Hypocholesterolemic, Antioxidative and Estrogenic Effects of Soybean Isoflavones

LEE Chung-hung
B. Sc. (Hons) in Biochemistry, CUHK

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Abstract

Cardiovascular heart disease (CHD) is a major health problem worldwide. Epidemiological studies show a direct association of CHD with levels of serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) but an inverse association with high-density lipoprotein cholesterol (HDL-C). Growing evidence shows that consumption of soy foods reduces risk of CHD. Isoflavones and their glycosides, which are present in soybean, might be responsible for this health benefit. However, up to date, the mechanisms by which the soybean isoflavones have such health benefit are poorly understood.

In the present study, the chemical composition and some health benefits of soybean isoflavones and their glycosides were investigated. By using column chromatography, high performance liquid chromatography (HPLC) and mass spectrometry (MS), seven different soybean isoflavones and their glycosides were successfully isolated and purified from soybeans.

The hypocholesterolemic effect of soymilk was firstly examined. Hamsters were divided into 3 groups. They were all fed a 0.1% cholesterol diet but they drank either water (W) or soybean milk (SM) or cow's milk (CM) as the drinking fluid. Serum TC, serum triglyceride (TG), HDL-C and non- HDL-C were measured. It was found that the SM group had lowest levels of TC, TG, non-HDL-C among the

three groups. The data suggest that soymilk has the hypocholesterolemic activity at least in hamsters.

Another experiment used ovariectomized hamsters as an animal model in determining the estrogenic property of soybean isoflavones. Ovariectomization leads to a significant elevation of serum TC because LDL receptor activity is drastically decreased in little or absence of estrogen. An oral dose of soymilk (OVX-SM) or soybean extract (OVX-SE) had a favorable effect on the serum lipoprotein profile. Both soymilk and soybean extract could significant decrease serum TC and non-HDL-C, leading to a decrease in the ratio of non-HDL-C to HDL-C, in ovariectomized hamsters. In addition, consumption of soymilk and soybean extract was associated with a decrease in serum TG. However, soymilk and soybean extract had no effect on the concentration of cholesterol in the liver and other tissues in these hamsters. These results showed that the hypocholesterolemic effect of soymilk and soybean extract may attributable to the estrogenic activity of the soybean isoflavones.

Soybean isoflavones possessed antioxidant activity. The Cu^{2+}- mediated human LDL oxidation, ferric reducing ability of plasma (FRAP), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays, were used to assess the antioxidative effects of individual soybean isoflavones and their glycosides. The seven

individual soybean isoflavones and their glycosides showed a weaker antioxidant activity in Cu^{2+}-mediated LDL oxidation compared with a major tea antioxidant, epicatechin. A similar result was also observed in the FRAP assay. For the DPPH assay, the seven soybean isoflavones and their glycosides also were less effective in free radical-scavenging than α-tocopherol. The present results clearly demonstrated that individual soybean isoflavones and their glycosides possessed antioxidant activity but the potency was weaker compared with that of epicatechin and α-tocopherol.

In conclusion, soymilk may serve as a health beverage to treat the hyperlipidemia patients, especially for the postmenopausal women and individuals whose cholesterol level is marginally high not to warrant the prescription of cholesterol lowering drugs.

摘要

心血管疾病是世界範圍的主要疾病。流行病學研究指出心血管疾病與血清總膽固醇和低密度脂蛋白膽固醇的水平成正比關係，而與高密度脂蛋白膽固醇的濃度成反比關係。食用豆製品食物有利於防治心血管疾病。大豆內的異黃酮(isoflavones)及其之糖甙，是防治心血管疾病的主要成份之一，到目前爲止，有關異黃酮對於防治心血管疾病的機理仍在研究當中。

在本研究中，對大豆異黃酮及其糖甙的化學成份和藥理活性進行了研究。通過提取分離高效液相制備，及質譜分析，從大豆內分離純化了七個異黃酮及其糖甙。

在豆奶降血脂的研究中，實驗蒼鼠分成三組，0.1%膽固醇的飼料分別配以水，豆奶及牛奶三種不同的飲料來飼養牠們。研究結果發現在豆奶蒼鼠組中，牠們的總膽固醇，血清甘油三脂及非高密度脂蛋白膽固醇爲最低的一組。結果証明豆奶能有效降低血脂。

此外，我們利用卵巢切除雌性蒼鼠作爲實驗模型研究大豆異黃酮的雌激素作用。卵巢切除導致體內雌激素減少，低密度脂蛋白受體活性降低，體內血清總膽固醇及甘油三脂升高。實驗以灌胃的方法給予蒼鼠豆奶 (OVX-SM) 及大豆提取物 (OVX-SE)。研究結果表明豆奶和大豆提取物都能顯著降低血清總膽固醇及非高密度脂蛋白膽固醇，從而減少牠們的非高密度脂蛋白和高密度脂蛋白膽固醇的比例。同時豆奶和大豆提取物可以降低血清中的三脂甘油。對於肝臟及

其他組織中的膽固醇含量則沒有影響。以上結果表明豆奶和大豆提取物的雌激素作用具有降低膽固醇的生理活性。

在測試從大豆分離出來的七種異黃酮及其糖甙抗氧化活性的實驗中，利用了銅離子誘導的低密度脂蛋白氧化和鐵還原這兩種方法進行測試。結果顯示它們的抗氧化能力弱於茶葉中的兒茶素。自由基清除實驗表明這七種異黃酮及其糖甙的自由基清除能力弱於維生素 E。由此証明大豆異黃酮及其糖甙具有抗氧化活性，但是與兒茶素及維生素 E 相比，它們的抗氧化能力較弱。

