METHOD VALIDATION OF DELPHINIDIN-3-O-GLUCOSIDE CHLORIDE IN RAT PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

NUR ATIELAH NABILA BT MOHD SHAFAWI

UNIVERSITI SAINS MALAYSIA

2022

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by

NUR ATIELAH NABILA BT MOHD SHAFAWI

Dissertation submitted in partial fulfillment of
the requirements of the degree of
Master of Science (Biomedicine) Mixed Mode

SEPTEMBER 2022

ACKNOWLEDGEMENT

Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis. First and foremost, I would like to express my deepest appreciation to my supervisor, Associate Professor Dr Wan Amir Nizam Wan Ahmad for providing me with the opportunity to do this research project under his supervision. Without his guidance in this project and throughout the process, this thesis would have never been accomplished. My appreciation is also dedicated to my co-supervisor, Dr Ruzilawati Abu Bakar for her valuable guidance throughout the project. I am extremely thankful for the time she has given on research work discussion and thesis preparation. Her expertise and knowledge were truly a great help and has steered me in completing this research.

I extend my gratitude to all the staff from Theurapeutic Drug Monitoring Laboratory (TDML) and Pharmacology Laboratory (School of Medical Sciences), particularly Encik Azaha, Mrs Halijah Miran, Mr Wan Mohd Hafiz and Mr Mohd Lukman for their help and useful technical advice. I greatly appreciate the time they have taken to share their knowledge with me.

I have been blessed to have my friends, Nurul Iffah and Iffah Izzati who has been through thick and thin with me throughout the project. I would like to thank them for their advice and support which contributed so much to this research. Finally, my deep and sincere gratitude to my beloved family for their continuous support and encouragement. This accomplishment would not have been possible without them. This research was financially supported by the Ministry of Agriculture's Herbal Research Grant Scheme

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LIST OF SYMBOLS

%	Percentage
<	Less than
>	More than
^{0}C	Degree Celsius
µg	Microgram
µL	Microliter
ng	Nanogram
g	gram
cm	Centimetre
g/mol	Gram per moles
kJ	Kilojoule
kcal	Kilocalories
mg	Milligram
mL	Millilitre
ng/mL	Nanogram per millilitre
mL/min	Millilitre per minute
min	Minutes
rpm	Revolutions per minute
et al.	*et alii* – 'and others'
R^2	Coefficient determinination

LIST OF ABBREVIATIONS

ACH	Acetylcholine
BRIS	Beach Ridges Interspersed with Swales
Cy3G	Cyanidin-3-O-glucoside
D3S	Delphinidin-3-sambubioside
FDA	Food Drug Administration
GSH	Glutathione
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
pH	Potential of hydrogen
PCA	Protocatechuic acid
PGA	phloroglucinaldehyde
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SPE	Solid-phase extraction
TAC	Total anthocyanin content

PENGESAHAN KAEDAH DELPHINIDIN-3-O-GLUKOSIDA KLORIDA DALAM PLASMA TIKUS OLEH KROMATOGRAFI CECAIR BERPRESTASI TINGGI (HPLC)

ABSTRAK

Hibiscus sabdariffa (Roselle) adalah salah satu tumbuhan perubatan yang sangat dikaji kerana tinggi dengan antosianin, yang penting dalam merawat pelbagai penyakit. Kajian ini dijalankan ke atas kaedah kromatografi cecair berprestasi tinggi (HPLC) untuk mengesan dan mengukur delphinidin-3-0-glucoside klorida, salah satu antosianin yang terdapat dalam H.*sabdariffa* dalam plasma tikus. Tambahan pula, kaedah kromatografi cecair berprestasi tinggi (HPLC) telah disahkan mengikut kaedah pengekstrakan fasa pepejal untuk menunjukkan bahawa kaedah analisis ini sesuai dan memastikan kebolehpercayaannya untuk menjalankan kajian lanjut farmakokinetik *H.sabdariffa* dan bioavailabiliti H.*sabdariffa* dalam plasma tikus. Enam parameter pengesahan dinilai mengikut garis panduan FDA: kelinearan, pemulihan, ketepatan, kejituan, had pengesanan, dan had pengkuantifikasi. Lengkung penentukuran delphinidin-3-0-glucoside klorida mempunyai penentuan pekali (R^2) ≥95.0%. Peratusan pemulihan delphinidin3-0-glucoside adalah 80.377%. Delphinidin-3-0-glucoside klorida juga menunjukkan ketepatan dan kejituan yang baik. Had pengesanan dan pengkuantifikasi kaedah untuk delphinidin-3-0-glucoside klorida masing-masing adalah 47ng/mL dan 213ng/mL. Kesimpulannya, kaedah HPLC yang disahkan dalam kajian ini boleh digunakan untuk menentukan tahap antosianin dalam kajian farmakokinetik roselle masa depan.

METHOD VALIDATION OF DELPHINIDIN-3-O-GLUCOSIDE CHLORIDE IN RAT PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

ABSTRACT

Hibiscus sabdariffa (Roselle) is one of the enormously explored medicinal plants with high anthocyanin, which is essential in treating various diseases. This study was conducted on the high-performance liquid chromatography (HPLC) method for detecting and quantifying delphinidin-3-0-glucoside chloride, one of the anthocyanins present in *Hibiscus sabdariffa* in the rat plasma. In addition, the HPLC method was validated following the solid-phase extraction method to demonstrate that this analytical method is suitable and to assure its reliability of H.*sabdariffa* pharmacokinetics and bioavailability study in rat plasma. Six validation parameters were assessed according to FDA guidelines: linearity, recovery, precision, accuracy, limit of detection, and limit of quantification. The calibration curve of delphinidin-3-0-glucoside chloride had a coefficient determination (R2) of ⩾95.0%. The recovery percentage of delphinidin3-0-glucoside was found to be 80.377%. Delphinidin-3-0-glucoside chloride also showed good precision and accuracy. The method detection and quantification limits for delphinidin-3-0-glucoside chloride were 47ng/mL and 213ng/mL, respectively. In conclusion, the validated HPLC method in this study can be used to determine anthocyanins in the future pharmacokinetic study of roselle.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Medicinal plants using natural resources have been utilised extensively by humankind for centuries. It has also become the oldest form of healthcare known to humans. The World Health Organization (WHO, 2004) notes that the development of novel herbal medicines, phytonutrients, or nutraceutical products has increased in recent decades. About 80% of the world population living in this developing world still have faith in herbal medicines products as their primary sources of healthcare (Ekor, 2014). The therapeutic potential of herbal drugs relies on their forms, such as the isolation of active constituents, the parts of a plant, or simple extracts. Herbal medicine often synergises portions of plants, simple extracts, or isolated active constituents. Therefore, one of the vital requirements for drug development is to define the composition of medicines before they are distributed to the population.

