# ORAL EPIGALLOCATECHIN GALLATE DECREASES BIOAVAILABILITY OF NADOLOL VIA MODULATION OF ILEAL AND HEPATIC OATP1A5, MDR1A AND OCT1 mRNA LEVELS IN SPONTANEOUSLY HYPERTENSIVE RATS

By

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A dissertation submitted to the Department of Pre-clinical Sciences,

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## ABSTRACT

# ORAL EPIGALLOCATECHIN GALLATE DECREASES BIOAVAILABILITY OF NADOLOL VIA MODULATION OF ILEAL AND HEPATIC OATP1A5, MDR1A AND OCT1 mRNA LEVELS IN SPONTANEOUSLY HYPERTENSIVE RATS

**Tan Hong Jie**

Concurrent use of epigallocatechin-3-gallate (EGCG) and medication may induce herb-drug interactions, which lead to therapeutic failure or drug toxicity if leave negligence. It has been reported that EGCG reduces bioavailability of plasma nadolol in normotensive models. Nevertheless, evidence on the effects of EGCG on hypertensive model, and the possible underlying mechanism have not been elucidated. This study aims (i) to investigate the effects of EGCG on bioavailability of nadolol (maximum plasma concentration, time required to reach maximum concentration in plasma, area under the time-plasma concentration curve, plasma half-life and total clearance) and subsequently its impact on blood pressure control; and (ii) to identify transcriptional regulatory roles of EGCG on the nadolol intestinal and hepatic drug-transporters in spontaneously hypertensive rats. Male SHR were pre-treated with EGCG (10 mg/kg body weight, i.g.), once daily dose for consecutively 13 days. At day-14, a single dose of nadolol (10 mg/kg body weight) was given to the rats 30 minutes after the last dose of EGCG administration. Systolic blood pressure (SBP) was measured at 6-h and 22-h post-nadolol administration. Plasma and

urinary excreted nadolol concentrations were quantified via high performance liquid chromatography, and the pharmacokinetic parameters were analysed by using non-compartmental analysis. Hepatic and ileal *Oatp1a5, Mdrla,* and *Oct1* mRNA expressions were determined by real-time PCR. SBP of SHR pre-treated with EGCG and received nadolol was significantly higher than those which were not pre-treated with EGCG but received nadolol. Pre-treatment of EGCG resulted in a marked reduction of plasma nadolol maximum concentration ($C_{max}$) and area under the time-plasma concentration curve (AUC) by 53% and 51% compared to its control. The 14-day treatment with oral EGCG led to a significant downregulation of mRNA levels of ileal *Oatp1a5, Mdr1a,* and *Oct1* genes by 4.03-, 8.01- and 4.03-fold; and hepatic *Mdr1a,* and *Oct1* genes by 2.61- and 2.66-fold. These data concluded that exposure to EGCG reduces bioavailability of nadolol and caused uncontrolled raised blood pressure with increased risks of cardiovascular events. Our data suggest that the reduced bioavailability of nadolol associates with the downregulation of ileal *Oatp1a5* and *Oct1* mRNA levels that subsequently lead to poor absorption of nadolol to the systemic circulation.

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**APPROVAL SHEET**

This dissertation entitled "**ORAL EPIGALLOCATECHIN GALLATE DECREASES BIOAVAILABILITY OF NADOLOL VIA MODULATION OF ILEAL AND HEPATIC OATP1A5, MDR1A AND OCT1 mRNA LEVELS IN SPONTANEOUSLY HYPERTENSIVE RATS**" was prepared by TAN HONG JIE and submitted as partial fulfilment of the requirements for the degree of Master of Medical Science at Universiti Tunku Abdul Rahman.

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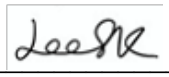

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It is hereby certified that **TAN HONG JIE** (ID No: **19UMM00010**) has completed this dissertation entitled "**ORAL EPIGALLOCATECHIN GALLATE DECREASES BIOAVAILABILITY OF NADOLOL VIA MODULATION OF ILEAL AND HEPATIC OATP1A5, MDR1A AND OCT1 mRNA LEVELS IN SPONTANEOUSLY HYPERTENSIVE RATS**" under the supervision of Dr. Lee Siew Keah (Supervisor) from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences, and Dr. Ling Wei Chih (Co-Supervisor) from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences.

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## DECLARATION

I TAN HONG JIE hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

(TAN HONG JIE)

Date: 04 Mar 2022

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## LIST OF ABBREVATIONS

| | |
|---|---|
| ABPM | Ambulatory blood pressure |
| ACEI | angiotensin-converting enzyme inhibitors |
| ADME | Absorption, distribution, metabolism, and excretion |
| AE | Adverse events |
| AHA/ACC | American Heart Association and American College of Cardiology |
| Ang II | Angiotensin II |
| AP-1 | Activator protein 1 |
| ARB | angiotensin receptor blockers |
| AUC | Area under curve |
| BB | beta blockers |
| BP | Blood pressure |
| BSP | Bromosulphophthalein |
| CCB | calcium channel blockers |
| cGMP | Cyclic guanosine monophosphate |
| CHD | Coronary heart disease |
| $CL_R$ | Renal clearance |
| CO | Cardiac output |
| COPD | Chronic obstructive pulmonary disease |
| CVD | Cardiovascular diseases |
| CYP | Cytochrome P450 |
| CYP | Cytochrome P450 |
| DALYs | Disability-adjusted life years |

| | |
|---|---|
| DBP | Diastolic blood pressure |
| EC | Epicatechin |
| ECG | Epicatechin-3-gallate |
| ECM | Extracellular matrix |
| EDCF | Endothelial derived contracting factors |
| EGC | Epigallocatechin |
| EGCG | Epigallocatechin gallate |
| ESC/ESH | European Society of Cardiology and European Society of Hypertension |
| ET | endothelin |
| GBD | Global burden of disease |
| GIT | Gastro-intestinal tract |
| GSH | Glutathione |
| GTE | Green tea extract |
| GTP | Guanosine triphosphate |
| GWAS | Genome-wide association studies |
| HBPM | Home blood pressure monitoring |
| HDI | Herb-drug interactions |
| HPLC | High performance liquid chromatography |
| HR | heart rate |
| IHD | Ischemic heart disease |
| IM | Intramuscular injection |
| IT | Intrathecal injection |
| IV | Intravenous injection |
| LIFE | Losartan Intervention for Endpoint |

| | |
|---|---|
| MDR1 | Multidrug resistance protein 1 |
| MI | Myocardial infarction |
| miRNA | Micro-RNA |
| $MMP^+$ | Methylphenyl pyridinium |
| NCA | Non-compartmental Analysis |
| NCD | Non-communicable disease |
| NHMS | National Health and Morbidity Survey |
| NICE | National Institute for Health and Care Excellence |
| NO | Nitric oxide |
| OAT | Organic anion transporters |
| OATP | Organic anion transporting polypeptide |
| OCT | Organic cation transporter |
| PBS | Phosphate-buffered saline |
| PCFT | Proton-coupled Folate Transporter |
| P-gp | P-glycoprotein |
| PNS | Parasympathetic Nervous System |
| RAAS | Renin-angiotensin-aldosterone system |
| RCT | Randomised controlled trials |
| RCULAC | Responsible Care and Use for Laboratory Animal Course |
| Ren-2 TGR | (mREN2)2 transgenic rat |
| ROS | Reactive oxygen species |
| RR | Relative Risk |
| SBP | Systolic blood pressure |
| SD | Sprague-Dawley |
| SEM | Standard error mean |

| | |
|---|---|
| sGC | Soluble guanylyl cyclase |
| SHR | Spontaneously hypertensive rats |
| SNS | Sympathetic nervous system |
| SQ | Subcutaneous injection |
| SV | stroke volume |
| $T_{1/2}$ | Half-life |
| TZ | Thiazide diuretics |
| UGT | Uridine 5’-diphospho-glucuronosyltransferase |
| USDA | United States department of agriculture |
| VPR | Volume-pressure recording |
| VSMC | Vascular smooth muscle cell |
| WKY | Wistar-Kyoto |

# CHAPTER 1

## INTRODUCTION

Hypertension or persistently raised blood pressure is the most prominent risk factor for cardiovascular diseases, which attributes to 17.79 million global deaths yearly, as reported in the Global Burden of Disease (GBD) study 2017, which is published in *Lancet* (Roth et al., 2018). Hypertension is a manageable and treatable disease, however, less than 13% of hypertensive patients achieved blood pressure control (<140/90 mm Hg) with active prescription or treatment (Attaei et al., 2017, Lu et al., 2017). Lifestyle, environmental factors, and/or dietary habits and patients' low compliance to treatment have been hypothesised as the factors contributing to the low effectiveness in hypertension management (Clement, 2017). Herbal medicine (or phytomedicine) is gaining popularity among hypertensive patients. It is not unexpected that herb-drug interactions could lead to reduced therapeutic or toxic effects.

Some herbal products and their active compounds modulate drug metabolising enzymes (cytochrome P450 and phase II conjugate enzymes), as well as drug transporters (Won et al., 2012, Zhou et al., 2004, Albassam and Markowitz, 2017). Modulations on the drug metabolising enzymes and/or drug transporters might lead to an increased risk of drug toxicity or therapeutic failure. Unfortunately, the recognition on drug interactions in today's practice is not

widespread and often being neglected. In fact, herb-drug interactions will continue to propagate with the common use of herbal medications (Albassam and Markowitz, 2017, Fugh-Berman, 2000).

Epigallocatechin gallate (EGCG) is one of the most abundant catechin compounds that belong to the flavonoid family, which is extracted from the green tea plant, *Camellia sinensis*. Green tea extract (GTE) and EGCG have been extensively studied for their therapeutic effects. Research findings suggest that they might be useful in the treatment and prevention of numerous diseases, including cardiovascular diseases, metabolic diseases, neurodegenerative disease and cancer (Liu et al., 2014, Dou et al., 2008, Wang et al., 2014). The anti-hypertensive effect of EGCG has been well-established in pre-clinical studies and has been frequently recommended as an anti-hypertensive therapeutic molecule (Potenza et al., 2007, Lorenz et al., 2015). As of reported potential health beneficial effects, GTE and purified EGCG are available in retails since recent years, mainly are registered as an herbal supplement, and they have become the popular choices among consumers (Won et al., 2012, Albassam and Markowitz, 2017). It is unavoidable that hypertensive patients might be taking highly concentrated GTE or purified EGCG as a part of their complementary treatment with or without the knowledge of their medical consultant. This scenario gains a health concern when accumulating consumers assuming these products are ‘natural’ and therefore are not aware of potential interactions of these with other medicines that they are taking.

A number of *in-vitro* and *ex-vivo* studies have shown that EGCG modulates cytochrome P450 (CYP), and drug transporters, such as proton-coupled folate transporter (PCFT), multidrug resistance protein 1 / p-glycoprotein (MDR1/P-gp), organic anion transporting polypeptide (OATP) etc., therefore affects the plasma concentrations of relevant substrate (Knop et al., 2015, Kissei et al., 2014). It has been reported that EGCG possesses inhibitory activities on MDR1 efflux and hepatic CYP3A subfamilies, and resulted in increased plasma concentration of the drug subtracts like verapamil, nicardipine, diltiazem, tamoxifen, erlotinib, etc. (Chung et al., 2009, Choi and Burm, 2009, Li and Choi, 2008, Shin and Choi, 2009, Zhang et al., 2008). Nevertheless, these *in-vitro* and *ex-vivo* studies do not represent multicellular responses and *in-vivo* studies are required to further confirm the roles of EGCG on drug metabolisms in biological entities.

Studies have demonstrated that either ingestion of green tea beverage, oral administration of green tea extract or EGCG in normotensive volunteers or rodents could significantly reduce the bioavailability of nadolol (Misaka et al., 2013c, Abe et al., 2018, O.Abe et al., 2017). Those research findings suggest that patients on nadolol treatment should stop taking green tea, GTE or EGCG to avoid therapeutic failure. However, can these findings be generalised to the hypertensive population? Besides, what is the impact of reduced bioavailability of nadolol on the pharmacological action in hypertensive conditions? It is well established that physiological actions of the cardiovascular functions in normotensive and hypertensive subjects are dissimilar. In addition, the vascular permeability and/or the vulnerability of transmembrane fluxes including drug-

transporters towards green tea extract of EGCG could be very different (Haddy, 1983). In addition, previous studies did not clarify the underlying mechanism involved in reduced bioavailability of nadolol in GTE or EGCG supplemented subjects/experimental models.

Therefore, by using a genetically hypertensive rodent model i.e. spontaneously hypertensive rats (SHR) - the closest animal model that represents human essential hypertension, the present study aims (i) to investigate the effects of EGCG on bioavailability of nadolol (maximum plasma concentration, time to achieve maximum concentration, area under the time-plasma concentration curve, plasma half-life and total clearance) and subsequently its impact on blood pressure control and (ii) to identify transcriptional regulatory roles of EGCG on intestinal and hepatic drug-transporters in SHR. The findings of this study will provide us with scientific evidence for a better understanding of the impacts of EGCG on pharmacokinetics and pharmacological actions of nadolol in hypertensive model. Underlying foundations of the observable facts i.e., regulatory actions of EGCG in the modulation of intestinal and hepatic drug-transporters at the transcriptional and translational levels can be elucidated as well. This evidence is significant when considering nadolol dose adjustment or use of alternative agents among EGCG or habitual green tea consumers.

**Research Objectives**

The study aimed: (a) to investigate the effects of EGCG on bioavailability of nadolol (maximum plasma concentration, time to achieve maximum concentration, area under the time-plasma concentration curve, plasma half-life and total clearance) and (b) to identify transcriptional regulatory roles of EGCG on *Oatp1a5*, *Mdr1a*, and *Oct1* intestinal and hepatic drug-transporters in SHR.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Cardiovascular Diseases

Cardiovascular diseases (CVD) are disorders whereby the blood vessels and heart are getting affected. CVD are categorised into 4 different types, there are a. coronary heart disease (CHD), including angina, heart attacks and heart failure; b. stroke and transient ischemic attack; c. peripheral arterial disease; and d. aortic disease. Although the aetiology of CVD is presently remains unclear, however, hypertension, hypercholesterolemia, diabetes have been outline as the major risk factors for CVD (Benjamin et al., 2019).

### 2.1.1 Epidemiology of Cardiovascular Diseases

Based on the data and statistics available from World Health Organisation, approximately 17.9 million people dies from CVDs in 2016, comprising 31.4% of deaths globally (World Health Organisation, 2017) and a raise of 25.2% (from 14,284,170 to 17,858,012) since the year 2000 (World Health Organisation, 2018). CHD and stroke are the main causes of global death (16.6% and 10.2% of all-causes death respectively) and contributed to the highest percentage in CVD mortality (52.8% and 32.4% respectively). In

addition, hypertensive heart diseases contributed to 5% of CVD mortality (897,680 death in 2016), which is the third-highest cause of death in CVD (World Health Organisation, 2018). Disability-Adjusted Life Years (DALYs) is a time-based measurement used to estimate the global burden of disease (GBD) by combining years of life lost due to disability and years of life lost due to premature mortality. The recent statistics showed that CVD contributed 393.11 million DALYs across 369 diseases and injuries from 204 countries, being 15.5% of total DALYs. Stroke and CHD are the main contributors to the worldwide DALYs, which comprising 7.2% and 5.7% of DALYs and has increased 50.4% and 32.4% respectively since the year 1990 (GBD 2019 Diseases and Injuries Collaborators, 2020a).

In Malaysia, CVD and other related circulatory diseases were the largest contributors to deaths in year 2014, which accounted for 34.8% of total death. Among all-causes of deaths, cerebrovascular diseases or strokes were the leading cause of deaths which contributed to 15.2% of the total deaths, followed by ischaemic heart disease or CHD, with 14.8% of total deaths. In addition, CVD is the main contributor to the total burden of disease and injury, with the highest DALYs compared to other causes, making up 20.8% (or 1,040,536) of total DALYs (or 4,992,646). Among total DALYs, CHD was the leading cause of total burden in Malaysia, contributing to 9.3% of the total DALYs, and strokes were the third highest, with 7.9% of total DALYs in the year 2014 (Institute for Public Health, 2017).

### 2.1.2 Risk Factors for CVD

The two main groups of CVD risk factors are modifiable and non-modifiable risk factors. Hypertension, obesity, physical exercises, smoking, eating behaviour, alcoholism profile and socioeconomic status are categorised as modifiable risk factors. Demographics, including age, gender, and ethnicity, family history, and diabetes are categorised as non-modifiable risk factors. The World Health Organisation has recognised hypertension as the most important global risk factor for morbidity and mortality, and it is the strongest risk factor amongst other factors contributing to cardiovascular diseases (Kjeldsen, 2018).

## 2.2 Hypertension

Blood pressure (BP) is the force against the walls of the blood vessel, especially arteries, exerted by the circulating blood in the body. The BP of an individual is maintained by peripheral vascular resistance and cardiac output. Peripheral vascular resistance is defined as the resistance produced to the blood flow in the peripheral arterial vessel, which involved a series of anatomical and functional modifications among the blood vessels, such as blood viscosity, tone of vascular musculature, and vessel diameter. Cardiac output (CO) is defined by the volume of blood in millilitres pumped from the left ventricle of the heart into the circulation in a minute, which is greatly influenced by the stroke volume, as well as the heart rate. The number of heartbeats in one minute is known as the heart rate (HR) while the amount of blood that is capable to be pumped out from the ventricle per beat is known as stroke volume (SV). Arterial pressure is

greatly controlled by the levels of plasma ions, specifically sodium, and renal function that is involved in the body fluid balance. For the local blood circulation, an auto-regulatory mechanism modulates the diameter of the blood vessel to achieve the optimal tissue perfusion. When auto-regulatory failed to respond, the peripheral vascular resistance will be elevated, subsequently, increasing and raising the BP. Persistent elevation of BP is diagnosed as hypertension (Giuseppe et al., 2014).

The blood pressure measurement methods utilised in *in-vivo* studies can be categorised into invasive method and non-invasive method. Invasive blood pressure (IBP) monitoring techniques which include artery cannulation and radio-telemetry methods, provide continuous blood pressure monitoring. However, general anaesthesia and surgery procedures are required in order to measure the blood pressure (BOGDAN et al., 2019).

IBP monitoring via artery cannulation is a procedure that involves the placement of a catheter into an artery, generally carotid artery, femoral artery or caudal ventral artery, and transmission of pressure to the calibrated transducer, which placed on left atrium. Data acquisition system is used to collect and amplify the signals from transducer, further translate the signal into digital signal with numeric value. The BP in numeric digit can be monitored in real-time manner during the measurement. The short-term BP can be done under sedation and long-term BP can be monitored in awaken animal with tunnelled catheters under the skin. While radio-telemetry method enables the continuous BP monitoring in freely moving animal with higher data reliability, however, a

radio transmitter implantation in the abdominal cavity is required. (Please elaborate more to balance the details you have elaborated for cannulation method)(BOGDAN et al., 2019).

Non-invasive blood pressure (NIBP) measurement involves the detection of blood flow that pass through the pressurised occlusion cuff. Typically, there are three types of sensor technologies used in NIBP, including piezoplethysmography, volume pressure recording and photoplethysmography. During the procedure, rodent model is immobilised in a stress-free condition without any form of distraction, follow by the application of the occlusion cuff and sensor to the tail. With the piezoplethysmography sensor, as known as Doppler system, the sensor with the acoustic gel detects the Korotkoff sound generated by the vibration of arterial wall and first blood flow through the artery; With the volume pressure system, differential transducer is used to measure the BP and has been reported to be the most consistent and accurate method to measure BP among other NIBP approaches; With the photoplethysmography system, blood pressure pulse is detected by photoelectric sensor at the tail temperature of 30-32℃. For all the three NIBP approaches, BP can be affected by several factor, including thermic stress, noise etc., and produces less reliable reading, hence, tail temperature and time of measurement should be standardised to overcome the inaccuracy caused (BOGDAN et al., 2019).

Despite the IBP produces higher data reliability, however, these techniques involves complicated surgical procedures, increased risk of morbidity due to the surgical errors. While NIBP is widely used in rodent blood

pressure measurement as it is non-invasive, with lesser stress induction, and easy to apply. The choices of IBP or NIBP are certainly based on the experimental designs and study requirements. (BOGDAN et al., 2019).