研究表明，豆奶可以作爲一種健康飲料用於治療高血脂患者，尤其適合更年期婦女及其膽固醇偏高的人群。

List of Abbreviations

AD	Alzheimer's disease
ALP	alkaline phosphatase activity
ANOVA	analysis of variance
APCI-MS	atmospheric pressure chemical ionization- mass spectrometry
Aβ	amyloid β protein
CE	cholesterol ester
CHD	coronary heart disease
CM	cow's milk
CTL	control
DMBA	7, 12-dimethylbenz[a]anthracene
DPPH	1,1-diphenyl-2-picrylhydrazyl
EFG	epidermal growth factor
ER	estrogen receptor
ERT	estrogen replacement therapy
ESR	electron spin resonance
FRAP	ferric reducing ability of plasma
GC	gas chromatography
GLC	gas liquid chromatography
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
HPLC	high performance liquid chromatography
HPLC-MS	HPLC-mass spectrometry

IDL	intermediate-density lipoprotein
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
MDA	malondialdehyde
MS	mass spectrometry
NMR	nuclear magnetic resonance
Non-HDL	non high-density lipoprotein
Non-HDL-C	non high-density lipoprotein cholesterol
OVX	ovariectomy
Ox-LDL	oxidized LDL
PTK	protein tyrosine kinase
PUFA	polyunsaturated fatty acid
SD	standard deviation
SE	soybean extract
SM	soymilk
SMC	smooth muscle cell
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TC	total cholesterol
TG	triglyceride
TMS	trimethylsilyl
TPA	12-*O*-tetradecanoyl phorbol-13-acetate
UV	ultraviolet
VEGF	vascular endothelial growth factor
VLDL	very low-density lipoprotein

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Chapter 1
General Introduction

1.1 History of soybean

The history of soybean (*Glycine max* L. Merr.) began over five thousand years ago on the windy plains of eastern Asia. According to the Chinese tradition, soybean was considered the most important cultivated legume and also was one of the "Wu Ku" or the five sacred crops named by Chinese emperor Sheng-Nung. Historians maintained that Sheng-Nung mentioned soybean in *Ben Tsao Gang Mu*, written in the year 2838 B.C. Others think soybean may have come on the scene a bit later, by 300 B.C. Soybean and millet were always mentioned in ancient texts as the two major food crops in northern China.

Once the soybean was cultivated by Chinese farmers, it spread gradually from the northern part to the southern part of China, then it spread into Korea, Japan, and Southeast Asia. Its spread to Japan was the eighth century A.D. It took one thousand years before it traveled west to Europe.

Soybean yields more usable protein per acre than does any other crop and is far more inexpensive protein than do animal foods. In China, with its shortage of pastureland and arable farmland, nutrient-rich soybean is an ideal food. Also, the Buddhist commitment to vegetarianism has led monks to create soy-based meat

substitutes.

Soybeans have revolutionized the diets of Asian countries. Inventive cooks have produced an amazing variety of soy products. The liquid squeezed from soaked soybeans has been used by elderly Chinese as an alternative to tea and has also been consumed by nursing mothers to stimulate milk production. In more recent years, this soymilk has begun to mirror cow's milk. In some Chinese cities, factories make early morning soymilk delivery to homes. And not too long ago, in Hong Kong, soymilk outsold Coca-Cola.

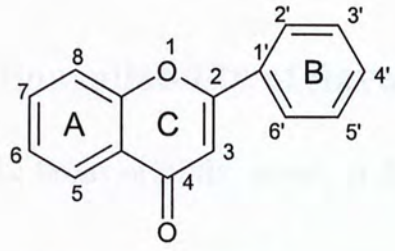
1.2 Health benefits of soybean

Recently, the health effects of soybean have been widely investigated. Epidemiological studies indicate that consumption of tofu and other soy foods is associated with the low incidence of breast cancer in Japanese women (Adlercreutz *et al.,* 1991; Adlercreutz, 1998). This discovery has led numerous researchers in recent years to search for the biochemical components in soybean that are responsible for the cancer risk-lowering effect. It is generally believed that isoflavones present in soybean are the active ingredients attributive to such benefit. Aside from the potential cancer prevention effect (Kennedy, 1998; Lamartiniere *et al.,* 1998; Thiagarajan *et al.,* 1998; Wei *et al.,* 1998; Wu *et al.,* 1998; Pollard and

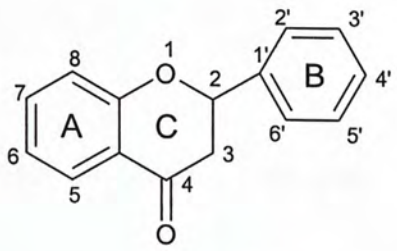
Wolter, 2000), isoflavones have also been found to have other potential health benefit, including heart disease prevention (Huff *et al.,* 1982; Wilcox *et al.,* 1995; Anthony *et al.,* 1996; Balmir *et al.,* 1996; Anthony *et al.,* 1998; Nilausen and Meinertz, 1998; Palacio *et al.,* 1998; Potter *et al.,* 1998a and 1998b; Wilson *et al.,* 1998; Wong *et al.,* 1998; Anderson *et al.,* 1999; Ho *et al.,* 2000 and Merz-Demlow *et al.,* 2000), osteoporosis reduction (Alekel *et al.,* 1998; Arjmandi *et al.,* 1998; Williams *et al.,* 1998) and alleviation of postmenopausal syndromes (Knight and Eden, 1996).

1.3 Introduction to flavonoids

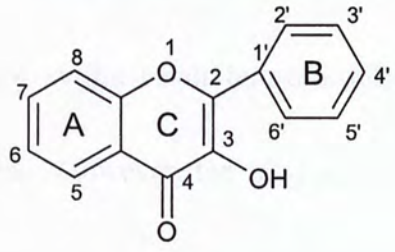
Flavonoids are distributed widely in plant foods such as vegetables and fruits. They possess a unique C6-C3-C6 structure (diphenylpropane structure) with varying number of phenolic OH groups. More than 4000 different flavonoids have been identified. Flavonoids can be classified as various subgroups including flavonols, flavones, flavanones, and isoflavones. All of them are structurally related to the parent compound, flavone (2-phenylbenzopyrone) (Figure 1.1).



