EFFECT OF GREEN TEA AND ITS BIOACTIVE COMPOUND, (-)-EPIGALLOCATECHIN-3-GALLATE, ON WEIGHT REDUCTION AND ON CYTOCHROME P450

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ABSTRACT

Obesity is presently considered to be an epidemic, affecting more than 10% of adult population worldwide since the 19th century. However, gene regulations that are involved in obesity are not clearly understood. Green tea (*Camelia sinensis*) and its main component, (-)-epigallocatechin-3-gallate (EGCG) have been claimed to have anti-obese property. In order to understand the genes that are involved in obesity, changes in gene expression during obesity development in a mouse model was examined. We aimed to understand how green tea extract and EGCG affect gene regulation during obesity development and since they have been claimed to possess anti-obese property, we also aimed to examine gene expression in the mouse during development of obesity. Since green tea is widely consumed, the question arises as to whether it can interact with cytochrome P450 (CYP) enzymes to cause herb-drug interactions. To examine this, the interaction of green tea extracts (methanol extract-GTME, water extract-GTWE and hexane extract-GTHE) and of EGCG with human recombinant CYP enzymes CYP3A4, CYP2D6, CYP1A2, CYP2C9 and CYP2C19 were investigated. Results of the animal study showed that high fat diet (HFD) induced weight gain (29.85 ± 2.34%) by 10% higher than the normal group (19.18 ± 1.95%) in 4 weeks. However, in the groups that were treated with green tea extract and EGCG, no obvious increase in weight were observed (5.74 ± 1.69% and 3.34 ± 2.27% respectively). For the study on gene expression, we found that genes that were upregulated or downregulated were related to metabolic syndrome directly or indirectly. A total of 1024 genes were irregulated in HFD suggested that these genes were involved in obesity development. A total of 1892 genes in HFD + GTE and 1817 genes in HFD + EGCG were irregulated in these samples, suggesting that the genes were involved in prevention of obesity. The overall results showed that green tea extract and EGCG are able to prevent obesity in mouse model. In the study on effects of green tea and EGCG

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on CYP enzymes, the order of potency of the green tea extracts and EGCG on CYPs is: EGCG > GTME > GTWE > GTHE. EGCG showed the strongest inhibitory effect on CYP1A2 (K_i: 0.94 µg/mL), CYP2C19 (K_i: 1.26 µg/mL), CYP3A4 (K_i: 3.28 µg/mL) and CYP2C9 (K_i: 6.79 µg/mL). GTME had strongest inhibitory effects on CYP2C19 (K_i: 2.08 µg/mL), CYP3A4 (K_i: 4.94 µg/mL) and CYP1A2 (K_i: 5.00 µg/mL) while GTWE had strongest inhibitory effects on CYP1A2 (K_i: 7.95 µg/mL) and CYP2C19 (K_i: 8.73 µg/mL). The CYP inhibition activities of the GTME and GTWE were suggested to be related to their EGCG content. Extracts with higher content of EGCG appeared to be more potent. Further investigation revealed that EGCG, GTME, GTHE and GTWE inhibited CYP1A2 and CYP2C19 non-competitively, and caused mixed competitive inhibition against CYP3A4. Specifically for CYP2D6, GTME and EGCG exhibited mixed competitive inhibition, while GTWE showed un-competitive inhibition. As for CYP2C9, GTME, GTWE and EGCG showed mixed competitive inhibition while GTHE showed un-competitive inhibition. These findings suggest that green tea extracts and EGCG are likely to contribute to herb-drug interactions when orally co-administered with drugs metabolized by CYP3A4, CYP2D6, CYP1A2, CYP2C9 and CYP2C19.

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ABSTRAK

Obesiti merupakan satu epidemik yang mempengaruhi sekurang-kurangnya 10% daripada populasi dewasa seluruh dunia semenjak kurun ke-19. Walaubagaimanapun, regulasi gen yang terlibat dalam perkembangan obesiti masih tidak diketahui. Teh hijau (*Camelia sinensis*) dan komponen utamanya, (-)-epigallocatechin-3-gallate (EGCG) didakwa dapat mengawal berat badan. Bagi tujuan untuk mengetahui gen yang terlibat dalam obesiti, regulasi gen untuk perkembangan obesiti dalam tikus telah dikaji. Selain daripada itu, kami juga ingin memahami bagaimana teh hijau dan EGCG mempengaruhi regulasi gen semasa perkembangan obesiti, oleh itu, regulasi gen masing-masing dalam tikus juga dikaji. Di samping itu, teh hijau berkemungkinan bertindak balas dengan enzim sitokrom P450 dan menyebabkan interaksi antara herbal dan ubat. Oleh itu, interaksi antara ekstrak teh hijau dengan menggunakan methanol (GTME), air (GTWE), hexane (GTHE) dan EGCG dengan enzim sitokrom CYP3A4, CYP2D6, CYP1A2, CYP2C9 and CYP2C19 telah dikaji. Dalam kajian haiwan, kami mendapati bahawa diet yang mempunyai lemak (HFD) mencetuskan kenaikan berat badan (29.85 ± 2.34%) sebanyak 10% berbanding dengan kumpulan normal (19.18 ± 1.95%). Walaubagaimanapun, tikus untuk kumpulan ekstrak teh hijau (GTE) dan kumpulan EGCG tidak menunjunkkan kenaikan berat badan yang nyata (masing-masing 5.74 ± 1.69% dan 3.34 ± 2.27%). Daripada keputusan kejujukan transkriptome, 1024 gen didapati diregulasi dalam kumpulan HFD, 1892 diregulasi dalam kumpulan HFD + GTE dan 1817 gen dalam kumpulan HFD + EGCG. Kesimpulannya, ekstrak teh hijau dan EGCG dapat mencegah obesiti dalam tikus. Dalam kajian kesan GTE dan EGCG ke atas enzim sitokrom pula, urutan ketoksikan adalah EGCG > GTME > GTWE > GTHE. EGCG memberikan kesan rencatan yang paling kuat kepada CYP1A2 (K_i: 0.94 µg/mL), CYP2C19 (K_i: 1.26 µg/mL), CYP3A4 (K_i: 3.28 µg/mL) dan CYP2C9 (K_i: 6.79 µg/mL). GTME pula mempunyai kesan rencatan yang paling kuat kepada CYP2C19 (K_i: 2.08

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μg/mL), CYP3A4 (K_i: 4.94 μg/mL) dan CYP1A2 (K_i: 5.00 μg/mL) sedangkan GTWE mempunyai kesan rencatan yang kuat kepada CYP1A2 (K_i: 7.95 μg/mL) dan CYP2C19 (K_i: 8.73 μg/mL). Kesan rencatan aktiviti CYP dari GTME dan GTWE adalah berkaitan dengan kandungan EGCG dalamnya. Ekstrak yang mempunyai kandungan EGCG yang tinggi memberi kesan rencatan yang lebih nyata. Kajian lanjutan mendedahkan EGCG, GTME, GTHE dan GTWE merencat CYP1A2 dan CYP2C19 dengan rencatan secara bukan bersaing, merencatkan CYP3A4 secara persaingan campuran. Untuk CYP2D6, GTME dan EGCG menunjukkan rencatan persaingan campuran, manakala GTWE menunjukkan rencatan secara tidak bersaing. Untuk CYP2C9, GTME, GTWE dan EGCG menunjukkan rencatan persaingan campuran sementara GTHE menunjukkan rencatan secara tidak bersaing. Penemuan ini menunjukkan ekstrak teh hijau dan EGCG mungkin berinteraksi dengan ubat yang dimetabolismekan oleh CYP3A4, CYP2D6, CYP1A2, CYP2C9 dan CYP2C19 apabila diambil pada masa yang sama.

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LIST OF SYMBOLS AND ABBREVIATIONS

EGCG	:	(−)-epigallocatechin-3-gallate
EC	:	(−)-epicatechin
EGC	:	(−)-epigallocatechin
ECG	:	(−)-epicatechin gallate
GCG	:	(−)-gallocatechin gallate
HPLC	:	high-performance liquid chromatography
8-OHdG	:	8-hydroxydeoxyguanosine
ATP	:	adenosine triphosphate
HDL	:	high-density lipoproteins
BMI	:	body mass index
MCR	:	melanocortin receptor
CPE	:	carboxypeptidase E
BDNF	:	brain-derived neurotrophic factor
PSWL	:	placebo-subtracted weight loss
PPAR	:	peroxisome proliferator-activated receptor
Mrap2	:	melanocortin 2 receptor accessory protein 2
DNA	:	deoxyribonucleic acid
Mc4r	:	melanocortin 4 receptor
VC	:	vehicle control
HFD	:	high-fat diet
GTE	:	green tea extract
RNA	:	ribonucleic acid
AUC	:	Area under the curve
SLCO1B3	:	solute carrier organic anion transporter family member 1B3

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SLC9B1 : solute carrier family 9 member B1

LTA : lymphotoxin alpha

TNF-Beta : tumor necrosis factor beta

ADH1C : alcohol dehydrogenase 1C (Class I), gamma polypeptide

DAO : D-amino acid oxidase

SLC39A5 : solute carrier family 39 member 5

IL1B : interleukin 1 beta

ITIH4 : inter-alpha-trypsin inhibitor heavy chain family member 4

ICOS : inducible T-cell costimulator

C3 : complement component 3

CNTF : ciliary neurotrophic factor

TAT : tyrosine aminotransferase

FTCD : formimidoyltransferase cyclodeaminase

GRIN2B : glutamate ionotropic receptor NMDA type subunit 2B

SLC6A2 : solute carrier family 6 member 2

TPH2 : tryptophan hydroxylase 2

MPZ : myelin protein zero

DBH : dopamine beta-hydroxylase

OCA2 : OCA2 melanosomal transmembrane protein

F5 : coagulation factor V

CDKN2B : cyclin dependent kinase inhibitor 2B

ADORA2A : adenosine A2a receptor

SLC26A3 : solute carrier family 26 member 3

TLR9 : toll like receptor 9

TF : tissue factor

mRNA : messenger RNA

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LF	:	low fat
HF	:	high fat
HFR	:	high-fat energy–restricted diet
WR	:	weight reduction
P450	:	cytochrome P450 system
CYP3A4	:	cytochrome P450 3A4
CYP2D6	:	cytochrome P450 2D6
CYP1A2	:	cytochrome P450 1A2
CYP2C9	:	cytochrome P450 2C9
CYP2C19	:	cytochrome P450 2C19
AMMC	:	3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin
BFC	:	7-benzyloxy-4-(trifluoromethyl)-coumarin
CEC	:	3-cyano-7-ethoxycoumarin
MFC	:	7-Methoxy-trifluoromethylcoumarin
HFC	:	7-hydroxy-4-(trifluoromethyl)-coumarin
NADPH	:	reduced nicotinamide adenine dinucleotide phosphate
NRS	:	regenerating system
GTME	:	green tea methanol extract
GTWE	:	green tea water extract
GTHE	:	green tea hexane extract
DMSO	:	dimethyl sulfoxide
ACN	:	acetonitrile
PBS	:	phosphate saline buffer
K_m	:	K_{max}
IC_{50}	:	half maximal inhibitory concentration
K_i	:	apparent inhibition constant

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Obesity

Obesity or being overweight is the state in which one has too much body fat. It is not considered to be a disease, but the implications may be serious; people with obesity are at a higher risk of developing a range of diseases such as cardiovascular diseases, stroke, hypertension, diabetes and cancer (Bray, 2007; Keller, 2008). Because of that, it should be no surprise that the life expectancy of an obese person is less than one without this condition (Barness et al., 2007). According to the World Health Organization (2016), obesity or overweight is defined as abnormal or excessive fat accumulation that may impair health. The Body Mass Index (BMI) is a measurement used to establish whether a person is obese or not. A person's BMI is calculated using the height and weight of the said person, and is presented in the unit kg/m^2. For Asians, adults with BMI greater than 25 are considered overweight, while 30 and above are considered obese.

Obesity is preventable, yet its prevalence is increasing globally every year. According to the WHO, obesity worldwide has nearly doubled since 1980. In 2008, more than 10% of the world's adult population (>1.4 billion people) were classified as obese. A more recent statistics from the WHO showed that in 2010, 60% of Malaysian adults (aged 18 and above) have BMIs of more than 25, putting them in the category of obese or at least overweight. Indeed, in adult obesity rate, Malaysia ranked number 6 in Asia and number 1 in Southeast Asia in 2010. This is a 17% increase from the 43% in 2006, and the obesity rate is expected to continue to increase. All of this is a cause for concern as more than half of the Malaysian adults are at health risks associated with obesity.

On a fundamental level, obesity results from an energy imbalance between calorie input and calorie output. This happens when excessive calories are consumed and

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excess body fat is produced (Keller, 2008). Factors that contribute to an individual's body weight include genetic, metabolic, behavioural, environmental, cultural and socioeconomic influences. Of these factors, the influence of genetics may be the most substantial; an estimated 60% of obese people may have a genetic predisposition to obesity. In fact, discoveries of the *obese* (*ob*) gene (Ingalls et al., 1950; Zhang et al., 1994) and *diabetes* (*db*) gene (Tartaglia et al., 1995) have opened up new framework on the importance of genes in regulating body weight. Since their discovery, other genes connected to obesity have been found. These genes include *agouti yellow* (A^y), an autosomal dominant gene that causes obesity (Lu et al., 1994); *fat* (*fat*) autosomal recessive mutation that causes obesity (Naggert et al., 1995); and *tubby* (*tub*), another autosomal recessive gene that causes obesity (Kleyn et al., 1996). More recently, brain-derived neurotrophic factor (BDNF) (Kernie et al., 2000), 5-HT_{2C} receptor gene (Yuan et al., 2000), peroxisome proliferator-activated receptor (PPARɣ) gene (Kadowaki et al., 2002), and fat mass and obesity (FTO) gene (Wahlen et al., 2008) were also found to be associated directly or indirectly with obesity. Future studies should be focused on other obesity-related genes to determine the extent to which genetics plays in obesity.

1.2 Green tea and its role in obesity treatment

Tea is a product of *Camellia sinensis* (L.) Kuntze (Fam. Theaceae) or more commonly known as tea plants. It is the most popular drink around the world after drinking water (Awasom, 2011). Its popularity comes from its unique flavour—a result of the methods of processing tea leaves. There are three main varieties of tea; i.e. black tea, oolong tea and green tea (Awasom, 2011; Graham, 1992). To produce black tea, fresh tea leaves are left aside to allow enzymatic oxidation (Graham, 1992). Unfortunately, this process tends to destroy potential medicinal compounds in the tea leaves. The production of Oolong tea is slightly better in this respect as it involves only

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partial enzymatic oxidation (Graham, 1992). The best however is green tea, Asia's most popular tea, where oxidation is prevented altogether by heating or steaming tea leaves soon after plucking (Graham, 1992). With this, the enzymes involved in oxidation are inactivated and valuable compounds such as catechins are preserved.

The main chemical components of green tea are polyphenols (up to 30% of dry weight), which mainly consists of catechins, i.e. (−)-epigallocatechin-48 3-gallate (EGCG), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-gallocatechin gallate (GCG) (Graham, 1992).

It has been shown that green tea and its most abundant catechin, EGCG have various pharmacological effects such as antioxidant (Benzie et al., 1999) (Cao, 1996; Langley-Evans, 2000; Luczaj et al., 2004; Prior & Cao, 1999), anticancer (Chung et al., 2003; Huang et al., 1999; Inoue et al., 1998; Jian et al., 2004; Laurie et al., 2005; Shibata et al., 2000; Yamamoto et al., 2003; Yu et al., 2004; Zhang et al., 2002), antihypertensive (Negishi et al., 2004), blood cholesterol reduction and cardiovascular disease prevention. More relevant to this study is that green tea and EGCG have been reported to possess anti-obesity properties (Miura et al., 2001; Murase et al., 2002; Negishi et al., 2004). This may be attributed to their ability to promote thermogenesis, fat oxidation (Boschmann & Thielecke, 2007) and inhibition of lipid peroxidation (Basu et al., 2010). Other than that, the role played by green tea in appetite suppression and nutrient absorption may also help in treating obesity (Rains et al., 2011).

With this, one question comes to mind: what roles do green tea and EGCG play in reducing weight? In order to answer this question, the role of green tea and EGCG in gene regulation will be identified.