### 2.2.1 Current Hypertension Measurement and Diagnosis

Human blood pressure can be measured by using a non-invasive sphygmomanometer with the arm outstretched and supported in the clinical setting. The upper arm is a preferred anatomical location for BP measurement, where the sphygmomanometer cuff and bladder are adjusted to fit an individual arm circumference. Generally, the unit of BP is expressed as millimetre mercury (mmHg) and the BP is recorded as (i) systolic blood pressure (SBP), which is defined as the maximum pressure against the blood vessel when ventricular contraction happens; and (ii) diastolic blood pressure (DBP) which is defined as the pressure applied to the vessel during relaxation and prior to the next contraction. Table 2.1 shows the definition of hypertension according to some selected guidelines, such as the Clinical Practice Guidelines on the Management of Hypertension (Ministry of Malaysia, 2018), the National Institute for Health and Care Excellence (NICE) in the UK (National Institute for Health and Care Excellence, 2019), the American Heart Association and American College of Cardiology (AHA/ACC) Hypertension Guidelines (Whelton et al., 2018) and the European Society of Cardiology and European Society of Hypertension (ESC/ESH) Guideline (Whelton et al., 2018). Generally, hypertension is diagnosed when clinic BP achieves a value of ≥140 / ≥90 mmHg (SBP/DBP). The latest ACC/AHA guideline recommends that hypertension should be

diagnosed when SBP ≥130 mmHg and/or DBP ≥80 mmHg instead of SBP ≥140 and/or DBP ≥90 mmHg. However, other guidelines remain the existing classification with the opinion that the proposed new definition (lower cut-off) by ACC/AHA does not change the management strategies, especially for patients with cardiovascular complications (Ministry of Health Malaysia, 2018).

Other than clinic BP, ambulatory blood pressure monitoring (ABPM) and home blood pressure monitoring (HBPM) are recommended by most of the guidelines, which both HBPM and ABPM have significant values to the hypertension prognosis (Eguchi et al., 2011). "White-Coat" hypertension, or known as isolated office hypertension, is a condition that elevated blood pressure was seen in clinic BP but not in HBPM and ABPM, whereas masked hypertension is a condition where clinic blood pressure is normal, but ABPM and HBPM shown an elevated blood pressure. In 2019, NICE has revised its hypertension guidelines to make ABPM a mandatory measurement to diagnose hypertension along with clinic BP, adding that HBPM could be an alternative to ABPM. ABPM of SBP ≥135 and/or DBP ≥85 could be diagnosed as hypertension. in the diagnosis of essential hypertension (National Institute for Health and Care Excellence, 2019).

**Table 2.1** Definition of hypertension based on the SBP and DBP values from different guidelines.

| Types | SBP (mmHg) | DBP (mmHg) | Remark |
|---|---|---|---|
| CPG Malaysia 2018 | | | |
| Clinic BP | ≥140 | ≥90 | Repeated Measurement |

| | | |
|---|---|---|
| NICE – UK 2019 | | |
| Clinic BP | ≥140 | ≥90 |
| ABPM/HBPM† | ≥135 | ≥85 |
| AHA/ACC -US 2017 | | |
| Clinic BP | ≥130 | ≥80 |
| ESC/ESH 2018 | | |
| Clinic BP | ≥140 | ≥90 |

†The ABPM/HBPM has been listed as a mandatory measurement in hypertension diagnosis in NICE Guidelines 2019

### 2.2.2 Burden of Hypertension in Worldwide and Malaysia

About 1.13 billion of the population worldwide is diagnosed with hypertension (World Health Organisation, 2019). Hypertension is the main risk factor that attributes to 25.8% of total death (10.8 million) and 14.57% of total DALYs, which stand for 369.52 million. In addition, hypertension attributes to 53.65% of total deaths in CVD, among which 53.2% was linked to ischemic heart disease (IHD), and 52.57% was related to stroke. Hypertension has been ranked as the 2nd and 3rd major risk factor for diseases in women and men, respectively, contributing to 7.78% and 9% of total DALYs in 2019, of which accountable for large association with CVD followed by diabetes and renal diseases (GBD 2019 Risk Factors Collaborators, 2020b).

In Malaysia, hypertension has a prevalence of 30.3% as reported in the National Health and Morbidity Survey (NHMS) 2015, and this prevalence was

the highest among ASEAN countries (Ministry of Health Malaysia, 2015). Mahadir Naidu et al. (2019) highlighted that the prevalence of hypertension in Malaysia could be as high as 66.8% if pre-hypertensive (SBP 130 – 139 mmHg and/or DBP 80 – 89 mmHg) were to be taken into consideration. It has been reported that the pre-hypertensive population accounted for 45.8% of the study population and a recent study has reported that the pre-hypertension in younger adults (<50 years old) have a higher prevalence to progress to the hypertensive stage (Kanegae et al., 2017).

### 2.2.3 Management of Hypertension

Studies has revealed that the reduction in BP is associated with a lower probability of CV morbidity and mortality, and all-cause mortality (Bundy et al., 2017, Ettehad et al., 2016). A decrease in 10 mmHg of SBP (5 mmHg of DBP), is linked to the reduced risk for major cardiovascular disease events, IHD, stroke and heart failure by relative risk (RR) of 0.80, 0.83, 0.73, and 0.72 respectively in the studied populations, and consequently led to a significant 13% reduction in all-cause mortality (Ettehad et al., 2016). A meta-analysis has revealed that every 5 mmHg reduction in DBP lowers the risk of IHD and stroke by 21% and 34% respectively, regardless of the BP baseline (Law et al., 2003). Hence, comprehensive, and effective BP control is crucial to prevent CV complications.

#### 2.2.3.1 Non-pharmacological Approach

Non-pharmacological approach, specifically lifestyle changes, should be offered at the first place, prior to prescribing antihypertensive agents for the patient in periodical treatment of hypertension. It is undeniable that an appropriate lifestyle modifications are crucial for hypertension prevention and control (Messerli et al., 2007). Salt intake reduction, DASH diet, physical activity, body weight reduction, restriction in alcohol consumption, smoking cessation and relaxation therapy have been recommended as the main components in lifestyle modifications approach in preventing and controlling hypertension (Ministry of Health Malaysia, 2018). Multiple lifestyle modifications is effective in controlling raised blood pressure and further reduce the risk of complications in pre-hypertension and Stage I hypertension (Elmer et al., 2006). Nevertheless, when the patient BP target is not achievable with lifestyle modifications, antihypertensive agent or therapy should be given, especially patients with one or more CV risk factors per definition listed in the **Table 2.2**.

#### 2.2.3.2 Pharmacological Approach

Antihypertensive agents that commonly prescribed in primary care and clinical setting include angiotensin-converting enzyme inhibitors (ACEI), angiotensin receptor blockers (ARB), beta blockers (BB), calcium channel blockers (CCB), diuretics (Messerli et al., 2007). These drugs exert the action on one or more pathophysiological sites of BP controls to achieve the desired

BP-controlling outcomes. ACEI, ARB, CCB, and thiazide diuretics (TZ) are recommended as the initial management of hypertension by CPG Malaysia, NICE, ESH/ESC while BB are not recommended to be prescribed as the first-line therapy. A study, namely " Losartan Intervention for Endpoint reduction in hypertension (LIFE)" has reported that treatment with atenolol (BB) increases the frequency of CV death, myocardial infarction (MI), and stroke, when compared to treatment with losartan (ARB) in a randomised trials consist of around 9,000 essential hypertensive patients with four-years of following-up (Dahlof et al., 2002). In conjunction with the new ACC/AHA guidelines, a network meta-analysis has revealed that BB has significantly less effectiveness than the TZ diuretics (Reboussin et al., 2018). BB has also been suggested to be prescribed to the younger-adult and adolescent patients who are allergic to ACEI or ARB, pregnant, and with an increase of sympathetic drive (National Institute for Health and Care Excellence, 2019). As aforementioned statements on the usage of BB, although it is not restricted, the use of BB will be always in the emergency or combined with multiple health conditions, thus, to ensure the effectiveness of BB is a crucial part to physicians to prevent therapeutic failure in patients.

**Table 2.2**: Risk stratification for CV events with the condition of raised BP and comorbidities.

| BP and Comorbidities | SBP 130-139 DBP 80-89 | SBP 140-159 DBP 90-99 | SBP 160-179 DBP100-109 | SBP ≥ 180 DBP ≥ 110 |
|---|---|---|---|---|
| No RF/TOD/TOC | Low | Low | Medium | High |
| TOD or RF (1-2), No TOC | Intermediate | Medium | High | Very High |
| TOC or RF (≥3) or atherosclerosis or CKD | High | High | Very High | Very High |
| History of MI / IHD, history of stroke/diabetes / CKD | Very High | Very High | Very High | Very High |

| Risk Level | Risk of Major CVD event in 10 years | Lifestyle modifications | Drug treatment |
|---|---|---|---|
| Low-intermediate | <10% | ✓ | x |
| Medium | 10 – 20% | ✓ | ✓ |
| High | 20 – 30% | ✓ | ✓ |
| Very High | >30% | ✓ | ✓ |

RF: Risk factors; TOD: Target organ damage; TOC: Target organ complications; MI: Myocardial infarction; CKD: Chronic kidney diseases.

### 2.3 Causes of Essential Hypertension

The aetiology for essential hypertension is multifactorial and there is no clearly identifiable cause. Complexity of the interactions between multiple genetic factors and environmental factors play an important role in determining

the risk of developing hypertension. There are more than 60 loci have been identified to be associated pathogenies of essential hypertension in GWAS studies, which involving mineralocorticoid pathway, sodium transport pathway, glucocorticoid pathway, sympathetic pathway, salt sensitivity-associated genes (Padmanabhan et al., 2015). Meanwhile, environmental factors related to lifestyle, such as physical inactivity, imbalance diet, alcoholism, and psychological stress lead to elevated BP (Reboussin et al., 2018). Renin-angiotensin-aldosterone system, sympathetic system overactivations, endothelial dysfunction, oxidative and inflammatory processes have been widely identified as main causes of pathogenesis of hypertension (Johnson et al., 2008).

### 2.3.1 Sympathetic Nervous System Over-activation and pathogenesis of hypertension

The sympathetic nervous system (SNS) and parasympathetic nervous system (PNS) are the component of the autonomic nervous system. PNS stimulates the heart and blood vessels, while the SNS acts directly and indirectly to control the cardiac and vascular function, also innervating the blood vessels, kidneys, adrenal gland, and heart. During fight-or-flight response driven by SNS, norepinephrine is released from post-ganglionic sympathetic neurons, and the adrenergic pathway is activated subsequently. Chromaffin cells in the adrenal gland function as the post-synaptic ganglion sympathetic neurons and release epinephrine. After the release and interaction of epinephrine and norepinephrine with the $\alpha_1$-adrenoceptors, vasoconstriction and elevation of heart rate are

triggered and consequently stimulating the release of renin. In contrast, presynaptic $\alpha_2$-adrenoceptors provide a negative feedback response to the $\alpha_1$-adrenoceptors by inhibiting the release of norepinephrine. In addition to the α-adrenoceptors, the β-adrenoceptors also contribute to the regulation of blood pressure. $\beta_1$- adrenoceptor activation increases heart rate and contractility, while $\beta_2$- adrenoceptors activation promotes vasodilation (Johnson et al., 2008). In physiological condition, the SNS rapidly responds to the changes of blood pressure via the arterial baroreceptors, to regulate (mainly increase) the heart rate, total peripheral resistance, and cardiac contractility. In response to chemoreceptors on the oxygen-carbon dioxide equilibrium in blood, SNS also increase arterial blood pressure (Singh et al., 2010). Study has revealed that SNS over-reactivity causes elevated arterial pressure, increased morbidity in heart failure and end stage renal disease (Fisher et al., 2009); (Smith et al., 2004).

### 2.3.2 Endothelial Dysfunction and pathogenesis of hypertension

Endothelial dysfunction is a reversible functional alteration on the endothelial cell constriction and relaxation activity. Endothelial dysfunction is resulting from the impairment of nitric oxide (NO) availability. NO is produced from the oxidation of L-arginine with the involvement of endothelial nitric oxide synthase (eNOS) or inducible derived nitric oxide synthase (iNOS) under pulsatile and shear stress, and hormonal signals (Kollau et al., 2018). NO interacts with the enzyme “soluble guanylyl cyclase (sGC)”, subsequently increase the conversion of “cyclic guanosine monophosphate (cGMP)” from “guanosine triphosphate (GTP)”. The action of cGMP on the protein effectors trigger the vascular dilation and relaxation, cardiac systole and immune-mediated inflammation (Lucas et al., 2000). NO also exerts direct vasodilation, regulating apoptosis, remodelling and angiogenesis via cGMP-independent activity. Decrease in the NO production and synthesis causes endothelial dysfunction and arterial stiffness (Chiba et al., 2019).

### 2.3.3 Renin-Angiotensin-Aldosterone System dysregulation and pathogenesis of Hypertension

RAAS modulates the vascular tone and fluid-electrolytes balance and subsequently achieved a BP regulation. Renin is released from granular cells from the kidney, specifically juxtaglomerular apparatus, in response to (1) decreasing in perfusion pressure determined by the renal baroreceptors located in afferent arteriole; (2) reducing in sodium in the distal tubule which detected

by macula densa cells; and (3) SNS innervating the $\beta_1$ receptor in kidney. Renin is cleaved by the angiotensinogen and formed angiotensin I, which further converted into angiotensin II (Ang II) mediated by angiotensin converting enzyme (ACE). The Ang II exerts its effect via binding to G-protein coupled receptors, predominantly $AT_1$ receptors. Ang II acts differently to different part of the body, such as arterioles (increases total peripheral resistance and renal vasoconstriction), kidney (increases sodium reabsorption), SNS (increases noradrenaline released), adrenal cortex (stimulates aldosterone release) and hypothalamus (increases anti-diuretic hormone release) (Schweda and Kurtz, 2011). Water and sodium retention lead to increased circulating volume and perfusion pressure in juxtaglomerular apparatus which in turn inhibits renin synthesis and release (Harrison-Bernard, 2009). Over-production of renin and production of Ang II are the main cause for the increased blood volume and peripheral resistance, hypertension and organ damage (Atlas, 2007).

## 2.4. Spontaneously Hypertensive Rat as an essential hypertension experimental model

The SHR, a genetically essential hypertensive model, is frequently used as the experimental model in the research related to essential hypertension and cardiovascular diseases. SHR is an inbred of Wistar-Kyoto (WKY) rats. SHR develops hypertension at around 4-6 weeks-old spontaneously without intervention (Pinto et al., 1998). In the early stage of hypertension in SHR, peripheral vascular resistance is at the normal range while the cardiac output is higher. At the stage with established hypertension, hypertrophic vessels lead to increased peripheral vascular resistance, with normal CO (Smith and Hutchins, 1979), and the cardiac remodelling develops (such as significantly elevation in myocyte cross-sectional area, left ventricular thickness, and significantly reduction in capillary density) (Engelmann et al., 1987). Like essential hypertension in human, the aetiology of the hypertension in SHR remains unknown, however, SHR shows alteration in SNS, modification in amount of NO and endothelial dysfunction. Expression and availability of sGC and cGMP were notably lesser in young SHR when compared to age matched WKY (Ndisang and Wang, 2003, Vapaatalo et al., 2000). SHR has an increased in arterial wall renin however Ang II level is not elevated, hence, Ang II sensitivity is still observed in the SHR as well (Arendshorst et al., 1990).

## 2.5 Herbal Medicine

Herbal medicine (herbs with therapeutic and healing properties) has been gaining popularity in many countries and has been integrating into the mainstream healthcare systems. Herbal medicine can be manufactured as therapeutic resources in several ways, including home remedies, crude extracts and purified active compound or purified pharmaceutical formulation. Phytopharmaceutical preparation can be derived solely from a whole plant or parts of plants (Mohamed et al., 2012). EGCG, the molecule of interest in this present study, has been widely recognised as herbal medicine.

### 2.5.1 Prevalence and Advantages of the Herbal Medicine Usage

It has been reported that the approximately 70% to 95%, of the developing countries are still relying on the herbal medicine as the primary source of treatment It has estimated that herbal medicine is the most sought for disease treatment, after the primary healthcare. (Pan et al., 2013).

Around twenty-five percent of prescribed medicine worldwide were originate from plants, which made up of 121 active compounds are being used as drug molecules (Wachtel-Galor and Benzie, 2011). Herbal medicine industry in China and India are well-established, while Germany leads the developed countries, with 54% of herbal medicine products being sold as medical prescriptions that can be covered by health insurance claims (Misra, 1998).

Herbal medicine usage has transcended geographical, gender, economic and socio-cultural divisions. Several reasons have adduced herbal medicine in the healthcare strategic position among the general population worldwide, especially developing countries, including (1) personal preferences, as the use of certain herbal medicine as primary source of treatment brings a successful outcome; (2) perception on the safety of using herbal medicine, where the uses of the herbal medicine always believed to be the most safety and carry no-risk or side effect approach compared to pharmaceutical drug; (3) accessibility, in which the access to conventional medical services is inconvenience (4) less expensive and affordable (5) efficacy of the treatment; and (6) as the last choice upon the failure on most of the present treatments (Philips, 2018).

### 2.5.2 Uses of Herbal Medicine in Hypertension Treatment

The following herbs including *Camellia sinensis*, the interest of this present study have been extensively studied and recommended as a cardioprotective agent owing to their antihypertensive effects.

a. *Gossypium barbadense* (Hasrat et al., 2004);
b. *Avena sativa* (Keenan et al., 2002);
c. *Blond psyllium* (Burke et al., 2001);
**d.** ***Camellia sinensis*** (Kurita et al., 2010, Potenza et al., 2007, Lorenz et al., 2015, Babu and Liu, 2008, Qian et al., 2018, Yang et al., 2004);
e. *Pinus pinaster* (Liu et al., 2004);
f. *Hibiscus sabdariffa* (Herrera-Arellano et al., 2004);

## 2.6 Epigallocatechins-3-Gallate (EGCG) in Green Tea

Green tea (*Camellia sinensis*) has been consumed as a health-promoting beverage since ancient times. Green tea catechins (GTCs), including epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and (−)-epigallocatechin-3-gallate (EGCG), are the major polyphenolic compounds found in the green tea.

According to the United States Department of Agriculture (USDA) Flavonoid Database, 1 g of brewed green tea leaf in 100mL infusion contains an average of 126.6 mg total catechins and 77.8 mg EGCG. The dry weights of EC, ECG, EGC and EGCG are 792 ± 3, 1702 ± 16, 1695 ± 1 and 8295 ± 92 mg/100 g in green tea, respectively (Bhagwat and Haytowitz, 2016). EGCG is the most abundance catechin in tea, and it is well studied for its biological effects in preclinical studies. It has been well established that EGCG possesses antioxidative, ant-lipogenesis, anti-thrombotic, anti-inflammatory, and anti-proliferative effects (Babu and Liu, 2008).

### 2.6.1 EGCG Medicinal Benefits in Treating Hypertension

The attenuation of antioxidant defence system and overproduction of reactive oxygen species (ROS) are crucial and influential in causing the progression of vascular disease and even, vascular damage. With the high concentration of ROS, deleterious effect on the vascular and cardiac myocytes is significant and also causes impairment to the cardiovascular function via inducing vascular smooth muscle cell (VSMC) proliferation, endothelial dysfunction, cardiac apoptosis and/or necrosis (Babu and Liu, 2008).

EGCG is the most potent and most abundance antioxidative compound found in the tea, which contributed by the ortho-trihydroxy group and 3-gallate esters. Both functional groups found on the EGCG play a notably role in antioxidant activity, specifically radical scavenging properties and preventing oxidative destruction of biological compounds. The hydroxyl groups of EGCG capable to scavenger free radicals and chelate metal ions, as a result, reduces the amount of ROS. In addition, studies have shown that EGCG inhibits NF-κB and activator protein 1 (AP-1) leads to a suppression on the redox active transcription factors. Besides, low to medium concentrations of EGCG capable to suppress pro-oxidant enzymes, i.e., iNOX and XO. EGCG increases the expression of eNOS (NO-dependent), prostacyclin, cAMP, and cGMP (NO-independent) that eventually leads to vasodilation in NO-dependent and NO-independent manner (Lorenz et al., 2015).

A recent meta-analysis on 24 randomised controlled trials, which involved 1697 subjects (859 subjects in green tea group and 838 subjects in placebo group), has revealed that <615 mg/dL catechins containing green tea or green tea extract significantly reduced the SBP by 1.79 mmHg and DBP by 1.69 mmHg (Xu et al., 2020).