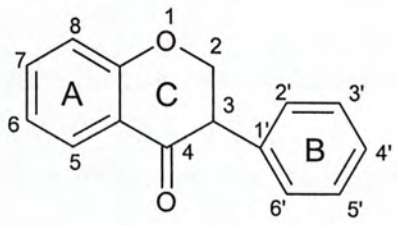
Flavone



Flavanone



Flavonol



Isoflavone

Figure 1.1 Chemical structures of some naturally occurring flavonoids.

	Substituent(s) at position:							
Flavonoid	5	6	7	8	2'	3'	4'	5'
Flavones								
Apigenin	OH		OH				OH	
Chrysin	OH		OH					
Tangeretin	OCH_3	OCH_3	OCH_3	OCH_3		OCH_3	OCH_3	
Nobiletin	OCH_3	OCH_3	OCH_3	OCH_3			OCH_3	OCH_3
Flavonols								
Kaempferol	OH		OH				OH	
Quercetin	OH		OH			OH	OH	
Galangin	OH		OH					
Fisetin			OH			OH	OH	
Myricetin	OH		OH			OH	OH	OH
Morin	OH		OH		OH		OH	
Flavanones								
Naringenin	OH		OH			OH		
Hesperetin	OH		OH			OH	OCH_3	
Isoflavones								
Biochanin A	OH		OH			OCH_3		
Genistein	OH		OH			OH		
Daidzein			OH			OH		

1.4 Bioavailability of flavonoids

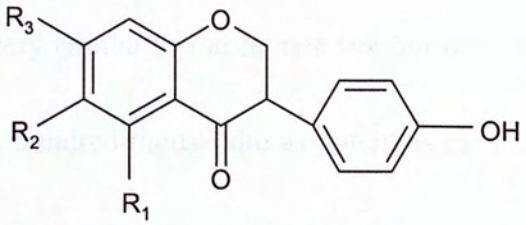
The behavior of flavonoids in the digestive tract still remains equivocal. The absorption and subsequent distribution, metabolism and excretion of flavonoids in humans are poorly understood. Flavonoid glycosides are likely to be hardly absorbed in the small intestine because their hydrophilicity lowers solubility in micelles. However, the β-glucosidase activity of microflora induces the hydrolysis of glycosides, resulting in aglycone in the large intestine (Tamura *et al.,* 1980). After such a conversion, flavonoids elevate lipophilicity, resulting in high solubility in bile acid micelles. A study in rats showed that absorption of rutin (quercetin-3-rutinoside) was slower than that of aglycone and quercetin (Manach *et al.*, 1997). This phenomenon was explained by the idea that hydrolysis in the large intestine was required for the absorption of rutin. However, Hollman *et al.* (1997) demonstrated that, from an experiment involving healthy ileostomy volunteers whose quercetin source resulted from the intake of quercetin glucoside-rich onion, quercetin glucosides were absorbed more easily than aglycone. His group also found that the quercetin (including metabolites and glycosides) level reached a maximum of 0.6 μM after the intake of fried onion (corresponding to 64 mg quercetin) in 2.9 hr (Hollman *et al.*, 1996). Paganga and Rice-Evans (1997) claimed that plasma from nonsupplemented humans contained quercetin and quercetin glycosides at the level of 0.5-1.6 μM. Andlauer *et al.* (2000)

showed that genistin and its glycoside, was partly absorbed without previous cleavage of the β-glycosidic bond. This observation was further confirmed in the study of Paganga and Rice-Evan (1997), who found the glycosides of quercetin was present in human plasma (Paganga and Rice-Evans, 1997). These studies indicated that flavonoid glycosides may be able to enter the human serum and exert their effect directly.

1.5 Chemistry of isoflavones

Isoflavones are a type of natural flavonoid and possess estrogen-like properties; hence they are sometimes referred to as phytoestrogens (Figure 1.2). They would likely be metabolized in relativity similar manners as other flavonoids, and therefore, may have similar health effects (Lee *et al.,* 1995; Levy *et al.,* 1984; Akiyama *et al.,* 1987; Adlercreutz *et al.,* 1993; Wang *et al.,* 1994).

In contrast to flavones, which exist widely in the plant kingdom, isoflavones are found predominately in a limited number of plants, such as legumes, particularly soybeans. Soy foods are the most important source of dietary isoflavones. Isoflavones are not essential nutrients, but they seem to play an important role in health maintenance.



	R_1	R_2	R_3
Genistein	OH	H	OH
Genistin	OH	H	O-Glucose
Daidzein	H	H	OH
Daidzin	H	H	O-Glucose
Glycitein	H	OCH_3	OH
Glycitin	H	OCH_3	O-Glucose

Figure 1.2 The chemical structure of soybean isoflavones.

1.6 Estrogenic property of isoflavones

Isoflavones look very similar to the female sex hormone estrogen. However, they are only about one hundred-thousandth as potent as the natural estrogen that is present in the blood (Farmakalidis *et al.,* 1985). But even though they are weak, they can interfere with the action of the more powerful natural estrogen in several important ways.

Isoflavones can compete the same receptors with estrogen. But when they bind to these receptors, they do not produce much of an effect because they are so weak. By competing with estrogen for the limited number of receptors, the isoflavones prevent estrogen from binding to these receptors. (Peterson and Barnes, 1991; Peterson and Barnes, 1993; Tang and Adams, 1980). So in the case of women, who normally produce a lot of estrogen, the isoflavones can actually act as antiestrogens.

1.7 Nutritional significance of isoflavones and their glycosides

There are many excellent reviews addressing the potential benefits of consuming diets containing isoflavones and their glycosides from soy foods (Messina *et al.,* 1994; Knight and Eden, 1996; Adlercreutz and Mazur, 1997; Kurzer and Xu, 1997; Bingham *et al.,* 1998; Humfrey, 1998; Setchell, 1998; Setchell and

Cassidy, 1999). These potential beneficial effects include being anticarcinogenic, antioxidative, cardioprotective, osteoprotective, neuroprotective and antiangiogenic.