Malaysia is not an exception becoming one of the countries that use medicinal plants as their primary sources of healthcare. Malaysia's forest is one of the world's 12 biodiversity abundance sites. The diversity of floral species found in Malaysia's tropical rainforest becomes a potential source for the use of medicinal plants (Lee et al., 2014). Roughly around 1300 species of medicinal plants had been recorded in Peninsular Malaysia and 7411 plant species in Sabah, Malaysia (Kulip et al., 2010). "Roselle," or its name, *Hibiscus sabdariffa*, is one of the enormously explored medicinal plants. It is part of the Malvaceous family and has more than 300 species scattered worldwide in subtropical and tropical regions. Moreover, roselle can grow well in various soil in warmer and humid climates. Therefore, roselle can be easily found in all friendly countries such as Malaysia, Indonesia, Philippines, Vietnam, Sudan, Mexico, Egypt, India, and Saudi Arabia (Singh et al., 2017). Calyx of Roselle has three types; green, red, and dark red, and it is highly cultivated as the red calyxes contain high concentrations of anthocyanin (Singh et al., 2017). Besides that, roselle also plays an essential role in treating other diseases, such as it can reduce bad cholesterols (LDL) in the blood (Kuriyan et al., 2010), diuretic agent (Jiménez-Ferrer

et al., 2012), anticancer (Md Akim et al., 2011), cardioprotective agent (Budin et al., 2019), and antibacterial properties (Chao & Yin, 2009).

Anthocyanins are naturally occurring, water-soluble pigments found in the vacuoles of vascular plants. Anthocyanins play a variety of functions in plants. They aid in pollinating plants by luring insects and animals with their colour and potent UV absorbance (Castañeda-Ovando et al., 2009). Anthocyanin gives out the red, purple, and blue pigments resulting from the conjugated bonds to the flowers, tubers, and fruits. It is classified as a flavonoid class and subclass of phenolic photochemical (Khoo et al., 2017). Anthocyanins are also water-soluble, and their stability varies on the pH, temperature, light, and structure (Laleh et al., 2006). Therefore, it will appear red in acidic conditions and blue in alkaline conditions. Cyanidin, delphinidin, pelargonidin, peonidin, malvidin, and petunidin are anthocyanins typically found in plants. The anthocyanins cyanidin, delphinidin, and pelargonidin are the most common ones found in nature. They are found in 80% of pigmented leaves, 69% of fruits and 50% of flowers (Castañeda-Ovando et al., 2009)

Delphinidin-3-sambubioside (D3S) and cyanidin-3-glucoside (C3S) are the majority of anthocyanins that contribute to the *H.sabdariffa* (Abdel-Moemin, 2016; Izquierdo-Vega et al., 2020). Delphinidin-3-sambubioside (D3S) and cyanidin-3-glucoside (C3S) are likewise thought to be the key players in the antihypertensive and hypercholesterolemic effects (Hopkins et al., 2013). In addition, delphinidin-3-sambubioside has been proven to be anticarcinogenic as it can promote apoptosis in neoplastic leukaemia cells through the p38-FasL and Bid pathway (Lo et al., 2007).

Anthocyanins like cyanidin-3-0-glucoside, delphinidin-3-0-glucoside, cyanidin-3-0-sambubioside, malvidin-3-0-glucoside, petunidin-3-0-glucoside, and acetylcholine (ACH) showed efficiency as antiradicals are abundant in the callus and calyx of roselle. Reactive oxygen species (ROS) and free radicals can harm cells, resulting in many chronic and degenerative diseases, including cancer, coronary heart disease, aging, and neurological disorders (Kouakou et al., 2015).

It is vital to understand how anthocyanin works in the human body. Therefore, it is crucial to grasp the anthocyanin pharmacokinetics and bioavailability by employing a specific and validated method or approach.

Solid phase extraction (SPE) is an extraction method that was used in this study to separate and purify the sample. Purification by SPE is a relatively simple method to eliminate non-phenolic impurities (Denev et al., 2010). Besides that, SPE also allows the removal of interfering biological matrix components and enhances the concentrations of analytes before being injected into High-performance liquid chromatography (HPLC). According to Hapsari et al., (2021), SPE is the most frequently employed technique to extract phenolic compounds from roselle calyx.

Additionally, liquid chromatography has been the method of choice when analysing the phenolic compounds in roselle calyces to identify or measure specific molecules, like anthocyanin (Hapsari et al., 2021). One of the most popular analysis techniques is HPLC. It is currently used for this research because it provides effective separation and identification of particular anthocyanin components. In addition to research, HPLC has a variety of other uses, such as testing for metabolites in blood samples for medical purposes, evaluating drugs in urine for legal purposes, and production purposes, such as checking pharmaceutical items for contaminants. It is suitable for both quantitative and qualitative analyses. Unlike qualitative studies, which rely on observable and descriptive data that cannot be quantified, quantitative analyses use data that can be measured. Due to its advantages over older liquid chromatography methods, which are mainly utilised for component identification, HPLC is now preferred (Kaiser et al., 2020).

