DEVELOPMENT AND OPTIMIZATION OF RAPID RESOLUTION LIQUID CHROMATOGRAPHY (RRLC) METHOD FOLLOWING SOLID-PHASE EXTRACTION FOR DETERMINATION OF ANTHOCYANIN IN RAT PLASMA

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DEVELOPMENT AND OPTIMIZATION OF RAPID RESOLUTION LIQUID CHROMATOGRAPHY (RRLC) METHOD FOLLOWING SOLID-PHASE EXTRACTION FOR DETERMINATION OF ANTHOCYANIN IN RAT PLASMA

by

NADIRATUL ASYIKIN BINTI SAUJI

Thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science (Biomedicine) (Mixed Mode)

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CERTIFICATE

This is to certify that the dissertation entitled "Development and optimization of Rapid Resolution Liquid Chromatography (RRLC) method following solid-phase extraction for determination of anthocyanin in rat plasma" is the bona fide record of the research work done Ms Nadiratul Asyikin binti Sauji during the period from March 2021 to August 2021 under my supervision. I have read this dissertation and that in my opinion it conforms to the acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfilment for the degree of Bachelor of Health Science (Honours) (Biomedicine).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purpose.

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LIST OF SYMBOLS

%	Percentage
λ	Lambda
°C	Degree celcius
±	Plus-minus
>	Greater than
<	Lesser than
cm	Centimetre
g	Gram
xg	Centrifugal force
g/mol	Gram per moles
kJ	Kilojoule
kcal	Kilocalories
µg	Microgram
µg/mL	Microgram per millilitre
µL	Microlitre
µmol/L	Micromoles per litre
µm	Micrometre
m	Metre
mg	Milligram
mL	Millilitre
mL/min	Millilitre per minute
mm	Millimetre
min	Minutes
ng/mL	Nanogram per millilitre

nm	Nanometre
nmol/L	Nanomoles per litre
rpm	Revolutions per minute
v/v	Volume per volume

LIST OF ABBREVIATIONS

C3S	Cyanidin-3-sambubioside
COX-2	Cyclooxygenase 2
Cy3G	Cyanidin-3-O-glucoside
DAD	Diode array detector
D3S	Delphinidin-3-sambubioside
HDL	High-density lipoprotein
HPLC	High Performance Liquid Chromatography
LC	Liquid chromatography
LDL	Low-density lipoprotein
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MAPK	Mitogen-activated protein kinase
MS	Mass spectrometry
MTBE	Methyl t-butyl ether
NMR	Nuclear magnetic resonance
PGE2	Prostaglandin E2
PCA	Protocatechuic acid
PGA	Phloroglucynaldehyde
pH	Potential of hydrogen
pKA	Acid dissociation constant
RRLC	Rapid Resolution Liquid Chromatography
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
SPE	Solid-phase extraction
UV	Ultraviolet
WHO	World Health Organization

PEMBANGUNAN DAN PENGOPTIMUMAN KAEDAH RESOLUSI PESAT KROMATOGRAFI CECAIR (RRLC) SELEPAS EKSTRAKSI FASA PADAT UNTUK PENENTUAN ANTOSIANIN DALAM PLASMA TIKUS

ABSTRAK

Kajian antosianin dalam rosel yang semakin meluas mengetengahkan kepentingan analisis sebatian tersebut untuk mengkaji ciri-ciri farmakologinya. Kaedah analisis yang sensitif dan spesifik diperlukan untuk menganalisis antosianin dengan tepat dalam sampel. Dalam kajian ini, resolusi pesat kromatografi cecair (RRLC) telah digunakan sebagai kaedah untuk menganalisis salah satu antosianin yang terdapat dalam rosel iaitu sianidin-3-O-glukosida klorida. Analisis spektrofotometri dan pengoptimuman keadaan kromatografi dilakukan bagi menentukan kaedah RRLC yang sesuai dan tepat untuk penentuan antosianin. Kaedah pengekstrakan cecair-cecair (LLE) dan pengekstrakan fasa padat (SPE) juga dijalankan untuk menilai kaedah pengekstrakan antosianin daripada sampel plasma tikus yang terbaik. Kaedah kromatografi yang telah dioptimumkan menunjukkan bahawa komposisi fasa gerak larutan akueus asid trifluoroasetik 0.1% dan asetonitril dalam nisbah 81:19, masing-masing dengan aliran 0.5 mL/min, pada suhu lajur 30°C dan pengesanan jarak gelombang 525 nm sesuai digunakan untuk analisis sianidin-3-O-glukosida klorida. SPE dipilih sebagai kaedah pengekstrakan antosianin terbaik dalam kajian ini kerana menunjukkan puncak yang lebih baik dalam kromatogram jika dibandingkan dengan LLE. Sebagai kesimpulan, kaedah RRLC yang diaplikasikan dalam kajian ini dapat digunakan untuk penentuan antosianin dalam kajian farmakokinetik rosel pada masa depan.