1.7.1 Anticarcinogenic activity

Several studies showed that the isoflavones had the anticarcinogenic activity (Peterson and Barnes, 1996; Wei *et al.,* 1998; Denis *et al.,* 1999; Griffiths *et al.,* 1999; Dimas *et al.,* 2000; Mukhtar and Ahmad, 2000; Steele *et al.,* 2000). Epidemiologic reports have associated soy isoflavones with a reduced incidence of breast and prostate cancers (Lee *et al.,* 1991; Messina *et al.,* 1994; Wu *et al.,* 1998).

Genistein is one of major isoflavones in soybean. Reports on genistein's ability to inhibit tyrosine kinases (Akiyama *et al.,* 1987), angiogenesis (Fotsis *et al.,* 1993), topoisomerase II (Okura *et al.,* 1988), diacylglycerol synthesis (Dean *et al.,* 1989), lipid peroxidation (Jha *et al.,* 1985), platelet-activating factor and epidermal growth factor–induced expression of c-fos (Tripathi *et al.,* 1992), as well as genistein's antioxidant property (Wei *et al.,* 1995), support the claim that genistein possesses anticancer activity. Barnes (1995a) showed that genistein decreased development of breast tumors in hamsters and slowed the growth of human breast cancer cells *in vitro*. Genistein inhibited both estrogen and growth factor-stimulated proliferation of human breast cancer lines MCF-7, T47D ER^+ and

T47D ER^- in culture with IC_{50} values ranging from 7.0 to 9.4 μM/ mL (Peterson and Barnes, 1996). Breast cancer growth is regulated by estrogen and peptide growth factors, such as epidermal growth factor (EGF), the receptor of which has intrinsic protein tyrosine kinase (PTK) activity. Akiyama *et al.* (1987) showed that genistein could act as a specific inhibitor of PTK. Therefore, genistein may block mammary epithelial cell growth by interfering with signal transduction events stimulated by estradiol or growth factors. Setchell *et al.* (1984) suggested that genistein might inhibit tumor cell growth by an antiestrogenic mechanism through competition with E_2 for occupancy of estrogen receptor (ER).

Genistein was also shown to inhibit 7, 12-dimethylbenz[a]anthracene (DMBA) -induced and 12-O-tetradecanoyl phorbol-13-acetate (TPA)-promoted skin carcinogenesis in mice (Wei *et al.,* 1998).

1.7.2 Antioxidative activity

Free radicals are chemical species, which have unpaired electrons. They can induce oxidation of macromolecules such as lipids, proteins and even DNA single-strand breakage. The damage to biosystem is one of the major processes that contribute to degenerative diseases such as cancer, cardiovascular disease and aging.

Antioxidant is a compound which is capable of inhibiting oxygen-mediated oxidation of diverse substrates, from simple molecules to polymers and complex biosystem. There are two types of antioxidants in general. The first type inhibits formation of free radicals, which may initiate oxidation. In most cases they are chelators of metal ions. The second type inhibits free-radicals chain-propagation reactions.

The antioxidative activity of isoflavones may be related to the phenol hydroxyl groups (Figure 1.3). Phenol hydroxyl groups can scavenge free radicals both *in vivo* and *in vitro*. Generally, the more hydroxyl groups, the greater the antioxidant capacity. Cao *et al.* (1997) suggested that these groups were the chemical basis for the antioxidant property of isoflavones. The reduction potential of these groups is influenced by the number and their positions on the molecule. Noroozi *et al.* (1998) showed that the aglycons, quercetin, kaempferol, luteolin and myricetin, had a greater antioxidative capacity than do the conjugate flavonoids. The high reductive capacity of kaempferol in Electron Spin Resonance (ESR) and Fluorescence Recovery After Photobleaching (FRAP) assay may be due to the presence of the 4'-OH on the B-ring and the 3-OH on the C-ring (Figure 1.1), which are connected via the π-orbital system and interact synergistically.

The antioxidative activities of these flavonoid compounds may change as a

result of solvent effects and pH, which can influence the state of protonation or deprotonation of the hydroxyl groups. Therefore, the antioxidant activity of isoflavones may differ significantly in various model systems depending on the reduction potentials of the hydroxyl moieties relative to that of the oxidizing radical, or transition metal ion, used in the system (Mitchell *et al.,* 1998).

There are many reports discussing the antioxidant activity of isoflavones. Some studies showed that flavonoids could inhibit LDL oxidation (Aviram and Fuhrman, 1998; Kerry and Abbey, 1998; Zhu *et al.,* 1999, 2000). Kirk *et al.* (1998) reported that low-density lipoprotein (LDL) oxidation-mediated by copper ion or free radicals was prevented by genistein and daidzein in a concentration-dependent manner.

Apart from the antioxidative ability of isoflavones in LDL, Wei *et al.* (1995) showed that genistein and, to a lesser extent, daidzein inhibited H_2O_2 formation. Also, various isoflavones were able to suppress $O_2^{-}\bullet$ production by xanthine/ xanthine oxidase. Genistein (20 μmol/ L) almost completely inhibited the production of $O_2^{-}\bullet$, while daidzein inhibited it by 80% at the same concentration. Genistein can also prevent some of the damage to cells caused by H_2O_2. Genistein, like the hydroxyl scavenger dimethylthiourea, could reduce the induction of adenylyl cyclase activity by H_2O_2 in A10 cells (a murine vascular smooth muscle

cell line) (Tan *et al.,* 1995) and prevented haemolysis of sheep red blood cells by dialuric acid or H_2O_2 *in vitro* (Pratt *et al.,* 1981).

Another report showed that genistein and daidzein could inhibit sister-chromatid exchanges of bone marrow cells and DNA adduct formation in mouse liver induced by 7,12-dimthylbenz[*a*]anthracene, a carcinogen (Giri and Lu, 1995).