1.2 Scope of the study

This study was conducted to validate the method of determining the presence of delphinidin in rat plasma by HPLC. Anthocyanin used in this study was delphinidin-3-0-glucoside chloride and its internal standard, delphinidin-3-5-0-diglucoside chloride. The validation methods will be helpful in pharmacokinetics studies.

First, before performing HPLC, solid-phase extraction was chosen as the extraction method for delphinidin and its internal standard. After that, the validation of the method was assessed. There were nine parameters of method validation, according to the CDER (2018), that were assessed, which are linearity, precision, accuracy, recovery, limit of detection (LOD), and limit of quantification (LOQ). In summary, this study was carried out to validate the solid-phase extraction method in determining the presence of delphinidin-3-0-glucoside chloride as the targeted anthocyanin found in the roselle using HPLC.

1.3 Problem statement and rationale of the study

Anthocyanin has become the centre of interest among researchers from various fields due to its health-promoting properties. According to Khoo et al., (2017), anthocyanin, widely found in plants, has traditionally been used as primary medicine for various diseases. However, studies of mechanisms associated with anthocyanin's absorption, metabolism, and pharmacokinetics are still unexplored (Kay, 2006).

Nevertheless, nowadays, studies of the pharmacological effects of anthocyanin in *H.sabdariffa* are being accomplished, such as their role as antidiabetic, anticancer (Izquierdo-Vega et al., 2020; Md Akim et al., 2011), anti-obesity, and anti-inflammatory, antibacterial effects (Chao & Yin, 2009) also, in the prevention of cardiovascular disease (Budin et al., 2019). Yet, the pharmacokinetics and its bioavailability had still been unexplored. Thus, the lack of information on anthocyanin lifted the need for validating methods to elucidate their health-promoting properties.

The validation method of the HPLC is essential to demonstrate that this analytical method is suitable and to assure its reliability for further studies of H.*sabdariffa* pharmacokinetics and bioavailability in rat plasma.

1.4 Aim of the study

The general objective of the study;

- To validate the HPLC method in elucidating delphinidin-3-0-glucoside chloride and its internal standard, cyanidin-3-5-0-diglucoside chloride concentrations in rat plasma.

The specific objectives of this study;

- To determine the linearity of delphinidin-3-0-glucoside chloride in rat plasma by HPLC.
- To determine the precision, accuracy, and recovery of delphinidin-3-0-glucoside chloride in rat plasma by HPLC.
- To determine the detection limit (LOD) and quantification limit (LOQ) of delphinidin-3-0-glucoside chloride in rat plasma by HPLC.

1.5 Research hypothesis

- Delphinidin-3-0-glucoside chloride and its internal standard, cyanidin-3-5-0-diglucoside chloride, will be quantitively detected in rat plasma by the HPLC method.

1.6 Study flowchart

The flowchart of the study is shown below.