Several pre-clinical studies shown the anti-hypertensive effect of EGCG in animal models. A study shown that oral EGCG (50 mg/kg twice daily) significantly attenuates the raised SBP induced by 8% of NaCl in Dahl rats (Luo et al., 2020). Besides, 200 mg/kg per day of oral EGCG significantly reduces the SBP in SHR (Potenza et al., 2007).

### 2.6.2 Potential Risks of Using EGCG

Despite the medicinal effects of EGCG extensively studies, safety profile and potential EGCG-drug interactions have not been well established. In a case report, a 16 years-old patient was admitted to the hospital with the new onset of jaundice, which the patient had a previous history of EGCG tablet consumption (400 mg daily) for 60 days. The patient was further diagnosed with acute liver injury (Patel et al., 2013) and earlier pre-clinical study has demonstrated that 100 mg/kg of intraperitoneal EGCG caused elevated level of ALT when compared to control (170 *vs.* 49 U/L) and died within 22h when given 150 and 300 mg/kg of EGCG (Galati et al., 2006).

## 2.7 General Concepts in Drug Absorption, Distribution, Metabolism and Transport

Pharmacokinetics is the study of drug absorption, distribution, metabolism, and excretion (ADME). ADME determine the speed of onset of drug action, the intensity of the drug effect, the drug amount reaches the systemic circulation (bioavailability) and the duration of drug action (drug half-life). Drug absorption is defined as the delivery of the unmetabolized drug from the site of administration to the systemic circulation. Drug distribution is a reversible process, in which the drug leaves the bloodstream and distributes into the interstitial and intracellular fluids. Drug metabolism is biotransformation of the drug into metabolites, primarily by the liver. Drug elimination is clearance of drug and its metabolites into urine, bile, or faeces. Route of drug administration is categorised into enteral (oral, sublingual), parenteral (intravenous, intramuscular, subcutaneous), and others like inhalation, intrathecal, topical, transdermal, and rectal.

Rate and efficiency of drug absorption of a drug depend on the site of absorption, chemical characterises and route of administration. Drug administration via oral route is the most preferred method as it is self-administered, easier to manage toxicity and carries low risk of systemic infections as comparing to parenteral route. Nevertheless, the bioavailability of an orally administered drugs is typically less than 100% due to (a) decomposition/degradation by gastric acid, digestive enzymes or microflora inactivation in GIT; (b) incomplete absorption; (c) first-pass metabolism effect

in intestinal wall and liver; and (d) drug efflux effect exerts by certain drug transporter, such as MDR1 or commonly known as P-glycoprotein (Ritter et al., 2008).

There are two main mechanisms involved in the drug absorption at the GIT, which are passive diffusion and active transport. Non-polar, hydrophobic, or lipid-soluble substances able to freely transport via passive diffusion and achieve well-absorption in the intestine. In contrast, the absorption of natural-occurring polar substances required specific transporter to facilitate the absorption process (Ritter et al., 2008).

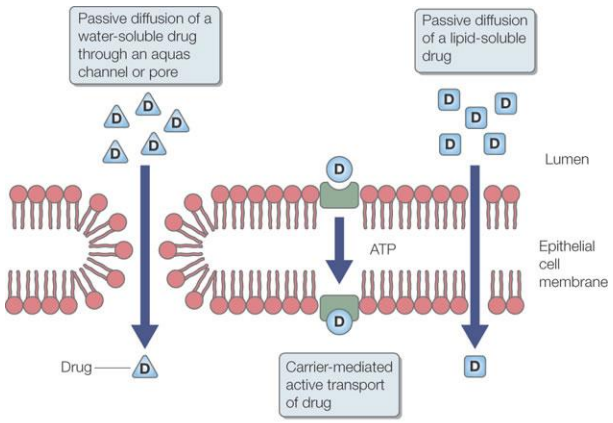



**Figure 2.1:** Modes of drug absorption from the GIT via oral route (adopted from Ritter et al. (2008))

Drug distribution describes the movement and transfer of the drug from and to the blood and multiple tissues and organs of the body. Hydrophilic or water-soluble drugs tends to stay within the blood or body fluid in the interstitial space; In contrast, lipid-soluble drugs tend to accumulate in the fat tissues. The distribution of the drug is mainly affected by protein-binding interactions and induction or inhibition of drug transporters. The binding of the drug to the

protein, particularly albumin, are varies enormously with some drugs are extremely highly bound. The binding of the drugs to plasma protein is reversible and to achieve an equilibrium between bound and unbound drug molecules, of which unbound drug molecules are remain active and bound drug molecules are pharmacological inactive. The bound drug molecules serve as important reservoir as they are avoided from being metabolised and excreted. Once the unbound drug molecules being metabolised, bound drug molecules will become unbound to be activated and exert the normal pharmacological action. One drug may compete with another drug to the binding site of the plasma protein, depend on the relative affinity to the binding sites, to increase the displacement of the drug with lower affinity from bound form into free form (Wisher, 2011).

Induction or inhibition of the drug transporters, such as MDR1, an efflux transporter, limit the drug distribution to the tissue like brain and testes. As MDR1 actively transport the substrate drug out of the cells, the inhibition of MDR1 could increase the uptake of the drug into the brain tissue, while induction of MDR1 could decline the uptake of the drug into brain tissue (Wisher, 2011).

Drug metabolism, known as biotransformation, where is a process that drug converted into metabolites via a series of processes, of which involving numerous mechanism and enzymatic activities in the body (Ionescu and Caira, 2006). There are two major types of reactions involved in the drug metabolism in the liver. Phase I reactions predominantly happen in endoplasmic reticulum. CYPs involve in metabolic modification of the drug via oxidation, reduction,

and hydrolysis processes. Phase II reactions involve the coupling of the drug with other substance, via series of processes, such as acetylation, glucuronidation, methylation, sulphation, mercapturic acid formation, amino acid reaction and glutathione conjugation, and further increase the polarity of phase I metabolite, eventually being inactivated and being excreted out from the body (Wisher, 2011).

CYPs are taking main responsibility for the metabolism of a variety of drugs. Despite more than 50 human CYPs have been identified, the most abundant families of CYP metabolising enzymes are CYP1A2 CYP2C, and CYP3A4. Among the main isoforms, most of the marketed medicines share drug metabolism with CYP3A4 which may result in drug-drug interactions. Phase II reactions occur by reacting with functional groups of a drug that were formed during the phase I reaction. The two main phase II reactions are glucuronidation and glutathione (GSH) conjugation. The main metabolising enzymes involved in phase II metabolism including UDP-glucuronosyltransferase (glucuronidation), glutathione-S-transferase (GSH conjugation), sulphotransferases (sulphation), methyltransferases (methylation) and CoA-S-acetyltransferase (acetylation) (Ritter et al., 2008). Nadolol, the drug interest of this present study, is a non-metabolised drug and it is excreted in an unchanged form (Misaka et al., 2020).

The processes of renal drug elimination are categorised into glomerular filtration, proximal tubular secretion, passive distal tubular reabsorption, and active tubular reabsorption **(Figure 2.2)**.

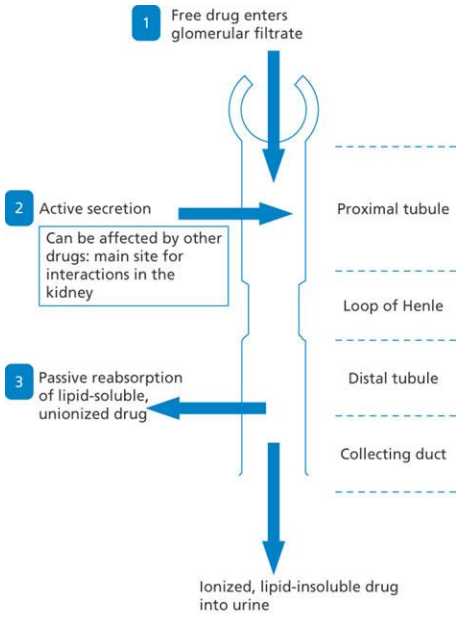



**Figure 2.2** Drug and drug metabolites renal elimination by glomerular filtration and tubular secretion and reabsorption (adopted from Ritter et al. (2008)).

In the process of glomerular filtration, the low molecular weight solutes able to pass through the glomerulus, while molecules with molecular weight greater than 66,000 Da could not pass through the glomerulus. Also, high albumin-binding drugs are not efficiently filtered through glomerulus. During proximal tubular secretion, active transport mechanism plays a pivotal role in transporting the organic anions and cations into the proximal tubule, facilitated by drug transporters such as organic anion transporters (OAT) and OCT. Each mechanism is saturable in theory, where a maximal rate of the drug transport is applied. However, the maximum rate of transportation is rarely reached in physiological condition, as the secretion drug is in a concentration gradient manner. The equilibrium between the bound and unbound drug could be interrupted, of which the bound drug turns into unbound form during the

transportation of the bound drug from peritubular fluid into the lumen. Hence, tubular secretion has high efficiency for the drug with high protein-binding properties (Ritter et al., 2008).

The passive distal tubular reabsorption is favourable to high lipid-soluble drug. The passive reabsorption is driven by the drug concentration gradient between tubular lumen and interstitial fluid and plasma. The passive drug reabsorption in the distal tubular is affected by the tubular filtrate flow rate and pH. At low pH, the weak-acidic drugs are in non-ionised lipid-soluble molecules and is more favourable to reabsorption. In contrast, when the tubular filtrate turns more alkaline, drugs tend to be ionised to a non-lipid soluble form and hinder the reabsorption process, and these drugs will be eliminated into the urine. (Ritter et al., 2008, Wisher, 2011).

## 2.8 Herb-Drug Interactions (HDI)

### 2.8.1 Introduction of HDI

Due to the popularity and regular use of herbal medicine and limited research in herb-drug interactions, the potential risk of herb-drug interaction that may lead to toxicity or therapeutic effects may be underreport.

Herb-drug interactions involve the drug metabolizing enzymes (DMEs) cytochrome P450 (CYP) and uridine 5'-diphospho-glucuronosyltransferase (UGT), as well as variety of drug transporters. The herb-drug interactions may occur through the alteration of pharmacokinetics, including drug absorption,

distribution, metabolism, and/or excretion. The modulation of intestinal enzymes, uptake transporters and efflux transporters by phytochemical supplements affect the rate and drug absorption. In addition, the modulation of hepatic and renal uptake transporters, efflux transporters and/or the inhibition/induction of DMEs can significantly affect the drugs' metabolism and excretion. Most of the reported hear-drug interactions are caused by the modulation of DMEs and/or transporters in the intestine and liver, which could lead to therapeutic failure or toxicity (Fugh-Berman, 2000).

In contrast, a controlled herb-drug or drug-drug interactions are being applied to enhance the bioavailability of the therapeutic drug via enhancing the intestinal absorption, reducing elimination, and modulating the DMEs activity to achieve the optimal therapeutic effect of the drugs. Modulation in the expressions or activities of membrane transporters could control the drug absorption and clearance rates. For example, inhibition of efflux transporters (e.g., *Mdr1a*) and/or upregulation of influx transporters is one of the strategies in drug bioavailability. The activities of DMEs must be modulated accordingly to the type of the therapeutic drug. Interactions are frequently used in the pharmaceutical industry, and yet, additional exposure of herbal medicines or herbal supplements could break the optimal designed interactions that has been optimized by pharmaceuticals company. Eventually, therapeutic failure or toxicity issues arise (Fugh-Berman, 2000).

### 2.8.2 EGCG-Drug Interactions

EGCG has been reported to have strong interactions with various of CYP enzymes, UGT enzymes, as well as membrane transporters (Fugh-Berman, 2000).

Muto et al. (2001) study has revealed that EGCG non-specifically inhibits CYP1A1, CYP1A2, CYP3A4, CYP2A6, CYP2C19, CYP2E1 via inhibition of human NADPH-cytochrome CYP reductase. Further study on EGCG – CYP interactions has shown that EGCG has a strong inhibitory effects on CYP3A4 and CYP1A2 and moderate inhibition on CYP2C9 via inhibition assay assessment (Satoh et al., 2016). EGCG showed a remarkable potency of inhibitory effects against CYP2B6 and CYP2C8 via competitive inhibition and non-competitive inhibition for CYP3A in *in-vitro* human liver and intestine microsome. In intestinal microsome, EGCG also shown non—competitive inhibition against CYP3A. In contrast, EGCG shows relatively weak inhibition against CYP2C19 and CYP2D6 (Misaka et al., 2013a). Clinical findings revealed that repeated high EGCG-containing (75%) GTE capsule administration significantly reduced 20% of the buspirone AUC via its inhibitory effect on CYP3A activity (Chow et al., 2006). In contrast, a case report has revealed that green tea consumption doubled the bioavailability of simvastatin, which is a substrate for CYP3A and CYP2C8 (Satoh et al., 2016).

In *in-vivo* studies, EGCG significantly increased the drug bioavailability of verapamil, nicardipine, diltiazem, tamoxifen (Chung et al., 2009); (Choi and Burm, 2009); Shin and Choi, 2009). In contrast, it has been

shown that EGCG reduced drug bioavailability of sunitinib and nadolol (Ge et al., 2011); (Misaka et al., 2013c)

A clinical study revealed that 800 mg/day of EGCG could effectively increase 20% AUC of the losartan and buspirone with minor decreased CYP3A4 activity (Chow et al., 2006). Another showed that EGCG reduced iron absorption by 14% and 27% with the dosage of 150 mg/day and 300 mg/day, respectively in human volunteers (Ullmann et al., 2005)

Table 2.3 and Table 2.4 summarise the effects of EGCG and GTE on drug availability and their effects in drug uptake and drug transporter expressions.

**Table 2.3:** Influences of EGCG/GTE on the drug bioavailability

| Compound | Dosage | Route | Drug | Model | Result | References |
|---|---|---|---|---|---|---|
| EGCG | 100 mg/kg | Oral | Sunitinib (30 mg/kg) | SD rats | ↓ 48% in $C_{max}$<br>↓ 52% in $AUC_{0-\infty}$ | (Ge et al., 2011) |
| EGCG | 4 mg/kg<br>12 mg/kg | Oral | Diltiazem (15 mg/kg) | SD rats | In 4 mg/kg,<br>↑ 1.65-fold in bioavailability<br>In 12 mg/kg,<br>↑ 1.76-fold in bioavailability<br>Both 4 mg/kg and 12 mg/kg,<br>↓ Total body clearance | (Li and Choi, 2008) |
| EGCG | 2 mg/kg<br>10 mg/kg | Oral | Verapamil (9 mg/kg) | SD rats | In 2 mg/kg,<br>↑ 74.3% verapamil AUC<br>↑ 51.5% norverapamil AUC<br>In 10 mg/kg,<br>↑ 111% verapamil AUC<br>↑ 87.2% norverapamil AUC | (Chung et al., 2009) |
| EGCG | 3 mg/kg<br>10 mg/kg | Oral | Nicardipine (12 mg/kg) | SD rats | In 3 mg/kg,<br>↑ 56.3% of nicardipine AUC<br>In 10 mg/kg<br>↑ 78.7% of nicardipine AUC | (Choi and Burm, 2009) |

| | | | | | | |
|---|---|---|---|---|---|---|
| EGCG | 3 mg/kg<br>10 mg/kg | Oral | Tamoxifen<br>(10 mg/kg) | SD rats | In 3 mg/kg,<br>↑ 48.4% in tamoxifen AUC<br>In 10 mg/kg,<br>↑ 77.0% in tamoxifen AUC<br>↑ 40.3% in metabolite AUC | (Shin and Choi, 2009) |
| EGCG | 150 mg/kg | Oral | Nadolol<br>(10 mg/kg) | SD rats | ↓ 81.0% in $C_{max}$<br>↓ 73.4% in AUC | (Misaka et al., 2013c) |
| EGCG | 30 mg/kg/day<br>(10 days) | Oral | Amlodipine | Rat liver microsome | ↑ 31.4% in $C_{max}$<br>↑ 48.5% in AUC | (Han et al., 2019) |
| GTE | 50 mg/kg | Oral | 5-Fluorouracil<br>(48 mg/kg) | SD rats | ↑ 151% in $C_{max}$<br>↑ 425% in AUC | (Qiao et al., 2011) |
| GTE | 25 mg/kg | I.V. | Doxorubicin | C57BL/6 | ↑ Doxorubicin concentration in the cancer cells. | (Jiang et al., 2019) |
| GTE | 175 mg/kg | Oral | Clozapine<br>(20 mg/kg) | SD rats | ↓ 43.2% in $C_{max}$<br>↓ 49.6% in AUC | (Jang et al., 2005) |
| GTE | 400 mg/kg | Oral | Simvastatin<br>(20 mg/kg) | SD rats | ↑ 3.4-fold in AUC<br>↑ 3.3-fold in $C_{max}$ | (Misaka et al., 2013b) |

| | | | | | | |
|---|---|---|---|---|---|---|
| GTE | 175 mg/kg | Oral | Quetiapine (25 mg/kg) | Wistar rats | ↓ 44.4% in $C_{max}$<br>↓ 38.6% in AUC | (Ezzeldin et al., 2015) |
| GTE | 400 mg/kg | Oral | Nadolol (10 mg/kg) | SD rats | ↓ 84.5% in $C_{max}$<br>↓ 74.4% in AUC | (Misaka et al., 2013c) |
| GTE | 500 mg/kg | Oral | Atenolol (50 mg/kg) | SD rats | ↓ 51.0% in $C_{max}$<br>↓ 38.7% in AUC | (Shan et al., 2016) |
| GTE | 200 mg/kg | Oral | Erlotinib (20 mg/kg) | Wistar rats | ↓ 67.6% in $C_{max}$<br>↓ 69.5% in AUC | (Maher et al., 2017) |
| GTE | 200 mg/kg | Oral | Lapatinib (25 mg/kg) | Wistar rats | ↓ 70.2% in $C_{max}$<br>↓ 74.0% in AUC | (Maher et al., 2017) |
| GTE | 400 mg/kg | Oral | Midazolam (20 mg/kg) | SD rats | ↑ 114.2% in $C_{max}$<br>↑ 196.4% in AUC | (Nishikawa et al., 2004) |
| Green tea | 2 mg/kg | Oral | Amoxicillin (100 mg/kg) | Wistar rats | ↓ in $C_{max}$<br>(PK data not presented) | (Kiss et al., 2019) |
| Green tea | 200 mg/kg | Oral | Chlorpromazine CRZ (15 mg/kg) | Wistar rats | Catatonic effect of CRZ is totally antagonised | (Cheeseman and Neal, 1981) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Green Tea | 2 mL/kg | Oral | Digoxin (0.3 mg/kg) | SD rats | ↑ 494.6% in $C_{max}$<br>↑ 353.6% in AUC | (Oda and Murakami, 2017) |

**Table 2.4:** Influences of EGCG/GTE on drug uptake via drug transporters expression modulation in cell line (*in-vitro*) studies.

| Compound | Concentration | Transporters | Substrate | Cell line | Result | References |
|---|---|---|---|---|---|---|
| EGCG | 10 µM<br>100 µM | OATP-B | Estrone-3-sulfate (E3F) | HEK/OATP-B | ↓ E3F by 30%<br>↓ E3F by 75% | (Yamaguchi et al., 1986) |
| EGCG | Various | OATP1A2<br>OATP1B1<br>OATP2B1 | Estrone-3-sulfate (E3F) | HEK/OATP1A2<br>CHO/OATP1B1<br>CHO/OATP2B1 | ↓ E3F on<br>OATP1A2 $IC_{50}$ 55 µM<br>OATP1B1 $IC_{50}$ 8 µM<br>OATP2B1 $IC_{50}$ 101 µM | (Roth et al., 2011) |
| EGCG | 100 µM | OATP1B1<br>OATP1B3 | Bromosulphophthalein (BSP)<br>Atorvastatin | HEK | ↓ BSP on<br>OATP1B1 by 36%<br>OATP1B3 by 88%<br>↓ Atorvastatin on<br>OATP1B1 by 31%<br>OATP1B3 by 57% | (Knop et al., 2015) |
| EGCG | 100 µM | MDR1/P-gp | R-123 | CHO | ↓ MDR1 photolabeling<br>↑ R-123 (4-fold) | (Jodoin et al., 2002) |
| EGCG | 100 µM | OCT1<br>OCT2<br>MATE1<br>MATE2-K | Metformin | HEK | ↓ Metformin on<br>OCT1 by 60%<br>OCT2 by 37%<br>MATE1 by 26%<br>MATE2-K by 32% | (Knop et al., 2015) |

| | | | | | | |
|---|---|---|---|---|---|---|
| GTE | Various | OATP1B1<br>OATP1B3 | BSP<br>Atorvastatin | HEK | ↓ BSP on<br>OATP1B1 $IC_{50}$ 2.60%<br>OATP1B3 $IC_{50}$ 0.39%<br>↓ Atorvastatin on<br>OATP1B1 $IC_{50}$ 1.90%<br>OATP1B3 $IC_{50}$ 1.00% | (Knop et al., 2015) |
| GTE | Various | OCT1<br>OCT2<br>MATE1 | Metformin | HEK | ↓ Metformin on<br>OCT1 $IC_{50}$ 1.40%<br>OCT2 $IC_{50}$ 7.00%<br>MATE1 $IC_{50}$ 4.90% | (Knop et al., 2015) |
| GTE | 1% (v/v) | MDR1/P-gp | Digoxin | Caco-2 | ↓ Digoxin by 25% | (Knop et al., 2015) |
| GTE | Various | OATP1A2 | Nadolol | HEK/OATP1A2 | ↓ Nadolol on<br>OATP1A2 $IC_{50}$ 1.36% | (Misaka et al., 2014) |
| GTE | 1 mg/mL | MRP2 | Methotrexate | MDCK/MRP2 | ↓ MRP2 mRNA<br>↑ Methotrexate (2-fold) | (Netsch et al., 2005) |
| GTE | 1% (v/v) | MDR1/P-gp | Digoxin | Caco-2 | ↓ Digoxin by 50% | (Knop et al., 2015) |
| GTE | Varies | rOct2 | Methylphenyl pyridinium ($MMP^+$) | S2/rOct2 | ↓ $MMP^+$ on<br>Oct2 $IC_{50}$ 1.9 mg/mL | (Jaiyen et al., 2016) |

## 2.9 Nadolol

Nadolol is a non-selective β-adrenergic antagonist (β-blocker) that has been used to treat hypertension and angina (AAPharma, 2016).