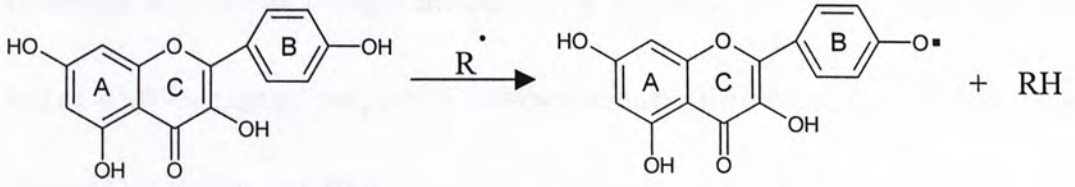


Figure 1.3 Free radical ($R^{\cdot}$) scavenged by flavonoids

1.7.3 Cardioprotective activity

The first epidemiological evidence that the intake of antioxidant isoflavones reduced the rate of coronary heart disease mortality was reported in 1993 for elderly Dutch men who were followed for five years (Hertog *et al.,* 1993a). Other epidemiological data also suggested that dietary isoflavones intake was inversely associated with mortality from coronary heart disease (Arai *et al.,* 2000; Ho *et al.,* 2000).

Evidence for an independent effect of isoflavones on blood cholesterol

concentrations has been demonstrated in rats, hamsters, nonhuman primates and humans (Anthony *et al.,* 1996; Balmir *et al.,* 1996; Cassidy *et al.,* 1995; Pelletier *et al.,* 1995). LDL oxidation is a critical event in atherogenesis; it promotes the formation of foam cells and proliferation of smooth muscle cells. This suggests that suppression of LDL oxidation by isoflavones may help prevent atherosclerosis. This claim is supported by recent reports indicating that isoflavones could reduce the levels of blood lipids including total cholesterol, LDL and apolipoprotein B (Anthony *et al.,* 1996, 1998; Merz-Demlow *et al.,* 2000). These effects may be due to the weak estrogenic property of soy isoflavones (Kuiper *et al.,* 1998), that share the LDL reducing and HDL increasing effect with human estrogen (Lilley *et al.,* 1998; Westerveld, 1998; Godsland, 2001).

1.7.4 Osteoprotective activity

Several studies have provided convincing data of the significant improvement of bone mass or other endpoints following soy feeding in experimental animals. Studies of bone mass in animal models support a biphasic effect of soy isoflavones on bone retention, with lower doses having improved skeletal retention of bone mass while high doses produce less benefit (Anderson *et al.,* 1998).

Arjmandi *et al.* (1996) showed that soy or isoflavone-enriched extracts improve

bone mass. Bone mass of ovariectomized rats fed soy protein compared to rats fed casein was significantly increased. Arjmandi *et al* (1998a,b) showed soy isoflavones could significantly increase the bone density of ovarian hormone-deficient rats. They suggested that the serum alkaline phosphatase activity (ALP), an index of bone formation, tended to be higher in soy-fed animals, suggesting a positive effect on bone formation. The proposed mechanism is that genistein could act as an inhibitor of tyrosine kinases (Akiyama *et al.,* 1987), which could directly modulate osteoclastic acid secretion. These acids secreted onto the bone matrix can dissolve the bone mineral. This process is under complex control related to the maintenance of serum calcium activity and bone strength. Thus, substances influencing bone resorption may be a potential antiosteoporotic agent (Williams *et al.,* 1998).

1.7.5 Neuroprotective activity

One of the first compelling pieces of evidence that postmenopausal estrogen replacement might protect against Alzheimer's disease (AD) in elderly women was an epidemiological study conducted by Tang *et al.* (1996), who showed a high correlation between estrogen use and lowered incidence of AD. In related studies, animal experiments demonstrated that ovariectomy-induced estrogen-loss without

replacement resulted in a measurable loss of cognitive function, that could be essentially prevented by estrogen-replacement (Green and Simpkins, 2000).

Amyloid β protein (Aβ), the major protein component of senile plaque, can elicit a toxic effect on neurons. It has been suggested to play an important role in pathogenesis of AD. Xu *et al.* (1998) demonstrated that estrogen inhibited Aβ generation in cultured neurons. The weak estrogenic property of soy isoflavones might mimick the effects of estrogen in neuroprotective actions.

1.7.6 Antiangiogenic activity

Angiogenesis, the generation of new capillaries, is virtually absent in the healthy adult organism and is restricted to a few conditions including wound healing and the formation of corpus luteum, endometrium, and placenta. However, if angiogenesis is not tightly regulated, it is obligatory for the growth and progression of solid cancers. Fotsis *et al.* (1993) showed that genistein was a potent inhibitor of endothelial cell proliferation. Another studies showed that flavonoid might inhibit angiogenesis through the inhibition on the secretion of a primary angiogenic cytokine, vascular endothelial growth factor, by human prostate and breast cancer epithelial cells (Jiang *et al.*, 2000). Therefore, flavonoids may inhibit the growth and progression of solid cancers through the inhibitory effect of angiogenesis.

Chapter 2
Composition of Soybean Isoflavones

2.1 Introduction

Flavonoids and their glycosides are compounds, which are widely distributed in fruits, vegetables and nuts. Naturally occurring flavonoids are classified as flavones, flavonols, flavanones, isoflavones and catechins. They are structurally related to the parent compound, flavone (2-phenylbenopyrone). The possibility that they may play a role in the prevention of several diseases, including cancer (Akiyama *et al.,* 1987; Fotsis *et al.,* 1993 and Peterson and Barnes, 1996), cardiovascular disease (Anthony *et al.,* 1998), and osteoporosis (Arjmandii *et al.,* 1998), is receiving great attention. Soy isoflavones are of particular interest. Although there have been previous studies on soybeans (Carroll *et al.,* 1978; Potter *et al.,* 1993), only recently has more comprehensive information begun to accumulate about the soy isoflavones in humans (Xu *et al.,* 1994; Tew *et al.,* 1996) and in experimental animals (Supko *et al.,* 1995; King *et al.,* 1996). In order to examine the possible roles of soy isoflavones in human health, it is important to separate and identify the different isoflavones and their glycosides in soybeans.