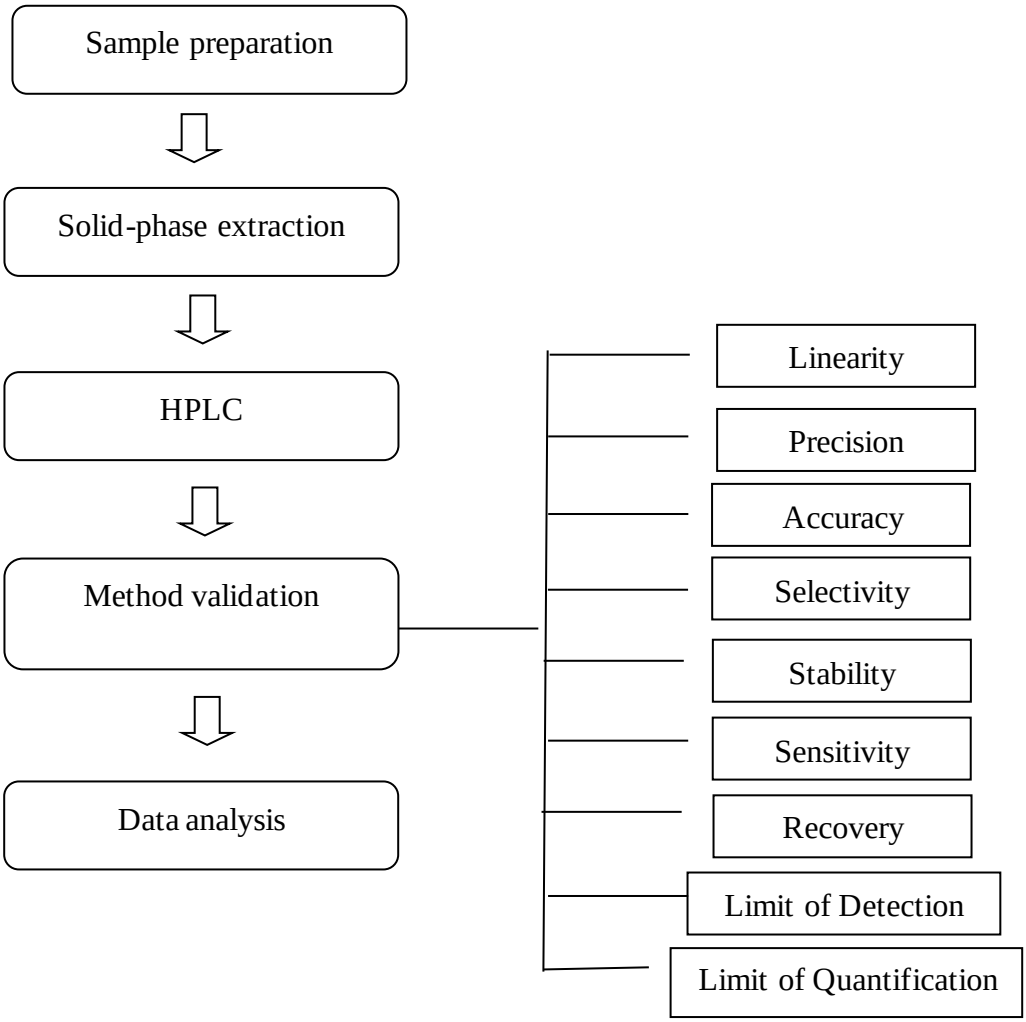

Figure 1.1 The flowchart of the study

CHAPTER TWO

LITERATURE REVIEW

2.1 *Hibiscus sabdariffa*

2.1.1 Geographical origin and distribution

Since people meet their humanity's basic needs for food, clothing, shelter, and medicines, plants have been significant in people's lives. They served as the foundation for many long-standing traditional medical systems, such as Ayurvedic, Unani, that continue to develop innovative treatments for humanity. Research on plants has received more attention recently on a global scale to uncover the enormous potential of medicinal herbs employed in diverse traditional systems. *Hibiscus sabdariffa*, a plant recognised for its delicacy and medicinal qualities that have several health benefits, is one of the medicinal plants examined that could be employed as effective phytochemical agents in the therapeutic of many ailments (Ali et al., 2005).

H.*sabdariffa* has a lovely blossom and is commonly cultivated in many developing nations. Tropical and subtropical climates are home to more than 300 species (Morton, 1987). It is commonly cultivated in Central and West Africa, South East Asia, and other regions such as India, Saudi Arabia, China, Malaysia, Indonesia, Philippines, Vietnam, Sudan, Egypt, Nigeria, and México. It is also known as karkadeh in Arabic, mei gui qie in China, asam belanda in Malaysia, Flor de Jamaica in Mexico, zobo in Nigeria, Karkade in Sudan, and krajeap in Thailand (Ali et al., 2005; Juhari et al., 2018). In english, it is commonly called as roselle.

Locally known as "Asam Paya" in Malaysia, roselle's name essentially translates to "sour" for "Asam" and "swamp" for "Paya." Other names include "Asam Susur" (for "sour rail"), "Asam Kumbang" (for "sour beetle"), and the infamous "Ribena Malaysia" (Mohd-Esa et al., 2010). The plant was first introduced to Malaysian farmers as a new agricultural sector by the Terengganu Agriculture Department in 1993. One of the replacements for tobacco plants in the BRIS (Beach Ridges Interspersed with Swales) soil is the Roselle plant, which has also been the subject of a new commercial plantation project in Malaysia. (Naimah et al., 2014). Roselle types

UMKL-1 (Terengganu) and UMKL-2 (Arab) are currently being intensively planted in Kedah, Pahang, Perak, and Johor (Ali et al., 2019).

Roselle is produced in numerous nations, although there are substantial quality variations. Yet, roselle's geographic origin significantly impacts its quality (Da-Costa-Rocha et al., 2014). For example, Sudan's roselle calyces are the world's best calyces despite their higher total anthocyanin content (TAC) (Tahir et al., 2020). Sudanese roselle is dark/orange, and German prices for Sudanese roselle peaked between 1993 and 1997.

2.1.2 Morphologyy

Roselle is originated from Malvaceae family. It is a 2-2.5 m tall woody-based subshrub or an annual or perennial herb on smooth, cylindrical red stems, which are grouped alternately. The stems are erect, solid, cylindrical, unbranched, mostly bristled, green, red, or regimented in various shades, and they can reach up to 5 meters (Brink, 2003; Islam, 2019).

The leaves are 3-5 palmately lobed and between 8 and 15 cm long. Roselle leaves reach maturity when they are green with red veins (Razali et al., 2014). The blooms have a robust fleshy calyx at the base that is 1-2 cm wide and grows to be 3-3.5 cm broad, meaty, and bright red as the fruit matures. The flowers are auxiliary or terminal, 8-10 cm in diameter, white to pale yellow, with a dark red mark at the base of each petal. The five sepalled crimson calyces, which have a collar, expand as the flowers ripen and enclose the velvety capsule., as illustrated in Figure 2.3. Eventually, the capsule turns brown and splits. It is vital to note that all the parts of the roselle plant, namely, the stems, leaves, and calyx, are acidic. The maturation period is roughly six months. Mid-April marks the start of the wet season, and roselle is planted. Its calyces are picked three weeks later, just before flowering (Riaz & Chopra, 2018).

Figure 2.1 The ripened roselle calyx (Castro et al., 2004)

Figure 2.2 The flowering season of roselle (Castro et al., 2004)

Figure 2.3 Botanical illustration of roselle (Brink, 2003)

2.1.3 Roselle as a plant remedy

Numerous traditional treatments contain roselle. It is prized for its moderate laxative properties, capacity to stimulate urine, relief from the heat, and treatment of blisters, cuts, and cracks in the feet (Duke, 2003; Qi et al., n.d.; Singh et al., 2017). Their Leaves, seeds, and ripe calyces are diuretic and antiscorbutic. Two diuretic ingredients, ascorbic acid and glycolic acids in roselle may contribute to the laxative effects.

Furthermore, the healing process can be expedited using heated leaves to boils, ulcers, and foot fissures. On sores and wounds, a lotion produced from leaves is applied. The brownish-yellow seed oil is reported to treat camel sores, and the seeds are said to have diuretic and tonic properties. In India, a decoction of the seeds is administered to treat minor debility, dyspepsia, strangulation, and dysuria cases, and the leaves are applied as a poultice to abscesses (Da-Costa-Rocha et al., 2014; Singh et al., 2017).

Brazilians use roselle's bitter roots for stomachic, emollient, and resolutive effects (Morton, 1987), while the Philippines use them as an aperitif and tonic (Duke, 2003). The calyx infusion, sometimes known as "Sudan tea," is used to treat coughs in East Africa. Biliousness is treated with roselle juice mixed with molasses, asafetida, salt, and pepper. (Duke, 2003; Qi et al., n.d.). Traditionally in Sudan, roselle has been used for relief of sour throat and healing wounds. In Burma, the leaves are emollient, and the seed is used to treat debility. Roselle leaves are used as a cough treatment and for their antibacterial, emollient, antipyretic, diuretic, anti-helmentic, and sedative effects in traditional African medicine.