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1.3 Effects of green tea on cytochrome

As mentioned earlier, tea is the most widely consumed drink, in which about 80% of the USA population are consuming tea after water. Green tea is well known for its nutraceutical properties where it has been used for medicinal purpose in China and Japan for thousands years (NCCIH, 2016). However, any beneficial substances have the potential to be toxic if overconsumed, yet we know so little about its toxicity. With this in mind, a portion of this study was dedicated to identifying the toxicity of different green tea extracts on human recombinant cytochrome (CYP) P450 enzymes.

Other than that, another concern is to determine whether green tea and EGCG inhibit enzyme metabolism. If they do, in what type of inhibition do these exhibit? In order to determine the possible mechanisms involved, possible modes of inhibition of various green tea extracts and EGCG on the CYP P450 enzymes were also tested.

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CHAPTER 2: LITERATURE REVIEW

2.1 Green tea

2.1.1 General description

Camellia sinensis (*C. sinensis*) is believed to have first appeared between 4,000 and 6,000 years ago (Mitscher et al., 1997). It is an evergreen shrub that can grow up to 30 feet in height in suitable conditions such as well drainage soil at tropical elevations where it grows naturally (Mitscher et al., 1997). Optimal growth conditions for *C. sinensis* include equatorial and humid climate with an annual temperature of 18 °C to 20 °C; high and evenly distributed rainfall; a minimum of 5 hours of sunshine per day; soil of pH 4.5 to 5.5, fertile, well-drained, and with a good layer of humus (Bonheure, 1990). In plantations, they are usually maintained as small bushes (2–3 feet) by trimming (Balentine et al., 1997). Tea is produced all around the world, but most noticeably in India, China, Sri Lanka, Turkey, Kenya, Russia, and Japan (Mitscher et al., 1997). Every year, approximately 2.5 million tons of tea leaves are produced, 20% of which is green tea. Green tea is consumed in Asia, some parts of North Africa, the United States, and Europe (Chacko et al., 2010).

2.1.2 Taxonomy hierarchy

The taxonomic hierarchy of *C. sinensis* is exhibited below. It is based on the Integrated Taxonomic Information System (ITIS), with Taxonomic Serial Number (TSN) 506801 (ITIS, 2016).

Kingdom : Plantae

Subkingdom : Viridaeplantae

Infrakingdom : Streptophyta

Division : Tracheophyta

Subdivision : Spermatophytina

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Infradivision : Angiospermae

Class : Magnoliopsida

Superorder : Asteranae

Order : Ericales

Family : Theaceae

Genus : Camellia L.

Species : *Camellia sinensis* (L.) Kuntze

2.1.3 Composition of green tea

The composition of tea may vary according to processing method. Green tea is said to retain most of its polyphenols due to its minimal processing. However, the exact chemical composition of green tea is still very complex.

Generally, green tea contains polyphenols (30% dry weight); proteins (15-20% dry weight); amino acids (1-4% dry weight), namely, theanine or 5-N-ethylglutamine, glutamic acid, tryptophan, glycine, serine, aspartic acid, tyrosine, valine, leucine, threonine, arginine, and lysine; carbohydrates (5-7% dry weight) i.e. cellulose, pectins, glucose, fructose, and sucrose; minerals and trace elements (5% dry weight), namely, calcium, magnesium, chromium, manganese, iron, copper, zinc, molybdenum, selenium, sodium, phosphorus, cobalt, strontium, nickel, potassium, fluorine, and aluminium; and trace amounts of lipids (linoleic and α-linolenic acids), sterols (stigmasterol), vitamins (B, C, E), methylxanthines (caffeine, theobromine and theophylline), pigments (chlorophyll, carotenoids), and volatile compounds (aldehydes, alcohols, esters, lactones, hydrocarbons) (Belitz, 1997; Graham, 1992). Other than that, fresh tea leaves contain some phenolic acids such as gallic acid (Graham, 1992).

Polyphenols (flavanols, flavandiols, flavonoids, and phenolic acids) are perhaps the biggest components in green tea, they account for up to 30% of green tea extract dry

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weight (Graham, 1992). Commercialized products of green tea can be found in forms of liquid or powder (extract), with typically 45% to 90% polyphenols and 0.4% to 10% caffeine. Most of the polyphenols are flavonols and many of them catechins. In fact, the biggest difference between green tea and black tea is the amounts of catechins; green tea contains more catechins than black tea or Oolong tea (Vinson, 2000). Five different types of catechins can be found in green tea, namely, (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-gallocatechin gallate (GCG) (Graham, 1992; Sano et al., 2001). The processing method of tea greatly affects the catechin retention both quantitatively and qualitatively (Khokhar & Magnusdottir, 2002).

2.2 (−)-epigallocatechin-3-gallate (EGCG)

EGCG is the most abundant catechin and the most biologically active compound in green tea, therefore it is widely studied for its biological functions (Sang et al., 2005). It has the molecular formula of $C_{22}H_{18}O_{11}$, with a molecular weight 458.37 g/mol. In its pure form, it looks like white powder with a hint of pink and is soluble in water. EGCG is categorised as a polyphenolic bioflavonoid, and it can be found in a variety of plants, and is especially abundant in green tea. Flavonoids are a class of natural products that contain the aromatic heterocyclic skeleton of flavan (2-Phenylbenzopyran) but without the nitrogen from plants. A flavonoid can be further categorised into one of six subgroups, one of them is flavonols, the subgroup in which EGCG belongs to. Bioflavonoids are also known as phytochemicals, Vitamin P, and Vitamin C2. Its chemical name is (2R,3R)-2-(3,4,5-Trihydroxyphenyl)- 3,4-dihydro-1 (2H)-benzopyran-3,5,7-triol 3-(3,4,5-trihydroxybenzoate).

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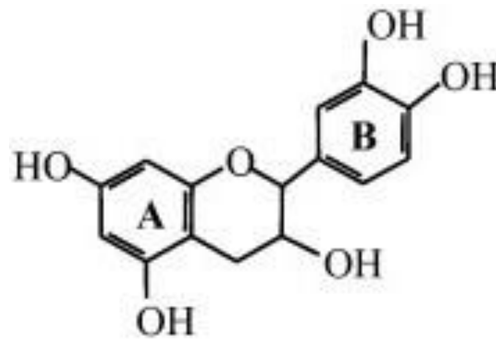

Figure 2.1 Catechin backbone

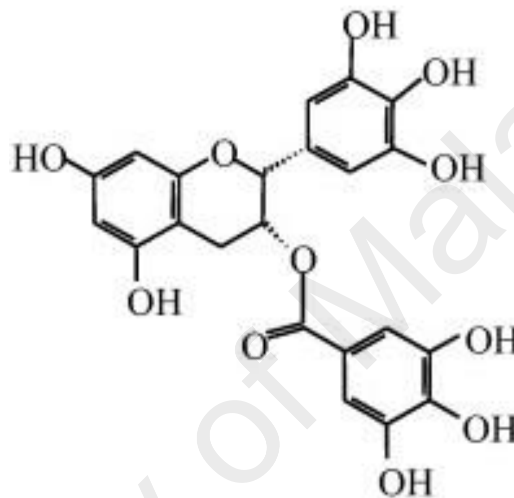

Figure 2.2 (-)-Epigallocatechin-3-gallate

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Pure EGCG can be extracted from green tea through a number of steps; first, tea is treated at 80 °C to 100 °C hot water, with 40% to 75% alcohol, or with 30% to 80% acetone. Tea is then washed with chloroform and transferred into an organic solvent, such as ethyl acetate, n-butanol, methyl isobutyl ketone or acetone. After that, the organic solvent is removed by distillation and the residue freeze-dried or spray-dried to produce catechins. The tea catechins are subjected to reverse-phase high-performance liquid chromatography (HPLC) using an eluting solution containing 0% to 25% acetone, 0% to 35% tetrahydrofuran and 65% to 85% water. After that, the product

(EGCG) can be concentrated, dried and powdered or purified via recrystallization (Hara, 1986).

2.3 Pharmacological potential of green tea and EGCG

Green tea has exhibited therapeutic properties against a variety of diseases such as cancer, heart diseases, hypertension and obesity; and it has been claimed that EGCG, its most abundant catechin, is responsible for these pharmacological effects. One hypothesis for green tea and EGCG's health benefits is that they are strong anti-oxidative agent (Rietveld & Wiseman, 2003). Indeed, the anti-oxidative properties of green tea and EGCG have been claimed to protect cells from nitric oxide (Tsai et al., 2007), DNA from UVB irradiation (Wei et al., 1999), Jurkat T cells from oxidative damage (Erba et al., 1999), bladder cancer and normal urothelium cells from hydrogen peroxide–induced cell death (Coyle et al., 2008), etc. Besides that, EGCG consumption was found to decrease lipid peroxidation and protein carbonylation in rats (Senthil Kumaran et al., 2008; Srividhya et al., 2008). In a pilot study by (Schwartz et al., 2005), oral cytology cell samples were taken from cigarette smokers and analysed for 8-hydroxydeoxyguanosine (8-OHdG), a marker for DNA oxidation damage. In the study, the number of 8-OHdG-positive cells from participants who also consumed total green tea extract was only 50% of the control group. This agrees with an *in vivo* study by (Xu et al., 1992) who has also shown that treatment of green tea extract and EGCG reduced the formation of 8-OHdG. Aside from that, a moderate dosage of tea polyphenols also suppresses oxidative stress, although high dosages of tea polyphenols can promote the formation of reactive oxygen species and induce toxicity (Tang et al., 2008).

Aside from that, it has also been suggested that green tea and EGCG have anti-microbial properties. According to Sirk et al. (2008), green tea catechins cause damage to bacterial lipid bilayer when bound to it. In *Escherichia coli* particularly, Cho et al.

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(2007) found changes in gene regulation that caused cell membrane damage after exposure to catechins in green tea. Other than that, green tea components especially EGCG were found to inhibit specific reductases (FabG, FabI) in bacterial type II fatty acid synthesis (Zhang & Rock, 2004). In fact, green tea catechins also have inhibitory effect on several enzymes such as protein tyrosine phosphatase, cysteine proteinases (Okamoto et al., 2003; Okamoto et al., 2004), DNA gyrase (plays a role in DNA replication) (Gradisar et al., 2007), dihydrofolate reductase (synthesize folate in bacteria and yeast) (Navarro-Martinez et al., 2006; Navarro-Martinez et al., 2005), and bacterial ATP synthase (help produce energy in microogranisms) (Chinnam et al., 2010).

Besides that, green tea and EGCG have been found to inhibit tumorigenesis and display chemopreventive effects on various types of cancers, including cancers of lung, oral cavity, esophagus, stomach, small intestine, colon, skin, liver, pancreas, bladder, prostate and mammary glands (Yang et al., Cancer prevention by tea: Evidence from laboratory studies, 2011). In fact, studies have shown that green tea inhibits chemically induced oral carcinogenesis in a hamster model and esophageal carcinogenesis in a rat model (Li et al., 2002; Wang et al., 1995). Another study shows that EGCG also inhibits tumorigenesis in rat stomach and forestomach induced by N-methyl-N'-nitro-N-nitrosoguanidine (Yamane et al., 1995). Administration of green tea and EGCG during the initiation stage or promotion stage was shown to significantly decrease (4-methylnitrosamino)-1-(3-pyridyl)-1-butanon-induced lung tumorigenesis in mice and hamsters (Liao et al., 2004; Mimoto et al., 2000; Schuller et al., 2004; Wang et al., 1992).

Due to the favourable results in animal models, the pharmaceutical use of green tea and EGCG had proceeded to clinical trials. Bettuzzi et al. (2006) have conducted such a study; 30 men with high-grade prostate intraepithelial neoplasia were given 600 mg green tea catechins daily for 12 months. Out of the 30 patients, only one developed

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prostate cancer whereas in the placebo group, 9 out of 30 patients developed prostate cancer (statistically significant). In Japan, for patients with their colorectal adenomas removed by polypectomy, the consumption of a green tea extract supplement (1.5 g / day) for 12 months was found to decrease the development of metachronous colorectal adenomas when compared to the control group (Shimizu et al., 2008).

One most important therapeutic properties of green tea and EGCG in this context is its anti-obesity properties. Hasegawa et al. (2003) have reported that oral administration of 130 mg powdered green tea daily to male Zucker rats (fed with a 50% sucrose diet containing 15% butter) resulted in reduction of body weight gain within 2 days and caused significant reduction in adipose tissue weight (5–9% decrease). In fact, in another study, a 2% green tea treatment in the diet effectively controlled body weight and reduced body fat accumulation in Sprague-Dawley rats (Choo, 2003). Another study carried out by Park et al. (2011) showed that treatment with 1% of green tea extract for 6 weeks can result in decreased body weight gain. In their study, green tea treatment caused reduction in adipose tissue mass (21%), hepatic lipids (13%) and serum alanine aminotransferase (25%) compared to the control mice. There were also a reduction of hepatic expression of inflammatory markers and tumour necrosis factors in liver and adipose tissue.

Other than green tea extract, anti-obesity studies have also been done on EGCG. In one study, treatment of EGCG has successfully reduced adipose tissue mass and ameliorates plasma lipid profiles in high-fat diet-induced obese mice (Lee et al., 2009). Besides that, Moreno et al. (2014) showed that administration of EGCG promoted a significant improvement in glucose tolerance, and a decrease in adipose tissue deposit weight mass, triacylglycerol, and high-density lipoprotein cholesterol levels for subjects with a high-fat diet. In another study, treatment of high-fat fed C57BL/6J mice with 0.32% dietary EGCG for 16 weeks reduced body weight gain by 33–41% and lowered

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total visceral adipose tissue by 37% (Bose et al., 2008). They also found that EGCG treatment for 4 weeks significantly lowered the weight of the mesenteric adipose depot (36% decrease). It was found in another study that Sprague–Dawley rats treated with 1% (w/w) Teavigo (90% EGCG) for 1 month showed reduction of subcutaneous adipose tissue (10% decrease), epididymal adipose tissue (5% decrease) and fed state triglyceride (11% decrease) (Wolfram et al., 2005). In contrast to the above findings, EGCG had been found to have no effect on body weight of Wistar rats fed with a high-fat, high cholesterol diet for 4 weeks, but it did reduce total plasma cholesterol, non-HDL cholesterol and hepatic total cholesterol concentration (Raederstorff et al., 2003).

In short, green tea extracts and EGCG exhibit unique pharmacological effects probably due to the uniqueness of EGCG's chemical structure and its strong anti-oxidative properties. A study in depth of green tea extracts and EGCG on various diseases will benefit human beings in future.

2.4 Green tea, EGCG & CYP P450 enzymes

When co-administrated with green tea, the effects of certain medications may be altered. For instance, green tea has an effect on the beta-lactams, a group of antibiotics used in reducing bacterial resistance (Ehmann et al., 2012); according to Stapleton et al. (2004), EGCG increases the effectiveness of the beta-lactams. Aside from that, (Cui et al., 2012) also proved that EGCG acts synergistically with cefotaxime in inhibiting extended-spectrum beta-lactamase-producing *Escherichia coli*. This indicates that the consumption of green tea together with the beta-lactams can directly increase the toxicity of the drug in the liver. However, in contrast with Stapleton's and Cui's results, green tea extract was found to weaken the effectiveness of amoxicillin in mice (Peng et al., 2010). Amoxicillin is one of the beta lactam antibitotics used to treat bacterial infections (Peng et al., 2010). According to Peng's study, the antibactierial effect of

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amoxicillin can be weakened *in vitro* with 0.25% green tea extract, and 5% *in vivo*. In any case, green tea and EGCG are not recommended to be consumed together with antibiotics.

Other than antibiotics, anticoagulants or blood thinning medications are not recommended for co-administration with green tea. This is because green tea is rich in vitamin K, and tends to inhibit anticoagulant action of warfarin (Taylor & Wilt, 1999). Also, aspirin should not be taken together with green tea or EGCG as they prevent platelets from clotting (Kang et al., 1999).

In a study by Jang et al. (2005), green tea extract induces hepatic CYP1A2 production in rats and therefore reduces the effect of clozapine. Other than that, green tea tends to increase bioavailability of verapamil due to inhibitory effect of EGCG on P-glycoprotein (Chung et al., 2009). Besides that, EGCG also inhibits bortezomib (a proteasome inhibitor used to treat multiple myeloma) by forming a stable complex with bortezomib (Golden et al., 2009).