DEVELOPMENT AND OPTIMIZATION OF RAPID RESOLUTION LIQUID CHROMATOGRAPHY (RRLC) METHOD FOLLOWING SOLID-PHASE EXTRACTION FOR DETERMINATION OF ANTHOCYANIN IN RAT PLASMA

ABSTRACT

The growing interest of anthocyanins in roselle has brought upon the importance of the compound's analysis to investigate its pharmacological properties. Sensitive and specific analytical methods are required to accurately analyse the anthocyanin present in the sample. In this study, rapid resolution liquid chromatography (RRLC) was used to analyse cyanidin-3-O-glucoside chloride, one of the anthocyanins found in roselle. Spectrophotometric analysis and optimization of chromatographic conditions were performed to develop a suitable and precise RRLC method for the determination of the selected anthocyanin. Both liquid-liquid extraction (LLE) and solid-phase extraction (SPE) method were performed to evaluate the best extraction method of anthocyanin in the rat plasma sample. The optimised chromatographic method demonstrated that the mobile phase composition of 0.1% trifluoroacetic acid aqueous solution and acetonitrile in the ratio of 81:19, respectively with a flow rate of 0.5 mL/min, at 30°C column temperature and detection wavelength of 525 nm were suitable for RRLC analysis of cyanidin-3-O-glucoside chloride. SPE was chosen as the final extraction method in the study as it produced better peaks in the chromatogram compared to LLE. In conclusion, the developed RRLC method in this study can be used to determine anthocyanins in the future pharmacokinetic study of roselle.

CHAPTER 1CHAPTER 1

INTRODUCTION

1.1 Background of study

Natural resources such as medicinal plants have been widely utilised as the lead materials in the development of new drugs and used in 25% of modern medicines. (Luca et al., 2012). According to the World Health Organization (WHO), medicinal plants are plants that contain substances that can be utilised for therapeutic purposes and produce valuable drugs from the metabolites (World Health Organization, 2011). Recently, the research on medicinal plants for human health benefits has risen worldwide and gained attention from researchers worldwide including in Malaysia. Malaysia is known as a tropical country that is rich in nature and has an abundant species of medicinal plants. For instance, approximately 1300 medicinal plant species and 7411 plant species have been recorded in Peninsular Malaysia and Sabah, respectively (Kulip et al., 2010).

Based on the previous report, 15% out of 300,000 plant species in the world have been studied and investigated for their pharmacological activities (Bakar et al., 2018). In today's societies, herbal remedies made from medicinal plants have become more popular among people who seeks natural-based products to substitute for conventional western medicine for the treatment of minor ailments. Other than that, herbal remedies also have been the choice for those who could not afford the increasing costs of personal health maintenance (Sofowora et al., 2013).

One of the immensely studied medicinal plants is *Hibiscus sabdariffa* or commonly known as roselle. Research has shown that some Hibiscus species possess and exhibited specific medicinal properties, including *H. sabdariffa* (Chin et al., 2005). Parts of roselle including calyces, seeds, leaves, fruits and roots are being employed in various foods as well as in herbal medicine as a potential non-pharmacological treatment (Khan, 2017). Different extracts from roselle play an essential role in treating other medical problems including many cardiovascular disorders (Gowd et al., 2017), helminthic disease (Vanawati et al., 2021) and cancer (Khan, 2017). The plant is also a source of anthocyanins which mainly acts as free radical scavengers and inhibit lipid peroxidation (Aminul Islam et al., 2016).

Anthocyanins are polyphenolic pigments that belong to the flavonoid group (Wallace and Giusti, 2015). They are mainly responsible for the presence of bright and attractive colours in various plant organs such as fruits, flowers, and leaves (Pojer et al., 2013). Due to their nature as natural food colourants, interest and studies on anthocyanins have been risen particularly in diet, regarding their effects on health-promoting properties. In addition, these compounds have attracted much attention for their physiological activities. Their role has become a topic of interest in the relationship between health and human diet (Rakić et al., 2015). Numerous studies have reported the beneficial health effects of consuming dietary fruits and vegetables containing anthocyanins (Rakić et al., 2015). Studies have linked the positive association of anthocyanins with antioxidant effect (Aminul Islam et al., 2016), anti-inflammatory effect (Valenza et al., 2018), reduction in risk of getting diabetes (Gowd et al., 2017) as well as protection against heart diseases (Pojer et al., 2013) and certain types of

cancer such as liver cancer (Bishayee et al., 2011), blood cancer (Tsai et al., 2014) and cervical cancer (Rugină et al., 2012).

The anthocyanins present in the *H. sabdariffa* plant are responsible for the bright, red colour of roselle's calyces. Several investigations have reported that roselle's calyces has high content in anthocyanins (Diessana et al., 2015; Djaeni et al., 2018; Wu et al., 2018). In addition, the extracts from the plants' calyces have been linked to medicinal properties such as in diuretic and also sedative treatments (Wu et al., 2018). Presently, anthocyanins of *H. sabdariffa* are being studied on their effect as the cardioprotective agent by reducing oxidative stress and increasing antioxidant status (Budin et al., 2019). The anthocyanins extract from roselle also has been demonstrated to be capable of mitigating stress and increases the immune-related parameters (Jomeh et al., 2021). The most common types of anthocyanidins are cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin (Khoo et al., 2017).

It is important to understand whether anthocyanins are active in the human body. Therefore, it is crucial to comprehend their bioavailability and pharmacokinetics by applying a specific method or approach. Analytical methodologies that can adequately cover the process of the analysis process to determine the plant compounds present and their contents are thus necessary.

An analytical procedure is necessary for the identification and quantification of anthocyanins present in studied plants. The application of sensitive and appropriate analytical methods will significantly assist in analysing the composition of

anthocyanins contents in roselle. Hence, this can further evaluate the biological activity and beneficial effects of these phenolic compounds in roselle as potential pharmaceutical ingredients for human health (Hapsari et al., 2021).

