ANTIHYPERTENSIVE AND RENOPROTECTIVE EFFECTS OF *HIBISCUS SABDARIFFA* EXTRACTS ON DOCA-SALT INDUCED AND SPONTANEOUSLY HYPERTENSIVE RATS

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by

HO YOKE MEI

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ABTS	2,2 -azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensin converting enzyme
Ang I	Angiotensin I
Ang II	Angiotensin II
Ang III	Angiotensin III
Ang IV	Angiotensin IV
APA	Aminopeptidase
AT1	Ang II type 1
AT2	Ang II type 2
C3S	Cyanidin 3-sambubioside
CrCl	Creatinine clearance
D3S	Delphinidin 3-sambubioside
DBP	Diastolic blood pressure
DMC	DOCA-salt control
DOCA	Deoxycorticosterone acetate
DPC	DOCA-salt positive control
DSC	DOCA-sham control
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	Half maximal effective concentration
FAPGG	N-(3[2-Furyl] Acryloyl)-phe-gly-gly
FE_{Na+}	Fractional excretion of sodium
GA	Gum Arabic
GAE	Gallic acid equivalents
GFR	Glomerular filtration rate
H&E	Hematoxylin and eosin
HPLC	High performance liquid chromatography
HS	*Hibiscus sabdariffa* calyces
HSEW	*Hibiscus sabdariffa* calyces 50 % ethanolic extract
HSW	*Hibiscus sabdariffa* calyces water extract
HSWF	Formulated *Hibiscus sabdariffa* calyces water extract
HW/BW	Heart weight/ body weight

KW/BW	Kidney weight/ body weight
LOD	Limit of detection
LOQ	Limit of quantification
M	Maceration
MCC	Microcrystalline cellulose
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate
NCD	Noncommunicable diseases
NED	N-1-naphthylethylenediamine dihydrochloride
NHMS	National Health & Morbidity Survey
NO	Nitrite oxide
OD	Optical density
PWV	Pulse wave velocity
QE	Quercetin equivalents
R	Reflux boiling
RAS	Renin-angiotensin system
RH	Relative humidity
RNS	Reactive nitrogen species
ROS	Reactive oxidative species
ROTA	Rotary evaporation
SBP	Systolic blood pressure
SD	Spray dry
SHR	Spontaneous hypertensive rat
SOD	Superoxide dismutase
SPC	SHR positive control
T-AOC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substances
TFC	Total flavonoid content
TPC	Total phenolic content
UFR	Urine flow rate

LIST OF APPENDICES

KESAN ANTIHIPERTENSIF DAN PERLINDUNGAN RENAL EKSTRAK *HIBISCUS SABDARIFFA* TERHADAP MODEL TIKUS TERARUH GARAM-DOCA DAN TIKUS HIPERTENSI SPONTAN

ABSTRAK

Hibiscus sabdariffa banyak digunakan sebagai ubat tradisional bagi rawatan hipertensi di seluruh dunia. Kajian ini dijalankan untuk membangunkan bentuk dos oral yang terpiawai, mesra industri dan stabil dengan menggabungkan dos efektif ekstrak kaliks *Hibiscus sabdariffa* yang telah ditentukan melalui tiga model hipertensi haiwan, iaitu model tikus hipertensi spontan (SHR), model tikus hipertensi DOCA-garam dan model tikus normotensif. Delfinidin 3-sambubioside dan sianidin 3-sambubioside adalah antosianin utama dalam ekstrak kaliks *Hibiscus sabdariffa* dan sangat tidak stabil dalam keadaan penyimpanan yang berbeza. Kaedah pengekstrakan dan pemekatan yang paling sesuai untuk ekstrak air dan ekstrak etanol 50 % kaliks *Hibiscus sabdariffa* adalah kaedah maserasi dan pengeringan secara semburan kerana kaedah ini menghasilkan jumlah kandungan fenolik, flavonoid, antioksidan, dan antosianin yang lebih tinggi daripada kaedah pendidihan refluks dan penyejatan berputar. Kedua-dua ekstrak air dan etanol 50 % kaliks *Hibiscus sabdariffa* kemudiannya menjalani penilaian farmakologi dan telah didapati bahawa ekstrak air kaliks *Hibiscus sabdariffa* menunjukkan kesan antihipertensi yang lebih kuat. Ekstrak air kaliks *Hibiscus sabdariffa* pada 500 mg/kg adalah paling berkesan dalam mengurangkan tekanan darah berdasarkan tekanan darah sistolik dan diastolik dalam model SHR dan DOCA-garam. Dalam model tikus normotensif, kedua-dua ekstrak air dan etanol 50 % kaliks *Hibiscus sabdariffa* tidak mempunyai kesan hipotensi kerana tekanan darah sistolik dan diastolik tidak menurun dengan ketara. Ekstrak air kaliks

Hibiscus sabdariffa dipilih untuk diformulasikan dengan menggunakan kaedah pengeringan lapisan terbendalir kerana kaedah ini menghasilkan formulasi yang lebih stabil dengan kapasiti penyerapan kelembapan yang lebih rendah dan sifat pengaliran serbuk yang lebih baik berbanding kaedah pengeringan secara semburan. 3:7 nisbah gam arabik dan selulosa mikrohablur dipilih untuk merumuskan ekstrak air kaliks *Hibiscus sabdariffa* berdasarkan sifat pengaliran serbuk yang "baik" dan "cemerlang". Ekstrak air kaliks *Hibiscus sabdariffa* yang dikeringkan melalui kaedah pengeringan lapisan terbendalir dengan 3: 7 nisbah gam arabik dan selulosa mikrohablur dapat mencapai kestabilan yang lebih baik berbanding dengan ektrak air kaliks *Hibiscus sabdariffa* yang tidak diformulasi selepas penyimpanan pada suhu bilik 25 °C selama enam bulan, iaitu 67.29 % bagi kandungan delfinidin 3-sambubioside dan 54 % bagi kandungan sianidin 3-sambubioside. Formulasi ekstrak air kaliks *Hibiscus sabdariffa* yang bernisbah 3:7 berkemungkinan memberikan kesan antihipertensi dan perlindungan renal dengan pengurangan ketara dalam nisbah protein kencing kepada kreatinin, kekakuan arteri dan nisbah berat jantung kepada berat badan, serta peningkatan paras superoksida dismutase berbanding dengan ekstrak air kaliks *Hibiscus sabdariffa* yang tidak diformulasikan dalam model SHR. Oleh itu, ekstrak air kaliks *Hibiscus sabdariffa* yang diformulasikan berpotensi untuk berfungsi sebagai terapi tambahan untuk hipertensi.