A "Karkade" calyces infusion is also used in Egypt and Sudan to reduce body temperature by increasing blood flow to the skin's surface and dilating the pores to cool the skin (Leung & Foster, 1996; Qi et al., n.d.). In addition, it is used to treat hypertension and liver problems in traditional Chinese medicine (Morton, 1987). In Guatemala, roselle "ade" is a favourite remedy for the aftereffects of drunkenness as the roselle extract decreases the rate of absorption of alcohol, lessening the intensity of alcohol effects in chickens (Da-Costa-Rocha et al., 2014; Morton, 1987).

2.1.4 Nutritional value and phytochemical constituents

Numerous components with nutritional potential that can be found in plants, including proteins, lipids, vitamins, fibre, and amino acids, have been examined to determine the nutritional composition of roselle, as shown in Table 2.1 (Izquierdo-Vega et al., 2020; Puro et al., 2014). There have been numerous claims that the roselle calyx is abundant in calcium, niacin, riboflavin, iron, and vitamin C that is nine times as potent as an orange (Ismail et al., 2008; Salami & Afolayan, 2020). In addition, citric acid, malic acid, tartaric acid, and polyphenolic acids (hibiscus acid and protocatechuic acid) are abundant in roselle calyces (Riaz & Chopra, 2018). The plant is also rich in minerals such as potassium and magnesium (Islam, 2019). As a result of their high content of these nutritional components, roselle calyces can be used as culinary ingredients and nutritional supplements.

Plant sections varied in their nutritional compositions and nutritional components. Therefore, there are currently variations in research about the nutritional content of the roselle calyces, which may be influenced by different varieties or genotypes, plant habitats, and harvesting circumstances (Salami & Afolayan, 2020). For example, Ismail et al. (2008) have reported that 100 g of the calyces contain protein (1.9 g), fat (0.1 g), carbohydrates (12.3 g), and fibre (2.3 g). These differences in value were hypothesised as a result of the soil that might impact the ash and mineral content within the same species (Carvajal-Zarrabal et al., 2012).

Table 2.1 Nutritional value per 100 g of roselle (Islam, 2019)

Roselle/Mesta (raw)	
Energy	205KJ (49kcal)
Carbohydrates	11.31 g
Fat	0.64 g
Protein	0.96 g
Vitamins	
Vitamin A equiv.	14µg (2%)
Thiamine (B1)	0.011 mg (1%)
Riboflavin (B2)	0.028 mg (2%)
Niacin (B3)	0.31 mg (2%)
Vitamin C	12 mg (14%)
Trace metals	
Calcium	215 mg (22%)
Iron	1.48 mg (11%)
Magnesium	51 mg (14%)
Phosphorus	37 mg (5%)
Potassium	208 mg (4%)
Sodium	6 mg (0%)

The main phytochemicals in roselle are anthocyanins, flavonoids, and organic acids; mostly citric acid, hibiscus acid, malic acid, and glycosides (Izquierdo-Vega et al., 2020). Among them, studies on flavonoids and anthocyanins are frequently discussed because they are believed to possess advantageous pharmacological and physiological effects (Izquierdo-Vega et al., 2020; Salami & Afolayan, 2020).

Roselle has also reportedly been found to possess biologically active chemicals with therapeutic effects (Salami & Afolayan, 2020). Roselle's primary components relevant to its pharmacology include flavonoids, polysaccharides, organic acids, and anthocyanins (Da-Costa-Rocha et al., 2014). The phytochemicals in roselle calyces contained flavonoids (20.08%), tannins (17.00 %), saponins (0.96 %), phenols (1.10 %), alkaloids (2.14%), and glycosides (0.132 %), which contributes to its pharmacological properties (Okereke et al., 2015; Riaz & Chopra, 2018).

The red calyxes of the roselle are the most utilised due to their high anthocyanin content (Islam, 2019). The concentration of these phytochemicals affects the colour of the roselle calyces, which varies. For example, the anthocyanin concentration in the dark red roselle calyces is five to seven times higher than in the clear roselle calyces. On the other hand, these compounds are absent from the plant's green and yellow variants (Apáez-Barrios et al., 2018).

2.2 Anthocyanins

Anthocyanins are the most prevalent flavonoids in the average diet (Gowd et al., 2017). The Greek terms "anthos" and "kyáneos," which translate to flower and blue, respectively, are the origin of the English word "anthocyanins" (Wallace & Giusti, 2015). These plant pigments are a class of flavonoid-related, water-soluble polyphenolic chemicals found in the vacuolar sap of the epidermal tissues of fruits and flowers (Passeri et al., 2016; Saha & Garg, 2021). They provide plant parts, including fruits, flowers, leaves, and even vegetables, in various colours ranging from red-orange to blue-violet (Soumya et al., 2019). They are in charge of producing the lovely red calyces in roselle (Carvajal-Zarrabal et al., 2012; Salami & Afolayan, 2020).

Up to this point, approximately 700 anthocyanin compounds have been identified and indicated in the literature (Gowd et al., 2017; Saha & Garg, 2021). There are roughly 17 anthocyanidins total that have been identified in nature. Six of them are extensively dispersed and account for over 90% of all anthocyanins (Wallace & Giusti, 2015). They are pelargonidin, malvidin, petunidin, peonidin, delphinidin, and petunidin (Pojer et al., 2013)

2.2.1 Chemical structure of anthocyanins

The glycosylated versions of anthocyanidins (aglycones) are anthocyanins (Mattioli et al., 2020). They are stable, a glycosylated glycoside that naturally occurs in plants. In contrast, their anthocyanidin counterparts are unstable and hardly seen in nature (Wallace & Giusti, 2015). Anthocyanidins are frequently attached to sugars in mono-, di-, or trisaccharide forms, including glucose, galactose, arabinose, rutinose, rhamnose, and xylose (Fang, 2014; Pojer et al., 2013). An attractive option for natural water-soluble colourants is anthocyanins because they are safe and soluble in water (Castañeda-Ovando et al., 2009).