The development of combined extraction method, spectrophotometric and chromatographic methods are regards to hold the most significant impact on the analysis of anthocyanins (Rubinskiene et al., 2005). Chromatography has been applied mainly in the extraction, separation, characterization and quantification of anthocyanins (Khoo et al., 2017; Benvenuti et al., 2018; Saha et al., 2021). In addition, liquid chromatography has been the most employed technique in roselle-related studies to measure the phenolic compounds from roselle calyces, aiming to identify or quantify specific compounds, including anthocyanins (Hapsari et al., 2021). High-performance liquid chromatography (HPLC) is one of the most common methods used for the analyses. In addition, an advanced and improved method of HPLC such as rapid resolution liquid chromatography (RRLC) is currently applied for the research as it offers better separation and identification of specific components of anthocyanins.

1.2 Scope of Study

This study was conducted to determine the presence of cyanidin in rat plasma by utilising a specific combination of analytical techniques such as extraction, spectrophotometric and chromatographic methods. The method developed from this study will be used as a standard in future pharmacokinetics studies. Anthocyanin tested in this study was Cyanidin-3-O-glucoside chloride. Cyanidin-3,5-O-diglucoside chloride was chosen as the internal standard.

The cyanidin and its internal standard were first analysed by spectrophotometry to detect their ultraviolet (UV) absorbances. Then, the cyanidin and internal standard were introduced to the liquid chromatography, RRLC method for optimisation of chromatographic conditions such as percentages of organic solvent, column temperatures, mobile phase, flow rates, detection wavelength and organic solvent. In addition, optimisation of extraction methods using rat plasma has been performed by liquid-liquid extraction and solid-phase extraction.

Apart from optimisation of chromatographic conditions and extraction methods, RRLC method validation has also been conducted to validate the chromatographic method developed. As a summary, this study was carried out to determine and quantitate the presence of targeted roselle anthocyanin, cyanidin using RRLC analysis following the optimised extraction methods.

1.3 Problem statement and rationale of the study

The ever-increasing evidence about anthocyanins has drawn many researchers from various types of fields to investigate more about these plant flavonoids. In pharmacological studies, interest in research about the health-promoting properties of anthocyanins is intensifying. Nevertheless, most studies are based on *in vitro*, thus leaving mechanisms associated with absorption, metabolism and pharmacokinetics largely unexplored and not well characterised (Kay, 2006).

Currently, studies are being performed on anthocyanins of *H. sabdariffa* for their various pharmacological effects such as their role in decreasing body weight, hyperlipidemia and obesity complications (Morales-Luna et al., 2019; Noordin et al., 2019). However, despite plenty of studies conducted on anthocyanins, the bioavailability of these roselle anthocyanins has not been well explored yet. Thus, there is limited understanding of their bioavailability and pharmacokinetics. Additionally, the increasing importance of anthocyanins analysis in elucidating their health-promoting properties has raised the need for specific tasks for their determination methods.

The development and validation of the RRLC method following extraction techniques are essential to determine the most appropriate analysis of one of the major anthocyanins in *H. sabdariffa,* cyanidin or specifically cyanidin-3-O-glucoside in rat plasma. This study also aimed to provide an efficient developed method to be used for future pharmacokinetics and bioavailability studies of *H. sabdariffa* in rat plasma.

1.4 Aim of the Research

The study was aimed to achieve the following general and specific objectives.

1.4.1 General objective

The general objective of this study was to develop and optimize rapid resolution liquid chromatography (RRLC) method following solid-phase extraction for the determination of anthocyanin in rat plasma.

1.4.2 Specific objectives

- To establish the rapid resolution liquid chromatography (RRLC) method for determining cyanidin-3-O-glycoside concentrations in rat plasma.
- To determine the best extraction method of specified anthocyanin from rat plasma, solid-phase extraction (SPE) or liquid-liquid extraction (LLE).
- To optimize the solid-phase extraction (SPE) method of specified anthocyanin from rat plasma.
- To validate the rapid resolution liquid chromatography (RRLC) method for determining cyanidin-3-O-glycoside concentrations in rat plasma.

1.5 Research hypothesis

- The specified anthocyanin would be detected in rat plasma using RRLC developed method.
- Solid-phase extraction method would give high recovery percentage of specified anthocyanin introduced in rat plasma