ANTIHYPERTENSIVE AND RENOPROTECTIVE EFFECTS OF *HIBISCUS SABDARIFFA* EXTRACTS ON DOCA-SALT INDUCED AND SPONTANEOUSLY HYPERTENSIVE RATS

ABSTRACT

Hibiscus sabdariffa is widely used as a folk medicine for hypertension treatment throughout the world. The present study was carried out to develop a standardised, industry friendly and stable oral dosage form incorporating the effective dose of *Hibiscus sabdariffa* calyces extract, as determined through three animal models, namely spontaneous hypertensive rat model (SHR), DOCA-salt induced hypertensive model and normotensive rat model. Delphinidin 3-sambubioside and cyanidin 3-sambubioside are the major anthocyanins in *Hibiscus sabdariffa* calyces extract and were known to be very unstable under different storage conditions. The most suitable extraction and concentration methods for *Hibiscus sabdariffa* calyces water and 50 % ethanolic extracts were maceration and spray dry methods because these methods produced higher total phenolic contents, total flavonoid contents, antioxidant capacity, and anthocyanin contents than reflux boiling and rotary evaporation methods. Both *Hibiscus sabdariffa* calyces water and 50 % ethanolic extracts were then subjected to pharmacological study and it was found that *Hibiscus sabdariffa* calyces water extract showed a more potent anti-hypertensive effect. *Hibiscus sabdariffa* calyces water extract at 500 mg/kg dose was the most effective in reducing blood pressure based on the systolic and diastolic blood pressures in the SHR model and DOCA-salt induced hypertensive model. In the normotensive rat model, both *Hibiscus sabdariffa* calyces water and 50 % ethanolic extract had no hypotensive effect as the systolic and diastolic blood pressures did not reduce significantly.

Hibiscus sabdariffa calyces water extract was selected for formulation by using fluid-bed dry method because this method produced a more stable preparation with lower moisture sorption capacity and better powder flow properties than the spray dry method. Ratio 3:7 for gum arabic and microcrystalline cellulose powder was selected to formulate fluid-bed dried *Hibiscus sabdariffa* calyces water extract based on its "good" to "excellent" powder flow properties. Fluid-bed dried *Hibiscus sabdariffa* calyces water extract with ratio 3:7 for gum arabic and microcrystalline cellulose was able to achieve better stability compared with unformulated *Hibiscus sabdariffa* calyces extract under room temperature 25 °C or 6 months, which is 67.29 % for delphinidin 3-sambubioside content dan 54 % for cyanidin 3-sambubioside content, respectively. The ratio 3:7 formulation of *Hibiscus sabdariffa* calyces water extract possibly exerted antihypertensive and renal protective effects with significant reduction in urinary protein to creatinine ratio, arterial stiffness and heart weight to body weight ratio, as well as an increase in superoxide dismutase level as compared to the unformulated *Hibiscus sabdariffa* calyces water extract in the SHR model. Thus, the formulated *Hibiscus sabdariffa* calyces water extract may serve as a potential adjuvant therapy for hypertension.

CHAPTER 1

INTRODUCTION

1.1 Noncommunicable diseases (NCDs)

Noncommunicable diseases (NCDs) are caused by a combination of genetic, physiological, environmental and behavioral factors, and mainly manifest as chronic conditions. The fundamental types of NCDs are cardiovascular diseases, cancers, chronic respiratory diseases and diabetes. These diseases are the results of the vicious cycle of rapid urbanization, the globalization of unhealthy lifestyles and population ageing. Subsequently, metabolic risk factors manifest in people as elevated blood pressure, obesity, raised blood glucose and blood lipids that can lead to cardiovascular disease, the leading NCD in terms of premature deaths (Forouzanfar *et al.*, 2016).

NCDs are estimated to account for 74 % of all deaths in Malaysia (World Health Organization, 2018a). Institute for Public Health (2015) ranked hypertension, smoking, diabetes, high cholesterol and high BMI as the biggest contributors to both disability adjusted life-years (DALY) and deaths. National Health & Morbidity Survey (NHMS) 2011 showed that at least 63 % of adults aged 18 years and above had at least one NCD risk factor. In brief, majority of Malaysian adults estimated to be 7,124793 (57.78 % males and 42.22 % females) are now “sick” or “at risk” (Ministry of Health Malaysia, 2020). At the 65th World Health Assembly in May 2013, Malaysia has already set the national targets for NCDs based on the voluntary global targets for the year 2025 in order to reduce hypertension prevalence to less than 24.0 %.

1.2 Hypertension

The history of hypertension research started with the invention of blood pressure measuring apparatus and technique (Kotchen, 2011). Reverend Stephen Hales is the

first person to measure arterial pressure in 1733 while Riva Rocci invented the first conventional sphygmomanometer in 1896 (Ward & Langton, 2007).

In modern days, hypertension or high blood pressure is a long-term medical condition in which the blood pressure in the arteries is persistently elevated above 140 mmHg systolic blood pressure and 90 mmHg diastolic blood pressure (Kaplan, 2010). Having blood pressure between 140/90 mmHg-159/99 mmHg is classified as stage 1 hypertension while blood pressure equal to or higher than 160/100 mmHg is classified as stage 2 hypertension. Meanwhile, adults with prehypertension (120/80 mmHg-139/89 mmHg) are at increased risk for them to progress to hypertension (Chobanian *et al.*, 2003; Mahadir *et al.*, 2019).

Table 1.1 Classification of blood pressure for adults

Blood Pressure Classification	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)
Normal	<120	and <80
Prehypertension	120-139	or 81-89
Stage 1 Hypertension	140-159	or 90-99
Stage 2 Hypertension	≥ 160	or ≥100

The force of blood pushing against the blood vessels wall as the heart pumps creates blood pressure. The higher the pressure the harder the heart has to pump. The regulation of blood pressure involves a multi-organ system response including the central nervous system (CNS), circulatory system, kidneys, and adrenal glands. If left unattended, hypertension can lead to myocardial infarction, an enlargement of the heart and eventual heart failure. This is the reason why hypertension is also known as the silent killer as it rarely causes any symptom for early detection (World Health Organization, 2013). Besides that, aneurysms may develop due to high pressure, making them prone to clogging and bursting. When this happens in the brain it will result in a stroke. Uncontrolled hypertension will eventually give rise to complications

like chronic kidney disease, cognitive impairment and blindness (Kilander *et al.*, 1998; Wong & Mitchell, 2004; Atkins, 2005; Rossignol *et al.*, 2015).

Essential or primary hypertension accounts for 95 % of all cases of hypertension. It is an idiopathic and heterogeneous disorder where causal factors vary for different individuals (Cormick *et al.*, 2021). Increased systemic blood pressure caused by an underlying medical condition like obstructive sleep apnoea, renal parenchymal disease, renal artery stenosis and primary aldosteronism, is known as secondary hypertension (Chiong *et al.*, 2008; Rimoldi *et al.*, 2014). Secondary hypertension can also occur during pregnancy (Malha & August, 2015). Irreversible alterations in the systemic vasculature can be minimised via early detection and appropriate treatment (Rimoldi *et al.*, 2014).

1.2.1 Pathophysiology of hypertension

Hypertension is a multifactorial and complex disease with different mechanisms closely bound up with its development. There is no absolute stand alone cause for the development of hypertension (Shams *et al.*, 2022).