### 2.9.1 Mechanism of Action of Nadolol

Nadolol is a non-selective β-receptors antagonist, or commonly known as β-blocker binds to both $\beta_1$ and $\beta_2$ receptors, but not $\beta_3$ (Cernecka et al., 2014). Antagonism of nadolol to the $\beta_1$ receptors in the heart and blood vessels blocks the binding of other adrenergic neurotransmitters, such as catecholamine to the receptors. Blockade of $\beta_1$ receptors in cardiac muscle reduces HR and CO, subsequently reduce the splanchnic circulation. Hence, the inhibitory effects lead to lower peripheral vascular resistance and eventually reduces the blood pressure. Inhibition of $\beta_1$ receptors in the juxtaglomerular apparatus inhibits the production of renin and subsequently reduces aldosterone-dependent water retention, angiotensin II-dependent vasoconstriction, release of adrenaline, and eventually leads to vasodilation and increased in water and sodium excretion (Gorre and Vandekerckhove, 2010).

### 2.9.2 Pharmacokinetics of Nadolol

Most of the exogenous beta-blockers are metabolised by CYP enzymes, and the elimination mainly via hepatic metabolism and/or renal excretion of the unchanged drug. Nadolol and atenolol are the only beta-blockers that appear to

be excreted in the unchanged forms (Zisaki et al., 2015). The following literature review focuses on the drug of study interest, nadolol.

In humans, approximately 34% of orally administered nadolol is absorbed into the systemic circulation. About 30% of the plasma nadolol is reversibly bound to plasma proteins and extensively distributed to the extravascular tissues. Maximum plasma concentrations ($C_{max}$) are achieved in 2-4 hours after oral administration. The half-life ($T_{1/2}$) is approximately 20-24 hours. Nadolol is non-metabolised by the body, whereby approximately 20% of the oral administrated dose is excreted into urine and around 70% is eliminated into faeces (including both unabsorbed and liver eliminated portion) (AAPharma, 2016). For the intravenous nadolol, approximately 73% and 23% is eliminated via kidney and faeces, respectively (Dreyfuss et al., 1977).

### 2.9.3 Nadolol Metabolism

Nadolol is a non-hepatically metabolised β-blocker, and its pharmacokinetics and bioavailability are solely associated with its membrane transporters rather than hepatic metabolism (CYP). In an *in-vitro* study, Misaka et al. (2016) revealed that nadolol is a substrate for MDR1A, OATP1A2, OCT1, OCT2, MATE1, and MATE2-K. The same research team has concluded that OATP1B1 and OATP1B3 are not involved in nadolol distribution (Misaka et al., 2016). Due to its non-metabolised property, nadolol is frequently being utilised as a probe drug to elucidate the systemic effect mediated by active transport.

### 2.9.4 Role of Membrane Transporters in Nadolol Distribution

Organic anion transporting polypeptide (OATP/Oatp) family transporters are encoded by the genes in *SLCO/Slco* superfamily (formerly known as *SLC21A)* and consisted of 11 identified OATP isoforms in human, 15 identifies Oatp isoforms in rodent (Roth et al., 2012). OATPs/Oatps are expressed in various tissues, such as kidney, liver, intestine, and brain, which mediate the transmembrane transport of a wide range of acidic organic compounds including bile salts, organic dyes, steroid conjugates, thyroid hormones, anionic oligopeptides, and many drug molecules. OATPs/Oatps are expected to play important roles in the absorption, tissue distribution and elimination of acidic drug molecules (Hagenbuch and Meier, 2004). Human OATP1A2/OATP-A, OATP2B1/ OATP-B and rat Oatp1a5/Oatp3 are

suggested to be involved in the intestinal absorption of drugs, including fexofenadine, and quinolone antibacterial agents (Kikuchi et al., 2006, Maeda et al., 2007). OATP1A2 protein is widely found on the brush border membrane of enterocytes (Glaeser et al., 2007),. In the hepatocytes, OATP1A2 is exclusively expressed in cholangiocytes, and the function remains ambiguous. It is believed that OATP1A2 reabsorbs drugs from the bile. In the kidneys, OATP1A2 is expressed at the apical membrane of the distal nephron, and it is believed that it is involved in both drug reabsorption and secretion (Lee et al., 2005).

Organic cation transporters (OCTs/Octs) are the multi-specific uptake transporters in multiple types of epithelia throughout the body. OCTs are encoded by the genes in *SLC22*/*Slc22* superfamily and consists of OCT1/Oct1, OCT2/Oct2, OCT3/Oct3, OCTN1/Octn1, and OCTN2/Octn2. OCTs/Octs mediates the transportation of low molecular weight substances, such as steroid hormone conjugates, biogenic amines and various drugs and toxins (Roth et al., 2012). OCT1 is usually considered to be a liver-specific transporter and found predominantly in the basolateral membrane if hepatocytes (Nies et al., 2008). Low levels of OCT1 mRNA have been found other tissues, such as intestine, heart, skeletal muscle, kidney, brain, and placenta (Gorboulev et al., 1997, Green et al., 1999). OCT1 is predominantly found in the apical membrane of the enterocytes (Han et al., 2013).

Drug efflux transporters P-glycoprotein (P-gp), is known as ATP-binding cassette subfamily B member 1 (ABCB1) protein or multidrug

resistance protein 1 (MDR1), are membrane-integrated proteins that limit intracellular concentration of substrate agents by pumping them out of cell through an active, energy dependent mechanism. MDR1 contains two ATP-binding sites, where ATP will bind in presence of a MDR1 substrate. Consequently, ATPase is activated and hydrolyse ATP. The energy produced by the ATP hydrolysis will allow the MDR1 to transport numerous substrates across cellular membranes (Watanabe et al., 2012). MDR1 protein is encoded by the only gene *MDR1* in human, while there are by two different genotype *Mdr1a* and *Mdr1b* in rodents. The amino acid sequences of Mdr1a and Mdr1b are highly similar with different tissue distributions. In rats, Mdr1a is mainly expressed in brush boarder side of enterocytes in intestines, while Mdr1b is mainly expressed in lung and intestines. Meanwhile, they are all expressed in the bile-facing canaliculi of the liver, urine-facing side of the brush boarder membrane of proximal tubules in the kidney, and apical or serosa side of the blood-brain, blood-cerebral spinal fluid, blood-placenta and blood-testis barriers (Liang et al., 2019). *Mdr1a* recognizes and effluxes a multitude of structurally and biochemically unrelated substrates (cyclic, linear, basic, uncharged, zwitterionic, negatively charged, hydrophobic, aromatic, nonaromatic, amphipathic) from 250 to 4000 kDa molecular weight, including drugs, endogenous compounds, steroid hormones, lipid, pharmaceuticals, nutraceuticals, dietary compounds, and others. Modulation of *Mdr1a* gene expression and/or Mdr1a activity by various mechanisms consequently influences Mdr1a-mediated drug disposition. In the intestine, Mdr1a effluxes the substrate into intestinal lumen. In the liver, Mdr1a excretes the substrate into

bile duct; while in the kidneys, Mdr1a eliminates the substrate into the urine (Hodges et al., 2011).

### 2.9.5 Elimination & Clearance of Nadolol

Nadolol is excreted into the urine in an unchanged form. Based on the data of 9 healthy subjects, around 60% of the nadolol is eliminated into the urine and 15% into the faeces after 72 hours of 1, 2, and 4 mg intravenous nadolol administration (Morrison et al., 1988). The total body clearance of nadolol is around 219 – 250 mL/min, renal clearance is 131 – 151 mL/min, and half-life of 8.8 – 9.4 h in healthy subjects (Morrison et al., 1988). However, in the patient with chronic renal failure, the half-life of nadolol is prolonged to 39.2 ± 5.1 h and total body clearance of 46.4 – 102 mL/min (Herrera et al., 1979).

### 2.9.6 Adverse Events of Nadolol

Nadolol is generally safe to use with minimum of side effects. It has been reported that nadolol administration led to mild to moderate increase of hepatic aspartate transaminase and alanine transaminase in a cohort patient with no other clinical symptoms (Merkel et al., 1986). Common side effects of nadolol include dizziness, numbness, feeling tired, upset stomach, vision problems, mood swings. Bronchospasm, severe hypotension, and hypersensitivity, which the rare side effects of nadolol but these are life-threatening and required immediate medical attention (Gopal and Mandiga, 2021).

### 2.9.7 EGCG-Nadolol Interaction

EGCG-nadolol interactions greatly reduces in bioavailability of nadolol in Sprague Dawley (SD) rats (Misaka et al., 2013c). Authors have extended their findings onto healthy human subjects and resulted in 700 mL/day of green tea ingestion (equivalent to 322 mg/day of EGCG) for 14 days significantly reduces nadolol AUC by 85% and $C_{max}$ by 85.3% (Misaka et al., 2014). Furthermore, concomitant of nadolol and a single ingestion of 150 mL brewed green tea (equivalent to 166 mg of EGCG) in healthy subject showed a significantly reduction in nadolol AUC by 56.8% and 45.4% respectively (Misaka et al., 2020); Consistent with Abe et al. (2018) study, which shown a significantly reduction in AUC by 28.1% and 40.0% with single infusion of low-dose (equivalent to 48.72 mg of EGCG) and high-dose (equivalent to 146.6 mg of EGCG) EGCG-concentrated GTE solution, respectively, in healthy volunteers. These studies further hypothesized that GTE-interaction could be due to its most abundance catechin, EGCG. Therefore, this present study aims to complete the research gap by examining the effects of EGCG on bioavailability of nadolol in a hypertensive model.

# CHAPTER 3

# MATERIAL AND METHODS

## 3.1 Reagents and Chemicals

EGCG with purity ≥98%, nadolol with European pharmacopoeia reference standard, metoprolol and all the other solvents and reagents utilised in the study were commercially available and were pharmaceutical or high-performance liquid chromatography (HPLC) graded. Besides, a complete list of materials and equipment was listed in Appendix A.

## 3.2 Experimental Design

The experimental protocol in this study was approved by the UTAR Scientific and Ethical Review Board (U/SERC/87/2018, Appendix B) and was carried out following the Institutional Animal Care and Use Committees Guidebook (National Research Council, 2010). Signs and symptoms of toxicity were observed according to OECD Guidelines for the Testing of Chemicals (OECD, 2008). The investigator has been trained and certified under Responsible Care and Use for Laboratory Animal Course (RCULAC) prior to animal handling for the present study.

### 3.2.1 Animal and Experimental Protocols

Male SHR (12 – 13 weeks-old) with bodyweight 250 - 265g were purchased from Monash University (Sunway Campus, Malaysia). All rats were housed in a well-ventilated holding room at a controlled temperature (24 ± 1°C) and controlled lightning (12 h dark/light cycle) with access to standard chow and water *ad libitum*. Animal were acclimatised to the new environment for at least 2 weeks prior to the commencement of the experiment. Animal were maintained at UTAR Animal Holding Facility throughout the experimental period.

SHR were randomly divided into four groups: Control (the vehicle – PBS was given, n = 6), Nadolol (a single dose of nadolol was given, n = 6), EGCG (14 days of EGCG was given, n = 6) and EGCG-Nadolol (14 days of EGCG followed by a single dose of nadolol were given). EGCG (10 mg/kg body weight/ day) was given using an oral gavage for consecutively 13 days at the same time of the day (0700-0730). The rats were fasted for a night (1900-0700). At day-14, a single dose of nadolol (10 mg/kg body weight) was given to the rats using an oral gavage 30 minutes after the last dose of EGCG administration at 0700-0730. SBP was measured at 6-h and 22-h post-nadolol administration.

**Table 3.1** Experimental groupings.

| **Group** | **Day 1- 14** | | **Day-14** | |
|---|---|---|---|---|
| | PBS | EGCG (10 mg/kg per day) | PBS | Nadolol (10 mg/kg) |
| Control | √ | | √ | |
| Nadolol | √ | | | √ |
| EGCG | | √ | √ | |
| EGCG-Nadolol | | √ | | √ |

EGCG (10 mg/kg body weight/ day) or PBS (vehicle) was given via oral gavage for consecutively 14 days and followed by a single dose of nadolol (10 mg/kg body weight) or phosphate-buffered saline (PBS) 30 min after the last dose of EGCG or PBS administration at day-14. SBP was measured at 6-h and 22-h after nadolol administration.

### 3.2.2 Blood Pressure Measurement

SHR were trained to familiarise with the restrainer for 15 – 30 min per day for at least 5 days before the SBP measurement. SBP measurement was done with the non-invasive volume-pressure recording (VPR) tail-cuff plethysmography method, which by using the CODA® Monitor. Briefly, the SHR were allowed to move freely into an acrylic rodent restrainer, attached with darkened nose cone, to minimise the stress introduce to the SHR. The restrained SHR were undergone pre-warming with warming pad wrapped around the restrainer, which temperature was set to 35ºC. The body temperature of SHR was monitored using a rectal temperature infrared probe. The tail temperature of SHR was monitored with infrared thermometer and the blood pressure

measurement was done at 33 - 35ºC. SHR with basal SBP of >160 mmHg was included into the study.

### 3.2.3 Blood and Tissues Collection

The time-point of the blood and urine collection were selected based on the pharmacokinetic of nadolol (Tmax, $T_{1/2}$) stated in the product data sheet as well as previous studies (AAPharma, 2016). ZeAAPharma, 2016). Zero point two millilitres of blood were periodically sampled from the saphenous vein over 12 h after nadolol administration, specifically at 1-, 2-, 3-, 5-, 7-, 9-, 12-h, into an EDTA-containing tube respectively. Zero point five to two millilitres of urine were cumulatively collected up to 22-h after nadolol administration in the fractional form of 0 to 4-h, 4 to 8-h, 8 to 12-h, and 12 to 22-h post-nadolol administration. Sampled blood was centrifuged at 1,200 × *g* for 15 minutes and plasma was transferred into a sterile microcentrifuge tube. Plasma and urine were stored in -80°C freezer until further analysis.

At the end of the experiment (24-h post-nadolol administration), the rats were exposed to excess carbon dioxide inhalation until loss of consciousness and cervical dislocation was performed to ensure successful euthanasia. Approximately 5 to 7 mL of blood was drawn from the inferior vena cava followed by liver (median lobe) and ileum procurement. Liver was washed with cold PBS for at least 3 times to remove the excessive blood. Ileum, was identified at 3 cm segment from the distal small intestine or the connection between caecum and small intestine (Patrick Sharp, 2012). Ileum was excised

and was gently flushed with cold PBS to remove chyme. Liver and ileum were stored in RNAlater™ stabilising solution at 4°C for overnight prior transfer into -80°C freezer for further analysis.

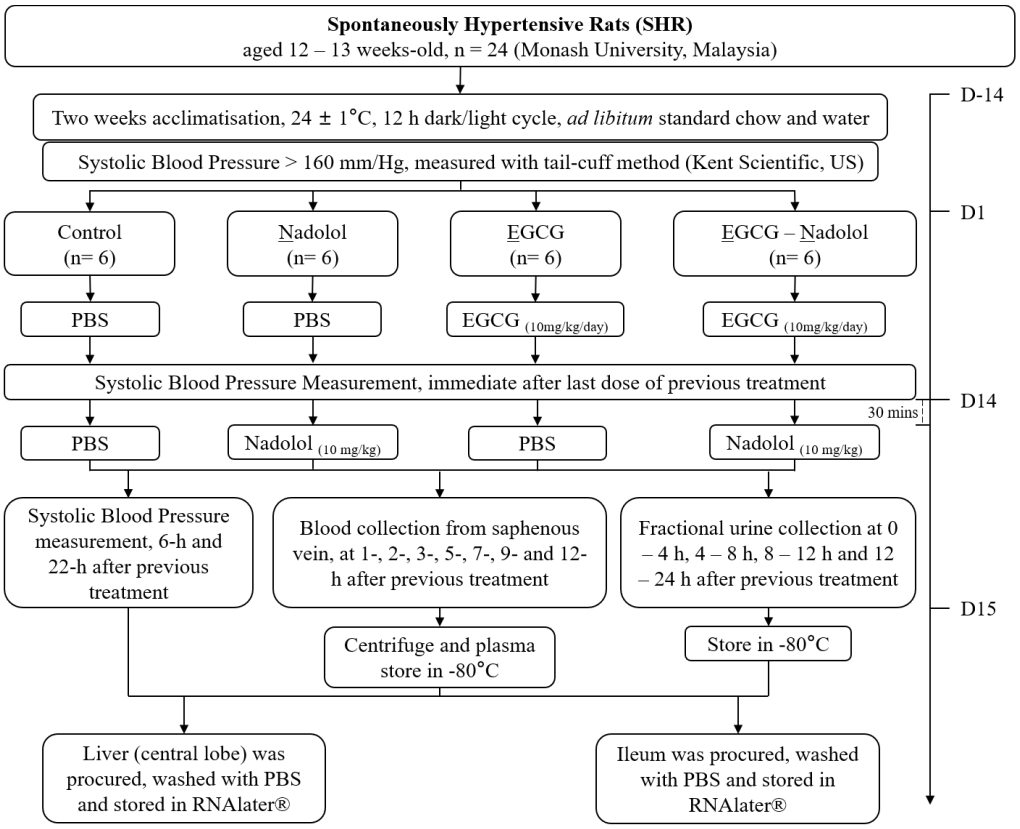



**Figure 3.1:** A schematic diagram of the study design and overall procedures.

### 3.3 Determination of Plasma and Urinary Nadolol Concentration

Nadolol in plasma and urine were extracted via liquid-liquid extraction as described in Delamoye et al. (2004) with minor modification. A total of 100 µL of plasma or 50-fold diluted urine samples were mixed with 500 µL of 1 M sodium hydroxide, 100 µL of metoprolol (100 ng/mL, internal standard) and 7 mL of chloroform-pentanol-diethyl ether solvent system (7:2:1). The mixture was shaken for 20 min at room temperature and centrifuged at 3,000 × *g* for 10

min. After centrifugation, the aqueous phase and protein precipitate were discarded and 200 μL of 0.05 M orthophosphoric acid (pH 2.1) was added into organic phase. Then, the mixture was shaken for 20 min and was subjected to centrifuge at 3,000 × *g* for 10 min. The aqueous phase was carefully collected and filtered through a 0.22 μm syringe filter. The samples were stored at -20°C until analysis.

Chromatography was performed with the Prominence LC-20A modular HPLC system with a UV-VIS detector and coupled with an auto-injector. Fifteen microliters of the sample solution were injected and separated on Symmetry® $C_{18}$ column (4.6 × 150 mm, 5 μm particle size) at 30°C. The mobile phase consisted of 35% of methanol in 50 mM NaCl solution with 0.1% of 0.1 N HCl and was isocratic run for 8 min at 1.0 mL/min. The detection of nadolol and metoprolol was set at 220 nm.