Although it has been shown that tea-drug interactions may occur when green tea is co-administered with drugs (Chung et al., 2009; Golden et al., 2009; Jang et al., 2005; Taylor & Wilt, 1999), detailed studies have yet to be performed to confirm this and identify the CYP P450 enzymes involved. Since green tea is gaining popularity worldwide as a health supplement, it is vital to understand its interaction with CYPs P450 enzymes.

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CHAPTER 3: EFFECTS OF GREEN TEA AND EGCG ON WEIGHT REDUCTION

3.1 Introduction

3.1.1 Obesity

Obesity is the silent killer that causes several metabolic symptoms. It affects the wellness of an individual as well as the quality of life in the long run. As genetics has been proven to be one of the most important factors that predisposes to obesity, it is crucial to have a deeper understanding of the relationship between obesity and genetics because the genetic profile of a person does not alter over the person's life time. In order to study this further, the powerful technologies available today can be utilised to transform information of obesity-linked genes into easily accessible information.

Meanwhile, drugs and supplements are being developed to reduce weight. Several natural foods have been found to assist in weight loss, and green tea is one of them. A study by Dulloo et al. (1999) showed that treatment with green tea extract results in an increase in energy expenditure for 24 hours. This study suggests that green tea promotes thermogenesis and fat oxidation. In fact, the relationship between green tea and thermogenesis was later proven by (Dulloo et al., 2000) who showed that brown adipose tissue thermogenesis was stimulated after the consumption of green tea.

The role that genetics plays in obesity development is most fascinating, and because of this, we have decided to study gene expression of obesity-linked genes in addition to investigating the effects of green tea extract and EGCG on obesity development.

3.1.2 Objectives

1. To identify the physiological and gene expression changes that occur during the development of high-fat diet-induced obesity.

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2. To study obesity prevention mechanisms caused by the consumption of green tea extract and EGCG treatment on mice fed with a high-fat diet.

3.2 Literature review

Genetics plays a crucial role in determining the appearances and behaviour of organisms. The roles which genetics play in obesity development in humans have been established from a series of family, twins, and adoption studies and the estimated heritability of BMI seems to range between 50% and 90% (Maes et al., 1997). Indeed, the data indicates that obesity is highly correlated with genetics. Since 1990, many studies have been conducted to identify genes linked to obesity in humans.

The relationship of genetics and obesity in house mice was accidentally discovered by Ingalls et al. (1950) when she and her colleagues discovered a single gene mutation that caused obesity and type II diabetes, and the gene was designated as *obese* (*ob*). They discovered that mice with the recessive mutation of *ob* tend to eat more and gain weight more rapidly. Decades later, Zhang et al. (1994) successfully sequenced the *ob* gene. The gene was found to be involved in the synthesis of leptin, a hormone that regulates food intake. The lack of *ob* leads to uncontrolled food intake, and hence causing obesity. Similar to *ob*, mice with the *db* gene are unable to encode the receptor for leptin (LEP-R), therefore resulting in obesity (Tartaglia et al., 1995). Another is the *fa* gene, with similar properties to the *db* gene, it was found in rats by Zucker (1961); the rats were named after the gene's discoverers. All 3 genes discussed are involved in the alteration of leptin or the leptin receptor. Since then, obesity genes are widely studied and many more genes have been discovered.

The *agouti* (*A*) gene serves as coat colour regulator and as an important gene model that causes pleiotropic effects with various dominant and recessive *agouti* locus alleles (Siracusa, 1991). There are a total of six autosomal dominant yellow A mutations in the

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A gene, namely *agouti yellow* (or lethal yellow) (A^y), viable yellow (A^{vy}), sienna yellow (A^{sy}), intermediate yellow (A^{iy}), hypervariable yellow (A^{hvy}) and intracisternal A particle yellow (A^{iapy}) alleles. All these autosomal dominant mutations are associated with obesity, hyperphagia, hyperinsulinemia, hypercorticosteronism and increased linear growth obesity (Lu et al., 1994; Yen et al., 1994). Homozygous A^y allele causes embryonic lethality in mice at 5.5 and 6.5 days postcoitum (Eaton, 1963). Heterozygous of A^y and A^{vy} cause obesity, hyperinsulinemia, hyperglycemia, and yellow fur commonly observed in the spontaneous obese yellow mutants (Klebig et al., 1995). It was found that products of the A^y gene, agouti-related proteins act as antagonists to central nervous system melanocortin receptor (MCR) binding, causing hyperphagia, obesity and hyperinsulinemia syndrome (Zemel, 1998). This may explain the role of agouti gene plays in obesity.

In contrast to the *agouti* gene, the mutation of the *fat* gene give rise to autosomal recessive obesity that causes obesity and hyperglycemia (Naggert et al., 1995). The *fat* gene was first found in a colony of HRS/J mice by Coleman and Eicher (1990), where the mice began developing obesity between 8 to 12 weeks old. The *fat* gene was later found to encode human carboxypeptidase E (CPE), a prohormone-processing exopeptidase in secretory granules of endocrine and neuroendocrine cells (Fricker, 1988). Mutant protein produced by Cpe^{fat} homozygous causes incomplete conversion of mouse proinsulins I and II to fully mature insulins and therefore causes hyperproinsulinemia (Varlamov et al., 1996). Aside from that, homozygous Cpe^{fat} also causes infertility in mice due to the loss of CPE enzymatic activity which halts the production of hypothalamic gonadotropin release hormone precursor (Naggert et al., 1995). These findings are consistent with a clinical study conducted by O'Rahilly et al. (1995) where patient with defect in the CPE gene also exhibits hyperproinsulinemia, hypoglycaemia, infertility and juvenile-onset obesity.

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Coleman and Eicher (1990) reported the discovery of the tubby (*tub*) gene at the same time as discovering *fat* gene in C57Bl/6J mice. Similar to *fat*, *tub* is an autosomal recessive mutation; however, unlike *fat*, *tub* is characterised by late-onset obesity (12 to 24 weeks old in mice) and accompanied by progressive retinal and cochlear degeneration (Ohlemiller et al., 1997). The *tub* gene is predominantly expressed in neuronal cells including those in the hypothalamus, therefore the late-onset of obesity in tubby mice may imply defects in neurons of the hypothalamic 'satiety center' (Kleyn et al., 1996). According to a study by (Koritschoner et al., 2001), *tub* is regulated by the major human metabolic regulators, tetraidothyronine (T4) and triiodothyronine (T3). T4 and T3, or commonly known as thyroid hormones, are vital hormones produced by the thyroid gland. Both T4 and T3 are important in energy metabolism, body growth, neuronal development and development of the cardiovascular system (Forrest & Vennstrom, 2000). According to (Koritschoner et al., 2001), *tub* is the target of thyroid hormones receptors (TRs); mutations of the gene cause tubby-associated symptoms, particularly aberrant energy metabolism and neuronal development.

Brain-derived neurotrophic factor (BDNF) is a growth factor that plays a crucial role in neuronal development and synaptic function (Kernie et al., 2000). Nonetheless, it is also found to be a key player in energy homeostasis (Wisse & Schwartz, 2003). In 1992, Lapchak and Hefti (1992) discovered that the infusion of BDNF in adult rat brains cause reduction in body weight. This agrees with the results of (Pelleymounter et al., 1995) where the injection of BDNF suppressed appetite and promoted weight loss in rats. Subsequently, more studies have been conducted to determine the relationship of BDNF and the regulation of food intake. Lyons et al. (1999) found that mice that carried BDNF heterozygously (Bdnf$^{+/-}$) exhibited severe obese phenotype due to overeating. Furthermore, patients that demonstrated BDNF haplo-insufficiency were all obese (Han et al., 2008). Besides that, studies in the molecular biology of obesity have also been

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conducted; a common single nucleotide polymorphism of BDNF, G196A has been linked to susceptibility to obesity in humans (Beckers et al., 2008). Indeed, when examined, G196A knock-in mice demonstrated weight gain.

Aside from that, there is the 5-HT_{2C} receptor gene, a gene in brain serotonin system that is important in the regulation of food intake and energy expenditure (Vickers et al., 2001). Mice with the mutation of 5-HT_{2C} receptor gene tend to consume more food and are unaffected by the level of leptin (Nonogaki et al., 1998). Nonogaki et al. (1998) conducted a study on 5-HT_{2C} receptor mutant gene mice. The mutant mice exhibited hyperphagia that lead to late-onset obesity; mRNA level of uncoupling protein-2 (UPC-2), which involves in regulation of sterol metabolism, was significantly higher in white adipose tissues (4-fold) and skeletal muscles (47%) in older obese mutant mice, whereas UCP-2 mRNA in liver were significantly higher in both young lean (54% increase) and older obese (52% increase) mutant mice. The role of 5-HT_{2C} receptor in regulating food intake was examined using 5-HT drugs; the drugs (fenfluramine (Pondimin, Ponderax, and Adifax), d-fenfluramine (Redux), and the SNRI sibutramine (Meridia, Reductil)) have caused placebo-subtracted weight loss (PSWL) ranging between 2.4 kg and 4.45 kg within 6–12 months (Arterburn et al., 2004; Haddock et al., 2002; Padwal et al., 2003).

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear proteins that belong to the nuclear hormone receptor family. There are three PPARs; PPARα, PPARβ and PPARγ. These mediate effects of small lipophilic compounds such as steroids, retinoids, bile acids and fatty acids on DNA transcription (Aranda & Pascual, 2001). More importantly, transgenic mice deficient in PPARα suffer from delayed onset obesity (Costet et al., 1998).

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The FTO gene was discovered by Frayling et al. (2007) and is associated with the regulation of body fat. It is highly expressed in the hypothalamus and pancreatic islets. Indeed, there is a strong link between the common FTO variant and BMI. According to Frayling et al (2007), a person with two copies of this FTO variant is likely to weigh on average 3 kg (6.6 pounds) more than a person without this FTO variant at all; a person with only one copy of this FTO variant is likely to weigh on average of 1.2 kg (2.6 pounds) more than a person without a single copy of this FTO variant. In genome-wide association studies conducted, FTO was found to be responsible for obesity-related traits in both children and adults (Dina et al., 2007; Rees et al., 2011; Scuteri et al., 2007). In fact, Fischer et al. (2009) found that in the absence of FTO, there were postnatal growth retardation and significant reduction in adipose tissue and lean body mass. In addition, another study conducted by Church et al. (2010) demonstrated that overexpression of the FTO gene caused an increase in food intake, and can consequently lead to obesity. FTO is the only obesity-linked gene to date to be consistently present in far-apart populations (Western and Asian) (Rees et al., 2011) and in both children and adults (Dina et al., 2007).

Besides that, melanocortin 2 receptor accessory protein 2 (Mrap2) a gene expressed in the brain was found to be involved in energy homeostasis regulation in zebrafish, rodents, as well as humans (Asai et al., 2013; Sebag et al., 2013). In another study, it was found that a mutation of melanocortin 4 receptor (Mc4r) is associated with early onset of obesity (Farooqi et al., 2003). Mc4r is part of a system in the brain involved with the control of appetite and food intake. It is a centrally expressed G protein–coupled receptor that decreases food intake whenever activated (Srinivasan et al., 2004). Mc4r gene mutations include frame shift, in-frame deletion nonsense, and missense mutations, located throughout the Mc4r gene. This gene plays an important role in regulating appetite suppression and energy storage in the body. Furthermore, recent

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studies by Asai et al. (2013) and Sebag et al. (2013) discovered that Mrap2 functions by altering signals through Mc4r, therefore the loss of Mrap2 gene will cause uncontrolled food consumption and lead to weight gain.

If leptin is referred to as the 'satiety hormone', then ghrelin is the 'hungry hormone'. Ghrelin has the opposite effect of leptin in that it triggers the desire to eat. Other than its role in meal initiation, it is also involved in energy homeostasis (Burger & Berner, 2014) and is a regulator of growth hormones (Kojima et al., 1999). Under normal conditions, secretion of ghrelin will increase before eating and decrease after eating. Although it is obvious that ghrelin is closely related to obesity, no study has yet proven the corresponding role of ghrelin in obese subjects. Interestingly, (Tschop et al., 2001) found that the level of ghrelin was not elevated but decreased in obese Caucasians. This is unexpected as the level of ghrelin was not associated with obesity. However, (Karra et al., 2013) found that the overexpression of FTO increases mRNA ghrelin, and may indirectly increase energy intake and hence cause obesity in humans. These contradictory results indicate that further investigation is required to determine the extent of ghrelin's role in body weight regulation.

Thus far, the genes associated with obesity and the mechanisms involved are not fully understood yet. A few studies have been done on gene expression, yet perhaps gene regulation plays a more crucial role in the physiological changes and in understanding the mechanisms involved. As such, we proposed to examine genes that may be regulated during the development of obesity. In short, we seek to identify and correlate the physiological, biochemical and gene expression changes that occur during the development of high-fat diet-induced obesity, and to study the changes that may occur to these parameters following intervention via treatment with green tea and EGCG.

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3.3 Methodology

3.3.1 Plant extraction

Fresh leaves of *C. sinensis* were collected from BOH Plantation at Bukit Cheeding, Selangor (Malaysia). Preparation of tea extracts were done according to reported methods (Hsu et al., 2011; Komes, 2010; Yin et al., 2009) with minor modifications. Fresh tea leaves were dried completely and grounded into powder. With a green tea powder to solvent ratio of 1:20, powder was then subjected to methanol for extraction at room temperature for 1 h with stirrer. After that, the mixture was filtered, and the filtrate was evaporated via rotary evaporation while the residue was discarded after filtration. After drying, the crude extract was stored in -20 °C for long term storage.

3.3.2 Animal work

A total of 96 × C57Bl/6 male mice at 7 weeks old were obtained from Monash University Sunway Campus, Subang Jaya. The mice were housed 3 animals per cage at 22–24 °C with a 12-h light/dark cycle in the animal facility. The mice were fed with normal chow and allowed to acclimatise to the facility for at least 1 week before the start of the experiment.

3.3.3 Treatment on mouse

The mice were categorized into four groups, with 24 mice per group, i.e. control group [VC, without treatment + normal chow (33 kcal/kg, refer to appendix for detailed content)], high-fat diet group (HFD, without treatment + 60% kcal fat D12492 high-fat diet), high-fat diet plus green tea extract treatment group (HFD + GTE, treatment with green tea methanol extract at 3 g/kg + 60% high-fat diet) and high-fat diet plus EGCG treatment group [HFD + EGCG, treatment with EGCG at 3 g/kg (97% purity, Toronto Research Chemical Inc, Canada) + 60% kcal high-fat diet]. For the VC group and HFD group, 0.9% saline were given. All groups were fed using oral gavage for 28 days. All

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mice were allowed free access to food and water during the experimental period. Food intake and body weight of the mice were recorded daily. A total of 6 mice from each group were sacrificed on the 8th, 15th, 22nd and 29th day. Aside from that, brain tissue and adipose tissues (epididymal, inguinal and brown adipose tissue) were collected, immediately immersed in RNAlater® solution (Thermo Scientific, New York) to stabilize the RNA content in the tissue, and stored at -80 °C before RNA isolation and gene analysis.