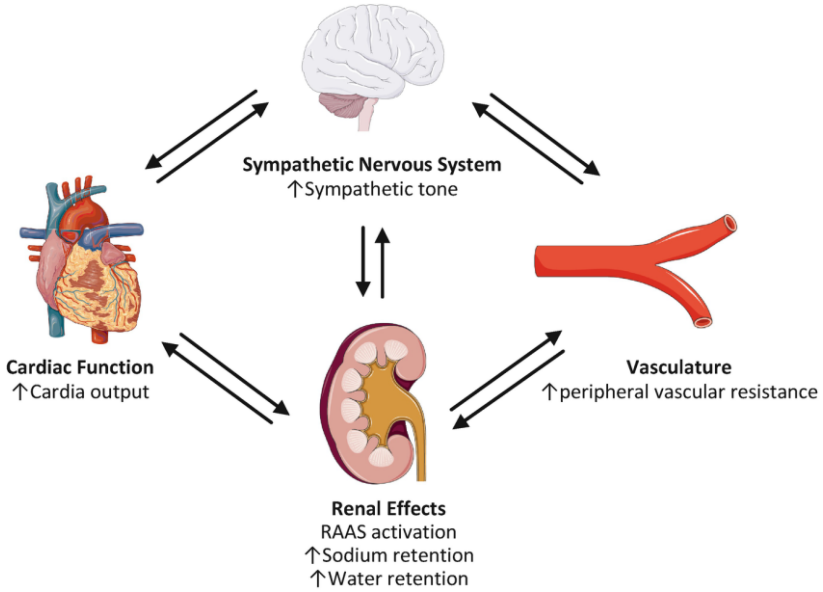


Figure 1.1 The complex pathophysiology of hypertension (adapted from Sousa *et al.* (2019))

1.2.1(a) Renin–angiotensin system (RAS)

Since renin was discovered as a pressor substance in 1898, the renin-angiotensin system has become the most important endocrine system closely related to the regulation of blood pressure (Lavoie & Sigmund, 2003; Sandeep *et al.*, 2022). When our body experiences sodium depletion due to decreased sodium intake or glomerular underperfusion, juxtaglomerular apparatus of the kidney will secrete renin. The sympathetic nervous system also triggers the release of renin via the action of norepinephrine on β_1-adrenoceptors (Saxena, 1992; Grossman-Rimon *et al.*, 2022). A series of enzymatic cascade reactions will begin with renin initiated conversion of its substrate (angiotensinogen) to angiotensin I (Ang I) , which is inactive on its own while angiotensin converting enzyme (ACE) in the lungs rapidly converts it to angiotensin II (Ang II). ACE also inactivates the vasodilator peptides bradykinin and kallidin which plays a role in exerting endothelial relaxation (Turner & Hooper, 2002; da Silva *et al.*, 2022). In the kidney Ang II is a potent vasoconstrictor directly and indirectly stimulating Ang II type 1 (AT1) receptors present on the vasculature and increasing sympathetic tone and arginine vasopressin release, thus raising blood pressure (Beevers *et al.*, 2001; Sparks *et al.*, 2011). Ang II is further metabolised by aminopeptidase A (APA) to angiotensin III (Ang III) which is then metabolised to angiotensin IV (Ang IV) by aminopeptidase N (APN). Angiotensin type 2 (AT2) receptor-mediated natriuresis is due to renal interstitial (RI) infusion of Ang III (Padia *et al.*, 2008). Researchers also discovered that chronic elevation of Ang IV in the brain will induce hypertension but it is reversible with Ang II AT1 receptor antagonists (Lochard *et al.*, 2004).

In recent years there has been considerable debate over whether local or paracrine RAS may exist in various tissues and might be a key regulator of blood

pressure. Tissue RAS exists in the brain, heart, vasculature, adipose tissue, pancreas, placenta, kidney and even gonads (Hagemann *et al.*, 1994; Speth *et al.*, 1999; Engeli *et al.*, 2000; Bader *et al.*, 2001; Sernia, 2001; Morimoto & Sigmund, 2002). Among all, the intrarenal RAS is hypothesised to play the main role in regulating blood pressure and renal function as Ang I and Ang II concentrations in proximal tubular fluid in the kidney frequently surpass those in plasma, and RAS components in the kidney can be controlled independently of circulating levels of the plasma renin, angiotensin, and aldosterone (Navar *et al.*, 1997; Roman *et al.*, 2016). Besides, brain RAS can synthesize all of the circulatory RAS components while facilitating neurotransmission in the brain and stimulating the release of vasopressin (Steckelings *et al.*, 1992; Cosardereliogl u *et al.*, 2020).

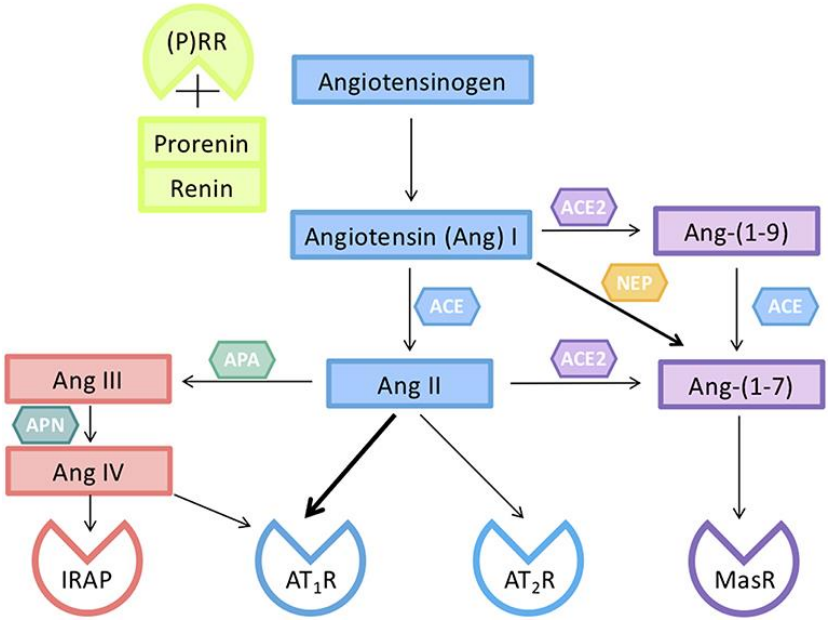


Figure 1.2 The renin-angiotensin system (RAS) [adapted from Lumbers *et al.* (2019)]

1.2.1(b) Arterial stiffness

The Seventh Report of the Joint National Committee reported that more than two-thirds of individuals over 65 years of age experience hypertension (Chobanian *et*

al., 2003). Aging is the main cause for increased stiffness of the large conduit arteries, leading to elevated systolic blood pressure and increased pulse pressure (Safar, 1999; Ferreira *et al.*, 2012). Arterial stiffness is characterised by luminal enlargement with thickened walls and reduced elastic properties. Long term arterial pulsation in the central artery causes gradual fragmentation and loss of elastin fiber while accumulation of stiffer collagen fiber in the media of large arteries (Izzo Jr & Shykoff, 2001; Pierce *et al.*, 2022). Arterial stiffness is strongly associated with atherosclerosis at various sites in the vascular tree (van Popele *et al.*, 2001; Wilkinson *et al.*, 2009; Di Marco *et al.*, 2022). Stiffness often occurs throughout the vascular tree in a patchy pattern rather than uniformly distributed (Zieman *et al.*, 2005; Weinberg, 2022). Obesity, sedentary lifestyles and smoking also linked to increased oxidative stress and thus worsen the state of arterial stiffness. Smoking-induced oxidative stress promotes the release of serotonin, which may contribute to subsequent vascular inflammation. (Lessiani *et al.*, 2016; Sugiura *et al.*, 2017).