A non-compartmental pharmacokinetic analysis (NCA) was performed by using PKSolver (Zhang et al., 2010) with plasma concentration *vs* time data. Renal clearance ($CL_R$) was calculated from the cumulative amount of unchanged nadolol excreted into urine divided by $AUC_{0\text{-}12}$ (Wang et al., 2002). The reason of NCA was chosen instead of other models is due to NCA is a standard, efficient, and effective method for estimating PK parameters and is indispensable for characterizing PK within a single study, also is the most preferably approach in PK, bioavailability study. In contrast, compartmental analysis was used for characterising PK across multiple studies, and to explore the PK variability on different factors of analysis (such as age, gender, race etc.),

hence multiple assumption is required to fit the data in linear or non-linear regression manner.

### 3.4 Determination of *Oatp1a5*, *Mdr1a*, and *Oct1* mRNA Levels

Total RNA was extracted with Monarch® Total RNA Miniprep Kit. Thirty milligrams of RNAlater™ fixed and frozen ileum and liver samples were incubated with Proteinase K at 37°C for 5 min and followed by tissue homogenisation in DNA/RNA protection reagent. The lysate was subjected to on-column gDNA removal followed by RNA on-column purification. Residual gDNA was further removed with on-column DNase I treatment. Purified RNA was eluted with 50 µL of nucleases-free water into a sterile microcentrifuge tube.

Quantity and quality of the extracted RNA were assessed by NanoPhotometer® based on the ratio of $A_{260}/A_{280}$ and $A_{260}/A_{230}$. The integrity of the extracted RNA was evaluated following agarose gel electrophoresis. Briefly, around 500 ng of RNA was run on 1% (*w/v*) bleach gel (Aranda et al., 2012) at 75 V for 45 min. A good quality of extracted RNA was determined by $A_{260}/A_{280}$ = 2.0 and $A_{260}/A_{230}$ = 2.0 – 2.2. An intact RNA integrity was determined by the visualization of two distinct bands (18s and 28s rRNA) with the intensity ratio of approximately 1:2.7 and without smearing. Only good quality and intact RNA was reverse transcribed with High-Capacity cDNA Reverse Transcription Kit in accordance with the manufacturer's protocol. Briefly, 2 µg of total RNA was added to a mixture of MultiScribe™ reverse transcriptase, random primers and dNTP provided by the manufacturer. The

reaction mix was spun down and transferred into Veriti® 96-well thermocycler with the following setting: 25°C – 10 min, 37°C – 120 min, 85°C – 5 min and 4°C – ∞. The cDNA samples were stored at -20°C for downstream qPCR analysis.

Luna® Universal qPCR Master Mix was used for quantitative PCR on StepOnePlus™ Real-Time PCR System. The primers' sequences are shown in **Table 3.2** and *Gaphd* was used as the housekeeping gene. The reaction mix was prepared, and the thermocycler setting was set as "Fast ramp speed" according to the manufacturer's instructions (**Table 3.3** and **Table 3.4**) and each reaction was ran in technical triplicate manner. The relative mRNA expression was determined using the comparative Ct method by calculating $2^{-\Delta\Delta Ct}$.

**Table 3.2:** Primer sequences for *Gapdh*, *Oatp1a5*, *Mdr1a*, and *Oct1*.

| Gene | Forward Primer | Reverse Primer | Reference |
|---|---|---|---|
| *Gapdh* | ATGGGAAGCTGG TCATCAAC | GTGGTTCACAC CCATCACAA | (Urayama et al., 2009) |
| *Oatp1a5* | CTGCAGTCCTGG GGTTATGT | TGCCAGCGAAT ACTCTTGTG | (MacLean et al., 2010) |
| *Mdr1a* | GCAGGTTGGCTG GACAGATT | GGAGCGCAATT CCATGGATA | (Ros et al., 2003) |
| *Oct1* | GCATGAGCTGCA CTGGTTGA | CCCATACGGCC AAGACAGG | (Wang et al., 2008) |

**Table 3.3:** Reaction mix setup for qPCR.

| Reagent | Stock | Final Concentration | Volume |
|---|---|---|---|
| Master Mix | 2x | 1x | 10 µL |
| Primer (F) | 10 µM | 0.25 µM | 0.5 µL |
| Primer (R) | 10 µM | 0.25 µM | 0.5 µL |
| Template | 100 ng/uL | 100 ng | 1 µL |
| Nuclease-free water | - | - | 8 µL |
| | | **Total Volume** | **20 µL** |

**Table 3.4** Thermocycler setting for qPCR.

| Step | Temperature | Duration | Cycles |
|---|---|---|---|
| Initial Denaturation | 95ºC | 60 seconds | 1 |
| Denaturation | 95ºC | 15 seconds | 40 |
| Extension | 60ºC | 30 seconds | |
| Melt Curve | Instrument default setting | | 1 |

### 3.5 Statistical Analysis

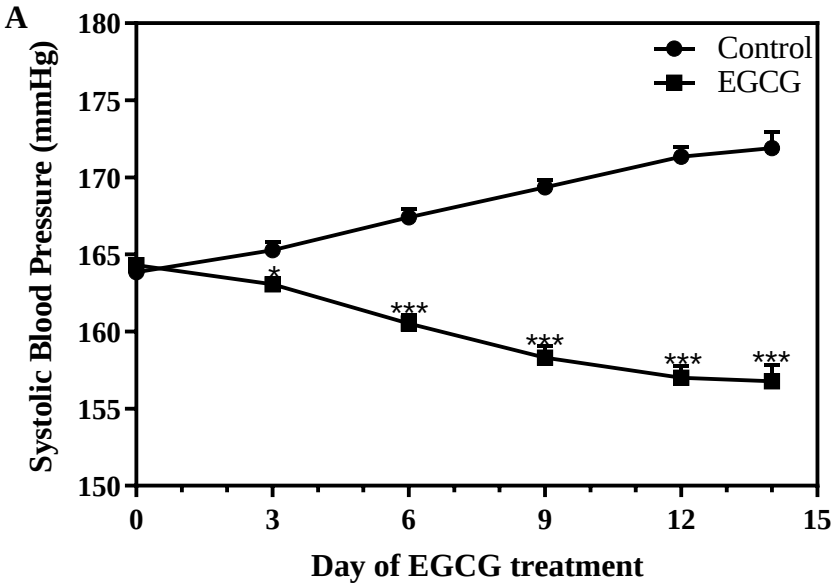
The data were presented as mean ± standard error mean (SEM). Systolic blood pressure was analysed using two-way ANOVA repeated measures followed by Tukey's post-hoc test. Urine and plasma concentrations of nadolol were analysed using one-way ANOVA followed by Tukey's post-hoc test. The mRNA levels were analysed using independent t-test. Pearson's correlation test was used to test the relationship of two selected parameters. All the statistical analysis were performed using SPSS® Statistics Software 26 (IBM, Armonk, NY, US). A *p*-value less than 0.05 ($p<0.05$) was considered to represent a statistical significance.

# CHAPTER 4

# RESULTS

## 4.1 Effect of EGCG on SBP and DBP during 14-days of EGCG supplementation in SHR

SBP of SHR received EGCG (EGCG) was consistently and significantly lower at day-3, 6, 9, 12, 14 compared to those did not receive EGCG (Control) (165 *vs.* 163 mmHg at day-3, 167.4 *vs.* 161 mmHg at day-6, 169 *vs.* 158 mmHg at day-9, 171 *vs.* 157 mmHg at day-12 and 172 *vs.* 157 mmHg at day-14) (**Figure 4.1 and Table 4.1)**



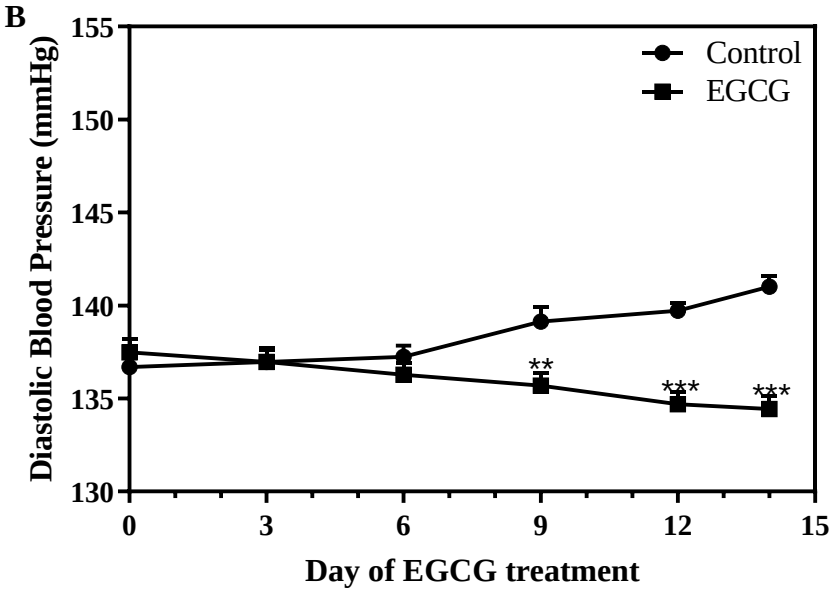



**Figure 4.1**: SBP (**A**) and DBP (**B**) at 0-, 3-, 6-, 9-, 12- and 14-day of EGCG supplementation. Each value represents the mean ± SEM (n = 12). *$p < 0.05$, ***$p < 0.001$ *vs* Control

**Table 4.1:** SBP and DBP at 0-, 3-, 6-, 9-, 12- and 14-day of EGCG supplementation

| Day- | SBP (mmHg) | | DBP (mmHg) | |
|---|---|---|---|---|
| | Control | EGCG | Control | EGCG |
| 0 | 163.9 ± 2.3 | 164.3 ± 1.2 | 136.7 ± 2.2 | 137.5 ± 2.4 |
| 3 | 165.3 ± 1.9 | 163.0 ± 1.5$^{*}$ | 136.9 ± 2.3 | 136.9 ± 2.6 |
| 6 | 167.4 ± 1.8 | 160.8 ± 1.6$^{***}$ | 137.2 ± 2.1 | 136.3 ± 2.2 |
| 9 | 169.3 ± 1.7 | 158.3 ± 2.6$^{***}$ | 139.1 ± 2.8 | 135.7 ± 2.4$^{**}$ |
| 12 | 171.3 ± 2.2 | 156.9 ± 2.7$^{***}$ | 139.7 ± 1.5 | 134.7 ± 2.2$^{***}$ |
| 14 | 171.9 ± 3.5 | 156.7 ± 3.7$^{***}$ | 141.0 ± 2.0 | 134.4 ± 2.4$^{***}$ |

Each value represents the mean ± SEM (n = 12). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* Control group.

### 4.2 Cage-side Observation

There were no signs of mis-behavioural activities (tremors, salivations, diarrhoea, and coma), changes in skin and fur condition, eye colour and mucous membranes, mortality have been observed throughout the experimental period. Upon dissection, the texture, colour, and the sizes of the thoracic/abdominal organs appeared normal.

### 4.3 Effect of EGCG on Water and Food intake

Water and food intake of EGCG treated SHR were comparable to those untreated group (**Figure 4.2)**.

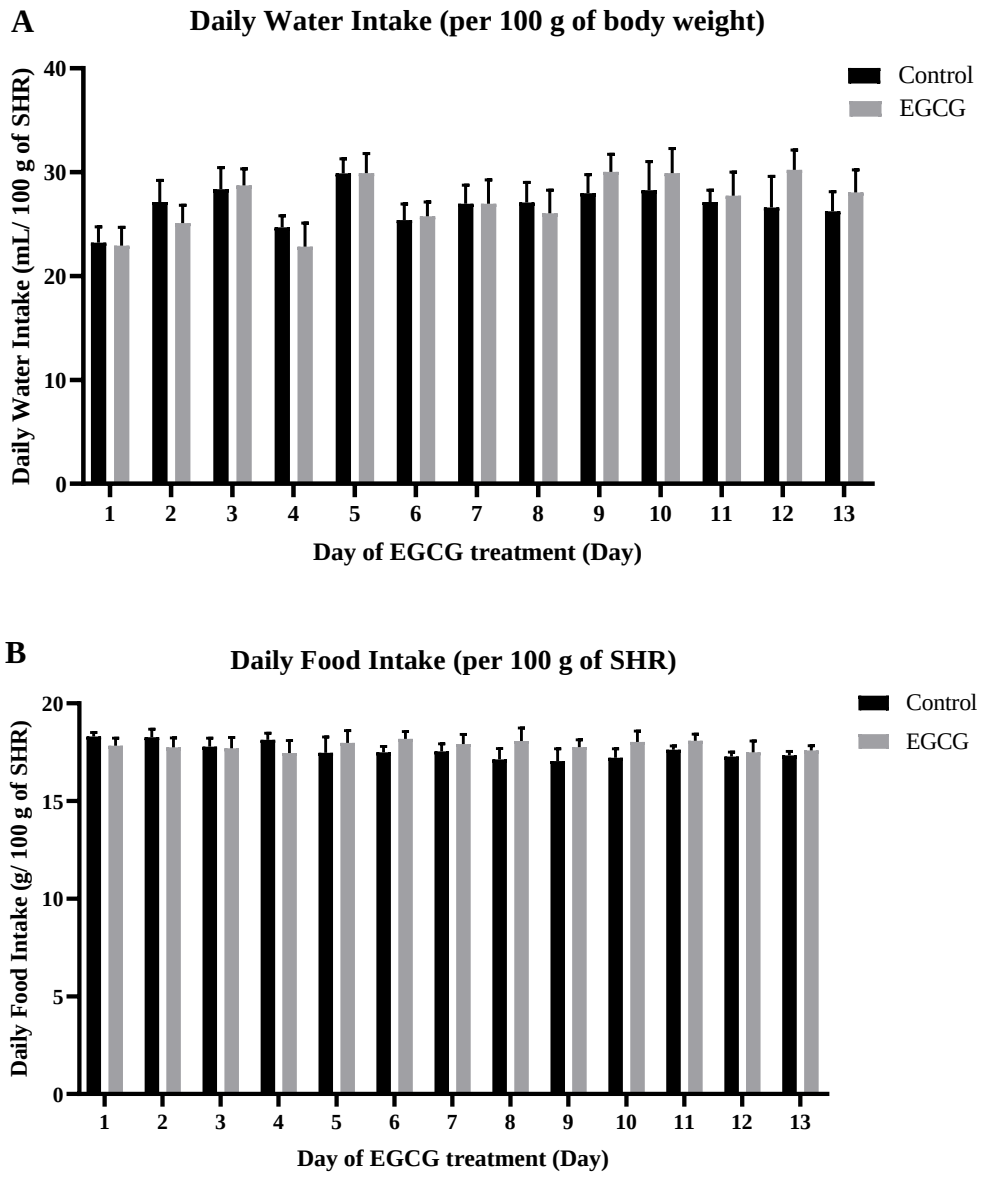



**Figure 4.2** Daily **(A)** water and **(B)** food intake of the SHR during EGCG treatment. The water intake (mL) and food intake (g) normalised to every 100 g b.w. of SHR.

### 4.4 Effect of EGCG on Body Weight

Body weight of SHR treated with EGCG was significantly lower ($p < 0.001$) from the Day-6 onwards when compared to control group (**Figure 4.3**).

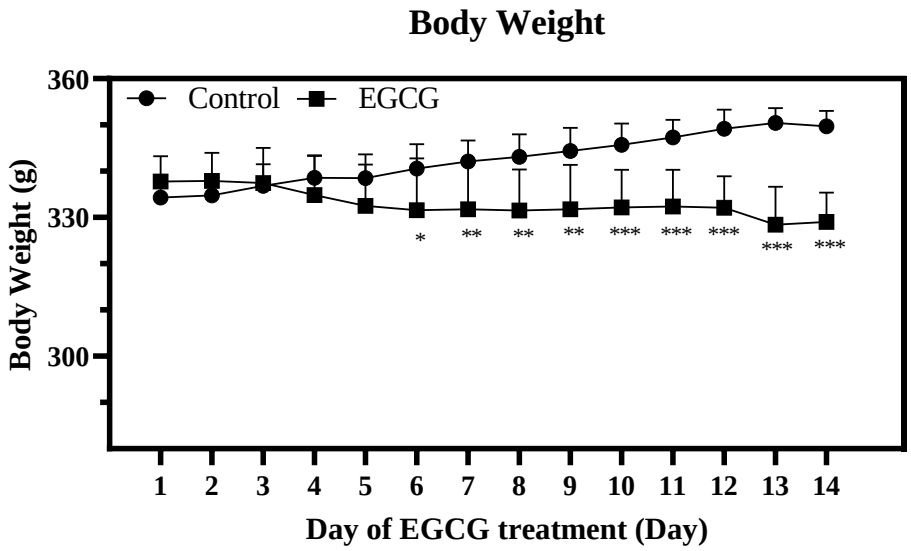



**Figure 4.3** Body weight of the SHR during EGCG treatment. Data were expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to Control.

## 4.5 Effect of EGCG and Nadolol Co-administration on Systolic Blood Pressure

SBP of the SHR received nadolol (Nadolol) were significantly lower at 6-h post nadolol administration, however, the antihypertensive effect of nadolol disappeared at 22-h post-nadolol administration compared to those that did not receive nadolol (Control) (**Figure 4.4**).

At 6-h post-nadolol, SBP of SHR pre-treated with EGCG and received a single dose of nadolol (EGCG-Nadolol) was significantly higher than those

received a single dose of nadolol but with no exposure to any EGCG (Nadolol) (128.6 ± 2.1 vs 110.6 ± 2.0 mmHg, $p < 0.001$). **(Figure 4.4)**

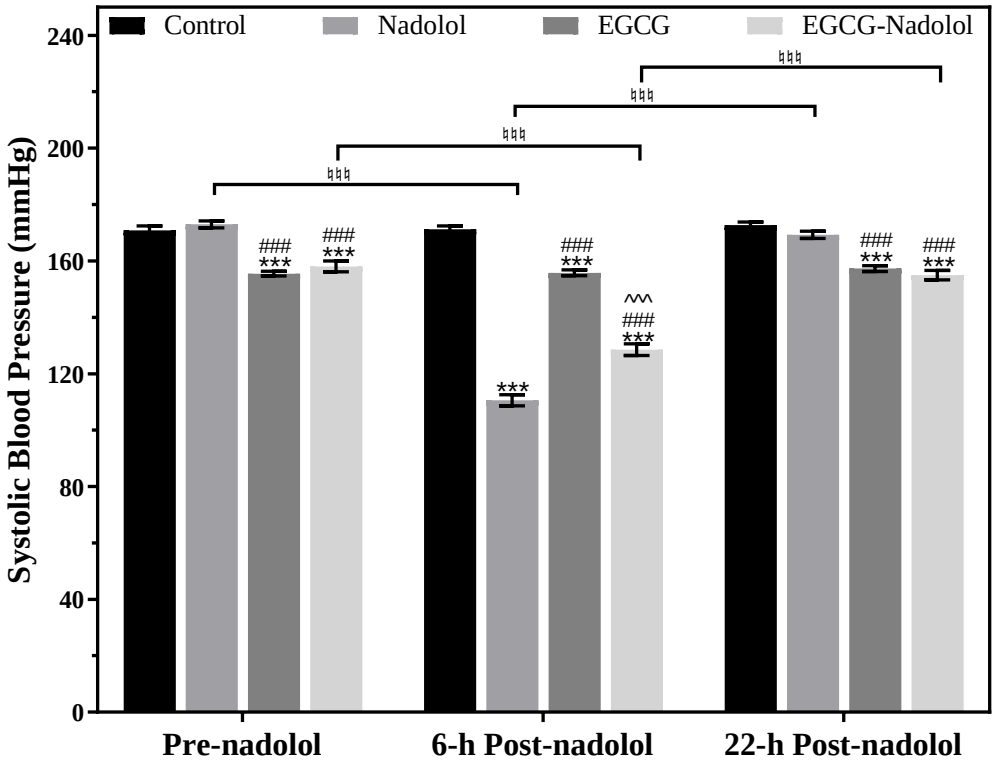



**Figure 4.4**: Systolic blood pressure of SHR received EGCG (EGCG), nadolol (Nadolol), EGCG and nadolol (EGCG-Nadolol) or placebo (Control). Data were expressed as mean ± SEM, n = 6, $^{***}p < 0.001$ *vs.* Control; $^{\#\#\#}p < 0.001$ *vs.* Nadolol; $^{\wedge\wedge\wedge}p < 0.001$ *vs.* EGCG; $^{\natural\natural\natural}p < 0.001$ *vs.* 6-h and 22-h post-nadolol.