3.3.4 RNA extraction

Brain tissues were thawed after being taken out from the -80 °C freezer. The tissues were then homogenized at 50-100 mg of tissue per 1 mL of TRIzol® Reagents (Thermo Scientific, New York) by using LabGEN 125 homogenizer (Cole Parmer, USA). The homogenized samples were incubated for 5 minutes at room temperature to allow complete dissociation of nucleoprotein complexes. A volume of 0.2 mL of chloroform per 1 mL of TRIzol® Reagents was added to the homogenized samples and shaken vigorously for 15 seconds. The samples were then incubated for 2 to 3 minutes at room temperature. The samples were centrifuged afterwards at 12,000 × g for 15 minutes at 4 °C. The centrifugation separated the samples into two phases, the upper aqueous phase was collected. Equal amount of ethanol (70%) was added to the tubes with the newly collected upper aqueous phase and mixed well by vortexing. A volume of 700 µL of the solution from each tube was then transferred to spin cartridges, and centrifuged at 12,000 × g for 15 seconds at room temperature. The flow-through was discarded and the column was reinserted into the same collection tube. Wash buffer I (700 µL) from PureLink® purification kit (Life Technologies, New York) was added to each spin cartridge and centrifuged at 12,000 × g for 15 seconds at room temperature. Again, the flow-through was discarded and the column was inserted into a new collection tube. A volume of 500 µL of wash buffer II with ethanol from PureLink® purification kits was

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added to the samples and centrifuged at 12,000 × g for 15 seconds at room temperature. This step was repeated once. After that, the samples were centrifuged at 12,000 × g for 1 minute at room temperature to dry the membrane with attached RNA. The collection tubes were then discarded and the spin cartridges were placed into 1.5 mL microcentrifuge tubes. 30 µL of RNase-free water was aliquot to centre of each tube for three times. The samples were then left to incubate for 1 minute at room temperature before extraction of total RNA into the microcentrifuge tubes via centrifugation at 12,000 × g for 2 minutes at room temperature. Following that, the spin cartridges were discarded and the extracted total RNAs were stored in a -80 °C freezer. Concentrations of total RNA were measured using Nanodrop ND-1000 and the quality of the RNA was examined through gel electrophoresis.

3.3.5 Gene expression study

A total of 24 RNA samples [control group (1 RNA sample per week), high fat diet group treatment group (1 RNA sample per week), green tea extract treatment group (2 RNA samples per week) and EGCG treatment group (2 RNA samples per week)] were sent out to Beijing Genome Institute (BGI) for transcriptome resequencing.

3.3.6 Data analysis

Microsoft Excel was used to calculate weight reduction in percentage and food intake of the 4 groups of mice. Student's t test was used to analyse the body weight area under the curve and food intake of the animals. Data from transcriptome resequencing was analysed using RStudio version 3.2.4.

3.4 Results

3.4.1 Green tea extract yield

The yield of leaf extract of *C. sinensis* in methanol was 10.85 mg per gram of green tea leaf powder.

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3.4.2 Weight reductions of mice with green tea extract and EGCG treatment

A total of 4 groups of animals were used in the study: VC, HFD, HFD + GTE and HFD + EGCG. The duration of the experiment was 28 days and the body weight was recorded on the daily basis. From Figure 3.1, it can be seen that the HFD group gained most weight compared to the other 3 groups. Body weight of HFD + EGCG group was similar to the VC group. HFD + GTE group gained the least weight during the experiment. On average, total weight gain (in area under the curve, AUC and percentage respectively) by the end of the experiment in VC group was 655.4 and 20.02 ± 2.31% per mouse, 703.1 and 29.85 ± 2.34% for the HFD group, 594.7 and 8.70 ± 3.06% for HFD + GTE group and 654.1 and 9.29 ± 2.69% for HFD + EGCG group (Figure 3.2 and Figure 3.3). AUC of VC vs HFD ($p < 0.05$), HFD vs HFD + GTE ($p < 0.005$) and HFD vs HFD + EGCG ($p < 0.05$) were significant under one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. The percentage of body weight gain were significantly different in VC vs HFD ($p < 0.005$), VC vs HFD + GTE ($p < 0.0005$) , VC vs HFD + EGCG ($p < 0.0005$), HFD vs HFD + GTE ($p < 0.0005$), HFD vs HFD + EGCG ($p < 0.0005$), after analysis with one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. The HFD group gained most weight compared to VC, HFD + GTE and HFD + EGCG group, while HFD + EGCG group gained least weight. Table 3.1 summarises the results for body weight changes in mice from day 1 to day 28.

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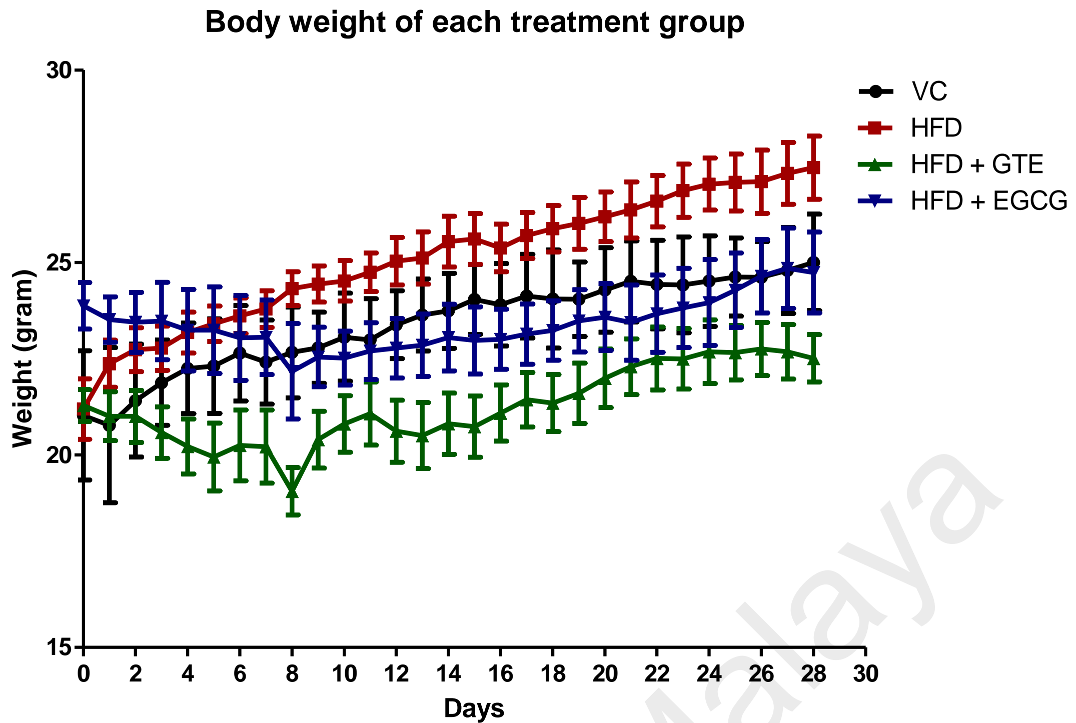

Figure 3.1 Body weight of mice from day 1 till day 28. VC: Vehicle control; HFD: high fat diet; HFD + GTE: high fat diet + green tea extract; HFD + EGCG: high fat diet + EGCG

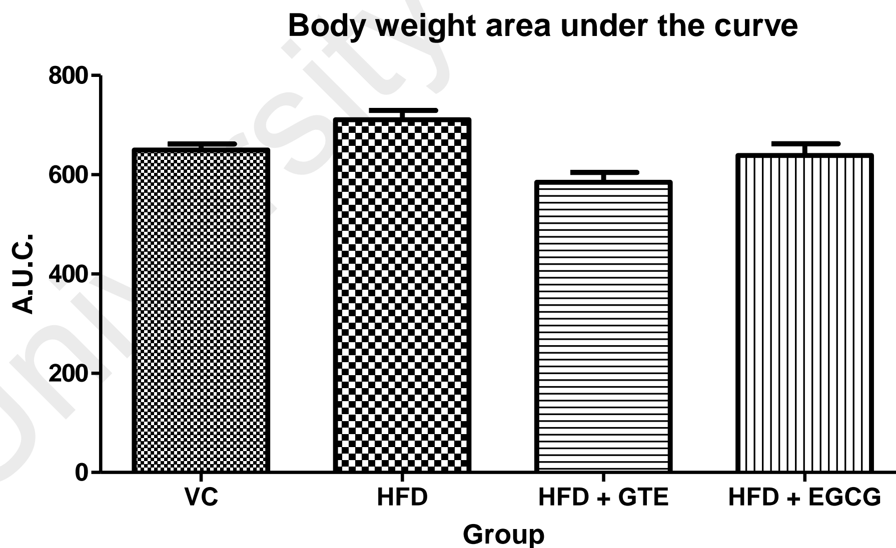

Figure 3.2 AUC values for body weight of each treatment group. The results were significantly different for VC vs HFD ($p < 0.05$), HFD vs HFD + GTE ($p < 0.005$) and HFD vs HFD + EGCG ($p < 0.05$) using One-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

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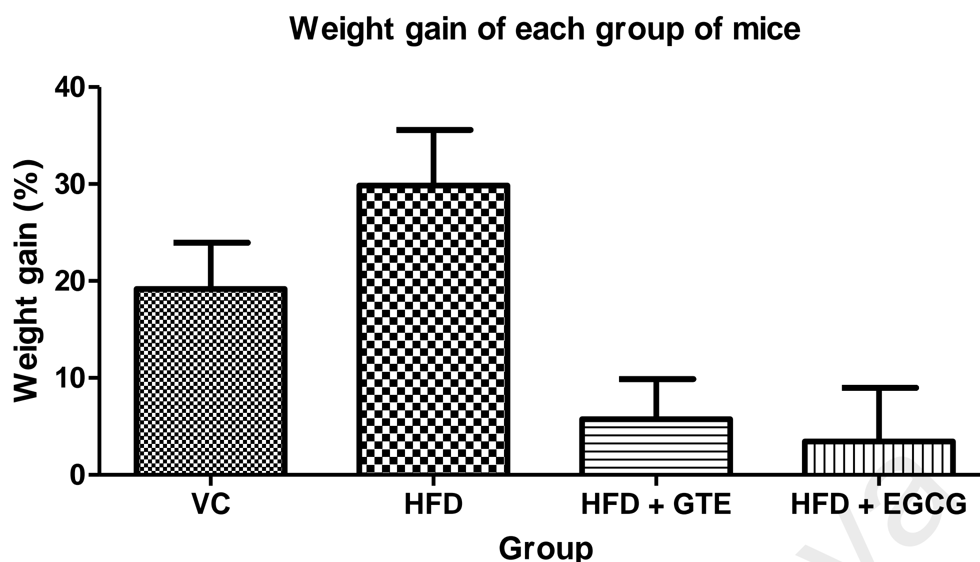

Figure 3.3 Body weight gain of each treatment group in percentage. The results were proven to be significantly different in VC vs HFD ($p < 0.005$), VC vs HFD + GTE ($p < 0.0005$) , VC vs HFD + EGCG ($p < 0.0005$), HFD vs HFD + GTE ($p < 0.0005$), HFD vs HFD + EGCG ($p < 0.0005$), after analysed with One-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

Table 3.1 Weight gain of different groups

	VC	HFD	HFD + GTE	HFD + EGCG
Area under the curve	655.4	703.1	594.7	654.1
Total weight gain per mouse	20.02 ± 2.31%	29.85 ± 2.34%	8.70 ± 3.06%	9.29 ± 2.69%
Mean weight gain	11.95 ± 1.01%	19.38 ± 1.36%	-0.19 ± 0.9%	-2.23 ± 0.52%

3.4.1 Food intake

Food intake was recorded on a daily basis for all four groups. Total food uptake for VC, HFD, HFD + GTE and HFD + EGCG are 662.82 g, 406.68 g, 317.71 g and 374.22 g, respectively. Food consumed by the VC group per mouse per day (3.95 ± 0.11 g) was significantly greater than the other groups (2.42 ± 0.06 g for HFD group, 1.89 ± 0.04 g for HFD + GTE group and HFD + EGCG group 2.23 ± 0.06 g, $p<0.0001$). Figure 3.4 shows total food intake of each group from day 1 till day 28. From the graph, total amount of food consumed by the control group is higher than the other three groups and amount of food consumed by high fat diet group and EGCG-treated group are comparable. The results also showed significance when the AUC of VC is compared to

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HFD ($p < 0.005$), VC compared to HFD + GTE ($p < 0.005$), VC compared to HFD + EGCG ($p < 0.005$), HFD compared to HFD + GTE ($p < 0.005$) and HFD + GTE compared to HFD + EGCG ($p < 0.05$), but not HFD compared to HFD + EGCG.

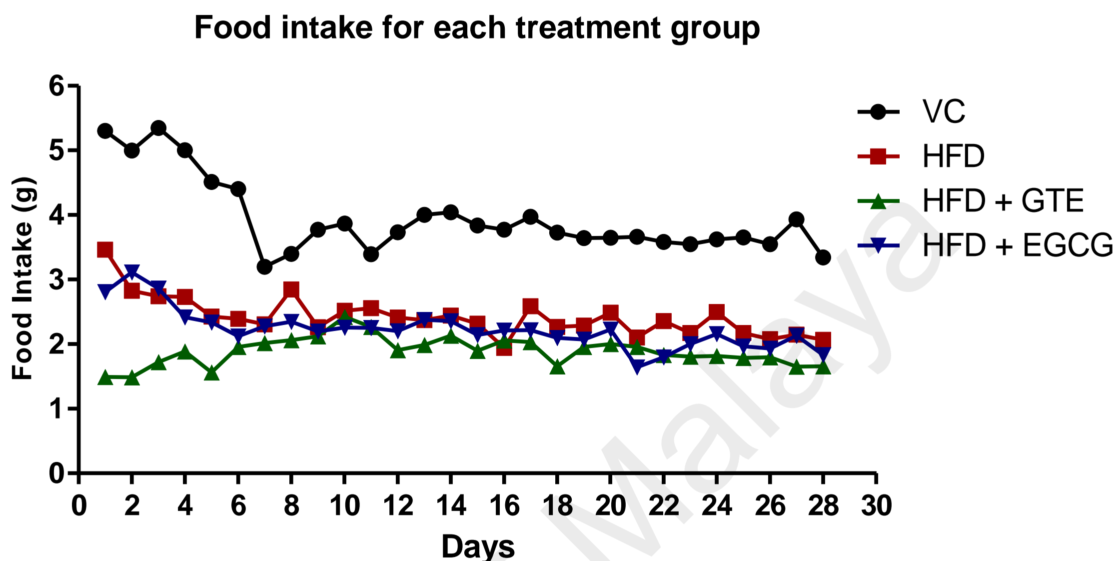

Figure 3.4 Total food intake of each group from day 1 to day 28.

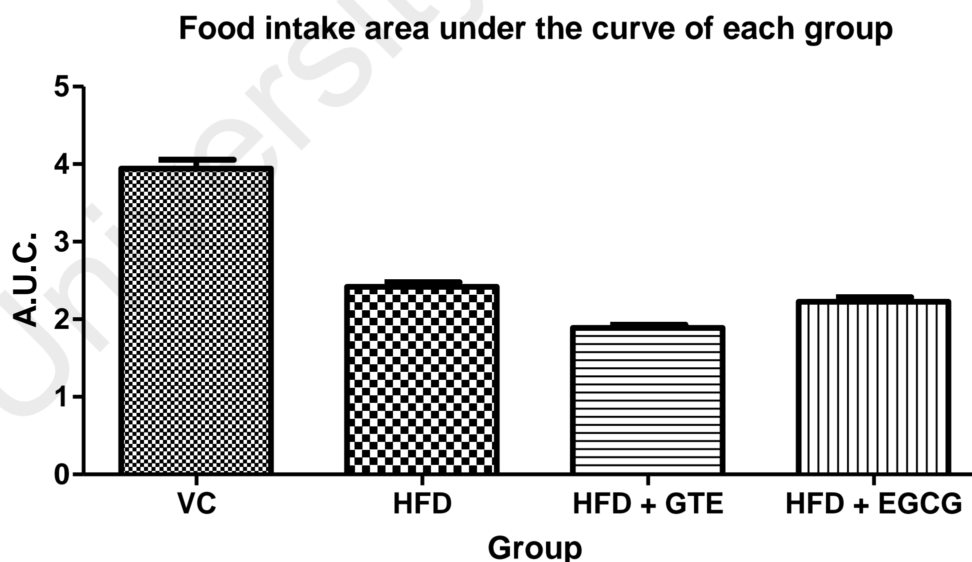

Figure 3.5 Food intake of each group in AUC. The food intake results are significance when the A.U.C. of VC is compared to HFD ($p < 0.005$), VC compared to HFD + GTE ($p < 0.005$), VC compared to HFD + EGCG ($p < 0.005$), HFD compared to HFD + GTE ($p < 0.005$) and HFD + GTE compared to HFD + EGCG ($p < 0.05$), but not HFD compared to HFD + EGCG.