1.6 Study flow chart

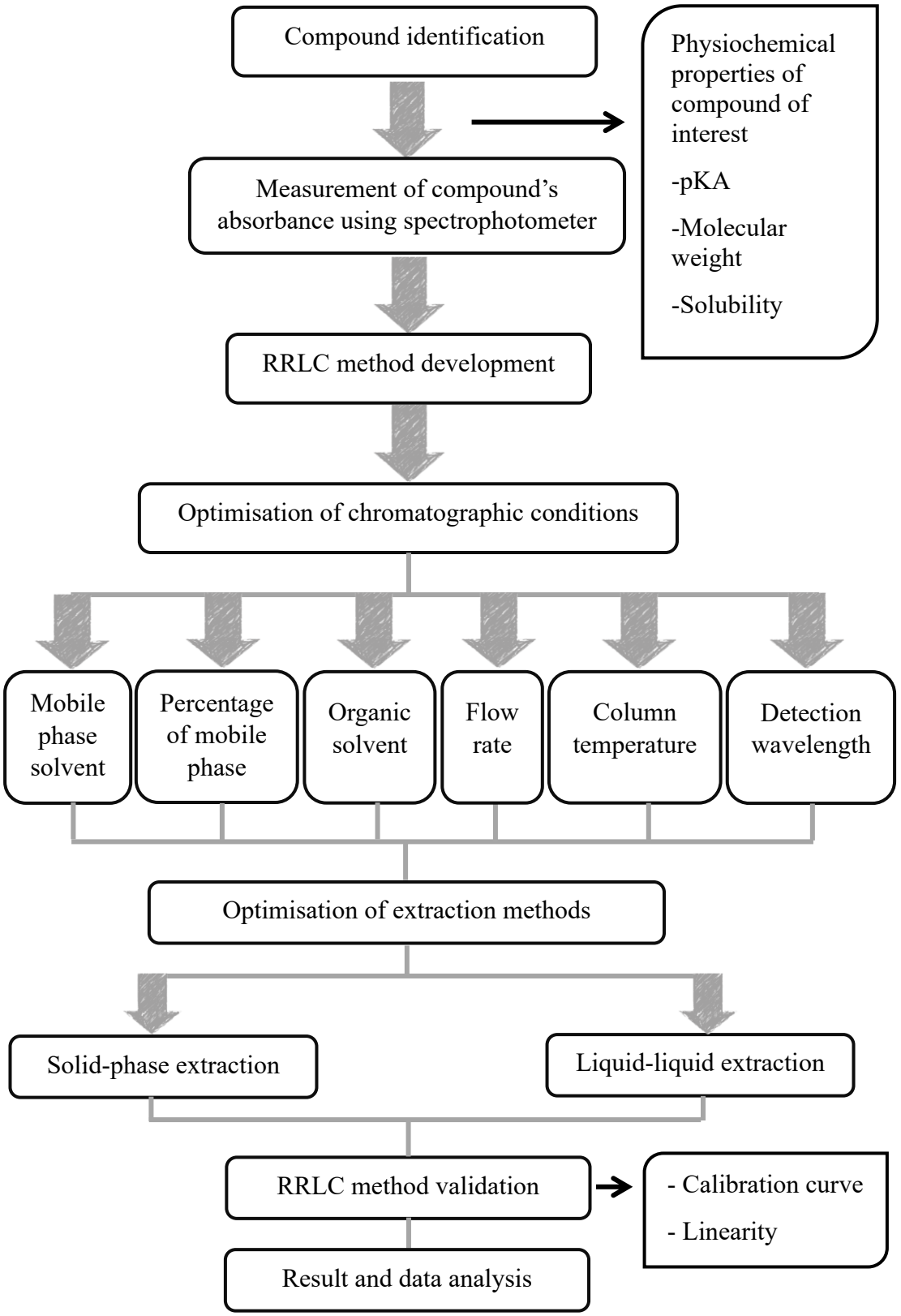

Figure 1.1 The flowchart of study

CHAPTER 2

LITERATURE REVIEW

2.1 Hibiscus sabdariffa

2.1.1 Origin and geographical distribution

Hibiscus is one of the most widely grown flower plants worldwide. It has more than three hundred species that are largely distributed in tropical and subtropical regions worldwide (Ismail et al., 2008). One of the species of Hibiscus that possesses medicinal and health-promoting properties is *Hibiscus sabdariffa. H. sabdariffa* is a tropical plant belonging to the Malvaceae family (Diessana et al., 2015). It is also known as sorrel, karkade or famously roselle (Salami and Afolayan, 2021).

Roselle is an annual herbaceous plant living in dry, subtropical, mountain climates (Guardiola and Mach, 2014). They are widely spread in warm and humid countries such as Malaysia, Indonesia, India, West Africa and many other tropical regions (Da-Costa-Rocha et al., 2014). In Malaysia, roselle is locally known as "asam kumbang", "asam susur" or "asam paya" (Omalsaad et al., 2014). Roselle was first introduced in the country by the agriculture department in Terengganu as one of the agriculture alternatives to substitute for tobacco plants in sandy soils (Ali et al., 2019). To date, it was reported that Johor is the state with the largest planted area and the highest production of roselle, followed by Penang, Selangor, Perak and Kedah (Keong et al., 2019).

In the Caribbean, Central America, Brazil, Australia, Hawaii, Florida, and the Philippines, roselle is commonly grown as a home garden crop (Shruthi and Ramachandra, 2019). Other than that, roselle is mostly grown for consumption and Egypt, Sudan, Mexico, Thailand and China are the main producers of roselle blossoms (Islam, 2019). Although the world largest producer is Thailand and China, the highest quality of roselle comes from Sudan (Aminul Islam et al., 2016).

2.1.2 Morphology

Roselle grows in a bush with many branches. It is a bushy, herbaceous subshrub with smooth or nearly smooth, cylindrical, typically red-coloured stems (Da-Costa-Rocha et al., 2014). The stems are erect, solid, cylindrical, unbranched, mostly bristled, green, red, or regimented in various shades (Islam, 2019). It can grow from 0.5 to 3 m and can reach up to 5 m (Ismail et al., 2008).

The leaves are green-coloured with reddish veins and come with long or short petioles. They can be measured up to 7.5 to 12.5 cm long (Da-Costa-Rocha et al., 2014). The flowers are comprised of white petals and a reddish centre at the base of the staminal column (Ismail et al., 2008). In addition, the flowers are made of 5 petal parts and attached to the base of the calyx (Islam, 2019).