Arteriosclerosis is a generic term used for hardening of arteries and arterioles. Atherosclerosis is a multifactorial disease of only the aorta and its major branches affected by atheromas. Atherosclerosis is the most common cause of arteriosclerosis (Damjanov, 2009; Rajamani & Fisher, 2017). Bazan *et al.* (2007) discovered that the elderly have significantly more aortic arch calcification if compared with younger patients. Arterial aging with vascular calcification and endothelial dysfunction also contributes to higher pulse wave velocity (Lee & Oh, 2010; Smith *et al.*, 2012).

Pulse wave velocity (PWV) is widely used as a marker of arterial stiffness and is a reliable predictor of cardiovascular events and a marker of asymptomatic organ damage (Vlachopoulos *et al.*, 2010; Mancia *et al.*, 2013). Koivistoinen *et al.* (2018)

recommended the use of PWV to predict the progression of blood pressure and it serves as a critical tool in predicting hypertension risk in young adults. Based on systematic review by Cecelja and Chowienczyk (2009), hypertension is closely associated with PWV and it is generally assumed that deterioration of the vascular wall due to hypertension ultimately leads to stiff arteries (Aatola *et al.*, 2010; Townsend *et al.*, 2015). Despite this hypothesis, Mitchell (2014) suggests that arterial stiffness is to blame rather than a consequence of hypertension.

In the state of inflammation, imbalance between collagen and elastin occurs and thus contributes to vascular stiffness (Johnson *et al.*, 2001). Consistently elevated luminal pressure also contributes to excessive collagen production (Xu *et al.*, 2000; Laurent & Boutouyrie, 2022). Besides that, Ang II also triggers collagen formation and reduces elastin synthesis. It also stimulates matrix remodeling and vascular hypertrophy, decreases nitric oxide-dependent signaling activity and increases oxidative stress (Pereira *et al.*, 2022). Furthermore, oxidative stress may contribute to vascular inflammation and lead to excessive cellular proliferation, which may eventually impair arterial elasticity (Csiszar *et al.*, 2002; Park & Lakatta, 2012).

Excessive dietary sodium intake has also been blamed for vascular stiffness, especially in older adults as it activates tissue Ang II and endogenous natriuretic sodium pump ligands-driven mechanism, affects not only the endothelial and vascular cell functions but structural remodeling, which cause arterial stiffening (Gu *et al.*, 1998; Bagrov & Lakatta, 2004; Edwards & Farquhar, 2022). A high salt diet was significantly associated with cardiovascular hypertrophy and increased arterial elastin and collagen. As a consequence, studies had shown that appropriate sodium restriction will help with

large elastic artery compliance in older stage 1 hypertension patients (Gates *et al.*, 2004).

Even without a significant hike in blood pressure, the relationship between high sodium intake and vascular alterations had been established by Maruyama *et al.* (2015) in spontaneously hypertensive rats. Pharmacological and genetic suppression of several vasodilating systems also account for higher salt sensitivity in animal models of hypertension (Lerman *et al.*, 2019). Blood pressure increased dramatically in genetically bradykinin-deficient brown Norway Katholiek rats after high salt diet was introduced, suggesting that the lack of urinary kinin generation caused diminished sodium and water excretion through urine, in turn promoting retention of sodium and water in the body and thus leading to hypertension (Majima *et al.*, 1993; Prieto *et al.*, 2021). In WKY rats, a high-salt diet significantly increased intra-arterial blood pressure while in SHR, a high-salt diet was closely related to cardiovascular hypertrophy and the situation was amplified with bradykinin B2 receptor blockade by long acting bradykinin-antagonist as evidenced by carotid hypertrophy, a rise in carotid elastin and collagen content, and excess collagen deposition in the aorta (Partovian *et al.*, 1998; Couture *et al.*, 2014).

1.2.1(c) Oxidative stress

Involvement of oxidative stress in hypertension has been hypothesised in recent studies (Duffy *et al.*, 1999; Rodrigo *et al.*, 2008; Tamadon *et al.*, 2014). Harrison and Gongora (2009) suggested that high levels of sodium, angiotensin II and other main hypertension markers in the body advocate reactive oxidative species (ROS) production in the brain, kidney, and vasculature and consequently results in hypertension. Researchers also believe that the imbalance between the generation of ROS and the

antioxidant defense systems is the main cause of oxidative stress (Baradaran *et al.*, 2014). Superoxide radical ($\cdot O_2^-$), hydroxyl radical ($\cdot HO$), lipid peroxyl ($\cdot LOO^-$) radical, alkoxy radicals ($\cdot LO^-$), hydrogen peroxide (H_2O_2), and hydroxyl anion (OH^-) are examples of ROS whereas nitric oxide (NO) and peroxynitrite ($ONOO^-$) are the reactive nitrogen species (RNS) which are known for their oxidation and reduction potential (Touyz & Schiffrin, 2004).

Oxidative injury in the hypertensive vasculature is well illustrated by plenty of studies. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and uncoupled NO synthase are found abundantly in vessels of hypertensive animals (Mohazzab *et al.*, 1994; Pagano *et al.*, 1995; Landmesser *et al.*, 2003). Inhibition of the NADPH oxidase reduces vascular collagen in aldosterone-induced mice. This reflects that ROS stimulates the secretion of collagen by vascular smooth muscle (Pu *et al.*, 2003; Virdis *et al.*, 2004; Patel *et al.*, 2006). Further stimulation of NADPH oxidase in the vascular smooth muscle cells by angiotensin II worsens the etiology of hypertension (Griendling *et al.*, 1994; Patel *et al.*, 2014).

In the kidney, endothelial damage in afferent arterioles is caused by NADPH oxidase, as Ang II stimulates superoxide formation which then augments cytosolic calcium ion response to Ang II (Wang *et al.*, 2003; Fellner & Arendshorst, 2005). Oxidative stress also disrupted the balance between angiotensin II and dopamine signaling, resulting in elevated proximal tubular sodium transport (Banday *et al.*, 2007; Banday *et al.*, 2008). Medullary superoxide also leads to vasa recta vasoconstriction and favors sodium influx, thus natriuresis becomes diminished and blood is increased (Dickhout *et al.*, 2002; Cowley Jr *et al.*, 2003). Furthermore, the accumulated superoxide in macula densa also inactivates NO and worsens afferent arteriolar

vasoconstriction and glomerular filtration rate (Liu *et al.*, 2004). The effectiveness of antioxidants like tempol in renal protection was evidenced in Dahl salt-sensitive rats fed with a high sodium diet, at the same time endorsing the detrimental role of ROS in the kidney (Meng *et al.*, 2003).

1.2.1(e) Endothelial dysfunction

Vascular endothelial cells are involved in cardiovascular regulation by secreting very potent local vasoactive agents like the vasodilator molecule NO and the vasoconstrictor peptide endothelin (Hadi *et al.*, 2005). Under normal homeostatic conditions, the endothelium retains normal vascular tone and blood fluidity. However, cardiovascular risk factors such as hypertension, aging, hypercholesterolemia, smoking, hyperglycemia, and genetic factors are all associated with alteration in endothelial function. Local production of NO is amplified by endothelial-dependent vasodilator bradykinin (Libby, 2001).