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3.4.2 Gene expression

Total RNA samples of the mice was extracted from the brain tissues after 28 days treatment. One total RNA sample from VC group per week and two total RNA samples were randomly picked from HFD, HFD + GTE and HFD + EGCG group per week to subject to transcriptome resequencing. Bioinformatical analysis was done to study the regulation of genes that are possibly involved in the development of obesity and prevention of obesity. Each set of genes was identified after the sequencing. Gene sets from HFD, HFD + GTE and HFD + EGCG were afterward normalized to gene set of VC. After normalization, a total of 1663 genes were found to be constantly regulated in the 28 days or 4 weeks treatment in HFD normalized to VC sample. A total of 1210 genes were found to be upregulated while 453 genes were found to be downregulated during the treatment period. The genes were then subjected to another filtration to find genes that were upregulated or downregulated at 2 or more folds. Number of genes regulated after filtration was 1220, 948 genes were upregulated and 272 genes were downregulated. For HFD + GTE, there were a total of 2059 genes which were regulated, 942 genes were upregulated and 1117 were downregulated. After filtration, 1264 genes were regulated while 516 genes were upregulated and 748 genes were downregulated. There were a total of 2211 genes that were regulated for sample HFD + EGCG after normalization. A total of 897 genes were upregulated and 1314 genes were downregulated. After filtration, the number of genes that were regulated were reduced to 1968 with 779 genes that were upregulated and 1189 genes were downregulated. The gene sets of HFD + GTE and HFD + EGCG were normalized to HFD to get more information on molecular changes that happens for prevention of obesity. There were 2619 genes that were regulated when HFD + GTE was normalized to HFD, with 842 genes that were upregulated and 1777 genes were downregulated. After filtration, 2260 genes were regulated with 682 genes which were upregulated and 1578 genes were

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downregulated. For HFD + EGCG sample, total genes regulated was 2484 genes, with 745 genes that were upregulated and 1739 genes were downregulated. After filtration total genes regulated was reduced to 2135 genes, with 537 genes that were upregulated and 1598 genes were downregulated. Another normalization was done to normalize HFD + EGCG to HFD + GTE to see if the effect of EGCG alone. Total genes regulated were 2417 genes, with 1074 genes were upregulated and 1343 genes were downregulated. After filtration, the total genes regulated were reduced to 2017 genes, with 881 genes that were upregulated and 1136 genes were downregulated. Table 3.2 shows the summary of the results from transcriptome resequencing analysis. Another filtration was done to map the gene list of each sequenced sample to human gene. Not all the genes from mouse have a counterpart in human and vice versa, therefore it is necessary to do the mapping. Table 3.3 to 3.7 showed the top 10 upregulated and 10 downregulated human equivalent genes and the fold change in respective sample. Overlapping genes between HFD + GTE and HFD + EGCG normalized to HFD were 582 genes in total, with 453 genes were downregulated and 129 genes were upregulated in both samples. Although these genes are commonly irregulated in both samples, the sequence of the genes based on fold change is slightly different. Table 3.8 shows the top 10 upregulated and 10 downregulated human equivalent genes in HFD + GTE and HFD + EGCG normalized to HFD samples.

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Table 3.2 Summary of results from trancriptome resequencing analysis

	HFD vs* VC	HFD + GTE vs VC	HFD + EGCG vs VC	HFD + GTE vs HFD	HFD + EGCG vs HFD	HFD + EGCG vs HFD + GTE
Total genes regulated	1663	2059	2211	2619	2484	2417
Upregulated genes	1210	942	897	842	745	1074
Downregulated genes	453	1117	1314	1777	1739	1343
Total genes that have more than 2 folds changes	1220	1264	1968	2260	2135	2017
Upregulated genes	948	516	779	682	537	881
Downregulated genes	272	748	1189	1578	1598	1136
No of human equivalent genes	1024	1007	1687	1892	1817	1767
Upregulated genes	806	463	689	630	472	738
Downregulated genes	218	544	998	1262	1345	1029

*vs in the table represent to which sample the genes normalized to

Table 3.3 Top 10 upregulated and 10 downregulated human equivalent genes and the fold change in HFD sample normalized to VC.

Gene	Fold change	Gene	Fold change
SLCO1B3	16.36	**DAO**	-9.39
SLC9B1	15.28	**CACNG6**	-7.44
EPS8L1	13.34	**SLC39A5**	-7.10
NLRP1	11.86	**RGS13**	-6.48
LTA	11.56	**ARHGAP8**	-5.88
ADH1C	11.01	**ZP1**	-5.69
CPA3	9.97	**METTL11B**	-5.22
ITGAX	9.61	**IL1B**	-4.69
CATSPERG	9.60	**GPX2**	-4.65
CLDN9	9.46	**MGST2**	-4.65

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Table 3.4 Top 10 upregulated and 10 downregulated human equivalent genes and the fold change in HFD + GTE sample normalized to VC.

Gene	Fold change	Gene	Fold change
ITIH4	19.58	**CLEC4M**	-17.20
MALL	14.53	**MGST2**	-15.70
HIST1H4D	13.28	**NXNL2**	-11.82
ICOS	12.92	**GPR65**	-10.39
MYBPC3	11.37	**OR10G2**	-9.84
C10orf82	10.33	**SPN**	-8.41
TRPM5	8.67	**CLDN8**	-7.57
CYP4B1	7.12	**TMEM236**	-7.26
LTA	6.19	**CNTF**	-7.04
C3	6.17	**SPATA25**	-6.64

Table 3.5 Top 10 upregulated and 10 downregulated human equivalent genes and the fold change in HFD + EGCG sample normalized to VC.

Gene	Fold change	Gene	Fold change
TAT	21.75	**CXorf22**	-31.26
TARM1	13.43	**IL1B**	-19.68
LBX2	11.73	**GRIN2B**	-12.51
CYP2A13	11.61	**TMEM182**	-11.85
CYP4B1	9.14	**TMEM211**	-10.98
PRSS22	8.31	**TEX13B**	-8.41
NLRC4	8.20	**GLRA4**	-7.75
FTCD	8.14	**CNPY1**	-7.45
LOC102723903	7.69	**TFAP2D**	-7.39
CLSPN	7.57	**PTX3**	-6.39

Table 3.6 Top 10 upregulated and 10 downregulated human equivalent genes and the fold change in HFD + GTE sample normalized to HFD.

Gene	Fold change	Gene	Fold change
FAT2	101.09	**IFI16**	-20.11
DBH	94.11	**OCA2**	-19.33
HOXA2	51.84	**KCNJ13**	-16.35
SLC6A2	42.37	**SYTL3**	-15.39
TPH2	35.18	**OR52W1**	-15.00
PCP2	32.97	**F5**	-14.64
MPZ	26.78	**GPR65**	-13.62
MYBPC3	26.11	**SPN**	-12.08
CD4	19.42	**C1orf162**	-11.96
IRX4	16.74	**CATSPERG**	-11.38

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Table 3.7 Top 10 upregulated and 10 downregulated human equivalent genes and the fold change in HFD + EGCG sample normalized to HFD.

Gene	Fold change	Gene	Fold change
OVOL2	53.28	**HOOK2**	-2.91
EGR2	14.87	**EBF4**	-3.25
SERPINB8	5.44	**GDPD2**	-3.05
GPR1	2.66	**PLXNA3**	-3.23
GSX1	2.37	**ASCC2**	-3.00
MEI4	3.82	**NCOA3**	-2.65
CDKN2B	2.47	**YEATS2**	-2.89
BAZ1A	3.19	**ULK3**	-2.39
IL12A	4.19	**PXN**	-2.80
CLSPN	6.90	**HDAC7**	-2.60

Table 3.8 Top 10 upregulated and 10 downregulated overlapping human equivalent genes and the fold change in HFD + EGCG sample normalized to HFD.

GTE	FCC	EGCG	FCC
IFI16	-20.11	**OR52W1**	-15.28
KCNJ13	-16.35	**SLC26A3**	-12.48
SYTL3	-15.39	**CLDN9**	-11.61
OR52W1	-15.00	**SYTL3**	-10.77
F5	-14.64	**SPTLC3**	-8.10
FOLR1	-11.15	**IFI16**	-7.89
SPTLC3	-9.97	**TLR9**	-7.30
ITGAX	-9.55	**F5**	-7.22
CLDN9	-8.61	**TMEM239**	-6.90
WDR86	-7.72	**ITGAX**	-6.81
MPZ	26.78	**MPZ**	64.77
MYBPC3	26.11	**CD4**	11.40
CD4	19.42	**GPR88**	9.10
ADORA2A	16.20	**ADORA2A**	9.06
SH3RF2	16.19	**MYBPC3**	7.95
GPR88	10.93	**PPP1R1B**	7.25
PPP1R1B	7.44	**CLSPN**	6.90
ANKRD63	7.39	**SH3RF2**	6.59
GPR6	6.78	**ARPP19**	4.65
SERPINA9	6.00	**GPR6**	4.63

3.5 Discussion

Weight gain of the HFD group is significantly higher than the VC group, the HFD + GTE and the HFD + EGCG groups. Although the HFD + GTE and the HFD + EGCG

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groups were fed with high-fat diet, the mean weight gain for these two groups was very low. Other than that, the food intake of VC was higher than HFD, HFD + GTE and HFD + EGCG group. However, HFD, HFD + GTE and HFD + EGCG had similar food intake throughout the treatment period. This rules out the possibility of low food consumption that had caused the low weight gain in HFD + GTE and HFD + EGCG. The low weight gain in HFD + GTE and HFD + EGCG is likely due to the treatment of green tea extract and EGCG; both green tea extract and EGCG may have anti-obesity properties that suppress weight gain in the treated mice. Based on the physiological changes (i.e. changes in body weight), we believe that there may be some alteration of gene regulation in obesity development during treatment of green tea extract and EGCG on mice.

In our study, we have found a number of regulated genes especially genes that are directly or indirectly related to obesity and metabolic syndrome in our treatments. Based on the treatments and supported by the physical evidence (i.e. the changes in body weight), we believe that each group of genes play important role in different process. We compared the gene list that we obtain to the online database, Obesity and Metabolic Syndrome Portal under Rat Genome Database (Shimoyama et al., 2015) in order to understand the development of obesity and prevention of obesity by GTE and EGCG treatment. For instance, the group of genes that is regulated in HFD normalized to VC, is probably involved in the obesity development. For instance, among the top 10 upregulated genes, the top gene, SLCO1B3, solute carrier organic anion transporter family member 1B3 that is normally expressed in liver, is important for bile acid and bilirubin transport (Hagenbuch & Gui, 2008) and is involved in metabolic pathway (Tamai et al., 2000; Yang et al., SLCO2B1 and SLCO1B3 may determine time to progression for patients receiving androgen deprivation therapy for prostate cancer, 2011). Overexpression of SLCO1B3 has been found to be associated with human

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carcinomas such as colon (Sun et al., 2014; Thakkar et al., 2013), lung (Sun et al., 2014) and pancreatic (Thakkar et al., 2013). The second top gene, SLC9B1, solute carrier family 9 member B1, is also involved in the transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds in liver. Although its function is not fully understood, its isoform, SLC9B2 is the important component in regulating insulin secretion (Deisl et al., 2013). More studies need to be done to understand the role of the gene in metabolic pathway. LTA (lymphotoxin alpha, fold change 11.56) or commonly known as tumor necrosis factor beta (TNF-Beta) is found to be overexpressed in type II diabetes melitus (Goldfarb & Ziyadeh, 2001; Pscherer et al., 2013). ADH1C, alcohol dehydrogenase 1C (Class I), gamma polypeptide, is one of the alcohol metabolism genes. This gene is believed to be associated with low level of high density lipoprotein (HDL) (Hines et al., 2001), where low HDL individuals are prone to have higher risk in developing metabolic syndrome (Liu et al., 2015). On the other hand, the top downregulated gene, DAO (D-amino acid oxidase) is possibly a detoxifying agent although the mechanisms underlying it is still unknown and is involved in metabolic pathway particularly in metabolism of food histamine (Maintz et al., 2008). SLC39A5 is involved in transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds. SLC39A5 (solute carrier family 39 member 5) also encodes a protein named Zip5 protein where it functions as a zinc transporter (Wang et al., 2004). Downregulation of SLC39A5 can cause zinc deficiency and hence lead to the development of metabolic syndrome (Jurowski et al., 2014). IL1B (interleukin 1 beta), one of the inflammatory mediators is known to be associated with metabolic syndrome such as obesity, insulin resistance, type II diabetes mellitus etc (Bing, 2015). The results suggested that the metabolic genes mentioned were regulated and hence trigger the development of obesity which supported by the physical increment in body weight.

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Among the regulated genes in GTE treated group normalized to VC, ITIH4 (inter-alpha-trypsin inhibitor heavy chain family member 4) was highly upregulated. ITIH4 is associated with hypercholesterolemia (Fujita et al., 2004). ICOS (inducible T-cell costimulator) was also upregulated in the sample. Upregulated of ICOS was found to be involved in regulating arteriosclerosis (Sharpe & Freeman, 2002). Similar to HFD, LTA was also upregulated. C3 (complement component 3) was found to associated with development of metabolic syndrome (Liu et al., 2016). Ciliary neurotrophic factor (CNTF) was found to induce weight loss and improve glucose tolerance in both humans and rodents (Watt et al., 2006). Upregulation of the metabolic genes is most probably due to the high percentage of fat in the food intake. Although the body weight of the group of animal did not rise as high as the HFD group, GTE treatment did not seem to suppress the expression of aforementioned metabolic genes. There are, however, non-metabolic genes in Table 3.4 that help in regulating the development of obesity in the animal.

In the EGCG treated sample, TAT (tyrosine aminotransferase) was found to be associated with tyrosinemia, in which the body cannot effectively break down the amino acid tyrosine (Natt et al., 1992). TAT was upregulated in EGCG treated group, and this showed that the treatment probably enhance the effect of TAT where it breaks down the amino acid tyrosine in more effective ways. FTCD (formimidoyltransferase cyclodeaminase) is another upregulated gene in EGCG treated group. Defect of FTCD is found to cause glutamate formiminotransferase deficiency (Hilton et al., 2003). IL1B was also found to be downregulated in EGCG treated group. GRIN2B (glutamate ionotropic receptor NMDA type subunit 2B), belongs to a class of ionotropic glutamate receptors, and is associated with type II diabetes mellitus (White et al., 2013) and hypoglycemia (Adams et al., 2014).

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In the GTE treated group normalized to HFD, we expected to find genes that are involved in regulation of body weight where GTE treated group was able to maintain the body weight throughout the treatment period. There were four upregulated genes (DBH, SLC6A2, TPH2 and MPZ) and two downregulated genes (OCA2 and F5) that are related to obesity/metabolic syndrome that were found. DBH (dopamine beta-hydroxylase) promotes anorexia (Roman et al., 2016), however there are evidence showing that DBH contributes to hypertension (Abe et al., 2005). Single-nucleotide polymorphism in the SLC6A2 (Solute Carrier Family 6 Member 2) was found to be associated with hypertension in the Japanese population (Ono et al., 2003). TPH2 (tryptophan hydroxylase 2) is associated with hypertension, where this gene is sensitive to stress response (Chen & Miller, 2012). MPZ (myelin protein zero) was shown to be downregulated in diabetic peripheral neuropathy (Cermenati et al., 2012). Mutation of OCA2 (OCA2 melanosomal transmembrane protein) causes type 2 oculocutaneous albinism (Hawkes et al., 2013) which is one of the metabolic disorder where it involves a complex reaction of melanin production through metabolic reactions (King et al., 2003). Last but not least, F5, or better known as coagulation factor V, is the blood coagulation factor. Increased level of F5 is associated with venous thromboembolism and cardiovascular diseases (Previtali et al., 2011).

For the EGCG treated group, only one metabolic gene was found to be upregulated, i.e CDKN2B, and one downregulated gene (NCOA3). This is probably a positive effect from the EGCG treatment where EGCG is able to normalize most of the obesity/metabolic syndrome related genes to their normal expression level. CDKN2B (cyclin dependent kinase inhibitor 2B) appears to be the regulator for subcutaneous adipose tissue and hence determinant for lipotoxicity and contribute to arteriosclerosis (Svensson et al., 2014).