The fruits are surrounded by enlarged fleshy calyces containing 22 to 34 seeds per capsule (Okereke, 2015). The calyx consists of 5 large sepals with a collar (epicalyx) of 8 to 12 slim and pointed bracts around the base. It enlarges at maturity and becomes edible with a fleshy, crisp and juicy taste (**Figure 2.1**) (Ismail et al., 2008; Da-Costa-

Rocha et al., 2014). The seeds are dark brown, with 4 to 6 cm long and weigh about 0.025 g (Okereke, 2015). **Figure 2.2** shows the botanical illustration of roselle.

Figure 2.1 *Hibiscus sabdariffa*

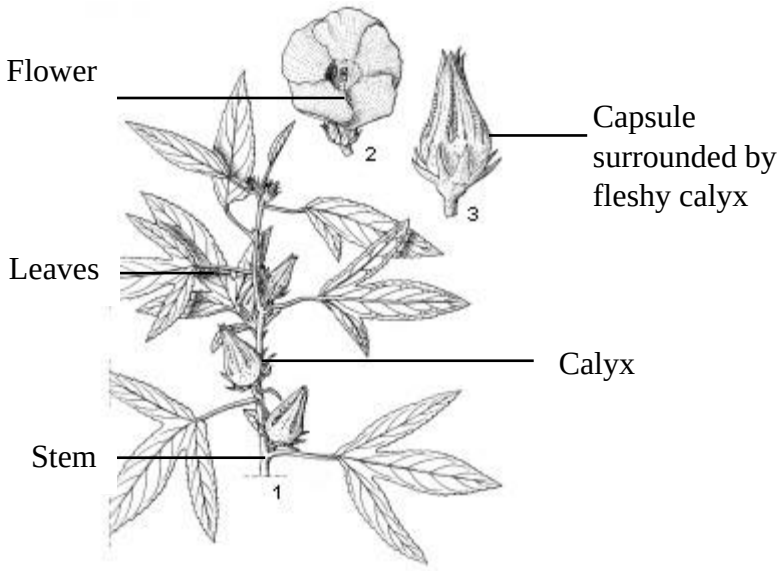

Figure 2.2 Botanical illustration of roselle
(Shamsuddin and van der Vossen, 2003)

2.1.3 Roselle as medicinal plant

2.1.3 (a) Roselle in local & traditional medicine

Various parts of roselle have been utilised in local and traditional medicines. The plant has been used to prevent diseases such as diabetics, cancer, hypertension and obesity as well as in the treatment of colds, toothaches, urinary tract infections and hangovers (Ashaye, 2013; Riaz and Chopra, 2018). Roselle juice with salt, pepper and molasses is applied to relieve coughs and remedy nausea (Mohamed et al., 2012). The plant is also regarded for its mild laxative effect, stimulating urination, providing relief during hot weather, and treating foot cracks, bilious, ulcers, and wounds. (Chin et al., 2005; Khan, 2017).

Other than that, the roselle plant is used as a folk remedy in the treatment of dyspepsia, dysuria, fever, heart ailments, scurvy, neurosis, and strangury (Nnam and Onyeke, 2003; Islam, 2019). Gemede et al., (2014) has reported that regular consumption of roselle may reduce nutritional deficiency complications such as night blindness, scurvy and rickets. The calyces extract added with common salt is believed to be beneficial to cure diarrhoea and dysentery of animals and humans (Riaz and Chopra, 2018). The leaves and flowers are used as a tonic to help with internal digestive and kidney functions. In addition, the seeds have diuretic, laxative, and tonic properties and are employed in the treatment of debility (Islam, 2019).

In African folk medicine, roselle leaves are used for antimicrobial, emollient, antipyretic, diuretic, anti-helminthic, sedative effect and a soothing cough remedy. In addition, the leaves or calyces infusions of roselle are traditionally used in India, Africa

and Mexico for their choleretic, febrifugal and hypotensive effects, reduction of blood viscosity and stimulation of intestinal peristalsis (Da-Costa-Rocha et al., 2014). In Sudan, roselle has been traditionally used for the relief of sore throat and healing wounds (Eman et al., 2007). It was claimed to be a Thai traditional medicine for kidney and urinary bladder stones while in Chinese folk medicine, it is used to treat liver disorders and high blood pressure (Maganha et al., 2010; Da-Costa-Rocha et al., 2014).

2.1.4 Nutritional value and phytochemical constituents

The nutritional composition of roselle has been studied on various compounds with nutritional capacity that have been found in the plant such as proteins, lipids, vitamins, fibre, amino acids as shown in **Table 2.1** (Puro et al., 2014; Izquierdo-Vega et al., 2020). Many have reported that the calyx of roselle is rich in calcium, niacin, riboflavin, iron, and vitamin C that is nine times stronger than an orange (Ismail et al., 2008; Salami and Afolayan, 2020). The roselle calyces are also rich in organic acids, citric acid, malic acid, tartaric acid and polyphenolic acids (hibiscus acid and protocatechuic acid) (Riaz and Chopra, 2018). The plant is also found to be rich in minerals such as potassium and magnesium (Islam, 2019). Therefore, roselle calyces can be used as nutritional supplements and also as a beneficial food ingredient as they are rich in these nutritional compositions.