Careful analysis of soy foods with reversed phase HPLC-mass spectrometry (HPLC-MS) and extraction protocols in the previous studies showed that most soy

foods contained a mixture of isoflavones and their glycosides (Barnes *et al.,* 1994; Wang *et al.,* 1994). The composition of isoflavones in soy foods was thought to be largely determined by the commercial processing of soybeans food products.

2.2 Objective

The present study was to isolate and purify the isoflavones and their glycosides from soybeans and to quantify the isoflavones in 19 traditional Chinese foods.

2.3 Materials and Methods

2.3.1 Extraction and isolation

2.3.1.1 Preparation of soybean butanol extract

Dried soybean powders (3 kg) were extracted with 18 liters of 70% ethanol three times at 60 ℃. Ethanol was evaporated under vacuum in a rotary evaporator to yield 1550 g of ethanol extract, which was then dissolved in 2 liters of distilled water. Then the ethanol extract was partitioned with chloroform in a ratio of 1:1 for three times. The chloroform fraction was dried down under vacuum to yield 298 g of chloroform extract. The remaining ethanol extract was further partitioned with butanol in a ratio of 1:1 for three times. The butanol and water fractions were dried down under vacuum to yield 78 g of butanol extract and 1080 g of water extract.

2.3.1.2 Preparation of isoflavones and their glycosides from soybean butanol extract

In brief, 78 g of soybean butanol extract was fractionated in a column packed with Sephadex LH-20 (100 μM, Pharmacia Fine Chemical Co., Ltd., Germany) and eluted with 5 liters of 70% ethanol (250 mL each time) to produce twenty fractions (F1 to F20). Fractions F1 to F6 and F17 to F20 contained mainly sugars, organic acids and some other components that had no UV absorption, while F7 - F16

contained mainly a mixture of glycosides. The total weight of the fractions F7 to F16 was 32 g. Each fraction of F7- F16 was then loaded onto a Sephadex LH-20 column. Pure ethanol was used to elute the column. The elutes were monitored using a high performance liquid chromatography (HPLC) equipped with a UV detector. These fractions containing isoflavones or glycosides were then loaded onto a C18 HPLC preparative column (Hypersil ODS, 250 x 22mm, 10 μm, Alltech, Deerfield, IL, USA) and eluted with 10% acetonitrile in 1% acetic acid solution, leading to purification of eight compounds. These glycosides were identified using their ultraviolet (UV) and mass spectrophotometry (MS). The purity of these flavonoid glycosides and genistin was greater than 95%, based on HPLC analysis.

2.3.2 HPLC analysis

2.3.2.1 Sample preparation for the HPLC analysis

All wet samples were freeze-dried before the HPLC analysis. One gram of dried, finely ground samples was placed in a 100 mL conical flask containing 40 mL of methanol, 20 mL of 0.1 N HCl and 0.2 mL of flavone (11.25 mM in methanol). The mixture was sonicated for 10 minutes and then stirred at room temperature for 2 hours using a magnetic stirrer. Extractants were centrifuged at 850 g for 10 minutes. The supernatant of sample was filtered through a 0.45 μm PTEE filter

[poly(tetrafluoroethylene), Alltech Associates Inc., Deerfield, IL, USA]. The supernatant was transferred into a 1.5 mL sample vial (Hewlett Packard, Palo Alto, CA, USA) and then subjected to HPLC analysis.

2.3.2.2 HPLC analysis

All the isoflavones and their glycosides were analyzed using a Hewlett Packard series 1100 HPLC (Hewlett Packard, palo alto, CA, USA) equipped with a binary pump delivery system (G1322A) and a diode array detector (G1946A).

In brief, 10 μL of the supernatant was loaded onto a C18 column (Hypersil ODS, 4.6 x 250 mm, 5 μM, Waters, Ireland) through an autosampler (G1313A). The diode array detector was set from 200 to 400 nm, and the eluting components were monitored at 260 nm. The quantitative analysis of the isoflavones and their glycosides were carried out according to the method of Wang and Murphy (1994) with some modifications. The mobile phase consisted of 1% acetic acid in water (v/v) (solvent A) and acetonitrile (solvent B). After injection of the sample, solvent B was increased from 10 to 20% in 40 min and then increased from 20 to 100% in the next 30 min. The flow rate was maintained at 0.8 mL/ min. The typical HPLC chromatogram of soybean was shown in Figures 2.1.