Anthocyanins are produced chemically from flavonol and, more precisely, the flavylium cation (2-phenylchromenylium) backbone (Khoo et al., 2017; Mattioli et al., 2020). Figure 2.4 depicts the overall molecular structure of anthocyanin. Anthocyanidins have a structure that consists of two benzoyl rings, A and B, separated by a heterocyclic ring, C (Pojer et al., 2013). The 3-position on the C-ring or the 5, 7-position on the A-ring are where the sugar moieties primarily attach (Fang, 2014).

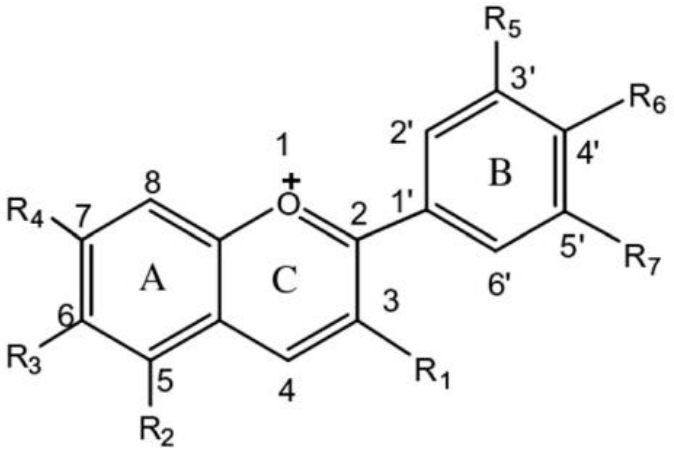

Figure 2.4 Structure of anthocyanin

2.2.2 Stability of anthocyanins

The stability of anthocyanins varies greatly (Fang, 2014). The type of anthocyanin structural pigments, light, temperature, pH, enzymes and many other variables affect how stable anthocyanins are (Laleh et al., 2006; Turturică et al., 2015).

2.2.2.1 Effect of pH

The pH of the solution can impact the stability and colour of anthocyanins as anthocyanins, which are produced from the flavylium cation, have an ionic molecular structure (Turturică et al., 2015). Therefore, in an aqueous solution, the anthocyanins alter structurally in response to pH changes.

Since flavylium ions are stable in extremely acidic circumstances, the anthocyanins are more stable at a lower pH solution (Khoo et al., 2017; Laleh et al., 2006). Anthocyanins have a red colour when the pH is acidic, a purple colour when the pH is neutral, and their colour gradually turns blue when the pH is increased (Khoo et al., 2017; Tanaka & Brugliera, 2013). For instance, cyanidin appears red in a solution with a pH of less than 3, violet at a pH of 7-8, and blue at a pH of more than 11 (Khoo et al., 2017). Anthocyanins are more severely destroyed when the pH rises. Most anthocyanin pigments, such as cyanidin and delphinidin, are highly stable in acidic environments, and breakdown occurs at higher pH levels (Khoo et al., 2017).

2.2.2.2 Effect of temperature

When solution temperatures are higher, anthocyanins are less stable (Khoo et al., 2017). This is due to the hydrolysation of the 3-glycoside structure, that act as a protective effect (Laleh et al., 2006). The heat treatment at a maximum temperature of 35 °C reduced the common grape's total anthocyanin concentration to less than half in the control berries at 25 °C (Mori et al., 2007). Even though the pH of the solution was low, anthocyanin's colour changed from red to orange at temperatures up to 40 °C (West & Mauer, 2013). Additionally, Le et al., (2019) found that when the temperature rises to 50 °C, the concentration of anthocyanin in the extract steadily drops.

2.2.2.3 Effect of light

Light is one of the most significant environmental elements affecting the formation of anthocyanins in plants (Zhou & Singh, 2004). Light has been shown to speed up the breakdown of anthocyanins in Barberis species (Laleh et al., 2006). The photochromes influence the biosynthesis of anthocyanin. Phytochromes A regulates plant responses to far-red light irradiation, whereas phytochrome B is predominant in responses to red light irradiation. Biosynthesis of anthocyanin has shown that red light and sunlight increased anthocyanin biosynthesis more than far-red light (Zhou & Singh, 2004). A study by Bakhshayeshi et al., (2006) proved that 50% of anthocyanin pigments destructed after it exposed to light, while at the same time, in the dark the level of destruction was 30%.

2.2.3 Types of anthocyanin in plants

Cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin are the anthocyanidins that are most frequently found in plants. According to Castañeda-Ovando et al. (2009), these anthocyanidins are distributed as follows: 50%, 12%, 12%, 12%, 12%, 7%, and 7%, respectively, in fruits and vegetables. 3-hydroxyanthocyanidins, 3-deoxyanthocyanidins, and O-methylated anthocyanidins are the three types of anthocyanidins. As opposed, acylated anthocyanins are found in anthocyanidin glycosides (Khoo et al., 2017). In particular, anthocyanins like delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside, and cyanidin-3-glucoside, which are potently hydrophilic antioxidants, are abundant in the calyces of roselle (Carvajal-Zarrabal et al., 2012).