Different parts of plants displayed different nutritional compounds and compositions. To date, the nutritional composition of fresh calyces of the plant differs from different studies, which may be due to different varieties or genotypes, plant environments, and harvesting conditions (Salami and Afolayan, 2020). For example, Ismail et al. (2008) has 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 the value were suggested due to the type of soil that may affect 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	205 kJ (49 kcal)
Carbohydrates	11.31 g
Fat	0.64 g
Protein	0.96 g
Vitamins	
Vitamin A equiv.	14 μg (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%)

Units: μg: Micrograms; mg: Milligrams; IU: International

In addition, roselle has been reported to contain biological active chemical substances with curative properties (Salami and Afolayan, 2020). The main constituents of roselle relevant in the context of its pharmacological are organic acids, anthocyanins, polysaccharides and flavonoids (Da-Costa-Rocha et al., 2014). According to Okereke, (2015), the phytochemicals in roselle calyces contained flavonoids (20.08%), tannins (17%), saponins (0.96%), phenols (1.10%), alkaloids (2.14%), and glycosides (0.132%), which contributes to its pharmacological properties (Riaz and Chopra, 2018). Among these phytochemicals, flavonoids and anthocyanins are commonly mentioned as the focus of studies as they are believed to hold beneficial pharmacological and physiological properties (Izquierdo-Vega et al., 2020; Salami and Afolayan, 2020).

Among different parts of roselle, the red calyxes are the most used for their concentration of anthocyanin (Islam, 2019). The colour of roselle calyces varies related to the content of these phytochemicals. The dark red calyces of roselle possess an anthocyanin concentration five to seven times greater than the clear roselle calyces. Conversely, the green and yellow varieties of the plant lack these compounds (Apáez-Barrios et al., 2018).

2.2 Anthocyanins

Anthocyanins are among the most widely consumed flavonoids in the daily diet (Gowd et al., 2017). The word anthocyanins originated from the Greek words; “anthos” and “kyáneos” which translates to flower and blue, respectively (Wallace and Giusti, 2015). These plant pigments represent a group of water-soluble polyphenolics compounds that belong to the flavonoid group and are present in the vacuolar sap of the epidermal tissues of flowers and fruit. (Passeri et al., 2016; Saha et al., 2021). They are responsible for various colours, ranging from red-orange to blue-violet of plant organs such as fruits, flowers, leaves, and also present in vegetables (Soumya et al., 2019). In roselle, they are responsible for the attractive red colours of calyces (Carvajal-Zarrabal et al., 2012; Salami and Afolayan, 2020).

Up until now, more or less 700 anthocyanin compounds have been stated and reported in the existing literature (Gowd et al., 2017; Saha et al., 2021). From these, there are approximately 17 anthocyanidins that exist in nature, of which six of them are widely distributed and represent approximately 90% of all anthocyanins identified (Wallace and Giusti, 2015). They are cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin (Pojer et al., 2013).

2.2.1 Chemical structure of anthocyanins

Anthocyanins are the glycosylated forms of anthocyanidins (aglycones) (Mattioli et al., 2020). They naturally occur in plants as a glycoside in the stable, glycosylated form. In contrast, their anthocyanidin counterparts are not stable and are rarely found in nature (Wallace and Giusti, 2015). Anthocyanidins are commonly bonded to sugars such as glucose, galactose, arabinose, rutinose, rhamnose, and xylose as mono-, di-, or

trisaccharide forms (Pojer et al., 2013; Fang, 2014). Anthocyanins are harmless and water-soluble, making them an interesting choice for natural water-soluble colourants (Castañeda-Ovando et al., 2009).

Chemically, anthocyanins are derived from flavanol or specifically flavylium cation (2-phenylchromenylium) backbone (Khoo et al., 2017; Mattioli et al., 2020). The general molecular structure of anthocyanin is shown in **Figure 2.3**. The structure of anthocyanidins consists of 2 benzoyl rings, denoted with A and B and separated by a heterocyclic ring, labelled with C (Pojer et al., 2013). The sugar moieties mainly attach to the 3-position on the C-ring or the 5, 7-position on the A-ring (Fang, 2014).

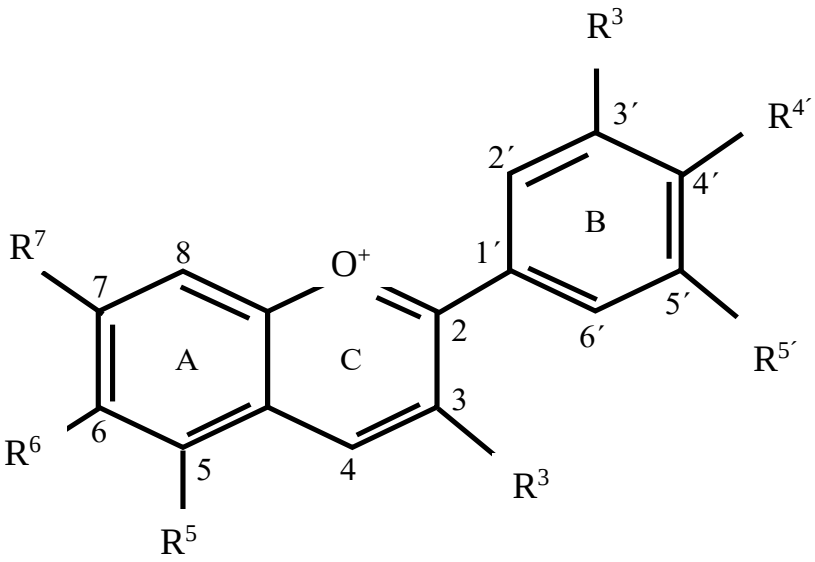

Figure 2.3 Basic structure of anthocyanin
Footnotes: A and B, benzoyl rings; C, heterocyclic ring

2.2.2 Stability of anthocyanins

Anthocyanins vary widely in stability (Fang, 2014). The stability of anthocyanins is dependent on several factors such as the type of anthocyanin structure pigments, light, temperature, pH, enzymes and many more (Laleh et al., 2006; Turturică et al., 2015).