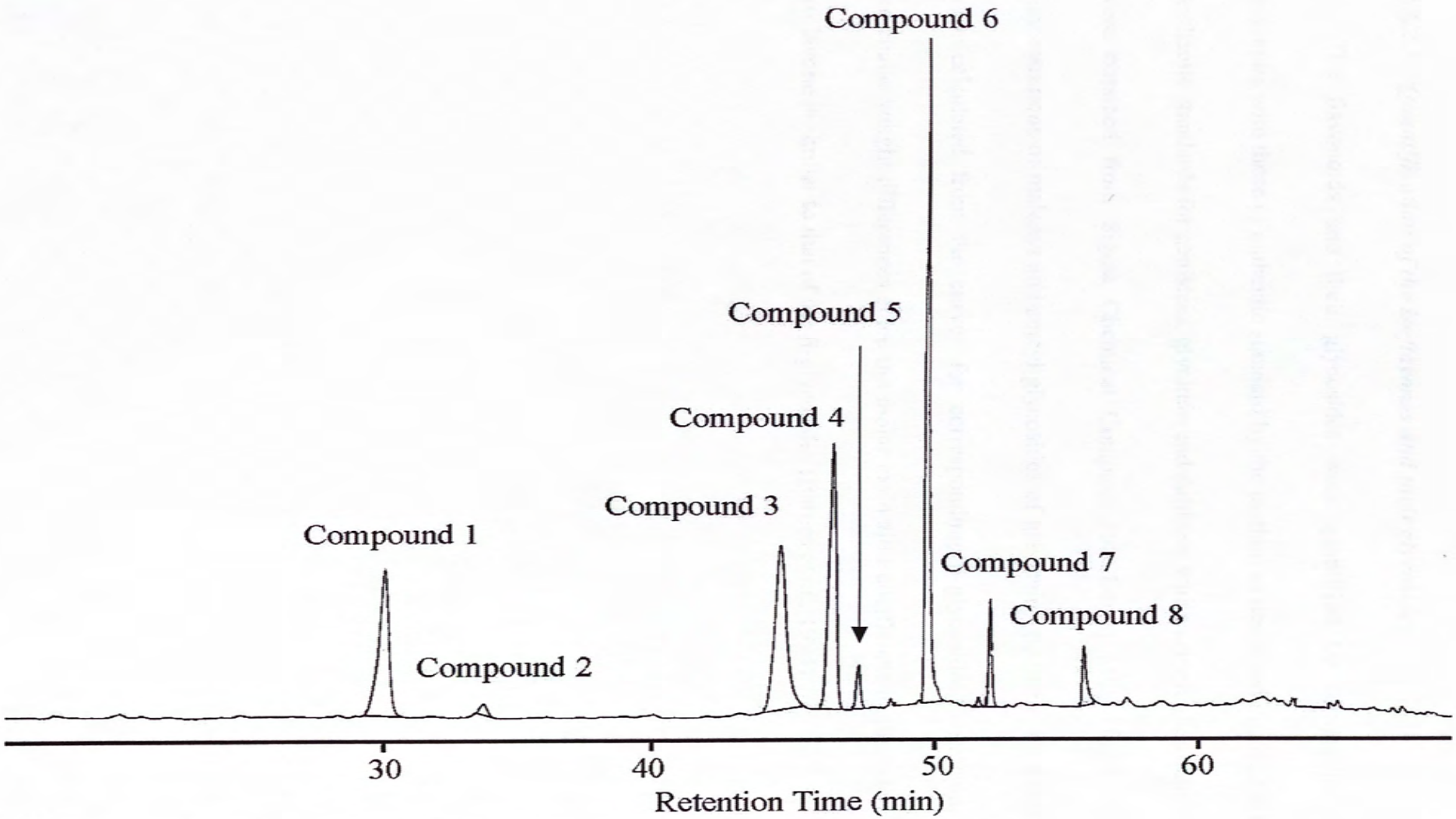


Figure 2.1 Typical HPLC chromatogram of soybean seed extract

2.3.2.3 Quantification of the isoflavones and their glycosides

The flavonoids and their glycosides were quantified by comparing area responses with those of authentic standard by the method as described in part ***2.3.1.2***. Authentic standards for genistein, glycitein and daidzein with purity higher than 98% were obtained from Sigma Chemical Company (St. Louis, MO, USA). The concentrations of malonyl and acetyl glycosides of genistein, glycitein and daidzein were calculated from the curves for corresponding β-glycosides, corrected for molecular weight differences since the molar extinction coefficient of the esterified isoflavone is similar to that of the β-glycosides (Barnes *et al.,* 1994).

2.4 Results

2.4.1 Structural Identification

The eight compounds were identified by studying their various spectra of UV and mass spectrometry. The results were compared with those obtained from Barnes *et al.* (1994) and the authentic standards of various isoflavones and their glucosides in soybeans. The characteristics of each compound were described below.

2.4.1.1 Compound 1

Compound 1, was isolated as a yellow amorphous powder, m.p. 233-235 ℃, $[\alpha]^{20}_{D}$ –36.4 (0.02*M*, KOH). The $[M+1]^+$ peak at *m/z* 417 in the APCI-MS (Atmospheric Pressure Chemical Ionization- Mass Spectrometry), indicated that the formula weight was 416. The APCI-MS spectrum of compound 1 revealed a peak at *m/z* 255 $[M\text{-glucosyl}+1]^+$, suggesting that compound 1 was a glycoside containing sugar. The UV absorption was maximum at 256 nm. After the comparison, compound 1 was deduced as daidzin with the percentage composition of C 60.58%; H 4.84%; O 34.58% and molecular formula $C_{21}H_{20}O_9$. (Figure 2.2).

2.4.1.2 Compound 2

Compound 2, was isolated as a yellow amorphous powder, m.p. 192-195 ℃, $[\alpha]^{20}_{D}$ –13.4 (c, 3.65 in DMF). The $[M+1]^+$ peak at *m/z* 447 in the APCI-MS (Atmospheric Pressure Chemical Ionization- Mass Spectrometry), indicated that the formula weight was 446. The APCI-MS spectrum of compound 2 revealed a peak at *m/z* 285 $[M\text{-glucosyl}+1]^+$, suggesting that compound 2 was a glycoside containing sugar. The UV absorption was maximum at 259 nm. After the comparison, compound 2 was deduced as glycitin with the percentage composition of C 59.19%; H 4.97%; O 35.84% and molecular formula $C_{22}H_{22}O_{10.}$ (Figure 2.2).

2.4.1.3 Compound 3

Compound 3, was isolated as a yellow amorphous powder, m.p. 254-256 ℃, $[\alpha]^{21}_{D}$ –27.7 (MeOH aq.). The $[M+1]^+$ peak at *m/z* 433 in the APCI-MS (Atmospheric Pressure Chemical Ionization- Mass Spectrometry), indicated that the formula weight was 432. The APCI-MS spectrum of compound 3 revealed a peak at *m/z* 271 $[M\text{-glucosyl}+1]^+$, suggesting that compound 3 was a glycoside containing sugar. The UV absorption was maximum at 263 nm. After the comparison, compound 3 was deduced as genistin with the percentage composition of C 58.34%; H 4.66%; O 37.00% and molecular formula $C_{21}H_{20}O_{10.}$ (Figure 2.2).