2.2.3.1 Delphinidin

Six anthocyanins were detected in the callus of roselle, while four anthocyanins were detected in the calyx of this plant. The six anthocyanins were cyanidin-3-0-glucoside, delphinidin-3-0-glucoside, cyanidin-3-0-sambubioside, delphinidin-3-0-sambubioside, malvidin-3-0-glucoside, and petunidin-3-0-glucoside (Kouakou, et al., 2015). The major anthocyanin found in roselle calyces is delphinidin-3-sambubioside, which occupies 70% of its total anthocyanins (Chang et al., 2012). It is readily

degraded in hydrolysis and hydrogenation at temperatures more than 40°C. Delphinidin is responsible for the dark red or purple colour appearance of the fruits

2.2.4 Anthocyanins' health advantages

The documented potential health benefits of anthocyanin pigments have increased interest in researchers. The health benefits include dietary antioxidants, antidiabetic, anti-inflammatory, aid in eyesight enhancement, and help lower cardiovascular disease risk. (Pojer et al., 2013; Soumya et al., 2019).

2.2.4.1 Antioxidant activity

Anthocyanins and anthocyanidins are essential in counteracting harmful oxidants such as reactive oxygen and nitrogen species (ROS and RNS) (Mattioli et al., 2020; Nimse & Pal, 2015). A previous study by Masheta & Al-Azzawi, (2018) reported that delphinidin reduced the mortality of C6 glial cells by increasing the activity of glutathione (GSH). This primary antioxidant defence enzyme is responsible for scavenging free radicals. The chemical properties of anthocyanin, including the number of hydroxyl groups, catechol moiety in the B ring, oxonium ion in the C ring, hydroxylation and methylation pattern, acylation, and glycosylation, influence the anthocyanins' antioxidant capacity (Pojer et al., 2013; Yang et al., 2011).

Glycosylation, for instance, decreased the antioxidant capacity of anthocyanin since it reduced its capacity to delocalised electrons (Pojer et al., 2013; Zhao et al., 2014). In addition, the antioxidant activity decreases with the number of sugar units at the C3 and C5 locations in the heterocyclic C-ring (Khoo et al., 2017). However, anthocyanin's glycosylated B-ring structure may help explain its powerful antioxidant action (Tena et al., 2020).

2.2.4.2 Antitumour and anticancer property

Generally, anthocyanin can be divided into at least six common types: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, according to the different substituent groups on the flavylium B-ring (Holton & Cornish, 1995). The research has indicated that the *ortho*-dihydroxyphenyl structure on the B-ring is the active site that inhibits tumour growth and metastasis (M. Xu et al., 2010). During the initial stage of tumorigenesis, anthocyanin scavenges free radicals that might damage the genome of normal cells. Therefore, preventing the occurrence of the tumour. Meanwhile, during the cancer formation, delphinidin could block the Ras–ERK MAPK and PI3K/Akt pathways, which are the oncogenes in cancer formation. Furthermore, anthocyanins can inhibit cancer cell proliferation mainly by arresting the cell cycle at different division phases via up-regulating the expressions of anti-oncogenes and down-regulating the expressions of oncogenes, accompanied by the expressions of different cyclins and their partners CDKs and/or CDKIs (Lin et al., 2017).

2.2.4.3 Antidiabetic activity

Diabetes mellitus is a chronic metabolic illness that affects millions of people globally. High blood glucose levels are a hallmark of type-2 diabetes, characterised by insulin resistance and relative insulin shortage (Pojer et al., 2013). Therefore, to adequately control diabetes, it's critical to avoid excessive postprandial blood glucose increases and to improve insulin resistance.

Anthocyanins may improve insulin resistance, protect β cells, increase insulin secretion, and slow down the small intestine's ability to digest sweets (Sancho & Pastore, 2012). Strugała et al., (2019) reported that the acylated anthocyanin petunidin-3-O-p-coumaryl-rutinoside-5-O-glucoside present in purple potato extract could lower fasting sugar levels in streptozotocin-induced diabetic rats. Additionally, in streptozotocin-induced diabetic Wistar rats, there was an increase in insulin secretion after the administration of one dosage of pelargonidin-3-glucoside (Roy et al., 2008). Anthocyanins, particularly delphinidin-3-sambubioside (D3S) and cyanidin-3-sambubioside (C3S), are thought to be the active ingredients responsible for the antihypertensive and hypocholesterolemic actions of roselle, probably because they are abundant in the aqueous extract (Hopkins et al., 2013).

2.2.4.4 Cardiovascular health

Platelet aggregation, hypertension, elevated plasma low-density lipoprotein (LDL) cholesterol, and vascular endothelium dysfunction can all lead to cardiovascular disease (Pojer et al., 2013). Delphinidin and cyanidin glucosides have been utilised to inhibit platelet aggregation due to their preventive effect in the early stages of thrombi formation, in the treatment of some diseases associated with poor microcirculation brought on by capillary fragility, as well as to stop the oxidation of LDLs (Yan et al., 2010). Alvarez-Suarez et al., (2014)reported that daily anthocyanin ingestion from strawberries improved the lipid profile by lowering total cholesterol, (LDL) cholesterol, and triglyceride levels, while high-density lipoprotein (HDL) cholesterol remained constant.

2.2.5 Bioavailability of anthocyanins

The bioavailability of anthocyanins, which is regulated by absorption, metabolism, distribution, and excretion, is one factor that determines how well they protect individuals against chronic disorders (Eker et al., 2019).

Despite having various molecular sizes and types of sugar or acylated groups attached, anthocyanins can be absorbed intact (Stalmach et al., 2012). Glycone, the sugar moiety, and acylated groups have a key role in the chemical structure, pace, and extent of anthocyanin absorption (Pojer et al., 2013). Anthocyanins may begin to absorb in the stomach and enter the bloodstream shortly after consumption (Pojer et al., 2013). It can take 3 hours for anthocyanin to reach its peak concentration in a person's circulatory system, indicating that anthocyanins can be quickly absorbed from the stomach (Fang, 2014; Speciale et al., 2014). Because the ingested anthocyanins did not show signs of breakdown in simulated acidic gastric juice, it is believed that they were absorbed intact without undergoing any metabolic alterations (Stalmach et al., 2012). However, recent information indicates that anthocyanins are metabolised and distributed in human serum and urine (Kay, 2006; Pojer et al., 2013). After absorption, anthocyanins are processed by phase I and phase II enzymes, creating compounds primarily found in the liver, kidneys, and enterocytes that are hydroxylated, glucuronidated, sulfated, and methylated (Mattioli et al., 2020).