2.2.2 (a) Effect of pH

The pH of the solution can affect the stability and colour of anthocyanins. This is due to the ionic nature of the molecular structure of anthocyanins, which are derived from flavylium cation (Turturică et al., 2015). The anthocyanins undergo structural rearrangements in an aqueous solution in response to changes in pH.

The anthocyanins are more stable at a lower pH solution as flavylium ion are stable in highly acidic conditions (Laleh et al., 2006; Khoo et al., 2017). Anthocyanins appear red in acidic conditions, purple in neutral pH and the colour gradually changes to blue in an increasing pH condition (Tanaka and Brugliera, 2013; Khoo et al., 2017). For example, at a lower pH solution (pH <3), cyanidin appears to be red, violet at pH 7–8; it is blue at a very high pH (pH >11) (Khoo et al., 2017). Increasing pH cause greater destruction of anthocyanins. Most of the anthocyanin pigments such as cyanidin and delphinidin have high stability in acidic conditions and their degradation occurs at higher pH (Khoo et al., 2017).

2.2.2 (b) Effect of temperature

Anthocyanins are less stable at higher solution temperatures (Khoo et al., 2017). A previous study reports that heat treatment at a maximum of 35°C reduced the total anthocyanin content in the common grape to less than half the amount in control berries at 25°C (Mori et al., 2007). In addition, the colour of anthocyanin changes from red to orange at up to 40°C, although the pH of the solution was low (West and Mauer, 2013). In addition, a study by Le et al., (2019) demonstrated that the concentration of anthocyanin in the extract gradually decreases as the temperature reaches up to 50°C.

2.2.2 (c) Effect of light

Light is one of the most important environmental factors influencing anthocyanin biosynthesis in plants (Zhou and Singh, 2004). The presence of light has been demonstrated to accelerate the destruction of anthocyanins in *Barberis* species (Laleh et al., 2006). The increasing light intensity also causes the gradual loss of monomeric anthocyanins, cyanidin-3-glucoside (Contreras-Lopez et al., 2014).

2.2.3 Types of anthocyanin in plants

The most common types of anthocyanidins are cyanidin, delphinidin, pelargonidin, peonidin, petunidin and malvidin. The distribution of these anthocyanidins in fruits and vegetables is 50, 12, 12, 12, 7 and 7%, respectively (Castañeda-Ovando et al., 2009). Anthocyanidins are grouped into 3-hydroxyanthocyanidins, 3-deoxyanthocyanidins, and O-methylated anthocyanidins. On the other hand, anthocyanins are in the anthocyanidin glycosides and acylated anthocyanins forms (Khoo et al., 2017).

The calyces of roselle are rich in polyphenols especially in anthocyanins like delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside and cyanidin-3-glucoside which are strongly hydrophilic antioxidants (Carvajal-Zarrabal et al., 2012).

2.2.3 (a) Cyanidin

There are six anthocyanins detected in the callus of *H. sabdariffa* while four anthocyanins were detected in the calyx of this plant (Kouakou et al., 2015). Four of the anthocyanins were cyanidin-3-O-glucoside, cyanidin-3-O-sambubioside, delphinidin-3-O-sambubioside and delphinidin-3-O-glucoside (Riaz and Chopra, 2018). In another study, roselle calyx has demonstrated to contain cyanidin-3-glucoside (Nuryanti et al., 2012).

Cyanidin-3-O-glucoside (Cy3G) also known as chrysanthemin or kuromanin, is a major flavonoid anthocyanin in plant-based foods (Olivas-Aguirre et al., 2016). Cyanidin-3-glucoside is usually formed in plants as a result of low pH (Khoo et al., 2017). This suggests that roselle calyces that contain glycosides of cyanidin probably do so due to the acidic nature of the roselle. As previously stated, Cy3G is vulnerable to degradation by various physicochemical variables such as pH, light, solvents and temperature (Hernández-Herrero and Frutos, 2015).

2.2.4 Health benefits of anthocyanins

Interest in anthocyanin pigments has intensified because of their possible health benefits as dietary antioxidants, anti-diabetic, anti-inflammatory, aid in vision improvement and help to reduce the risk of cardiovascular illnesses (Pojer et al., 2013; Soumya et al., 2019).