### 4.6 Effect of EGCG on Bioavailability of Nadolol

Plasma nadolol concentrations were determined at 1-, 2-, 3-, 5-, 7-, 9- and 12-h post-nadolol administration in Nadolol and EGCG-Nadolol groups. Pharmacokinetic parameters of nadolol are summarised in **Table 4.2**. The results showed a marked decreased bioavailability of plasma nadolol and total urinary excreted nadolol amount in EGCG-Nadolol as compared to Nadolol group (**Figure 4.5 and Figure 4.6**). The $C_{max}$ and area under the time-plasma concentration curve ($AUC_{0-\infty}$) in EGCG-Nadolol were significantly lower, by 52.71% and 50.63% compared to Nadolol group. However, there were no significant differences in $T_{max}$, $T_{1/2}$, and $CL_R$ in between EGCG-Nadolol and Nadolol groups. Plasma nadolol concentrations at 12-h and 24-h were untraceable in both groups **(Figure 4.5)**.

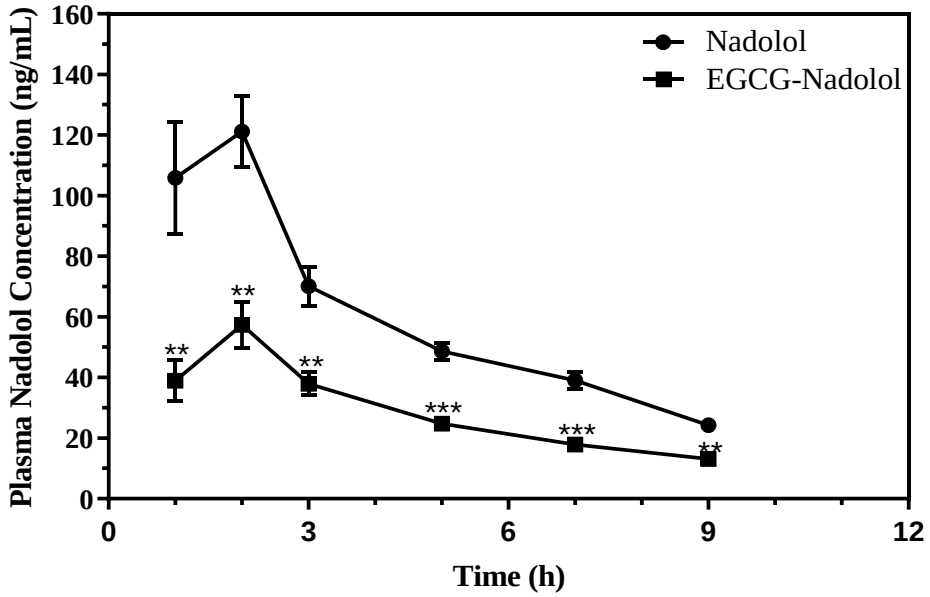



**Figure 4.5** Plasma concentration of nadolol in SHR received nadolol (Nadolol), EGCG and nadolol (EGCG-Nadolol). Plasma concentrations were measured at

1-, 2-, 3-, 5-, 7-, 9-, 12-, and 24-h after nadolol administration. The value at 12-h and 24-h was untraceable by HPLC. The values were expressed as mean ± SEM, n = 6. $^{*}p < 0.05$, $^{***}p < 0.001$ *vs.* Nadolol.

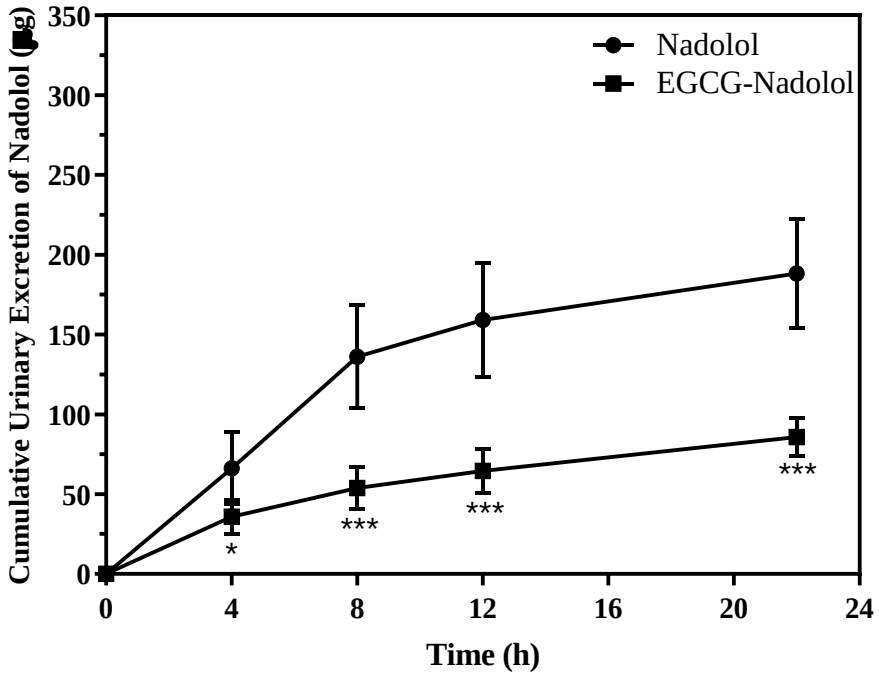



**Figure 4.6**: Time – the cumulative amount of nadolol in urine profile of SHR received nadolol (Nadolol), EGCG and nadolol (EGCG-Nadolol). The urine was collected cumulatively up to 22-h in fractions from 0-4 h, 4 – 8 h, 8 – 12 h and 12 – 22 h. The values were expressed mean ± SEM, n = 6. $^{***}p < 0.001$ *vs.* Nadolol.

**Table 4.2**: Pharmacokinetics parameters of nadolol in SHR

| | Nadolol | EGCG-Nadolol |
|---|---|---|
| $C_{max}$ (ng/mL) | 136.1 ± 13.5 | 64.4 ± 4.8*** |
| $T_{max}$ (h) | 2 (1 – 2) | 2 (1 – 2) |
| $T_{1/2}$ (h) | 3.96 ± 0.12 | 4.21 ± 0.10 |
| $AUC_{0-12}$ (h·ng/mL) | 531.7 ± 43.3 | 251.7 ± 16.8*** |
| $AUC_{0-\infty}$ (h·ng/mL) | 671.0 ± 54.7 | 331.3 ± 19.4*** |
| $CL_R$ (mL/min) | 5.71 ± 0.08 | 5.94 ± 0.20 |

$C_{max}$: maximum plasma concentration of nadolol; $T_{max}$: time to reach $C_{max}$; $T_{1/2}$: terminal half-life; AUC: area under the time – plasma nadolol concentration curve. The values of $C_{max}$, $T_{1/2}$, AUC and $CL_R$ were expressed as mean ± SEM, $T_{max}$ was expressed as median and range. ***$p < 0.001$ *vs.* Nadolol.

### 4.7 Effects of EGCG on Ileal and Hepatic *Oatp1a5*, *Mdr1a*, and *Oct1* mRNA Levels

The mRNA expressions of ileal *Oatp1a5*, *Mdr1a*, and *Oct1* were significantly lower in EGCG and EGCG-Nadolol than the non-EGCG treated group (Control and Nadolol). The mRNA expression of hepatic *Mdr1a* and *Oct1* were significantly lower in EGCG and EGCG-Nadolol than the non-EGCG treated group (Control and Nadolol) **(Table 4.3)**.

No significant difference was found in the mRNA expression of ileal *Oatp1a5*, *Mdr1a*, and *Oct1* and hepatic *Mdr1a* and *Oct1* between Nadolol and Control group.

As a side note, the yield of extracted total RNA of liver and ileum tissues were 131.6 ± 6.0 and 80.2 ± 5.6 µg/100 µL. The purity of extracted RNA samples was assessed by nanophotometer, with $A_{260/280}$ of 2.005 ± 0.003 and 2.012 ± 0.004; $A_{260/230}$ of 2.051 ± 0.025 and 2.061 ± 0.017 for liver and ileum samples respectively (**Appendix D**). The integrity of RNA was assessed by agarose gel electrophoresis on 1% bleach gel (Aranda et al., 2012), where two distinct bands representing 18S RNA and 28S RNA were observed in both liver and ileum samples (**Appendix D**). Quantitative PCR master mix was tested for the efficacy and each PCR amplicons were analysed with melt-curve (**Appendix E**). Both tests had proven an optimised and high efficiency master mix, primers and thermocycler settings were used in the experiment. The amplicons were specific which supported by gel electrophoresis (**Appendix E)**.

**Table 4.3**: Fold-change of mRNA levels in SHR received EGCG (EGCG), nadolol (Nadolol), EGCG and nadolol (EGCG-Nadolol) or PBS (Control).

| | Relative mRNA Levels (fold change) | | | |
|---|---|---|---|---|
| | Control | EGCG | Nadolol | EGCG-Nadolol |
| Ileum | | | | |
| *Oatp1a5* | 1.00 ± 0.21 | 0.23 ± 0.03$^{*}$ | 1.00 ± 0.25 | 0.27 ± 0.05$^{\#}$ |
| *Mdr1a* | 1.00 ± 0.31 | 0.12 ± 0.01$^{*}$ | 1.00 ± 0.24 | 0.13 ± 0.01$^{\#}$ |
| *Oct1* | 1.00 ± 0.19 | 0.27 ± 0.03$^{**}$ | 1.00 ± 0.28 | 0.23 ± 0.02$^{\#}$ |
| Liver | | | | |
| *Mdr1a* | 1.00 ± 0.19 | 0.36 ± 0.08$^{*}$ | 1.00 ± 0.09 | 0.41 ± 0.08$^{\#\#\#}$ |
| *Oct1* | 1.00 ± 0.11 | 0.42 ± 0.05$^{***}$ | 1.00 ± 0.29 | 0.34 ± 0.04$^{\#}$ |

Data were expressed as mean ± SEM. Independent T-test was performed between Control *vs* EGCG and Nadolol *vs* EGCG-Nadolol. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* Control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ *vs* Nadolol.

## 4.8 Association of Reduced Bioavailability, Drug Transporters mRNA Levels and Blood Pressure

The reduced mRNA levels of ileal *Oatp1a5* and *Oct1* were positively correlated with the reduced bioavailability of nadolol ($r^2$ = 0.898 and 0.631, $p$ <0.01 and <0.05 respectively) (**Table 4.4**). The reduced bioavailability of nadolol also associated with the increased SBP ($r^2$ = -0.758, p < 0.01) (**Table 4.5**).

**Table 4.4**: Association of drug transporter mRNA levels with nadolol AUC.

| mRNA Expression | $AUC_{0-12}$ | | $AUC_{0-\infty}$ | |
|---|---|---|---|---|
| | $r^2$ | *P* | $r^2$ | *P* |
| **Ileum** | | | | |
| *Oatp1a5* | $0.898^{***}$ | 0.000 | $0.901^{***}$ | 0.000 |
| *Mdr1a* | 0.436 | 0.157 | 0.419 | 0.175 |
| *Oct1* | $0.631^{*}$ | 0.028 | $0.604^{*}$ | 0.038 |
| **Liver** | | | | |
| *Mdr1a* | 0.526 | 0.079 | 0.517 | 0.085 |
| *Oct1* | 0.546 | 0.066 | 0.520 | 0.083 |

Pearson correlation test showed that mRNA levels of ileal *Oatp1a5* and *Oct1* were positively correlated with nadolol $AUC_{0-12}$ and $AUC_{0-\infty}$. n= 6. $^{*}p < 0.05$ and $^{***}p < 0.001$.

**Table 4.5**: Association of SBP at 6-h post-nadolol administration and bioavailability of nadolol.

| Bioavailability of nadolol | SBP at 6h post-nadolol administration | |
|---|---|---|
| | $r^2$ | *P* |
| $AUC_{0-12}$ | $-0.758^{**}$ | 0.004 |
| $AUC_{0-\infty}$ | $-0.752^{**}$ | 0.005 |

Pearson's correlation test showed that SBP is negatively associated nadolol $AUC_{0-12}$ and $AUC_{0-\infty}$, n = 6. $^{**}p < 0.01$.

# CHAPTER 5

# DISCUSSION

The key findings in this present study suggest that reduced drug bioavailability could be due to poor intestinal absorption, rapid metabolism, or fast elimination from the body system. In this study, metabolism and elimination are less likely to contribute to the reduced bioavailability of nadolol as it is a non-metabolizing drug and the renal clearance in EGCG-treated and non-treated groups are comparable. On the other hand, this study suggests that EGCG downregulates the expression of the drug transporters (*Oatp1a5* and *Oct1*) lining the intestinal membrane and subsequently leads to poor nadolol absorption, low bioavailability of nadolol and eventually lower antihypertensive effect in SHR. Other than these major findings, supplementary data have been included in a chronological order in the following sections.

## 5.1 EGCG Supplementation Reduces Elevated Blood Pressure

Although investigating the antihypertensive effect of EGCG was not the main objective of this present study, it is worth pointing out that EGCG reduced SBP in SHR throughout the experimental period, and its antihypertensive effect persisted even approximately 23 hours after withdrawal of treatment. It has been shown that EGCG exhibits its anti-hypertensive effect via its potent anti-oxidative activity, angiotensin II and ACE inhibitory activity and also direct

vasodilatation effect (Potenza et al., 2007, Qian et al., 2018, Lorenz et al., 2015). It seems that the antihypertensive effect of EGCG in pre-clinical studies is promising and it also has frequently been promoted as a novel antihypertensive molecule (Khan and Mukhtar, 2018). Clinical trials have been proposed to establish its antihypertensive effect further. Therefore, our findings highlight that the potential EGCG-nadolol interaction should be taken into serious consideration in future clinical trials.

### 5.2 EGCG Supplementation Did Not Cause Observable Toxic Effects

EGCG is generally considered as "safe" when it is taken in a low dose. In this present study, oral EGCG at 10 mg/kg b.w. for 14 days did not lead to any cage-side-observable toxic effects, as well as any macroscopic changes in the thoracic and abdominal organs in SHR.

In a Phase IV clinical trial, a single oral dose of 135 mg and 270 mg of dietary EGCG supplement was shown to be well tolerated and with no severe side effects in 24 healthy young adults (Wightman et al., 2012). In addition, a daily dose of 800 mg EGCG for 4 weeks was shown to be safe and well tolerated in healthy human subjects (Chow et al., 2003). Nevertheless, it has been found that ingestion of EGCG is associated with various side effects such as inhibition of spontaneous excitatory synaptic transmission (Vignes et al., 2006), suppression of hepatic gluconeogenesis (Collins et al., 2007), induction of hypochromic anaemia via iron absorption inhibition (Ryan and Hynes, 2007), liver injury, gastrointestinal bleeding, and kidney damage (Mazzanti et al., 2009,

Younes et al., 2018). Cases of hepatotoxicity have been associated with consumption of high doses (800 – 1080 mg/day) of EGCG-containing dietary supplements. In most of the cases, patients presented with increased serum bilirubin and alanine aminotransferase (ALT) levels, and periportal and portal inflammation were found in several biopsies (Mazzanti et al., 2009, Younes et al., 2018).

In pre-clinical studies, it has been demonstrated that long-term (108 weeks) EGCG supplementation (25 mg/kg body weight/day, given via drinking water) extends lifespan of healthy rats by reducing aging-associated of increased blood pressure, liver, and kidney damage (Niu et al., 2013). In contrast, Lambert et al demonstrated that a single dose of EGCG (1500 mg/kg, i.g.) increased plasma ALT by 138-fold and reduced survival by 85%. 2-7 days of lower repeated-dose of EGCG given at 750 mg/kg body weight exhibited moderate to severe hepatic necrosis in CF-1 mice (Lambert et al., 2010). Few years later, Wang et al reported that 5-day- repeated-EGCG given at a dose of 75 mg/kg body weight intraperitoneally significantly evoke hepatoxicity in mice (Wang et al., 2015).

This present study concludes that 14-day of oral 10 mg/kg b.w. EGCG did not cause observable toxic effects in SHR, nevertheless, EGCG exposure as low as 10 mg/kg b.w. leads to EGCG-nadolol interactions as shown in the Chapter 4.

### 5.3 EGCG Supplementation Did Not Affect Food and Water Intakes

This present study shows that oral EGCG at a 10 mg/kg b.w. for 14 days did not affect the eating and drinking patterns of the animals. Food intake observation gives clues in growth, physical activity, heat production and closely related to physiological activities. While water intake gives clues in losses of water via evaporation, or by water excretion via urine and faeces. EGCG supplementation did not cause any changes in these physiological processes. There is limited evidence in showing effects of oral EGCG in food/water intakes. An isolated study demonstrated that 81 mg/kg b.w. of oral EGCG did not affect food intake, surprisingly, when the same amount of EGCG was given intraperitoneally, the food intake reduced by 50% along with reductions in body weight, testosterone, oestradiol, leptin, and insulin in Sprague-Dawley rats. The research team further concluded that EGCG may modulate the appetite-regulating pathway (Kao et al., 2000). Nevertheless, it is conclusive that food/water intakes are not associated with the changes in blood pressure and bioavailability of nadolol in SHR exposed to EGCG in this study.

## 5.4 EGCG Supplementation Reduces Body Weight

Consumption of EGCG or green tea has been frequently associated with reduction in body fat and body weight. A meta-analysis has demonstrated that daily consumption of green tea which contains 100-460 mg of EGCG for a period of 12 weeks shown greater effect in body fat and body weight reduction (Vazquez Cisneros et al., 2017). In an *in vivo* study, 50 and 100 mg/kg of oral EGCG significantly reduces body weight along with the Lee index (b.w. *0.33*1000 / body length) in high-fat-diet fed C57BL/6J mice (Li et al., 2018). It has been proposed that EGCG-led body weight reduction could be due to its effects in reducing food consumption, adipogenesis and lipogenesis (Huang et al., 2014). The differences could be due to (i) different experimental designs especially the physiological entity i.e., in human vs rodent model (ii) the amount, type, and purity of the EGCG given to the subjects/animal model. In the meta-analysis conducted by Vazquez Cisneros (2017), they summarised that the subjects were given green tea which contains 100-460mg of EGCG rather than EGCG in a pure form. The minimal treatment duration required in causing significant weight reduction has not been well established.

In this present study, Pearson correlation test showed that the body weight reduction in SHR exposed to EGCG is not associated with the food and water intake (Appendix E, Table E). In another word, the body weight reduction in the EGCG treated group could be due to the changes in internal environment e.g., changes in metabolism pathways rather than food consumption.

Nevertheless, it is certainly that more studies are required to validate these research questions.

### 5.5 Repeated Oral EGCG Supplementation Reduces Bioavailability of Nadolol and its Antihypertensive Effect in SHR

The data of this present study shows that EGCG exposure significantly reduces bioavailability of nadolol, Cmax, AUC with no changes in nadolol half-life, Tmax and renal clearance in SHR. Statistical analysis shows that reduced bioavailability of nadolol is closely associated with the loss of its antihypertensive effects in SHR. Our findings are consistent with (Misaka et al., 2013c) study, which SD rats were used. In their study, significantly reduces bioavailability, $C_{max}$, AUC with no changes in half-life and $T_{max}$ of nadolol, however, renal clearance was not disclosed.

Nadolol is a blood pressure lowering drug, and its antihypertensive effect persists for approximately 24 hours after dosing (Food and Drug Administration., 2011). Consistent with our current data, plasma nadolol was not traceable at 22 hours after dosing. Simultaneously, its antihypertensive effect was not observable in SHR received a single dose of nadolol. Interestingly, nadolol lost its antihypertensive as early as at 6-h post-nadolol administration in SHR pre-exposed to EGCG supplementation.

It has been claimed that repeated consumption of green tea (700 mL/day for 14 days) significantly reduced the plasma nadolol concentration in healthy

volunteers (Misaka et al., 2014). The same team later demonstrated that a single infusion of brewed green tea also significantly reduced the bioavailability of nadolol in healthy volunteers (O.Abe et al., 2017). The exact amount of ingested green tea catechins or EGCG were not clarified in these studies. In another *in-vivo* study, the team concluded that a single dose of GTE (400 mg/kg body weight, i.g) and high amount of EGCG (150 mg/kg body weight i.g.) significantly reduced the bioavailability of plasma nadolol in Sprague Dawley rats (Misaka et al., 2013c). The impact of green tea/EGCG on pharmacological actions of nadolol, and the possible mechanisms that led to reduced bioavailability of nadolol were not examined in these studies. This present study demonstrated that EGCG as low as 10 mg/kg b.w. for 28 days could significantly lead to reduced bioavailability of nadolol as well as suppressed pharmacological effects in SHR.