Anthocyanins, such as delphinidin, can alter their structure and go through alterations in response to shifting pH levels in the gastrointestinal tract's various pH zones, which can affect their bioaccessibility, bioavailability, and subsequent bioactivity (Pojer et al., 2013; Xie et al., 2016). For instance, anthocyanins are mostly found in the most stable form, the flavylium cation, in the stomach (at low pH). On the other hand, other less stable anthocyanin metabolites will be present in the small and large intestines at neutral pH.

Anthocyanins are rarely ingested in amounts that would be considered therapeutically active, even in high doses (Mattioli et al., 2020). Anthocyanins' pharmacokinetics indicate that they are poor in bioavailability (Pojer et al., 2013). The concentration of anthocyanins' metabolites may have been below detection limits. The carbinol and chalcone forms of anthocyanins that exist at neutral pH in blood and urine may not be detected during analysis. This is because they could not reform into flavylium cation upon acidification, contributing to the low bioavailability of anthocyanins (Pojer et al., 2013).

According to Braga et al., (2018), anthocyanin bioavailability can be assessed by utilising *in vivo*, *ex vivo*, or *in vitro* experiments. The most significant plasma concentrations recorded in humans ranged between 1.4 and 592 nmol/L and occurred 30 minutes to 4 hours after taking dosages ranging from 68 to 1300 mg (Kay, 2006). Eating anthocyanin-rich fruits caused the maximal plasma concentration between 30 and 2 hours later. Additionally, animal studies suggest that anthocyanins have a systemic bioavailability of 0.26 to 1.8 percent (Fang, 2014).

However, anthocyanins are now known to be more bioavailable than previously thought (Czank et al., 2013). After around 48 hours of oral ingestion, the metabolites of the anthocyanins are still in the blood. Following the injection of 500 mg of isotopically tagged Cy3G, the absorption, distribution, metabolism, and excretion (ADME) of a C5-labeled anthocyanin in eight human male volunteers were studied. The C and C-labeled metabolite concentrations in the biological samples of blood, breath, urine, and feces were assessed using isotope-ratio mass spectrometry and liquid chromatography-tandem mass spectrometry over 48 hours. The result of relative

bioavailability was 12.4 ± 1.4 %. Maximum rates of C elimination were achieved 30 minutes after ingestion, whereas C-labeled metabolites peaked (maximum serum concentration: 5.97 ± 2.14 μmol/L) at 10.2 ± 4.1 hours. The half-life for C-labeled metabolites, identified as degradation products, phenolic, hippuric, phenylacetic, and phenylpropenoic acids, ranged between 12.4 ± 4.2 and 51.6 ± 22.6 hours.

2.2.6 High-performance liquid chromatography and its principle

HPLC has been cited extensively in the analysis of pharmaceutical compounds in the literature. In column chromatography, known as HPLC, a sample (analyte) that has been dissolved in a solvent (mobile phase) is pumped at high pressure through a column with chromatographic packing material that has been immobilised (stationary phase). The characteristics of the sample, the solvent, and the stationary phase, as well as how quickly the analytes travel through the column, determine the analytes' retention time. Analytes with the most robust interactions with the stationary phase exit the column the slowest as the sample moves through the column, resulting in the most extended retention durations. As a result of their rapid elution, samples having limited interaction with the column material, on the other hand, have short retention periods. (Petrova & Sauer, 2017)

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Materials

The chemicals, reagents, apparatus, consumables, and equipment used in this research project are listed in Table 3.1-3.4

Table 3.1 List of chemicals and reagents used in this research

Reagents	**Manufacturers/suppliers**
Acetonitrile	Merck / Darmstadt, Germany
Delphinidin-3-0-glucoside chloride	ChemFaces / Wuhan, China
Cyanidin-3-5-0-diglucoside chloride	ChemFaces / Wuhan, China
Formic acid (HCOOH)	Merck / Darmstadt, Germany
Methanol (MeOH)	Merck / Darmstadt, Germany
Trifluorocacetic acid (TFA)	Merck / Darmstadt, Germany

Table 3.2 List of apparatus used in this research

Apparatus	**Manufacturers/suppliers**
2 mL screw cap vial	Agilent / California, USA
2 mL screw cap amber vial	Agilent / California, USA
100 mL measuring cylinder	Simax / Praha, Czech Republic
500 mL Schott bottle	Duran / Mainz, Germany

Table 3.3 List of consumables used in this research

Consumables	**Manufacturers/ Suppliers**
1000 µL pipette tips	Axygen® / California, USA
200 µL pipette tips	Biologix / Selangor, Malaysia
200 µL vial inserts	Agilent / California, USA
2 mL microcentrifuge tubes	Eppendorf / Hamburg, Germany
Needle (21G x 1)	Terumo / Tokyo, Japan
Nylon membrane filters	Bioflow / Selangor, Malaysia
Sep-Pak tC18 cartridges	Waters / Massachusetts, USA
Syringe (5 cc/ml)	Terumo / Tokyo, Japan

Table 3.4 List of equipments used in this research

Equipment	**Model/ Manufacturer**
High-performance liquid chromatography	Gilson
Deep freezer (-20ºC)	Snow / Kedah, Malaysia
Fume hood	Esco / Oregon, USA
Minishaker / Vortexer	Merck / Darmstadt, Germany
pH meter	Thermo Fisher Scientific / New Hampshire, USA
Solid-phase extraction manifold	Thermo Fisher Scientific / New Hampshire, USA
Vacuum pump	GE Motors / Houston, USA
Vacuum filtration apparatus	Varian / California, USA
Water bath	Memmert / Schwabach, Germany
Weighing balance	Sartorius / Göttingen, Germany