Panza *et. al.* (1993) found out that clinically effective antihypertensive therapy is not able to restore the impaired endothelial dependent vascular relaxation in patients with essential hypertension and thus denoted that endothelial dysfunction is irreversible once hypertensive events are underway. However, with advancement in medical research, recent studies show that the new generation of β-blockers, including nebivolol and carvedilol can improve endothelial function in hypertensive patients owing to their antioxidant properties (Zepeda *et al.*, 2012). The trial on reversing endothelial dysfunction (TREND) showed that six months treatment with quinapril improved endothelial dysfunction as assessed by coronary artery diameter response to intracoronary acetylcholine infusion in normotensive patients with coronary artery disease (Mancini *et al.*, 1996). In the BANFF trial, eight weeks treatment with 20 mg

quinapril in 80 patients with coronary disease was showing improved flow mediated dilatation compared to baseline (Anderson *et al.*, 2000). The presence of the deletion allele has been associated with higher levels of circulating and tissue ACE while the BANFF trial shows quinapril was highly related to the presence of the insertion allele of the ACE genotype (Winkelmann *et al.*, 1996; Anderson *et al.*, 2000).

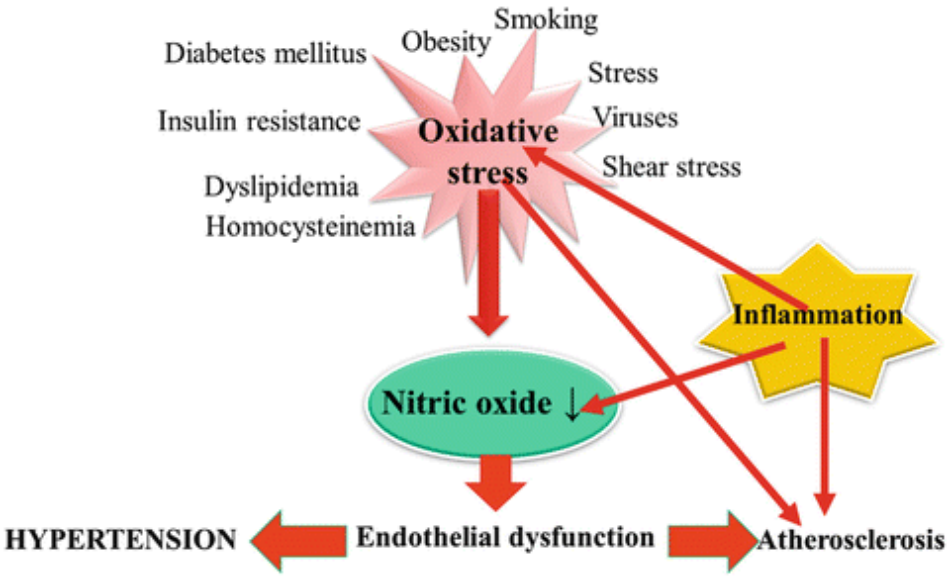


Figure 1.3 The relationship of oxidative stress and endothelial dysfunction to hypertension. [adapted from Konukoglu and Uzun (2016)]

1.3 Animal model for hypertension study

The journey of scientific research which aims to search for new ways to prevent, diagnose and treat diseases never ends. In order to achieve this, it is important to optimize the understanding of the human body and resolve various molecular mechanisms that cause disease. Currently, it is impossible to attain this goal without conducting animal studies (Lerman *et al.*, 2019). Over the past 70 years, a variety of animal models of hypertension mostly involving rats, have been developed for the study of the signaling pathways and molecular mechanisms involved in the pathophysiology of hypertension (Pinto *et al.*, 1998; Reyes-García *et al.*, 2022). It allows scientists to perform tests on vascular function and blood pressure regulation by various therapeutic

agents (Mitchell *et al.*, 2007). Lerman *et al.* (2005) classified animal models for hypertension study as non genetic and genetic models of hypertension.

1.3.1 Genetic model

Spontaneously hypertensive rats are so far the best model mimicking human essential hypertension (Mitchell *et al.*, 2007). Okamoto and Aoki (1963) were the first to introduce spontaneous hypertensive rats (SHR) that do not require physiological, pharmacological or surgical intervention. SHR is obtained by selective breeding of animals with the desired phenotype and inbreeding of the following generations has continued to achieve genetic homogeneity. After three generations of selective inbreeding, the rats were entirely hypertensive and the blood pressure retained at a significantly high level even after 15 weeks (Yamori, 2013). Studies showed that both SHR and essential hypertension patients do share some similarities such as multifactorial inheritance factor and the neural vasomotor tone and non-neural structural alterations which cause increased vascular resistance (Trippodo & Frohlich, 1981; Conrad *et al.*, 1995; Badyal *et al.*, 2003). SHR also becomes the base animal in developing the stroke prone SHR in which 80 % of the progeny will have cerebrovascular lesions and shorter life span imitating stroke in humans (Yamori *et al.*, 1976; Nabika *et al.*, 2012).

1.3.2 Non-genetic model

1.3.2(a) Deoxycorticosterone acetate (DOCA)-salt induced hypertension

DOCA-salt induced hypertension was first experimented on rats in early 1940s and is a secondary hypertension model which imitates the effects of mineralocorticoid- and glucocorticoid-induced hypertension (Selye *et al.*, 1943; Lin *et al.*, 2016). This

model is volume dependent as DOCA causes sodium and water retention in the body while overloading happens as only one kidney is left to cope with the changes. Since neither administration of DOCA nor partial removal of renal mass is effective in creating hypertension as this model is salt dependent, saline is usually substituted for the drinking water (Tajima *et al.*, 1983; Titze *et al.*, 2005). Extracellular volume expansion due to further reabsorption of salt and water, causes blood volume and blood pressure to increase. This model also results in different degrees of end organ damage involving heart, kidney and vascular system (Lafferty *et al.*, 1991; Kirchner *et al.*, 1993; Besse *et al.*, 1994; Temiz-Resitoglu, 2022). This is also one of the very few models that demonstrates low-renin hypertension and is very useful for the study of diuretics (Pinto *et al.*, 1998). Studies also related increased secretion of vasopressin to water retention and vasoconstriction while altering the activity of renin–angiotensin–aldosterone system leading to increased sympathetic sensitivity. Meanwhile, studies conducted by Iyer *et al.* (2010) provided further evidence that DOCA-salt hypertension in an animal is a reliable model of oxidative stress and inflammation based on pathophysiological and biochemical responses toward compounds that lower the concentrations of reactive free radical species.