In addition, previous studies had shown that EGCG as low as 2 to 4 mg/kg b.w. modulated bioavailability of sunitinib, diltiazem, verapamil and nicardipine (Choi and Burm, 2009, Chung et al., 2009, Ge et al., 2011, Li and Choi, 2008). Our data showed that nadolol lost its antihypertensive effect as early as at 6-hour post administration in SHR exposed to EGCG. This scenario is worrying as uncontrolled high blood pressure certainly increases the risk of cardiovascular events and end-organ damage (Ettehad et al., 2016). Physicians and patients prescribed with nadolol should be alerted that concurrent consumption of EGCG supplement or EGCG-contained products should be avoided.

This study further hypothesised that the reduced nadolol plasma concentration could be due to poor drug transportation rather than changes in its elimination half-life ($T_{1/2}$) and renal clearance ($CL_R$) (**Table 4.2**).

## 5.6 EGCG Modulates Nadolol Drug Transporters mRNA Expression thus Reduces Its Bioavailability

The key findings of this study suggest that EGCG downregulates the expression of the drug transporters (*Oatp1a5* and *Oct1*) lining the intestinal membrane and subsequently leads to poor nadolol absorption, low bioavailability of nadolol and eventually lower antihypertensive effect in SHR. A detailed schematic diagram below has summed up the findings of the study, from the impact of EGCG-nadolol interactions to the decipherment of the possible mechanism involving the transporters expression leading to such phenomenon **(Figure 5.1)**.

Pearson correlation test has shown that the reduced nadolol plasma concentrations are significantly associated with downregulated mRNA abundance of ileal nadolol influxes, i.e., *Oatp1a5* and *Oct1*. The roles of EGCG in epigenetic modulation via interaction with DNA methyltransferase and histone deacetylases has been demonstrated (Negri et al., 2018). This study proposes that transcriptional levels of ileal *Oatp1a5* and *Oct1* are susceptible to EGCG epigenetic modulation, and our study further postulates that EGCG inhibits the expression of ileal *Oatp1a5* and *Oct1* via its inhibitory activities on HNF4α promoter transactivation (Wang et al., 2020a) which ultimately leads to

poor nadolol transportation across the enterocytes. In physiological condition, the transcriptional expression of *Oatp1a5* and *Oct1* is dependent on the expression of the HNF4α, which two HNF4α molecules bind to the promoter region of *Oct1* and *Oatp1a5* and transactivate both *Oct1* and *Oatp1a5* transcriptional activity (Roth et al., 2012). Furthermore, a study has demonstrated that the expression of *HNF4α* is downregulated with the activation of ERK1/2 signalling pathway (Veto et al., 2017). EGCG has been reported to have the ERK1/2 signalling pathway activation properties (Wang et al., 2020a), which is anticipated to downregulate the *HNF4α* expression and further reduce both *Oatp1a5* and *Oct1* expression.

Even though *Mdr1a* efflux was downregulated in EGCG exposed rats, statistical correlation tests showed no association between the plasma nadolol concentrations and *Mdr1a* mRNA levels. These findings conclude that the reduced bioavailability of nadolol could be due to the poor intestinal absorption rather than modulation in the efflux system.

Influence of EGCG on suppression of *Mdr1a* activity or gene expression level has been reported in various laboratory models, for examples colorectal cancer cell line and intestinal epithelial cells model - Caco-2 (Jodoin et al., 2002), hepatocellular carcinoma cell line - HepG2 (Satonaka et al., 2017), pancreatic carcinoma cell line – PANC-1 (Zhu et al., 2012) etc. These studies mainly focus on the multi-drug resistance with the aims in investigating the beneficial effects of EGCG in cancer therapy. Consistent with these previous studies, this present study found that EGCG reduces gene expression of ileal

*Mdr1a* in SHR. Nevertheless, statistical correlation tests show there is no association between the plasma nadolol concentrations and *Mdr1a* mRNA levels. These findings draw a conclusion that the reduced bioavailability of nadolol could be due to the poor intestinal absorption rather than modulation in efflux system. Several studies have also shown that EGCG modulates CYPs (Misaka et al., 2013a) however, as nadolol is not metabolised by CYPs, this study did not intend to investigate the roles of EGCG on CYPs modulation and nadolol metabolization.

An *in-vitro* study has demonstrated EGCG inhibits transport activities of *Oct*, *Mdr1a*, and *Oatp* regardless of its location – either in enterocytes, hepatocytes, or renal proximal tubular cells (Knop et al., 2015). This present study confirms that EGCG reduces mRNA expression of *Mdr1a* and *Oct1* in both enterocytes and hepatocytes in SHR. Changes in mRNA levels of *Mdr1a* and O*ct1* could affect the hepatic clearance rate of nadolol; nevertheless, the elimination half-life ($T_{1/2}$) and $CL_R$ were comparable in EGCG treated SHR compared to those untreated groups. It seems further studies are required to elucidate the roles of these hepatic drug transporters and metabolisation of nadolol. Hepatic *Oatp1a5* was not measured in this present study as previous studies have shown that *Oatp1a1* and *Oatp1b1* are the dominant Oatp transporters for the hepatic uptake in rats and the role of *Oatp1a5* in liver has not been elucidated (Klaassen and Aleksunes, 2010).

Miyazaki et al (2013) investigated the involvement of *Mdr1a* efflux and *Oatp1a5* influx in the pharmacokinetics of nadolol by administrations of *Mdr1a*

inducer (dexamethasone), *Mdr1a* inhibitor (itraconazole), or *Oatp1a5* inhibitor (naringin) to normotensive Sprague Dawley rats (Miyazaki et al., 2013). They revealed that both *Mdr1a* inducer and inhibitor significantly altered the plasma nadolol levels. Plasma and urine nadolol levels were found non-statistically but consistently lower in *Oatp1a5* inhibitor treated group. The team then concluded that *Mdr1a* plays a relative determinant role in pharmacokinetics of nadolol over *Oatp1a5*. In contrast, our data suggest that EGCG modulates multiple drug transporters of nadolol i.e., *Mdr1a, Oct1* and *Oatp1a5* mRNA expression levels, and eventually leads to decreased plasma nadolol levels in a hypertensive rodent model. Further correlation statistical analysis showed that ileal *Oatp1a5* and *Oct1* mRNA abundance play the determinant role in affecting plasma nadolol levels and subsequently modulate blood pressure regulation. More functional studies are required to establish the relative determinant roles of *P-gp*, *Oatp1a5* in bioavailability of nadolol.

The present findings have showed that lower plasma nadolol concentrations is statistically associated with the lower expression levels of intestinal *Oatp1a5* and  but not associated with the intestinal *Mdr1a* levels. Hence, current data suggests that modulation of intestinal *Oatp1a5* and *Oct1* play the determinant role in nadolol absorption relatively to *Mdr1a* in SHR pre-exposed to EGCG. Protein expression analysis and functional assays could be performed to reconfirm the role of intestinal *Oatp1a5* and *Oct1* in bioavailability of nadolol in SHR.

This present study further demonstrated that exposure of EGCG as low as 10 mg/kg b.w. for 14 days suppresses the mRNA expression of the major nadolol transporters, which is significantly associated with reduced nadolol plasma concentration and therefore causing a lack of drug effect in controlling the raised blood pressure in SHR. It is uncertain if the genes downregulation is perpetual or reversible via cessation of EGCG exposure. Nevertheless, we believe that simultaneous administration of EGCG with either acute or chronic nadolol will affect the bioavailability of nadolol. In addition, it is crucial to highlight that long term EGCG exposure may affect intestinal and hepatic transportation of many other drugs that require *Mdr1a*, *Oatp1a5*, and *Oct1* membrane transporters. Further studies are required to investigate these research hypotheses.

Furthermore, the findings of present study could be helpful in raising awareness to the public and physicians that, the intake of either EGCG or green tea-related products potentially modulates the absorption of the therapeutic agents that are involved the drug transporters OATP1A2, OCT1 and MDR1. Hence, an investigation on the patient's history of EGCG-related intake could be helpful in preventing the undesired adverse effect brings to patient during the therapy. Public should also be aware on the intake of EGCG, or green tea-related product while there are ongoing therapy or medication treatment, and public are encouraged to consult the physicians if necessary.

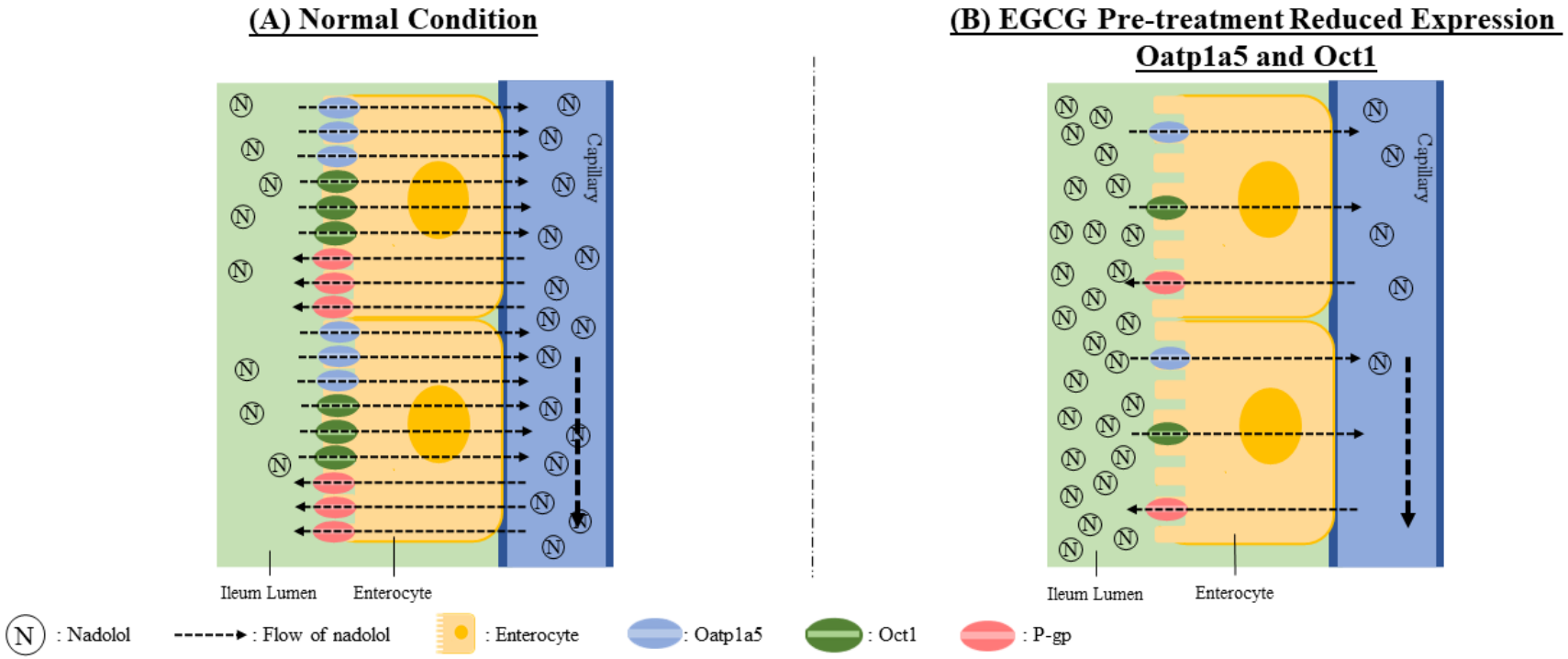



**Figure 5.1** A schematic diagram of the proposed mechanism on the effect of EGCG on nadolol transportation.

# CHAPTER 6

## CONCLUSION

In conclusion, EGCG may lead to poor nadolol absorption across the enterocytes, and subsequently, lead to reduced bioavailability of nadolol, and potential nadolol therapeutic failure and increased hypertension-related morbidity and mortality. This scenario is worrying as uncontrolled high blood pressure certainly increases the risk of cardiovascular events and end-organ damage. Physicians and patients prescribed with nadolol should be alerted that concurrent consumption of EGCG supplement should be prohibited. Therefore, considerations on dose adjustment of the prescribed nadolol or use of alternative antihypertensive agents among EGCG consumers or habitual green tea drinkers should be taken seriously.

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# APPENDICES

## APPENDIX A

**Table A:** Summary of items and brands for the chemicals, reagents, plasticwares, consumables, equipment, software, and kits used in the study.

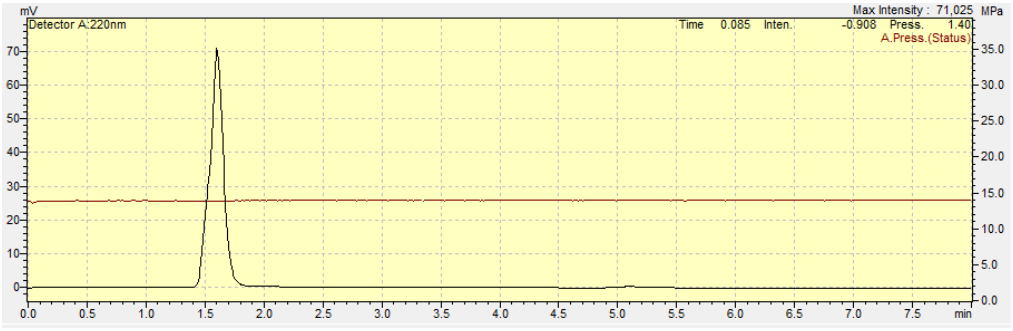
| Items | Brand | Country |
|---|---|---|
| | Chemicals and Reagents | |
| (-)-Epigallocatechins-3-Gallate | Cayman Chemical | Michigan, US |
| 50x TAE buffer | Thermofisher | Massachusetts, US |
| Agarose | Thermofisher | Massachusetts, US |
| Bleach | Clorox® | California, US |
| Chloroform | Merck | New Jersey, US |
| Diethyl ether | Merck | New Jersey, US |
| Hydrochloric acid (HCl) | Merck | New Jersey, US |
| Metoprolol | Tokyo Chemical Industry | Tokyo, Japan |
| Nadolol European pharmacopoeia (EP) reference standard | Sigma-Aldrich | Missouri, US |
| ortho-Phosphoric acid 85% (EMSURE® ACS) | Merck | New Jersey, US |
| PCR Primers | Integrated DNA Technologies (IDT) | Iowa, US |
| Pentanol | Merck | New Jersey, US |
| Potassium chloride (KCl) | Sigma-Aldrich | Missouri, US |
| Potassium phosphate monobasic ($KH_2PO_4$) | Sigma-Aldrich | Missouri, US |

| | | |
|---|---|---|
| RNA stabilising solution (RNAlater™) | Ambion | Texas, US |
| Sodium chloride (NaCl) | Sigma-Aldrich | Missouri, US |
| Sodium hydroxide (NaOH) | Sigma-Aldrich | Missouri, US |
| Sodium phosphate dibasic dihydrate ($Na_2HPO_4$) | Sigma-Aldrich | Missouri, US |
| Standard chow for rodent | Goldcoin | Singapore |
| <u>Plasticwares and Consumables</u> | | |
| 0.5 mL conical tubes | Corning | Massachusetts, US |
| 1 mL syringe | Terumo | Tokyo, Japan |
| 1.5 mL conical tubes | Corning | Massachusetts, US |
| 10 mL syringe | Terumo | Tokyo, Japan |
| 15 mL conical tubes | SPL Life Sciences | South Korea |
| 50 mL conical tubes | SPL Life Sciences | South Korea |
| Analytical column (Symmetry® $C_{18}$) | Waters Corp. | Massachusetts, US |
| Blade | B. Braun | Melsungen, Germany |
| Guard column (Symmetry® $C_{18}$) | Waters Corp. | Massachusetts, US |
| HPLC micro-inserts (250 μL) | ChromineX | Malaysia |
| HPLC micro-vials | ChromineX | Malaysia |
| Micropipette tips (P-1000, 100, 10) | Labcon | California, US |
| Needles (21G and 25G) | B. Braun | Melsungen, Germany |
| Nylon syringe filter (0.22 μm) | Membrane Solutions | Washington, US |
| qPCR strip tubes | Applied Biosystems | California, US |

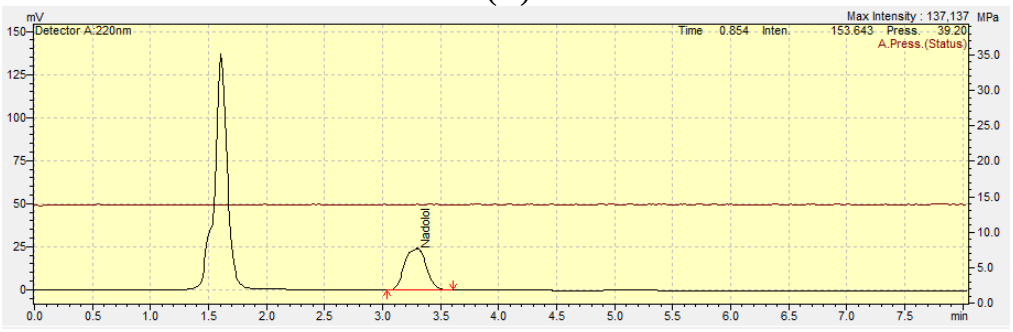
| Laboratories Equipment | | |
|---|---|---|
| -20°C freezer | Haier | Qingdao, China |
| 4°C refrigerator | HiTec | Malaysia |
| -80°C freezer | Sanyo | Osaka, Japan |
| Analytical balance (BSA224S) | Sartorius | Göttingen, Germany |
| Benchtop centrifuge machine (Sorvall Legend X1) | Thermofisher | Massachusetts, US |
| Gel Electrophoresis system (Mini-Sub® Cell GT) | Bio-Rad | California, US |
| High-capacity balance (ELB2000) | Shimadzu | Kyoto, Japan |
| HPLC system (Prominence LC-20A) | Shimadzu | Kyoto, Japan |
| Microcentrifuge machine (Heraeus™ Fresco 21) | Thermofisher | Massachusetts, US |
| NanoPhotometer® | Implen GmbH | München, Germany |
| Non-invasive blood pressure system (CODA™ Monitor) | Kent Scientific | Connecticut, US |
| Real-time PCR system (StepOnePlus™) | Applied Biosystems | California, US |
| Thermocycler (Veriti® 96-well) | Applied Biosystems | California, US |
| UV transilluminator & imaging (UVP BioSpectrum® 410) | Analytik Jena | California, US |
| Water Purification System (Milli-Q® Integral 5) | Millipore | Massachusetts, US |

| | | |
|---|---|---|
| | Software | |
| GraphPad Prism 8 | GraphPad Software | California, US |
| ImageJ | NIH | Maryland, US |
| LabSolutions | Shimadzu | Kyoto, Japan |
| Office 365 | Microsoft | Washington, US |
| SPSS 26 | IBM | New York, US |
| StepOne™ Software | Applied Biosystems | California, US |
| VisionWorks® | Analytik Jena | California, US |
| | Assays & Kits | |
| 5x DNA loading buffer Tricolour | Bioline | London, UK |
| EtB"Out" Nucleic Acid Staining Solution | Yeastern Biotech | Taipei, Taiwan |
| High-Capacity cDNA Reverse Transcription Kit | Applied Biosystems | California, US |
| Luna® Universal qPCR Master Mix | New England Biolabs | Massachusetts, US |
| Monarch® Total RNA Miniprep Kit | New England Biolabs | Massachusetts, US |

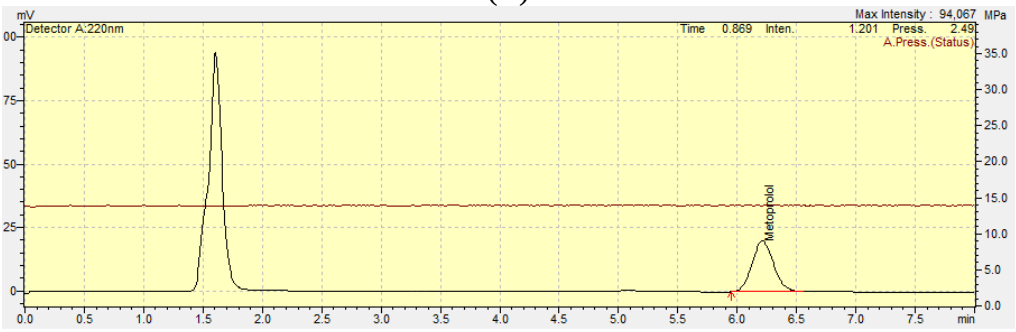
# APPENDIX B



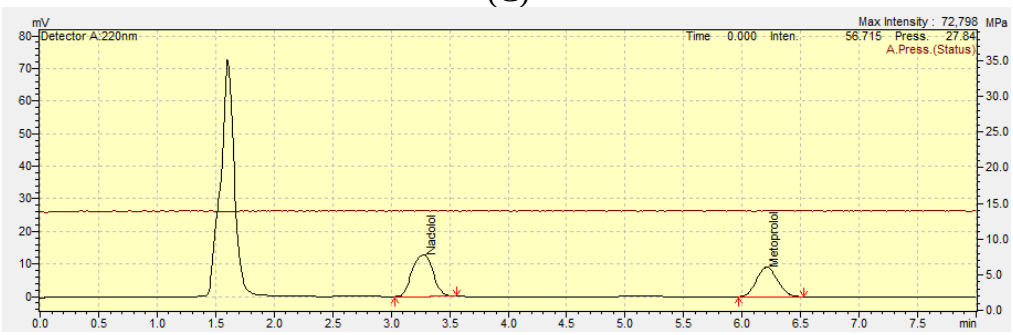

(A)




(B)




(C)




(D)

**Figure B1:** Chromatograms of analytes from (A) Vehicle (0.05 M orthophosphoric acid; (B) Pure nadolol in 0.05 M orthophosphoric acid; (C) Pure metoprolol in 0.05 M orthophosphoric acid; (D) Extracted sample. Chromatograms were extracted from LC Solution Software.