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Commonly expressed genes between GTE treated and EGCG treated group are MPZ, ADORA2A, F5, SLC26A3 and TLR9. MPZ and ADORA2A were upregulated, while F5, SLC26A3 and TLR9 were downregulated in both GTE and EGCG treated group. As mentioned previously, MPZ is associated with diabetes. Lack of ADORA2A, adenosine A2a receptor, was found to be associated with hypertension (Molero et al., 2013). Other than that, mutation of SLC26A3, (solute carrier family 26 member 3) was the cause of congenital chloride diarrhea (Dorwart et al., 2008). Nevertheless, TLR9 (toll like receptor 9) was found to play an important role in regulating adipose tissue inflammation and obesity-related metabolic disorders (Hong et al., 2015). These overlapping genes are probably due to the action of EGCG as EGCG is the major component in GTE.

Other than that, there are, however, many genes that might interact or be associated or be involved in the development of obesity and in the prevention of obesity. Each gene that is listed in the table should be studied thoroughly in order to understand the aforementioned processes.

The reason we chose a mouse model for our study was due to the close genetic and physiological characteristics of mice to humans. Laboratory mice have been selectively bred to produce certain desired traits useful in the genetics research. In a study conducted by Kim et al. (2005), obesity genes were studied in a mice model. In the study, *tabw2* congenic mice (which exhibit increased adiposity and hyperleptinemia) and control mice were bred and fed with a high-fat-sucrose diet for 10 weeks. After that, the *tabw2* mice were euthanized and gene expression in the liver and adipose tissues were analysed. The results showed that 1026 genes were up-regulated and 308 were down-regulated in the liver, whereas 393 were up-regulated and 187 were down-regulated in adipose tissue in *tabw2* congenic mice compared to that of the control mice. The top 50 regulated genes were found in the adipose tissue. Among the genes

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expressed within the congenic interval (47.0–137.3 Mb), 45 genes were expressed in the liver, 32 genes in adipose tissue, and 7 genes in both.

Koza et al. (2006) have also conducted a similar study using C57BL/6J mice. In their study, 3 weeks old mice were fed with alternating diets in the following order: low-fat chow diet for 5 weeks, high-fat diet for 6 weeks, low-fat chow diet for 2 weeks, and high-fat diet for 6 weeks. The mice were then euthanized; their hypothalamus and inguinal fat depots were analysed via microarray analysis for gene expression. During the course of the study, the changes in body weight were also recorded; it was found that the body weight of mice during the second low-fat chow diet were higher than their body weight before being fed with high-fat diets for the first time. Moreover, the rate of body weight gain was 2.2 times greater on their second exposure to high-fat diets. For gene expression, only 8 genes showed altered expression in the hypothalamus as compared to the 1902 genes (792 genes were up-regulated, 1110 were down-regulated) in inguinal fat depots.

Aside from that, there has been a study that investigated tissue factor (TF) gene expression in obesity. The study was carried out by Samad et al. (2001). In the study, tissues from the brain, lung, heart, kidney, adipose and liver were collected and TF mRNA levels was compared between obese mice and lean mice (injected with insulin). The results shows that TF mRNA was promoted in all tissues (4.7 folds in the brain, 5 folds in the lung, 5 folds in the heart, 6 folds in the kidney, 3.5 folds in adipose and 3.7 folds in the liver). *In situ* hybridization analysis indicates that TF mRNA was elevated in bronchial epithelial cells in the lungs, in myocytes in the heart, and in adventitial cells lining the arteries including the aortic wall. Administration of insulin to lean mice induced TF mRNA in the kidneys, brain, lungs and adipose tissue.

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Besides that, Miller et al. (2008) have done a study on the effects of different diets on gene expression of adipocytes. In their study, mice were divided into 4 groups; one group fed with a low-fat diet (LF; 10% fat energy), one group with an unrestricted high-fat diet (HF; 60% fat energy), one group with a high-fat energy–restricted diet (HFR; 60% fat energy) and one group with a HF diet followed by weight reduction (WR). The results showed that 587 genes were altered in HF but not in LF and HFR; the expression of these genes did not change with moderate obesity (HFR) but did with severe obesity (HF). Compared to LF, 59 genes that code for extracellular proteins were expressed differentially in HF and WR.

3.6 Conclusion

There are four treatment groups in total, VC, HFD, HFD + GTE and HFD + EGCG. After a treatment period of 28 days, the HFD group significantly gained more weight (29.85 ± 2.34%) than VC, HFD + GTE and HFD + EGCG groups. The HFD + GTE and HFD + EGCG groups gained 8.70 ± 3.06% and 9.29 ± 2.69% respectively. Food intake is not the cause of low weight gain in HFD + GTE and HFD + EGCG as the food intake for HFD, HFD + GTE and HFD + EGCG was similar. After the analysis of the transcriptome resequencing data, we were able to obtain a gene list on HFD normalized to VC group. The list of the genes is suggested to be related to the development of obesity in mouse, and potentially depicted the development of obesity in human as the genetic background of both the organisms is highly similar. With the treatment of GTE and EGCG, we were also able to find out that the genes were regulated due to respective treatment and the overlapping genes that are being regulated in both treatment groups. With the gene lists obtained as a result of the respective treatment, the mechanism of action underlying can be uncovered. The overlapping genes are probably due to the action of EGCG as EGCG is the major component in GTE.

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CHAPTER 4: EFFECTS OF GREEN TEA AND EGCG ON CYTOCHROME P450 ENZYMES

4.1 Introduction

4.1.1 Cytochrome P450

The cytochrome P450 system (P450) is a large group of hemoprotein enzymes with thiolate groups. They are located in smooth endoplasmic reticulum and mitochondria of the cells of several tissues, particularly in liver tissues (Nelson, 2009). They are monooxygenases that catalyse reactions based on the activation and heterolytic cleavage of molecular oxygen, with the insertion of one oxygen atom into the substrate and the reduction of the other to form water (Bernhardt, 2006; Mansuy, 1998; Werck-Reichhart & Feyereisen, 2000).

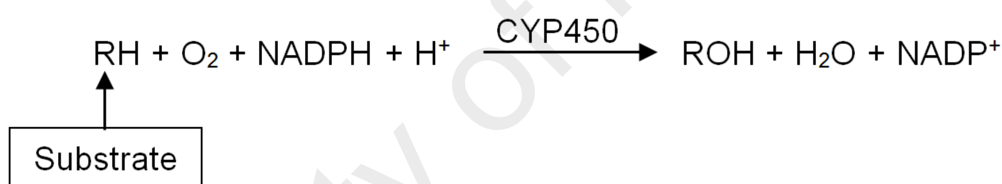

Figure 4.1 Reduction process when a substrate is bound to CYP450 enzyme

The reduced form of the enzymes (after reaction) binds to carbon monoxide, and this results in an absorption peak of wavelength 450 nm, and hence the name **P**igment absorbing at **450** nm (**P450**) (Omura & Sato, 1964).

Up until now, 18 families of CYP P450, 57 genes and more than 59 pseudogenes have been discovered in humans (Nelson, 2009). The enzymes are named in the following manner; all P450 genes begin with the root abbreviation CYP, followed by a number designating the family, followed by a letter for designating the subfamily, and ending with another number designating the gene; for instance, CYP2A1.

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The significance of P450 was only uncovered when advances in technologies made biochemical characterisation possible. P450 is involved in several biosynthetic and xenobiotic pathways in humans; its role in drug metabolism is one of its most important functions to humans. There are three major phases in metabolism and P450 is the most important enzyme system in Phase I where oxidation and hydrolysis are carried out. Prior to the action of P450 (removal of foreign chemicals), drugs are metabolized by the addition or removal of certain chemical groups, this in turn generates powerful carcinogens. Left unchecked, the accumulation of these carcinogens would cause drug toxicity.

4.1.2 Objectives

1. To examine the effects of green tea extracts (methanol, aqueous and hexane) and EGCG on human recombinant CYP enzymes CYP3A4, CYP2D6, CYP1A2, CYP2C9 and CYP2C19.
2. To determine green tea extracts' and EGCG's mode of inhibition on CYP3A4, CYP2D6, CYP1A2, CYP2C9 and CYP2C19.

4.2 Literature Review

One side effect observed in subjects who consumed green tea is the alteration of drug hepatic metabolism, particularly cytochrome enzyme inhibition by certain components in green tea. In the human liver, there are at least 18 distinct CYPs, with 10 from families 1, 2, and 3 (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) responsible for hepatic metabolism of most marketed drugs (Penner et al., 2012). These 3 families are well known Phase 1 drug metabolising enzymes, or enzymes that insert or activate a functional group in the drug molecule. Among P450, CYP3A4, CYP1A2, CYP2D6, CYP2C9 and CYP2C19 are the more important enzymes that get attention as they are involved with drug metabolism in the liver.

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CYP3A4 is the most important enzyme in the body. It metabolizes approximately 50% of prescribed drugs in the market including acetaminophen, codeine, cyclosporin A, diazepam and erythromycin (Frye et al., 2004; Nebert & Jorge, 2002). CYP3A4 is also the most expressed P450 in intestinal enterocytes, contributing significantly to Phase I metabolism of orally administered drugs (Ding & Kaminsky, 2003; von Richter et al., 2004). Substrates metabolized by CYP3A4 include immunosuppressants, anti-cancer drugs, antidepressants, opioids, etc. (Hendrychova et al., 2011). Just as CYP3A4 metabolizes a long list of drugs, it is subjected to many inducers and inhibitors. CYP3A4 inducers such as buspirone, triazolam and verapamil tend to lower the concentrations of CYP3A4 substrates in plasma, resulting in reduced efficacy of the substrate (Horn, 2008). This indirectly causes the lack of patient response even when the drug is consumed at the prescribed dosage. On the other hand, inhibition of CYP3A4 will cause increased concentrations of CYP3A4 substrates and reduced CYP3A4 activity. Potent inhibitors of CYP3A4 include clarithromycin, itraconazole and ketoconazole (Horn, 2008).

CYP2D6 interacts with as many as 25% of the drugs in the market (Ingelman-Sundberg, 2004). Drugs metabolized by CYP2D6 include antidepressants, antipsychotics, analgesics and antitussives (Ingelman-Sundberg et al., 1999; Kirchheiner et al., 2004). As with CYP3A4, CYP2D6 activity can be increased or decreased with inducers and inhibitors. Other than that, CYP2D6's activity is greatly affected by genetic polymorphisms and therefore varies from individual to individual. Individuals can be categorized into one of 4 groups; poor metabolizers, intermediate metabolizers, extensive metabolizers and ultrarapid metabolizers (Bertilsson et al., 1993). This is due to the different allelic variants of CYP2D6. According to Bradford (2002), CYP2D6 allele frequency varies between racial/ethnic groups. For instance, *CYP2D6*4*, a gene encoded for enzyme with decreased function is commonly observed

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in European Caucasians. On the other hand, the common allele in Asians, *CYP2D6*10* is encoded for unstable CYP2D6 and has reduced affinity for substrates. These findings of CYP2D6 polymorphism are extremely important in drug screening to exclude drug-test candidates that exhibit alternative CYP2D6 activity after drug consumption.

The remaining marketed drugs (roughly 20%) are metabolized by CYP1A2, CYP2C9 and CYP2C19 (Frye et al., 2004; Nebert & Jorge, 2002). CYP1A2's substrates are mainly antipsychotics (Flockhart, 2007), endogenous substrates (Guengerich, 1993) and substrates that eliminate environmental toxins (Eaton et al., 1995). Aside from that, CYP1A2 was found to activate several aromatic amines which cause carcinogenesis and therefore is a key enzyme in chemical carcinogenesis (Eaton et al., 1995). On the other hand, CYP2C9 catalyses xenobiotics such as angiotensin II blockers, non-steroidal anti-inflammatory drugs, alkylating anticancer prodrugs, and sulfonylureas (Flockhart, 2007). Similar to CYP2C9, CYP2C19 metabolizes xenobiotics like mephenytoin, omeprazole, diazepam and some barbiturates (Flockhart, 2007). Like CYP2D6, CYP2C9 and CYP2C19 are also subjected to gene polymorphism (Bertilsson, 1995). Despite their importance in drug metabolism, research on CYP1A2, CYP2C9 and CYP2C19 are limited. It is important that more studies be done on these CYPs as more and more drugs are found to be metabolized by these enzymes.

Compounds that inhibit CYPs may potentially interact with other co-administered drugs, potentially causing adverse effects. Therefore, we aimed to evaluate the effects of various green tea extracts (methanol extract, aqueous extract and hexane extract) and its active compound, EGCG on CYPs. Besides that, we also aimed to determine their mode of inhibition using Crespi's high throughput *in vitro* fluorescent P450 assays. The potential inhibition effect of green tea extracts and EGCG on CYP3A4, CYP2D6, CYP1A2, CYP2C9 and CYP2C19 were examined in order to predict adverse drug interactions with other therapeutic products.

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4.3 Methodology

4.3.1 Chemicals

Epigallocatechin gallete (EGCG) of 97% purity was purchased from Toronto Research Chemical Inc. (North York, ON, Canada). All active recombinant human enzymes (CYP3A4, CYP2D6, CYP1A2, CYP2C9 and CYP2C19), marker substrates (3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin (AMMC), 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC), 3-cyano-7-ethoxycoumarin (CEC), 7-Methoxy-trifluoromethylcoumarin (MFC) and 7-hydroxy-4-(trifluoromethyl)-coumarin (HFC)), and the reduced nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system (NRS) were purchased from Gentest Corporation (Woburn, MA, USA). All other chemicals and standard references, including quinidine, ketoconazole, tranylcypromine, sulfaphenazole and furafylline were purchased from Sigma Aldrich (St. Louis, MO, USA).

4.3.2 Plant Extraction

Fresh leaves of *C. sinensis* were collected from BOH Plantation on Bukit Cheeding, Selangor, Malaysia. Preparation of tea extracts were done according to reported methods (Hsu et al., 2011; Komes, 2010; Yin et al., 2009) with minor modifications. Fresh tea leaves were dried completely and grounded into powder. The powder was then divided into 3 groups, each consecutively subjected to different solvent (water, methanol and hexane) for extraction. Extraction with methanol and hexane were done at room temperature while water extraction was done at 80 °C. The ratio of green tea powder to solvent is 1:20. After a period of 1 hour, the powder-solvent mixture was filtered; the filtrate was evaporated using a rotary evaporator and the residue was discarded after filtration. For water extraction, the filtrate was freeze-dried instead. The crude extracts were then stored at -20°C for long term storage.

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4.3.3 Fluorometric Enzyme Inhibition Assay

The inhibitory effects of EGCG and the green tea methanol extract (GTME), green tea water extract (GTWE) and green tea hexane extract (GTHE) on CYP enzymes were assayed using a modified Crespi method (Crespi et al., 1997; Kong et al., 2011). Stock solutions of EGCG, GTME, GTWE and GTHE were prepared using dimethyl sulfoxide (DMSO) diluted with acetonitrile (ACN) to ensure complete solubility of the solutes. The stock solutions were further diluted with phosphate saline buffer (PBS) when used in the experiments. In order to avoid any interference, the percentages of DMSO and ACN in the working solution were fixed in a way that did not exceed 0.25% and 1% respectively. Briefly, 20 µL of EGCG, GTME, GTWE and GTHE (0 to 1000 µg/mL), the negative controls (PBS) and the positive controls (standard inhibitors for each CYP enzymes tested) were added respectively to 30 µL enzyme/substrate mixture and 80 µL buffer (please refer to Table 1 for details) in 96-well Dynex Fluorolux^{TM} HB black, flat-bottom microplates (Chantilly, VA, USA). The mixtures were pre-warmed at 37°C for 10 min. Reaction was then initiated by adding 20 µL of NAPDH NRS into the mixture. The mixture was incubated for 20, 30 or 45 min, depending on the enzyme used. A stop solution was added at the end of the incubation to stop the reactions. The fluorescence intensity of the enzyme metabolites in each well was measured using Perkin Elmer VICTORTM X5 fluorescence plate scanner, (Waltham, MA, USA).