2.2.4 (a) Antioxidant activity

Anthocyanins and anthocyanidins play a crucial role as free radical scavengers against damaging oxidants such as reactive oxygen and nitrogen species (ROS and RNS) (Nimse and Satish, 2015; Mattioli et al., 2020). The antioxidant potential of anthocyanins is determined by the molecule's chemical structure, the number of hydroxyl groups, the catechol moiety in the B ring, the oxonium ion in the C ring, the hydroxylation and the methylation pattern, acylation; and glycosylation (Yang et al., 2011; Pojer et al., 2013). For example, the antioxidant potential of anthocyanins is influenced by glycosylation. Glycosylation will decrease the radical scavenger activity of anthocyanin, as it diminishes the ability of anthocyanin radicals to delocalize electrons (Pojer et al., 2013; Zhao et al., 2014). The more sugar units at the C3 and C5 positions in the heterocyclic C-ring, the lower the antioxidant activity is (Khoo et al., 2017). However, the glycosylated B-ring structure of anthocyanin can contribute to the high antioxidant activity (Tena et al., 2020).

In addition, hydroxylation at the B-ring also will enhance antioxidant capacity (−OH > −OCH3 >> −H) (Pojer et al., 2013). Hence, the antioxidant capacity of anthocyanidins is in the order of delphinidin > petunidin > malvidin = cyanidin >

peonidin > pelargonidin. Subsequently, the substitution of hydroxyl groups with methoxy groups in the B-ring will decrease the antioxidant capacity of anthocyanins (Bindhu and Jayaraj, 2020). In summary, the antioxidant activity of anthocyanins increases with the number of hydroxyl groups in the B-ring and decreases with the number of glycosyl groups bonded to the A and C rings.

2.2.4 (b) Antidiabetic properties

Diabetes mellitus is classified as a chronic metabolic disorder that affects millions of people worldwide. Type-2 diabetes is associated with insulin resistance and relative insulin deficiency and is characterized by a high blood glucose level (Pojer et al., 2013). It is important to prevent excess postprandial increases in the blood glucose level and improve insulin resistance to effectively manage diabetes.

Current published data suggest that anthocyanins may lower blood glucose by improving insulin resistance, protecting β cells, increasing insulin secretion and reducing digestion of sugars in the small intestine (Sancho and Pastore, 2012). For instance, acylated anthocyanin petunidin-3-O-p-coumaryl-rutinoside-5-O-glucoside found in purple potato extract was found to reduce the fasting sugar levels in streptozotocin-induced diabetic rats (Strugała et al., 2019). In addition, an *in vivo* study involving streptozotocin-induced diabetic Wistar rats that received one dose of pelargonidin-3-glucoside demonstrated an increase in insulin secretion (Roy et al., 2008).

Anthocyanins, particularly delphinidin-3-sambubioside (D3S) and cyanidin-3-sambubioside (C3S), are believed to be the active components responsible for the antihypertensive and hypocholesterolaemic effects of roselle, possibly because they are found in large amounts in aqueous extract (Hopkins et al., 2013).

2.2.4 (c) Anti-inflammatory effects

Inflammation is a complex biological response of vascular tissues to injuries, irritants, or stimulants, and is associated with the initiation, development, and progression of cancers or tumours (Pojer et al., 2013). The ability of anthocyanins to inhibit lipoxygenase and cyclooxygenase 2 (COX-2) enzymes has also been studied (Szymanowska and Baraniak, 2019). Dietary supplements of the anthocyanins-rich extract from wild mulberry and the cyanidin-3-glucoside are shown to be effective in suppressing carrageenan-induced oedema and peritonitis through the downregulation of COX-2 expression and inhibition of prostaglandin E2 (PGE2) production (Hassimotto et al., 2013). Delphinidin, in particular, can suppress the activation of the mitogen-activated protein kinase (MAPK) involved in directing cellular responses to a wide range of stimuli such as mitogens and proinflammatory cytokine (Pojer et al., 2013).

2.2.4 (d) Cardiovascular health

Cardiovascular disease can develop due to platelet aggregation, hypertension, high-plasma LDL cholesterol, and vascular endothelium dysfunction (Pojer et al., 2013). Glucosides of delphinidin and cyanidin have been used to inhibit platelet aggregation because of their preventive effect in the initial stage of the formation of thrombi, in the treatment of some diseases related to poor microcirculation resulting from capillary

fragility, and also to prevent the oxidation of the LDLs (Y. Yang et al., 2010). Other than that, Alvarez-Suarez et al., (2014) reported that daily anthocyanin consumption through strawberries improved the lipid profile reducing total cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides levels, while high-density lipoprotein (HDL) cholesterol remained unchanged.

2.2.5 Bioavailability of anthocyanins

The effectiveness of anthocyanins in protecting individuals against chronic illnesses depends on various parameters, including their bioavailability, which is determined by absorption, metabolism, distribution, and excretion (ADME) (Eker et al., 2020).

Anthocyanins can be absorbed intact despite having different molecular sizes and types of sugar or acylated groups attached (Stalmach et al., 2012). The rate and extent of absorption of anthocyanins are significantly affected by the chemical structure, glycone, sugar moiety, and acylated groups (Pojer et al., 2013). Anthocyanins absorption may start in the stomach and appear in the bloodstream in few minutes immediately after ingestion (Pojer et al., 2013). It can reach maximum concentration in the human's circulatory system within 3 hours, suggesting that the anthocyanins can be quickly absorbed from the stomach (Fang, 2014; Speciale et al., 2014). The ingested anthocyanins are thought to be absorbed intact without any metabolic changes as they did not exhibit degradation in simulated acidic gastric juice (Stalmach et al., 2012). However, new evidence suggests that the anthocyanins are absorbed and transported as metabolites in human serum and urine (Kay, 2006; Pojer et al., 2013). After absorption, anthocyanins are metabolized by phase I and phase II enzymes,