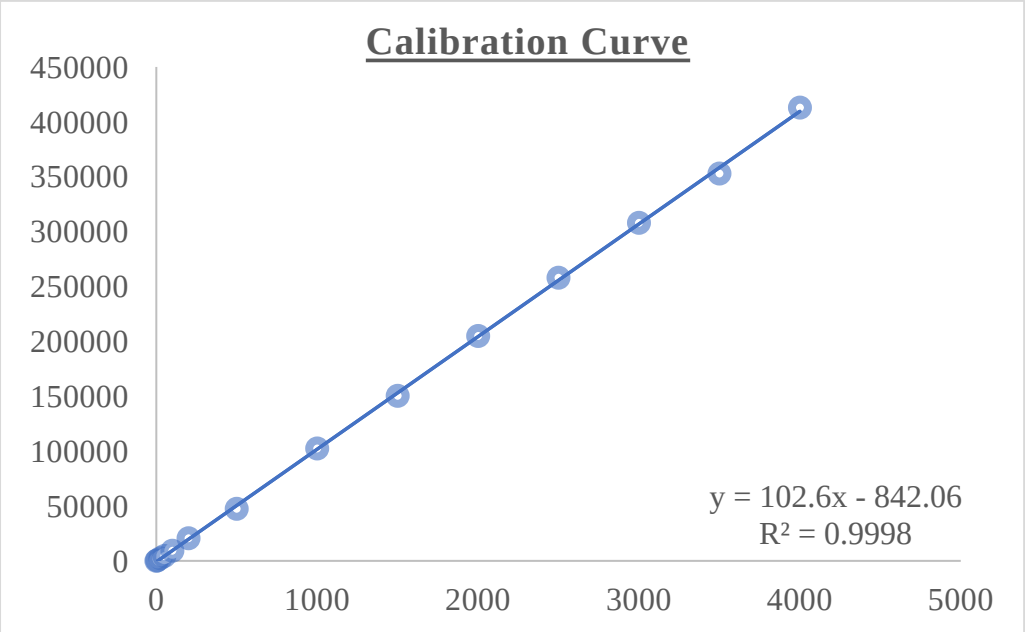



**Figure B2:** Calibration curve of HPLC to quantify concentrations of nadolol in the samples.

**Table B:** The performance analysis of liquid-liquid extraction and HPLC protocols

| **Parameters** | **Value** |
|---|---|
| Nadolol Peak (min) | 3.280 ± 0.039 |
| Metoprolol Peak (min) | 6.218 ± 0.053 |
| Recovery (%) | 100 ± 6.28 |
| Limit of Detection (ng/mL) | 7.511 |
| Limit of Quantitation (ng/mL) | 22.761 |

## APPENDIX C

**Table C:** The tissue input (mg), extracted RNA concentration, $A_{260/280}$ and $A_{260/230}$ of liver and ileum samples.

| Sample (Liver) | Input (mg) | Concentration (ng/µL) | $A_{260/280}$ | $A_{260/230}$ |
|---|---|---|---|---|
| 1 | 28.8 | 1664 | 2.024 | 2.219 |
| 2 | 28.8 | 1422 | 2.014 | 2.201 |
| 3 | 28.7 | 1210 | 2.010 | 2.153 |
| 4 | 27.8 | 2196 | 2.026 | 2.128 |
| 5 | 28.7 | 1218 | 2.010 | 2.037 |
| 6 | 28.8 | 1044 | 2.015 | 2.047 |
| 7 | 28.2 | 1328 | 2.018 | 2.199 |
| 8 | 28.0 | 1062 | 2.027 | 2.176 |
| 9 | 27.7 | 1374 | 1.992 | 2.095 |
| 10 | 27.9 | 1250 | 1.997 | 2.070 |
| 11 | 28.8 | 1364 | 1.998 | 2.042 |
| 12 | 28.7 | 1236 | 1.997 | 1.991 |
| 13 | 27.7 | 714 | 2.026 | 2.017 |
| 14 | 29.0 | 1220 | 2.020 | 2.186 |
| 15 | 28.2 | 1256 | 2.013 | 1.999 |
| 16 | 27.8 | 1718 | 1.990 | 1.935 |
| 17 | 28.9 | 1468 | 1.977 | 1.756 |
| 18 | 29.1 | 938 | 1.962 | 1.818 |
| 19 | 28.2 | 1074 | 2.011 | 2.139 |
| 20 | 28.3 | 1574 | 2.018 | 2.168 |
| 21 | 28.3 | 1568 | 1.998 | 1.960 |
| 22 | 28.0 | 1298 | 1.991 | 1.997 |
| 23 | 28.3 | 1170 | 1.997 | 1.924 |
| 24 | 27.9 | 1228 | 2.000 | 1.962 |
| **Mean** | 28.4 | 1316 | 2.005 | 2.051 |
| **SEM** | 0.1 | 60 | 0.003 | 0.025 |

| Sample (Ileum) | Input (mg) | Concentration (ng/µL) | $A_{260/280}$ | $A_{260/230}$ |
|---|---|---|---|---|
| 1 | 30.2 | 1670 | 1.998 | 2.147 |
| 2 | 30.8 | 830 | 2.054 | 2.173 |
| 3 | 30.3 | 532 | 2.000 | 1.973 |
| 4 | 29.2 | 644 | 2.009 | 2.064 |
| 5 | 31.0 | 1206 | 2.030 | 2.131 |
| 6 | 30.3 | 900 | 2.002 | 2.153 |
| 7 | 29.3 | 554 | 2.009 | 2.022 |
| 8 | 29.3 | 1050 | 2.027 | 2.092 |
| 9 | 30.1 | 704 | 2.035 | 2.108 |
| 10 | 31.5 | 868 | 2.009 | 2.097 |
| 11 | 31.4 | 646 | 2.006 | 2.019 |
| 12 | 30.5 | 914 | 2.031 | 2.156 |
| 13 | 30.1 | 1168 | 2.049 | 2.147 |
| 14 | 29.8 | 480 | 2.017 | 1.951 |
| 15 | 30.2 | 690 | 2.006 | 2.006 |
| 16 | 30.0 | 454 | 2.009 | 2.045 |
| 17 | 29.5 | 570 | 2.007 | 2.050 |
| 18 | 30.7 | 650 | 1.994 | 2.110 |
| 19 | 30.7 | 892 | 2.019 | 2.009 |
| 20 | 29.4 | 784 | 2.000 | 1.980 |
| 21 | 22.5 | 544 | 1.956 | 1.787 |
| 22 | 30.9 | 844 | 2.000 | 2.100 |
| 23 | 28.5 | 854 | 2.005 | 2.114 |
| 24 | 31.8 | 810 | 2.015 | 2.035 |
| **Mean** | 29.9 | 802 | 2.012 | 2.061 |
| **SEM** | 0.4 | 56 | 0.004 | 0.017 |

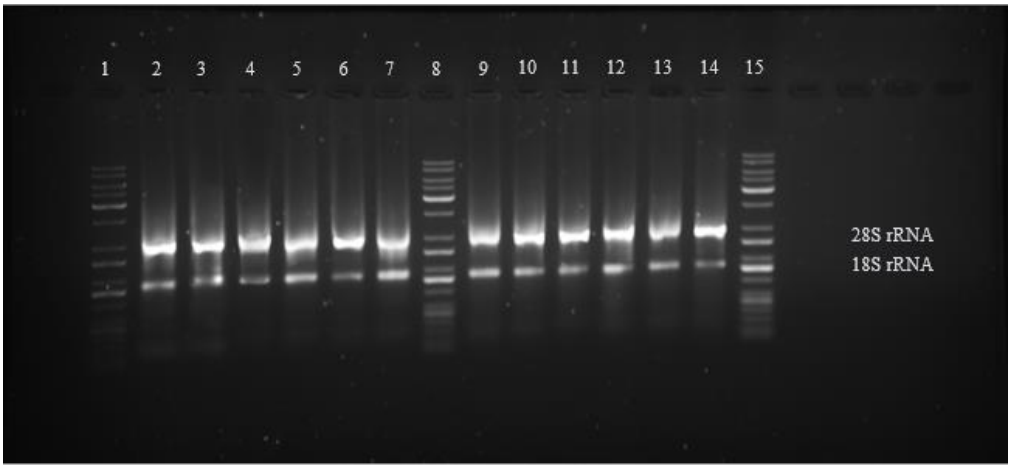



**Figure C:** The gel image of RNA integrity test on 1% bleach gel. Lanes 1,8 and 15 were 1 kb DNA ladder; Lane 2 to 7 were randomly chosen liver RNA samples; Lane 9 – 14 were randomly chosen ileum RNA samples. Loaded volume was standardised at 5 µL, which consisted of 1000 µg of RNA and 5x loading dye.

## APPENDIX D

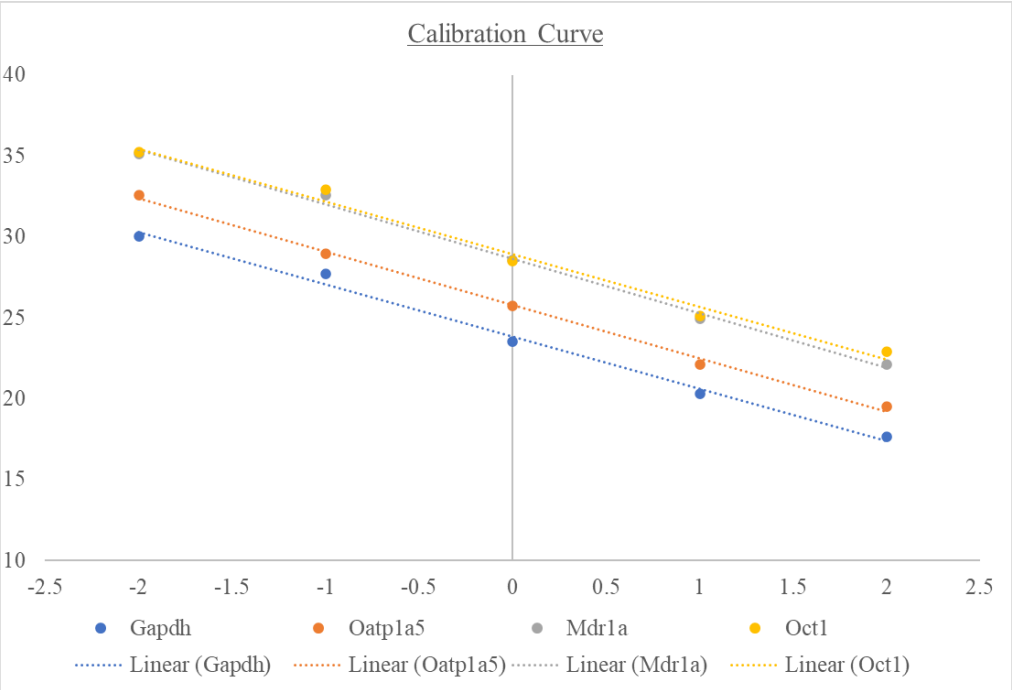
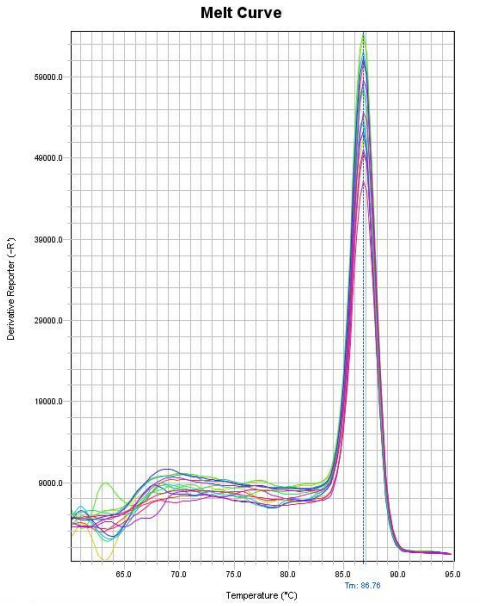


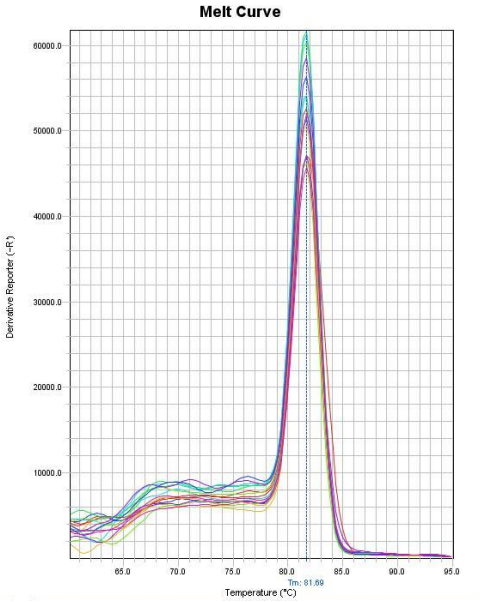

**Figure D1:** Calibration curve of *Gapdh*, *Oatp1a5*, *Mdr1a* and *Oct1* amplification efficiency in Luna® Universal qPCR Master Mix.

**Table D:** Summary of qPCR efficiency of each tested genes, templates were in 10-fold dilution.

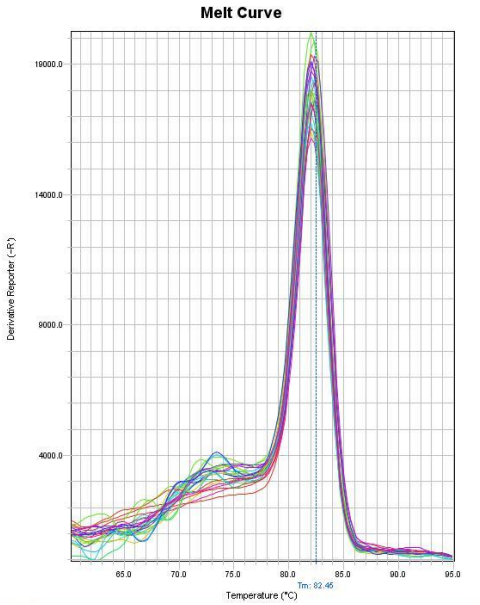
| Gene | Slope | $R^2$ | Efficiency |
|---|---|---|---|
| *Gapdh* | -3.2147 | 0.992 | **104.68%** |
| *Oatp1a5* | -3.2916 | 0.997 | **101.28%** |
| *Mdr1a* | -3.3648 | 0.996 | **98.24%** |
| *Oct1* | -3.2486 | 0.988 | **103.15%** |



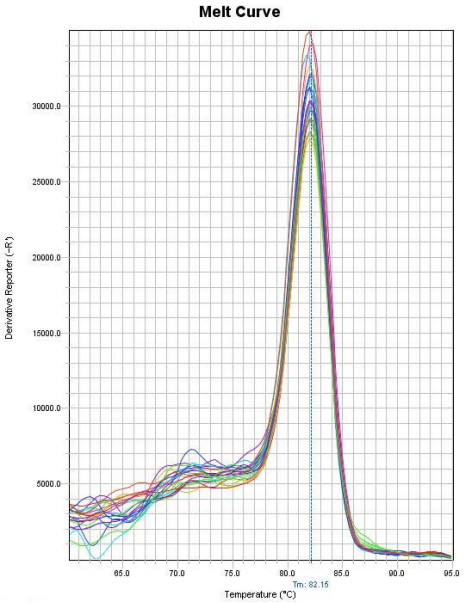

A. *Gapdh* ($T_m$ = 86.76ºC) [221 bp]




B. *Oatp1a5* ($T_m$ = 81.69ºC) [201 bp]




C. *Mdr1a* ($T_m$ = 82.45ºC) [70 bp]




D. *Oct1* ($T_m$ = 82.15ºC) [51 bp]

**Figure D2:** Melt curve analysis of *Gapdh*, *Oatp1a5*, *Mdr1a* and *Oct1* amplicons. Analysis was done with StepOne Software v2.3.

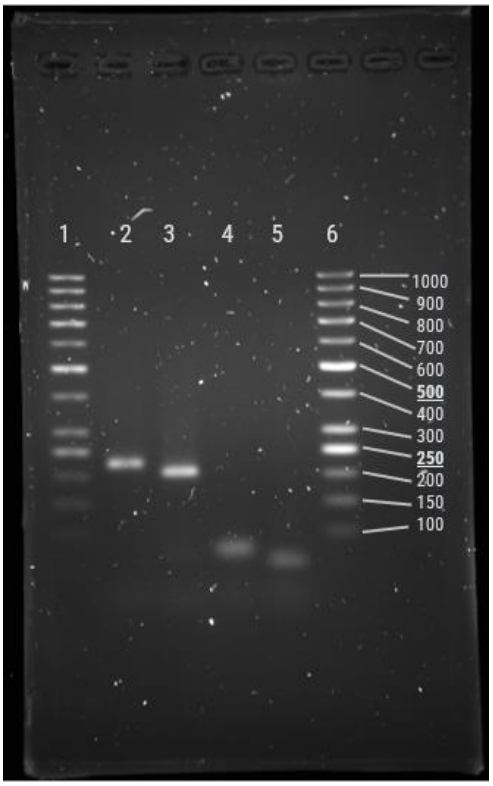



**Figure D3:** Gel image of qPCR amplicons of *Gapdh*, *Oatp1a5*, *Mdr1a* and *Oct1* under 2.5% TAE agarose gel. Lane 1 & 6: 50 bp DNA ladder; Lane 2: *Gapdh*, 221 bp; Lane 3: *Oatp1a5*, 201 bp; Lane 4: *Mdr1a*, 70 bp; Lane 5: *Oct1*, 51 bp. The amplicon length of each gene was predicted and determined by Primer-BLAST.

## APPENDIX E

**Table E:** Association of body weight with water intake and food intake.

| | **Water Intake** | | **Food Intake** | |
|---|---|---|---|---|
| | ***r*²** | ***P*** | ***r*²** | ***P*** |
| Body Weight | 0.050 | 0.377 | 0.058 | 0.303 |

Data were analysed with Pearson Correlation test. $p<0.05$ considered statistically significant

## LIST OF PUBLICATIONS

Tan, H.J., Ling, W.C., Chua, A.L. and Lee, S.K., 2021. Oral Epigallocatechin Gallate Reduces Intestinal Nadolol Absorption via Modulation of Oatp1a5 and Oct1 Transcriptional Levels in Spontaneously Hypertensive Rats. *Phytomedicine,* 90:153623.

Tan, H.J., Ling, W.C., and Lee, S.K., 2019. Epigallocatechin gallate supplementation suppressed antihypertensive effect of nadolol in spontaneously hypertensive rats. *International Journal of Cardiology*, *297*, p.26. (Abstract)

## LIST OF CONFERENCE PARTICIPATION

Tan, H.J., Ling, W.C., and Lee, S.K., 2019. Epigallocatechin gallate supplementation suppressed antihypertensive effect of nadolol in spontaneously hypertensive rats. Oral Presentation in National Heart Association of Malaysia Congress 2019 12-14 April 2019, Kuala Lumpur Convention Centre, Malaysia