1.3.2(b) Renovascular hypertension

Renovascular hypertension is defined as systemic hypertension resulting from vascular occlusion of the main renal artery (Textor, 2009). Induction of renovascular hypertension in the dog was first described by Goldblatt *et al.* (1934), and therefore it is often referred as “Goldblatt” hypertension. It is divided to three models: namely two kidney one clip model (2K1C) where both kidneys are preserved, but one of the renal artery is constricted; the one kidney one clip (1K1C) model where unilateral

nephrectomy was performed and the remaining artery was constricted; and two kidney two clips (2K2C) version which both renal arteries were occluded (Zeng *et al.*, 1998). In the two kidneys models, mild degree of ischemia and reduced renal perfusion pressure stimulates elevated renin synthesis and release from the clipped kidney. Meanwhile, the intact contralateral kidney responds to sodium retention by natriuresis. However, renovascular hypertension in rat models is a big challenge to researchers as neither all the animals develop stable hypertension nor survive through the experiment (Zeng *et al.*, 1998; Campos & Baltatu, 2012; Kasacka *et al.*, 2015). This model requires excessive use of animals as only 50 % of the animals will develop stable hypertension. While in the 1K1C model, an increase in blood pressure happens rapidly due to prompt salt and water retention because pressure diuresis and natriuresis do not take place as there is no collateral intact kidney. Plasma renin activity is usually normal and it soon becomes volume dependent (Wiesel *et al.*, 1997; Ibarz-Blanch *et al.*, 2022).

1.3.2(c) Dietary hypertension

The regulation of extracellular fluid volume by renal sodium excretion plays an important role in blood pressure homeostasis and the human kidney is able to maintain the salt load balance in the body (Ivy & Bailey, 2014). However, epidemiological data from different populations have shown that sodium intake is directly proportional to the prevalence of hypertension (He & MacGregor, 2009; Dong, 2018; Robinson *et al.*, 2019). Rat studies on the relationship between sodium intake and blood pressure in rats was well established by incorporating 0.9-8 % of sodium in their diet for 10 days to 15 months (Dobrian *et al.*, 2003; Gu *et al.*, 2008; Gomes *et al.*, 2017). A long duration of hypertension induction period was required in this model for stable hypertension condition. Besides that, several dog and rabbit studies proved that high salt intake

greatly increases both plasma and blood volume while augmenting arterial vasoconstrictor response (Korner *et al.*, 1980; Gupta *et al.*, 1981; Hainsworth *et al.*, 2003). Until now the mechanisms underlying sodium-induced hypertension are not fully understood, but alterations in renal function, fluid volume, fluid regulatory hormones, the vasculature, cardiac function, and the autonomic nervous system are believed to play a part in the disease prognosis (Farquhar *et al.*, 2015).

1.4 The importance of plants in allopathy and traditional medicine

Plants are widely used in herbal medicines throughout the world for health promotion and treatment of disease. The World Health Organization (2019) defined herbal medicines as herbs, herbal materials, herbal preparations and finished herbal products which contain parts of plants or its material, active ingredients, or combinations of it. In some countries, herbal medicines may also contain natural organic or inorganic active ingredients like animal and mineral materials added due to local traditional belief.

Herbal medicines are normally used to treat chronic illness or health promotion instead of to cure acute and life threatening conditions. Affordability and convenience are the main considerations in using herbal medicines, especially for the elderly. They are also concerned about the adverse effects of conventional medicines as herbal medicines are widely perceived as natural and safe. Sometimes, people also seek out herbal medicines when conventional medicine is ineffective in the treatment of disease, such as end stage cancer (Qato *et al.*, 2008; Wachtel-Galor & Benzie, 2011). Herbs are sometimes used as adjuvant therapy to conventional medicine. However, for about 80 % of people in many developing societies, herbal medicines are still the primary health

care that is readily available and affordable (World Health Organization, 2004; Rankoana, 2022).

Plants and natural sources are the fundamentals of modern medicine and play a crucial role in the pharmaceutical industry. Apart from being used as therapeutic agents directly, plants also serve as natural precursors for drug synthesis of pharmacologically active compounds (Li & Vederas, 2009). About 25 % of drugs prescribed worldwide and 11 % of the 252 WHO essential drugs list are plant derivatives (Rates, 2001). It is absolutely true as about 60 % of newly-synthesised antihypertensives are based on their natural product structures (Newman *et al.*, 2003).

Morphine, one of the well known pharmacologically active pure compounds is extracted from poppy seed *Papaver somniferum* (Klockgether-Radke, 2002). Besides morphine, others include the cardiac medication digoxin from *Digitalis purpurea* (Eichhorn & Gheorghiade, 2002), antimalarials quinine from Cinchona bark (Renslo, 2013), antipsychotic and antihypertensive drug reserpine from *Rauwolfia serpentina* and an aspirin precursor salicylic acid, derived from willow bark (Jack, 1997; Soni *et al.*, 2016).

1.4.1 Malaysian herbs and its contribution to health

Malaysia is well known as one of 12 megadiverse countries in the world with several G200 Ecoregions in East and West Malaysia with the tropical rainforests constitute the core of biodiversity in Malaysia (Ministry of Natural Resources and Environment, 2006, 2014). The herbs which are endemic to Malaysia are among the natural assets (Arifin, 2018). The Natural Product and Drug Development Centre (NPDC) under Malaysia Institute of Pharmaceuticals and Nutraceuticals (IPharm) (2019) is set up as the discovery hub of active therapeutic agents from Malaysia's rich

biodiversity natural resources. They are playing a vital role in developing the National Natural Products Repository (MyNature 50000) and isolation of potentially active compounds in the biopharmaceutical industry.

Between 1960 and 1981, 114 000 extracts of 35 000 plants were screened at the National Cancer Institute (Snader & McCloud). In 1986, specimens from the bintangor tree (*Calophyllum lanigerum*) were collected from Sarawak forest for cancer research (Ministry of Natural Resources and Environment, 2006). However, the researchers discovered bintangor tree contains calanolide A which is effective against the human immunodeficiency virus (HIV) (Patil *et al.*, 1993; Spino *et al.*, 1998). Due to very scarce yield, synthetic version of calanolide A, the (+)-calanolide A was developed and undergoing Phase II clinical trials (Mahomoodally & Gurib-Fakim, 2013). The search of possible drugs from the forest has never ended. Latex of *Calophyllum teysmanii* was found to contain a possible alternative to calanolide A, the costatolide which is slightly less active but can be isolated with 20-30 % higher in yield (Fuller *et al.*, 1994).

The herbal industry has been emphasised as a new source of economic growth contributor for Malaysia and herbs have been classified as potential agricultural commodities under the National Key Economic Area (NKEA) (Ministry of Agriculture and Agrobased Industry, 2011). Herbs have been considered as the crops of the future and several high value Malaysian herbs have been identified to be developed commercially in Malaysia and to cater to the increased demand of the local and export market. Among the herbs that were identified are tongkat ali (*Eurycoma longifolia*), kacip fatimah (*Labisia pumila*), misai kucing (*Orthosiphon stamineus*), pegaga (*Centella asiatica*), mengkudu (*Morinda citrifolia*), hempedu bumi (*Andrographis paniculata*), serai wangi (*Cymbopogon nardus*), roselle (*Hibiscus sabdariffa*), dukung

anak (*Phyllanthus niruri*) and mas cotek (*Ficus deltoidea*) (Mohd Hafizudin *et al.*, 2019).

1.4.2 The importance and challenges of herbal extract formulation

Scientific studies on herbal supplements by preclinical studies and clinical trials increase the confidence of the public towards the herbal extract formulation. The demand for taking commercial health supplements for health maintenance and disease prevention has increased over time (Ahmad *et al.*, 2015).