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Table 4.1 Summary of the components of the fluorometric inhibition assays

	CYP3A4	CYP1A2	CYP2C19	CYP2D6	CYP2C9
Substrate (final)	BFC: 50 µM	CEC: 5 µM	CEC: 25 µM	AMMC: 25 µM	MFC: 75 µM
Enzyme	1.0 pmol/well	0.5 pmol/well	0.5 pmol/well	1.5 pmol/well	2.0 pmol/well
Standard inhibitor	Ketocona-zole	Furafylline	Tranylcy-promine	Quinidine	Sulfaphena-zole
Buffer	Potassium phosphate buffer 200 mM	Potassium phosphate buffer 100 mM	Potassium phosphate buffer 50 mM	Potassium phosphate buffer 100 mM	Tris-HCl pH 7.5 buffer
Fluorescence Filter	Ex: 409 nm	Ex: 409 nm	Ex: 409 nm	Ex: 390 nm	Ex: 409 nm
	Em: 530 nm	Em: 460 nm	Em: 460 nm	Em: 460 nm	Em: 530 nm
Temperature			37 °C		
Incubation time	30 min	20 min	30 min	30 min	45 min

(Kong et al., 2011)

4.3.4 Mechanism-based inhibition study

K_m is the concentration of substrate which permits the enzyme to achieve half V_{max} while V_{max} is the maximum velocity or rate at which the enzyme catalyzed a reaction. By using K_m and V_{max} values for each CYP enzyme in our previous work (Kong et al., 2011) and IC_{50} from our previous assay as reference, the specific mechanism-based inhibition for each CYP were studied. This study was done by varying the concentration of both substrates (½ K_m, K_m, 2 K_m and 4 K_m) and samples (½ IC_{50}, IC_{50}, 2 IC_{50}, 4 IC_{50}). Incubation reagents and other experimental conditions were the same as in the fluorometric enzyme inhibition assay. Data obtained were analysed using Lineweaver-Burk double reciprocal plots and the experimental inhibition constant (K_i) for each sample were obtained.

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4.3.5 High Performance Liquid Chromatography (HPLC)

The percentage of EGCG in green tea extracts (GTME, GTWE and GTHE) were measured using high performance liquid chromatography by referring to Saito et al. (2006). EGCG was used as the standard. The HPLC system consists of the reservoir tray, degasser DGU-14A, pump A and B LC-10AD, auto sampler SIL-20A HT, column oven CTO-10A, communication bus module model CBM-20A and UV-vis detector SPD-10A (Shimadzu Corporation). The column used was Zorbax XDB C18 column (250 mm × 0.3 mm and 5 μM particle diameter (Agilent Technologies, US)). Detection was carried out at absorbance 280 nm. Mobile phase A was HPLC grade 0.1% trifluoroacetic acid (TFA) while mobile phase B was HPLC grade ACN. Stock solutions for the standard and the extracts were prepared at 1 mg/mL by dissolving in HPLC-grade methanol. The standard solutions were prepared in a series of concentrations (500 μg/mL, 250 μg/mL, 125 μg/mL and 62.5 μg/mL) to establish a calibration curve. Analyses of the samples were performed at the flow rate of 0.4 mL/min for 15 min. All chromatographic analyses were performed at 30 °C.

4.3.6 Data Analysis

The data were analysed using PRISM (version 5.04). IC_{50} were calculated using relative IC_{50} determination. K_m and V_{max} were obtained from Lineweaver Burk plots (Kong et al., 2011), Dixon plots and secondary reciprocal plots. Apparent inhibition constant (K_i) and mode of inhibition were determined from the analysed data.

4.4 Results

4.4.1 Inhibitory potency and K_i of green tea extracts and EGCG on CYPs' activities

The inhibition potency (IC_{50}) of the three types of green tea extracts (GTME, GTWE and GTHE) and EGCG on enzyme activities of CYP3A4, CYP2D6, CYP1A2, CYP2C19 and CYP2C9 are shown in Table 4.2 while their K_i are shown in Table 4.3.

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Table 4.2 IC_{50} of green tea extracts and EGCG, and the percentage of EGCG in green tea extracts.

	EGCG content	3A4	2D6	1A2	2C19	2C9
Substrate		BFC	AMMC	CEC	CEC	MFC
Standard inhibitor		ketoconazole 0.07 ± 0.004 µM	quinidine 4.84 ± 0.071 nM	furafylline 5.48 ± 0.027 µM	tranylcypromine 2.98 ± 0.064 µM	sulfaphenazole 0.26 ± 0.037 µM
GTME	46.46%	14.49 ± 0.29 µg/mL	56.07 ± 0.26 µg/mL	8.70 ± 0.27 µg/mL	2.13 ± 0.13 µg/mL	5.92 ± 0.19 µg/mL
GTWE	44.21%	23.04 ± 0.99 µg/mL	46.74 ± 0.97 µg/mL	15.50 ± 0.61 µg/mL	7.05 ± 0.49 µg/mL	11.37 ± 1.4 µg/mL
GTHE	2.58%	84.70 ± 0.77 µg/mL	*339.30 ± 7.5 µg/mL*	86.34 ± 0.41 µg/mL	15.27 ± 0.43 µg/mL	24.21 ± 0.53 µg/mL
EGCG	97%	2.13 ± 0.32 µg/mL	16.63 ± 0.58 µg/mL	1.89 ± 0.24 µg/mL	0.70 ± 0.05 µg/mL	3.02 ± 0.43 µg/mL

Table 4.3 K_i of green tea extracts and EGCG.

	3A4/BFC	2D6/AMMC	1A2/CEC	2C19/CEC	2C9/MFC
GTME	4.94 µg/mL	95.18 µg/mL	5.00 µg/mL	2.08 µg/mL	13.48 µg/mL
GTWE	22.47 µg/mL	149.20 µg/mL	7.95 µg/mL	8.73 µg/mL	31.04 µg/mL
GTHE	29.33 µg/mL	*	48.19 µg/mL	29.81 µg/mL	103.30 µg/mL
EGCG	3. 28 µg/mL	20.05 µg/mL	0.94 µg/mL	1.26 µg/mL	6.79 µg/mL

From the data, it can be established that EGCG, GTME and GTWE, but not GTHE, showed potent inhibitory effects on all CYP enzymes tested. EGCG showed the strongest inhibitory effect (lowest IC_{50} in all CYPs tested), followed by GTME (second lowest IC_{50} in 4 out of the 5 CYPs tested). For the K_i values, EGCG has the lowest K_i as expected, followed by GTME, GTWE and GTHE (Table 4.3). The low IC_{50} and K_i of GTME and GTWE correlate with their EGCG content where percentage of EGCG was found to be highest in GTME (46.46%) followed by GTWE (44.21%) while GTHE has

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the lowest percentage of EGCG (2.58%) (Table 4.2). This indicates that CYP inhibition by GTME and GTWE is likely attributed by their EGCG content.

By using the IC_{50} values, we were able to perform mechanism assays in an attempt to identify the modes of inhibition and K_i values induced by EGCG and the green tea extracts. In fact, K_i and IC_{50} can be used to compare relative potencies of inhibitors. K_i is a constant for a given compound on its action on a particular enzyme, while IC_{50} is a relative value whose magnitude depends on the concentration of the substrate used in the assay (Burlingham & Widlanski, 2003). We adapted K_m values that we have earlier established (Kong et al., 2011) in our current experiment to calculate the concentration of substrates used for each CYP. The results are summarised in Table 4.4. The data shows that all three green tea extracts and EGCG exerted a non-competitive inhibition on CYP1A2 (Figure 4.5)and CYP2C19 (Figure 4.6). In non-competitive inhibition, an inhibitor binds to an enzyme forming an enzyme-inhibitor complex; whenever a substrate binds to one of these enzyme-inhibitor complexes, it forms an enzyme-inhibitor-substrate complex and is unable to form the final product.

Mixed competitive inhibition was found to be the mode of inhibition of all green tea extracts and EGCG on CYP3A4 (Figure 4.2). The same inhibition mode was also shown in GTME and EGCG on CYP2D6 (Figure 4.4), and GTME, GTWE and EGCG on CYP2C9 (Figure 4.3). For mixed competitive inhibition, an inhibitor binds to an allosteric site of an enzyme and cause changes to the enzyme structure, thereby preventing the substrate from binding to the enzyme. The mixed-competitive inhibition is indicated by a particular pattern in a Michaelis-Menten plot—a decrease of V_{max} and increment of apparent K_m as concentrations of the particular extract or EGCG in the assays increased. On top of that, Lineweaver-Burk plots (Figure 4.2) and Dixon plots further indicated that the inhibition mode involved was mixed competitive inhibition.

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In contrast, uncompetitive inhibition was observed in the inhibition of CYP2C9 with GTHE (Figure 4.3), and the inhibition of CYP2D6 with GTWE (Figure 4.4) as indicated by Michaelis-Menten plots (both V_{max} and apparent K_m decrease), Lineweaver Burk plots (Figure 4.3 and 4.4) and Dixon plots. The characteristic of an un-competitive inhibitor is that it only binds to an enzyme-substrate complex and prevents the enzyme from reacting with substrate.

The effects of these three inhibitions can be lessened but not overcome, by adding more substrates to the assays. It is important to know the mode of inhibition to have a better idea when designing drugs, in order to reduce the side effects from drug-drug interactions. For GTHE, we did not test the mechanism of inhibition because its IC_{50} is higher than 300 μg/mL, indicating that the inhibition potential is negligible.

Table 4.4 Mode of Inhibition of green tea extracts and EGCG on CYP enzymes.

	3A4/BFC	2D6/AMMC	1A2/CEC	2C19/CEC	2C9/MFC
GTME	Mixed competitive	Mixed competitive	Non-competitive	Non-competitive	Mixed competitive
GTWE	Mixed competitive	Un-competitive	Non-competitive	Non-competitive	Mixed-competitive
GTHE	Mixed competitive	***	Non-competitive	Non-competitive	Un-competitive
EGCG	Mixed competitive	Mixed competitive	Non-competitive	Non-competitive	Mixed competitive

***K_i and mode of inhibition were not identified due to high IC_{50} (<300 μg/mL), which indicates that GTHE may not be an inhibitor to CYP2D6.

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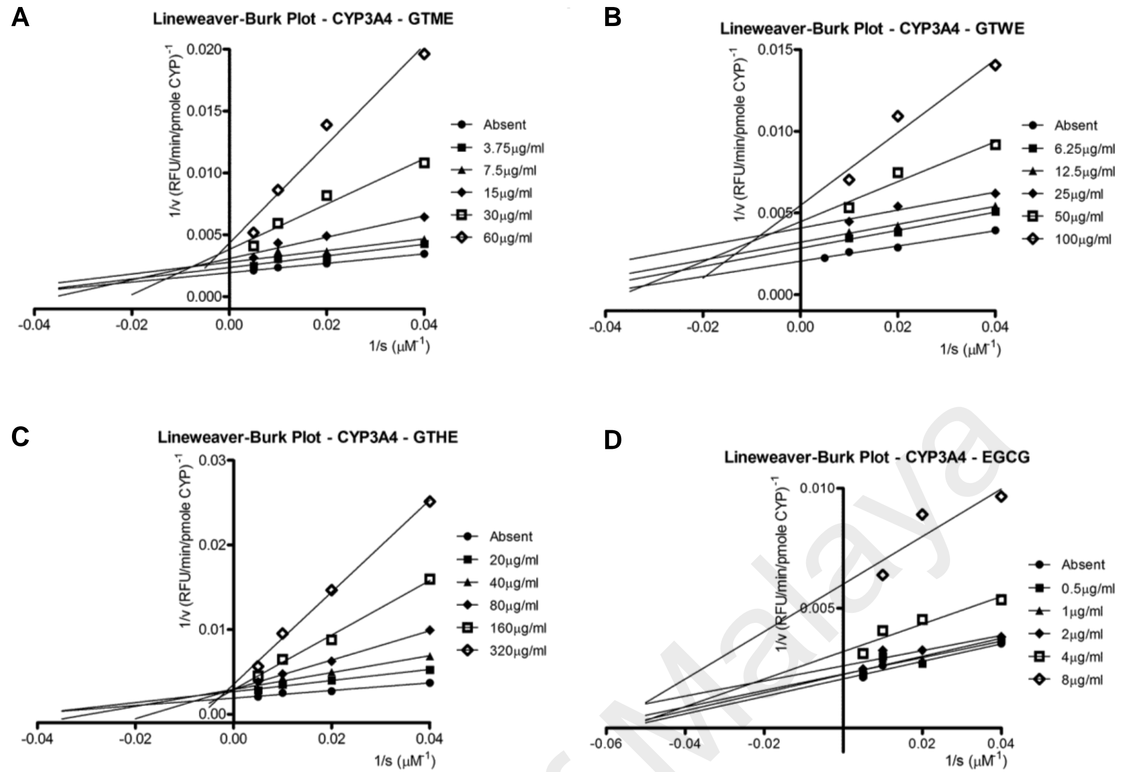

Figure 4.2 Inhibition of CYP3A4 by green tea extracts and EGCG. Lineweaver-Burk plot of the inhibitory effect of GTME (A), GTWE (B), GTHE (C) and EGCG (D) on CYP3A4. Intersection points are found at 2nd quadrant of the graph which depicted mixed competition inhibition happened.

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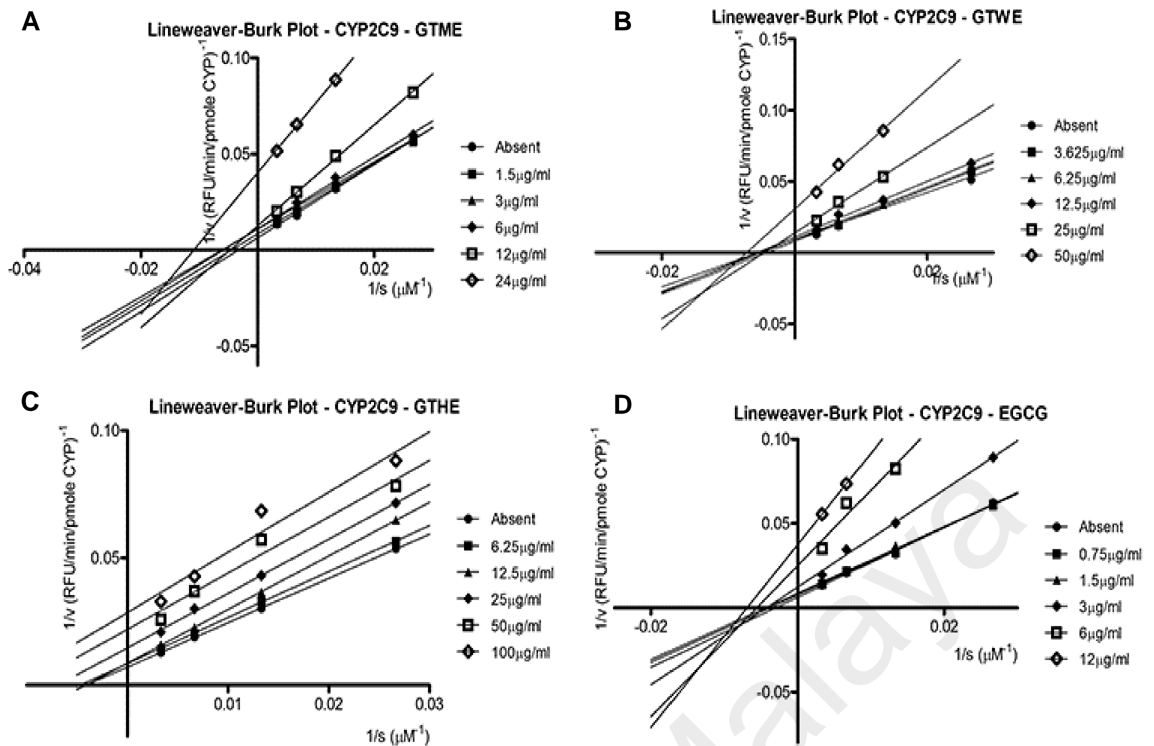

Figure 4.3 Inhibition of CYP2C9 by green tea extracts and EGCG. Lineweaver-Burk plot of the inhibitory effect of GTME (A), GTWE (B), GTHE (C) and EGCG (D) on CYP2C9. Same as Figure 1, intersections points in Figure 2 (A), (B) and (D) intersected at 2nd quadrant, while (C) showed the trend of un-competitive inhibition as no intersection point was shown.

