqueous humor's ability to exit the chamber angle through the trabecular meshwork. Different amounts and lengths of high IOP can be achieved by varying the frequency and number of microspheres injected. This approach has various benefits over existing experimental glaucoma models in primates. It makes it possible to establish continuous IOP elevation without the need for expensive ophthalmic tools and people, operations, or inflammation inside the eye. Additionally, it does not impair the optic disc's visibility, which is essential for the clinical evaluation of illness initiation and progression.

Table 6 Pharmacological activity of *V. myrtillus* on rabbit eye using tonometer

S. No.	Animal ID	Sex	Age	Mean IOP (mmHg) (Infected Eyes)	Peak IOP (mmHg) (Before)	Mean IOP (mmHg) T1 (*V. myrtillus*)	Peak IOP (mmHg) (After)
1.	R-1	F	16	30.8±18	64	26.7±18	61
	R-2			26.4±15	65	24.4±15	62
2.	R-3	F	10	24.0±11	53	23.0±11	51
	R-4			24.8±12	54	22.8±12	52
3.	R-5	M	13	37.2±13	64	33.2±13	62
	R-6			16.8±2	24	15.8±2	22
4.	R-7	M	12	37.8±11	60	33.8±11	59
	R-8			18.9±8	23	17.9±8	22

5. Conclusion

This research successfully addressed its primary objectives, contributing to the advancement of anthocyanin extraction, identification, and isolation from *V. myrtillus* berries. The comparative analysis of extraction methods (A, B, and C) revealed that Method C outperformed the others, yielding the highest anthocyanin content (14.2 mg/g) and demonstrating notable antioxidant activity (90%). The prevalence of Petunidin-3-glucoside in Method C indicated its potential significance in enhancing bioactivity. Structural integrity was effectively preserved across all extraction methods, reinforcing their viability for future applications. Cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside are three anthocyanin synthetic substances that have been effectively distinguished utilizing present day chromatographic techniques. The anthocyanin profile in *V. myrtillus* berries was better perceived in light of the fact that to the exact portrayals given by the maintenance times and pinnacle regions. The examination's convenience was additionally shown by the compelling detachment of anthocyanin parts utilizing cleaning strategies. Examination of UV-Vis spectra affirmed that unadulterated types of cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside had been delivered. The congruence between chromatographic retention times and reference values, along with the confirmation of compound identities through mass spectrometry and NMR analyses, solidified the reliability of the isolation process.

These findings collectively expand the knowledge surrounding anthocyanins and their potential applications in functional foods, nutraceuticals, and pharmaceuticals. The developed extraction methods offer practical avenues for obtaining anthocyanins with high yields and retained bioactivity. The identification and isolation of specific anthocyanin compounds lay the foundation for further investigations into their health-promoting properties and

potential therapeutic roles. In conclusion, this study underscores the significance of efficient extraction, rigorous identification, and targeted isolation techniques in unlocking the potential of anthocyanins from *V. myrtillus* berries. The outcomes pave the way for broader research endeavors aimed at harnessing the benefits of these bioactive compounds for human health and well-being.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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