World Health Organization (2018b) defined herbal dosage forms as the herbal products in various physical forms like liquid, solid or semi-solid, with or without excipients, in a particular formulation. Herbal dosage form can be consumed in various ways and forms, including whole herb, teas, syrup, or capsules and tablets that contain a ground or powdered form of the herb or its concentrated extract. Herbal ingredients can also be incorporated in ointments to use as agents. Different solvents like the most common water, ethanol and methanol are used to extract herbal extracts via different methods. Alcoholic extracts are known as tinctures, hot water extracts are tisanes, decoction involves long term boiling, while maceration is a cold infusion of plants (Kadiri *et al.*, 2010; World Health Organization, 2018b).

Unlike most of the over the counter medicines which are formulated with single compound chemicals, herbal medicines contain minimally processed plant or plant extracts which contain multiple phytochemicals. The innate characteristic of traditional medicine bestowed synergistic potential for treating complex diseases, but also increased the challenges to formulate plant extracts into dosage forms (Yang *et al.*, 2014). Plant extracts always contain a combination of valuable but little amounts of active compound and large amounts of secondary material like polysaccharides, salts,

sugars and acids that can significantly hinder the design of dosage form and the stability of the finished product (Bonati, 2017).

The efficacy of a herbal medicine can fluctuate from batch to batch due to variation in quality and quantity if there is no standardization of the potent components within it. Environmental factors, time of harvesting and post-harvesting conditions are the main causes of variation between batches of herbal products (Folashade *et al.*, 2012). Besides that low yield from the natural products also become one of the great challenges in dosage form formulation of herbal medicine. The formulation also involves a lot of excipients in order to enhance the stability of the herbal extract.

1.5 *Hibiscus sabdariffa*

Genus Hibiscus which belongs to the Malvaceae family has more than 300 known species which are widely cultivated as ornamental plants. *Hibiscus sabdariffa* (HS) is generally known as roselle or asam susur in Malaysia (Osman *et al.*, 2011). Its calyx is used more intensively than leaves, seeds and roots in traditional medicine. The seed oil is used to produce scrubs and soaps in Malaysia (Ismail *et al.*, 2008). In Sudan, furundu is a traditional dish prepared from fermented HS seed (Yagoub *et al.*, 2004). The leaves are used traditionally as an antiscorbutic while the bitter root contains tartaric acid and is suitable to make aperitif and tonic (Alegbejo *et al.*, 2003; Mungole & Chaturvedi, 2011).

Table 1.2 Taxonomic classification of *Hibiscus sabdariffa* (USDA, 2000)

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Dilleniidae
Order	Malvales
Family	Malvaceae
Genus	*Hibiscus*
Species	*Hibiscus sabdariffa*

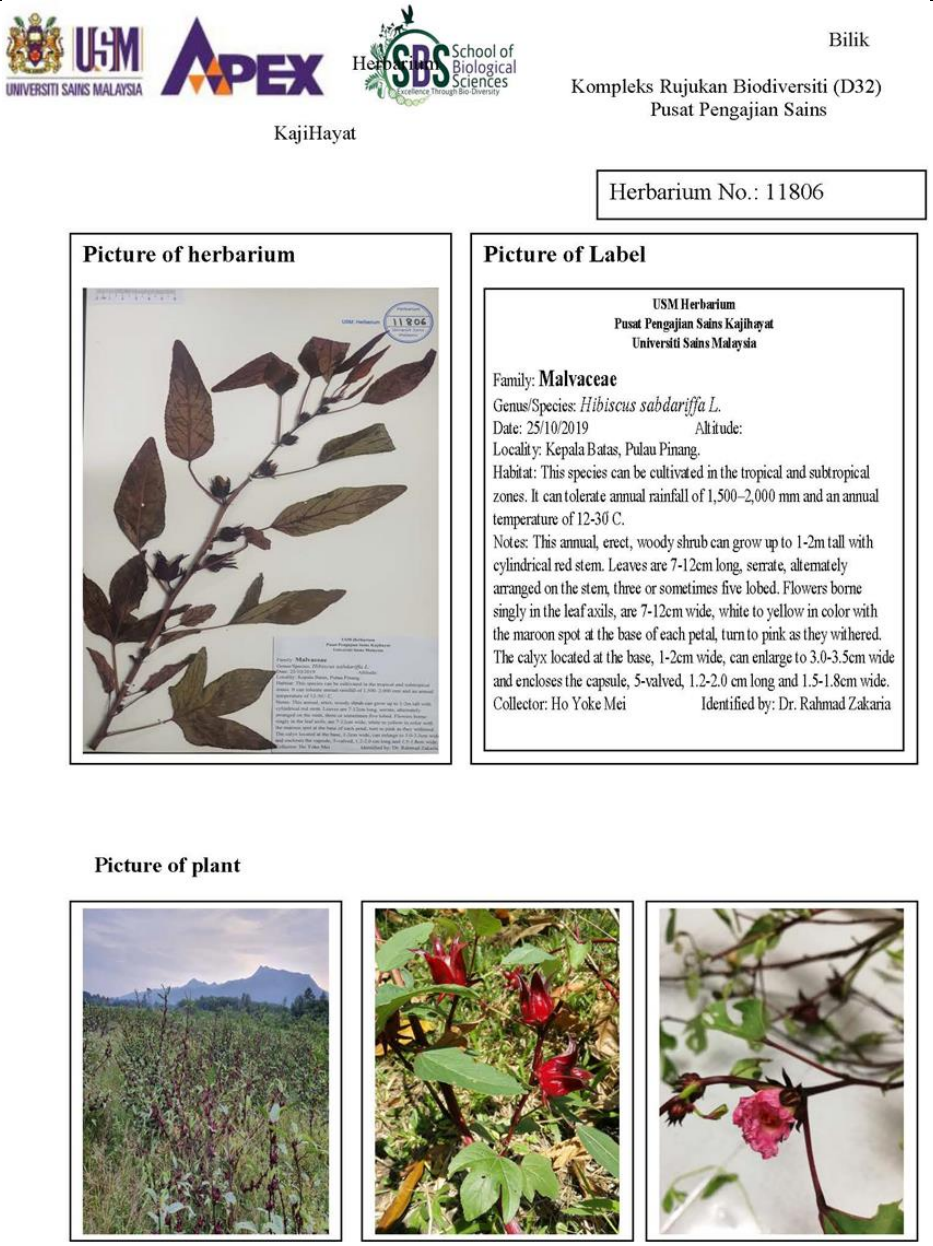


Figure 1.4 Voucher specimen of *Hibiscus sabdariffa* (11806) deposited at Herbarium, School of Biological Sciences USM.

1.5.1 General description

HS has broad leaves, predominantly yellow or red solitary and axillary flowers and reddish stems up to 1.1 m high with tapering root (Lim, 2014). HS adapts to a variety of soils in a warm and humid environment. However, crop improvement through conventional hybridization is not easy due to its cleistogamous flowers. Thus, the intensive mutation breeding program to produce a more superior genotype was introduced (Osman *et al.*, 2011). HS originated from West Africa and is a relatively new crop in Malaysia since first introduced in early 1990s. Terengganu has the very first commercial plantation and it has then spread to other states where Pahang and Johor are now leading in this plant cultivation (Siti, 2016). HS is promoted as a unique commodity export and its multiple harvests after three months planting are able to generate rapid income (Nik, 2010; Bernama, 2018).