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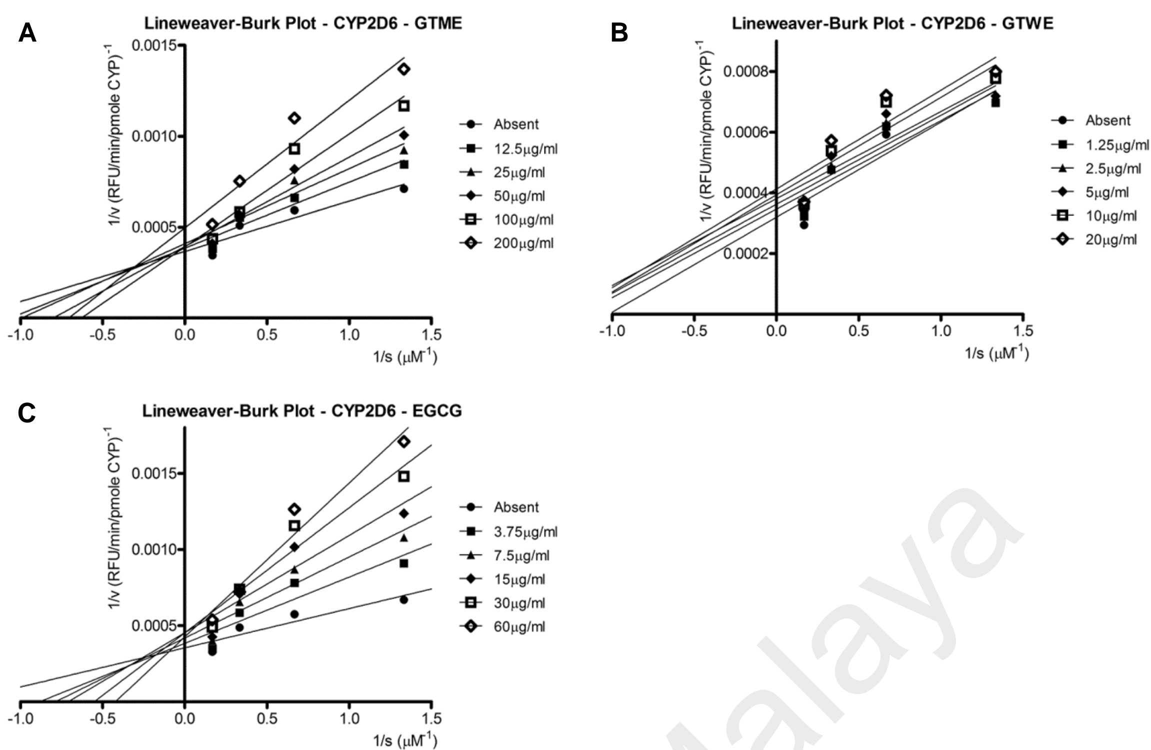

Figure 4.4 Inhibition of CYP2D6 by green tea extracts and EGCG. Lineweaver-Burk plot of the inhibitory effect of GTME (A), GTWE (B) and EGCG (C) on CYP2D6. Treatment of CYP2D6 with GTME (A) and EGCG (C) showed mixed-competitive inhibition while treatment with GTWE (B) demonstrated un-competitive inhibition.

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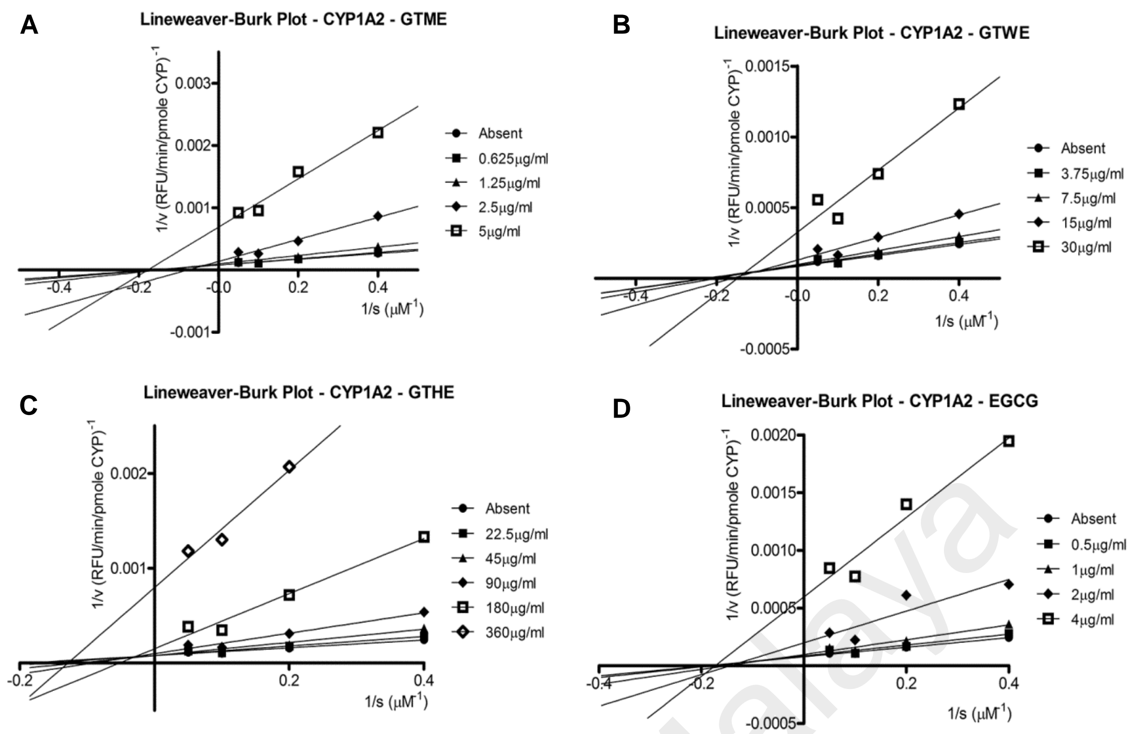

Figure 4.5 Inhibition of CYP1A2 by green tea extracts and EGCG. Lineweaver-Burk plot of the inhibitory effect of GTME (A), GTWE (B), GTHE (C) and EGCG (D) on CYP1A2 showed that all samples demonstrated non-competitive inhibitions.

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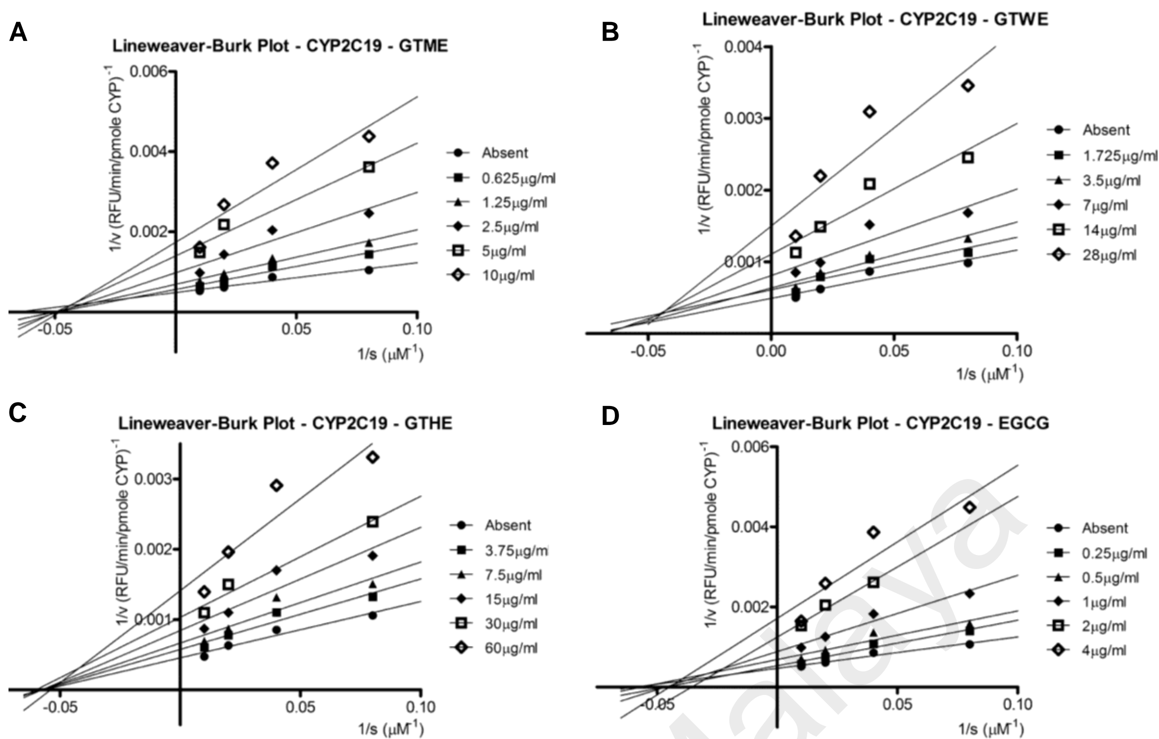

Figure 4.6 Inhibition of CYP2C19 by green tea extracts and EGCG. Lineweaver-Burk plot of the inhibitory effect of GTME (A), GTWE (B), GTHE (C) and EGCG (D) on CYP2C19. Same as Figure 4, all samples exhibited non-competitive inhibitions.

4.5 Discussion

Green tea aqueous extract has previously been found to be the most potent CYP3A4 inhibitor (IC_{50} = 73 µg/mL) (Engdal & Nilsen, 2009). This is not that different from our findings (IC_{50} = 46.7 µg/mL). Furthermore, EGCG effectively inhibited a mutation assay involving *Salmonella typhimurium (S. typhimurium*) TA1538 cells expressing human CYP3A4 (Muto et al., 2001). In addition, green tea aqueous ethanolic extract inhibited CYP3A4 in both *in vitro* and *in vivo* studies (Nishikawa et al., 2004). However, no clinical studies have validated the inhibition of CYP3A4 by green tea extracts and EGCG. With regard to clinical studies, there are two studies of interest. The first study (Donovan et al., 2004) found that decaffeinated green tea had no effect on CYP3A4 activity in 11 healthy volunteers who took it at daily dosages of 504 mg EGCG. However in the second study (Chow et al., 2006), a small reduction in CYP3A4 activity was observed in 42 healthy volunteers who took decaffeinated green tea at daily dosages of 800 mg EGCG (Much higher than Donovan's group). This suggests that the

dosage employed by Donovan's group was insufficient to cause an inhibitory effect on CYP3A4.

The inhibition of CYP3A4 was reported to impair the metabolism of drugs such as irinotecan (Lin et al., 2008; Mirkov et al., 2007) and midazolam (Muto et al., 2001), resulting in an increase of *in vivo* half-life and toxicity. In one study, EGCG was found to increase the bioavailability of tamoxifen (Shin & Choi, 2009). The reduction of CYP3A4-mediated liver and intestinal first-pass metabolism was suggested to be the possible underlying mechanism of action.

Another extensively studied CYP is CYP2D6, which is involved in the metabolism of 25% to 30% of all drugs in clinical practice, making it one of the most important CYPs studied (Nebert & Jorge, 2002). In fact, CYP2D6 was also found to be involved in stereospecific metabolism of several important groups of drugs, such as antiarrhythmics, antidepressants and neuroleptics (Bertilsson, 1995). Although a few studies have shown that EGCG cause no significant inhibition to CYP2D6 (Chow et al., 2006; Donovan et al., 2004), green tea aqueous ethanolic extract was capable of inhibiting CYP2D6 at 50 μg/mL in *in vitro* assay in another study (Nishikawa et al., 2004). And this is in accordance with our own findings (IC_{50} of 56.07 μg/mL).

As a major CYP2C in the liver, CYP2C9 has been shown to be involved in the metabolic clearance of several clinically important drugs such as phenytoin and warfarin (Goldstein, 2001; Lee et al., 2002). For green tea extracts' inhibition on CYP2C9, there was a difference in IC_{50} between our data and that of another group (Nishikawa et al., 2004); ours were lower. This may be due to the different extraction solvents used. In another study, brewed tea inhibited CYP2C9 in *in vitro* studies, though it did not show any effect in clinical studies (Greenblatt et al., 2006).

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CYP2C19 is known to metabolise drugs such as proton pump inhibitors (omeprazole) (Bertilsson, 1995), antidepressants (imipramine, diazepam) (Bertilsson, 1995), anticonvulsants (S-mephenytoin) (Goldstein & de Morais, 1994), hypnosedatives, muscle relaxants, and antimalarial drugs (Desta et al., 2002). To date, no other study has been conducted to investigate the inhibition effects of green tea extracts or EGCG on CYP2C19.

CYP1A2 occupies 15% of total cytochrome enzyme content (Shimada et al., 1994) and metabolises clinically important drugs such clozapine (Bertilsson et al., 1994), theophylline (Ha et al., 1995) and the antidepressant duloxetine (Lobo et al., 2008). Besides that, caffeine is also a substrate for *in vivo* CYP1A2 (Fuhr et al., 2007). Interestingly, EGCG inhibited mutagenic activation in *S. typhimurium* TA1538 cells expressing human CYP1A2 and oxidations catalysed by human CYP1A2 (Muto et al., 2001). However, EGCG did not alter CYP1A2 activities in another study (Chow et al., 2006).

Our findings on CYP2C19 and CYP1A2 inhibition by green tea extracts or EGCG, together with the findings on their mode of inhibition, may provide some preliminary insight into CYP2C19 and CYP1A2 inhibition by EGCG and EGCG-containing products, and ultimately serve as reference data for further investigations.

There were some limitations in this study, one of them is that we could not accurately determine green tea extracts' and EGCG's potency to humans. *In vivo* studies are needed to determine the bioavailability of each compound, and the effects of the said compounds on the CYPs in animals and in humans. In addition, we were unable to compare the green tea extracts that we used with brewed green tea consumed in real life given the varying conditions (ratio of tea leave to water, temperature of water, brewing duration, etc.) tailored to personal preferences. Thus, it is difficult to accurately deduce

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the effect of green tea on humans; clinical studies with a large numbers of volunteers are required for this.

4.6 Conclusion

The CYP inhibition studies revealed the relative inhibition potencies and content of EGCG in each extract. It was suggested that the CYP inhibitory effects are attributed mainly to EGCG. Besides that, our studies also showed green tea extracts' and EGCG's modes of inhibition, yet the actual mechanisms of inhibition remain unknown. More studies especially in *in vivo* settings are needed to narrow down the mechanisms of inhibition. This may help explain the discrepancy between results from the *in vitro* and clinical studies. Furthermore, caution is needed in drawing inferences of clinical drug interactions based on *in vitro* data involving natural products.

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CHAPTER 5: CONCLUSION AND RECOMMENDATION

The study on the physiological and gene expression changes that occur during the development of high fat diet-induced obesity has indicated that green tea and EGCG effectively suppress the development of obesity in mice. Based on our *in vivo* study, there is a high possibility that the gene regulation of obesity development was altered by the treatment with green tea extracts and EGCG. With the data from transcriptome resequencing, we found that metabolic genes are among the genes that are being regulated in HFD, HFD + GTE and HFD + EGCG group. A total of 1024 irregulated genes in HFD suggested that these genes were involved in obesity development. A total of 1892 genes in HFD + GTE and 1817 genes in HFD + EGCG were irregulated in these samples, and these suggested the genes that were involved in prevention of obesity. Further studies can be done in verifying the results through qPCR and western blotting.

The CYP inhibition studies revealed the relative inhibition potencies and the content of EGCG in each extract. The results also suggest the CYP inhibitory effects are mainly attributed to EGCG. Our studies showed the modes of enzyme inhibition of green tea extracts and EGCG, but the actual mechanisms of inhibition remain unknown. More studies especially in *in vivo* settings are needed to confirm the exact mechanisms of inhibition. The results from such studies may explain the discrepancies in the results obtained from *in vitro* studies and clinical studies. Furthermore, caution is needed in extrapolation of the results to clinical drug interactions based on *in vitro* data involving natural products.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Conference attended: Oral presentation at 26th Scientific Meeting of Malaysian Society of Pharmacology and Physiology (MSPP) 2012

Manuscript submitted:

Mei-Fong Ng, Lik-Voon Kiew, Wai-Mun Kong, Yan Pan, Umarani Subramaniam, Zahurin Mohamed. Evaluation of the effects of green tea extracts and its active component epigallocatechin gallate on cytochrome P450 enzymes using a high throughput assay.

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