HS calyces' role in the food industry is versatile. In Pakistan, HS calyces are used in the fruit-preserving industry as pectin while in America, Asia and Europe it is mainly used as a food colorant. In the Caribbean, Christmas celebrations without HS juice are considered incomplete. Carib Brewery Trinidad Limited produces a Shandy Sorrel HS flavored alcoholic drink (Lim, 2014).

1.5.2 Nutrient content

The United States Department of Agriculture Agricultural Research Service (2018) reported that raw HS contains the following nutrient content.

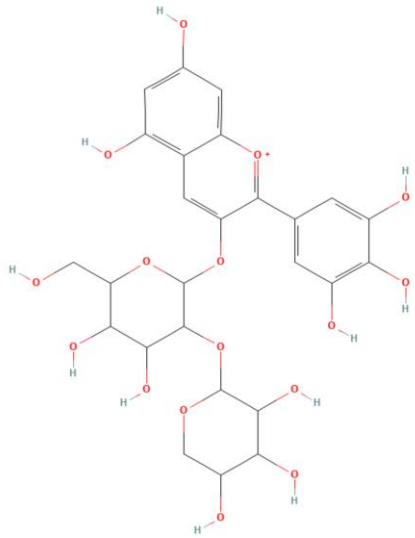
Table 1.3 Nutrient content of HS adapted from USDA (2018)

Nutrient	Unit	per 100 g
Proximates		
Water	g	86.58
Energy	kcal	49
Energy	kJ	205
Protein	g	0.96
Total lipid (fat)	g	0.64
Ash	g	0.51
Carbohydrate, by difference	g	11.31
Minerals		
Calcium, Ca	mg	215
Iron, Fe	mg	1.48
Magnesium, Mg	mg	51
Phosphorus, P	mg	37
Potassium, K	mg	208
Sodium, Na	mg	6
Vitamins		
Vitamin C, total ascorbic acid	mg	12
Thiamin	mg	0.011
Riboflavin	mg	0.028
Niacin	mg	0.31
Vitamin B-12	µg	0
Vitamin A, RAE	µg	14
Retinol	µg	0
Vitamin A, IU	IU	287
Lipids		
Fatty acids, total trans	g	0
Cholesterol	mg	0

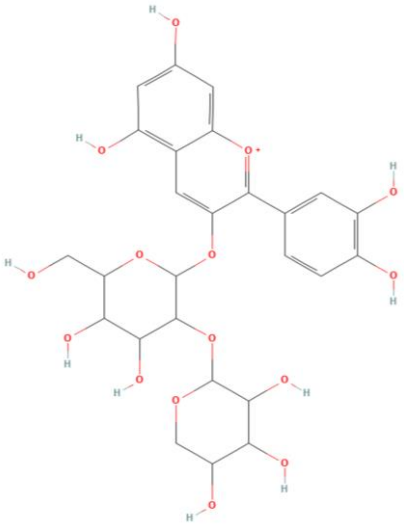
1.5.3 Phytochemicals

Phytochemical is a broad term defined as the bioactive non-nutritive components that present naturally in plants as the Greek word “phyto” means plant (Arendt & Zannini, 2013; Diep *et al.*, 2014). Within the plant, the phytochemicals play the role of granting the plant with its unique hue, scent, flavor and protect them from infection and predators (Watson *et al.*, 2018). Consumption of phytochemicals are believed to exert protective effects against various diseases and maintain general health in humans (Süntar & Yakıncı, 2020). The contribution of all researchers in phytochemicals screening and identification is undeniable. Their efforts have opened up possibilities of advancement in nutraceuticals and pharmaceuticals.

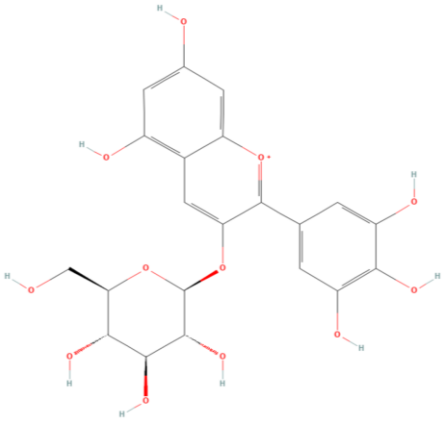
The most representative anthocyanins in the extract, namely delphinidin 3-sambubioside and cyanidin 3-sambubioside, are also accompanied by substantial amounts of phenolic compounds. The process of discovery of the particular anthocyanin in HS was very lengthy. At first, delphinidin 3-sambubioside was isolated as hiviscin by Yamamoto and Osima (1932) who then renamed it as delphinidin-pentoside-glucoside in 1936. In later years, delphinidin 3-glucoside, cyanidin 3-sambubioside (gossypicyanin) and cyanidin 3-glucoside (chrysanthemin) were isolated from HS which originated from Taiwan and Trinidad (Shibata & Furukawa, 1969; Du & Francis, 1973). Around that time, cyanidin 3,5-diglucoside and cyanidin-3-(2G-glucosylrutinoside) were discovered in HS var. altissima (Subramanian & Nair, 1972). Anthocyanin of HS is mainly distributed in the calyces (Degenhardt *et al.*, 2000; Alarcon-Aguilar *et al.*, 2007; Beltrán-Debón *et al.*, 2010; Alarcón-Alonso *et al.*, 2012) and also is present in leaves (Rodríguez‐Medina *et al.*, 2009), stems and roots (Rahim, 2014).



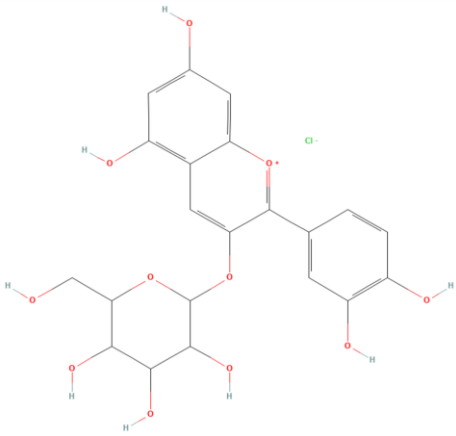
(a) Delphinidin 3-sambubioside



(b) Cyanidin 3-sambubioside



(c) Delphinidin 3-glucoside



(d) Cyanidin 3-glucoside

Figure 1.5 Anthocyanins that can be found in the calyces of *Hibiscus sabdariffa* abundantly are delphinidin 3-sambubioside, cyanidin 3-sambubioside, delphinidin 3-glucoside and cyanidin 3-glucoside.

Besides that, volatile compounds produce the distinctive aroma of HS. In a study conducted by Jirovetz *et al.* (1992), more than twenty five volatile compounds, mainly unsaturated hydrocarbons, alcohols and aldehydes from C8 to C13 were reported in the seed oil of HS. Afterward, thirty seven volatile compounds in HS calyces were characterised including fatty acid derivatives (such as 2-ethylfuran and hexanal), sugar derivatives (furfural and 5-methyl-2-furaldehyde), phenolic derivatives (eugenol), terpenes (such as 1,4-cineole, limonene) and miscellaneous compounds (e.g. acetic