2.4.1.4 Compound 4

Compound 4, was isolated as a yellow amorphous powder, m.p. 168 ℃, $[\alpha]_D$ –45.2 (c, 0.2 in DMSO). The $[M+1]^+$ peak at *m/z* 503 in the APCI-MS (Atmospheric Pressure Chemical Ionization- Mass Spectrometry), indicated that the formula weight was 502. The APCI-MS spectrum of compound 4 revealed a peak at *m/z* 255 $[M\text{-glucosyl}+1]^+$, indicating that compound 4 was a glycoside containing sugar. The UV absorption was maximum at 258 nm. After the comparison, compound 4 was deduced as 6"-*O*-malonyldaidzin with the percentage composition of C 57.37%; H 4.41%; O 38.21% and molecular formula $C_{24}H_{22}O_{12.}$ (Figure 2.2).

2.4.1.5 Compound 5

Compound 5, was isolated as a yellow amorphous powder, m.p. 162 ℃. The $[M+1]^+$ peak at *m/z* 533 in the APCI-MS (Atmospheric Pressure Chemical Ionization- Mass Spectrometry), indicated that the formula weight was 532. The APCI-MS spectrum of compound 5 revealed a peak at *m/z* 285 $[M\text{-glucosyl}+1]^+$, indicating that compound 5 was a glycoside containing sugar. The UV absorption was maximum at 260 nm. After the comparison, compound 5 was deduced as 6"-*O*-malonylglycitin with the percentage composition of C 56.39%; H 4.54%; O 39.06% and molecular formula $C_{25}H_{24}O_{13.}$ (Figure 2.2).

2.4.1.6 Compound 6

Compound 6, was isolated as a yellow amorphous powder, m.p. 145 ℃. The $[M+1]^+$ peak at *m/z* 519 in the APCI-MS (Atmospheric Pressure Chemical Ionization- Mass Spectrometry), indicated that the formula weight was 518. The APCI-MS spectrum of compound 6 revealed a peak at *m/z* 271 $[M\text{-glucosyl}+1]^+$, indicating that compound 6 was a glycoside containing sugar. The UV absorption was maximum at 260 nm. After the comparison, compound 6 was deduced as 6"-*O*-malonylgenistin with the percentage composition of C 55.60%; H 4.28%; O 40.12% and molecular formula $C_{24}H_{22}O_{13}$. (Figure 2.2).

2.4.1.7 Compound 7

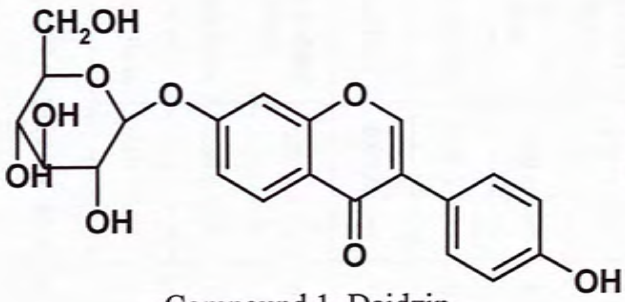
Compound 7, was isolated as a yellow amorphous powder, m.p. 330 ℃. The $[M+1]^+$ peak at *m/z* 255 in the APCI-MS (Atmospheric Pressure Chemical Ionization- Mass Spectrometry), indicated that the formula weight was 254. The UV absorption was maximum at 249 nm. After the comparison, compound 7 was deduced as daidzein with the percentage composition of C 70.86%; H 3.96%; O 25.17% and molecular formula $C_{15}H_{10}O_4$. (Figure 2.2).

2.4.1.8 Compound 8

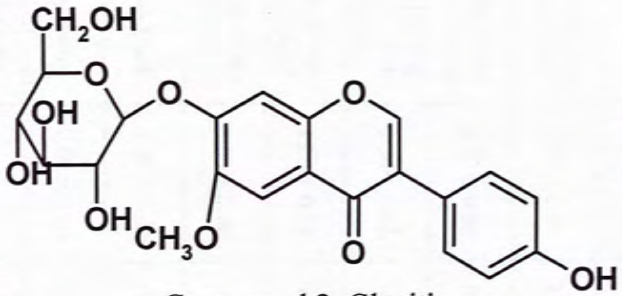
Compound 8, was isolated as a yellow amorphous powder, m.p. 302 ℃. The $[M+1]^+$ peak at *m/z* 263 in the APCI-MS (Atmospheric Pressure Chemical Ionization- Mass Spectrometry), indicated that the formula weight was 262. The UV absorption was maximum at 263 nm. After the comparison, compound 8 was deduced as genistein with the percentage composition of C 66.67%; H 3.73%; O 29.60% and molecular formula $C_{15}H_{10}O_5$. (Figure 2.2).

2.4.2 Quantification of isoflavones in traditional Chinese foods

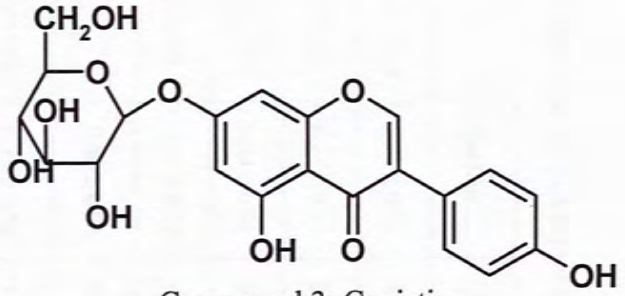
The isoflavones in 20 samples, including soybean and 19 other traditional Chinese foods (Tables 2.1), were quantified as previously described in parts ***2.3.2.1*** and ***2.3.2.2*** from the standard curves of their authentic standards or their pure compounds (purified in part 2.3.1.2) with flavone as an internal standard.



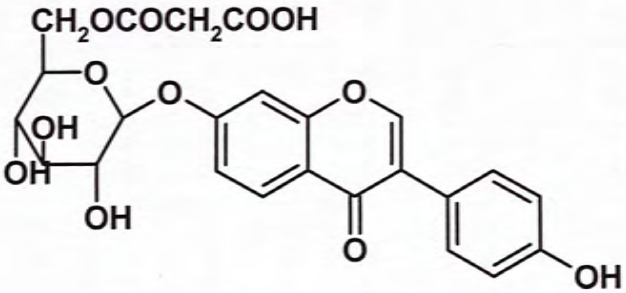
Compound 1. Daidzin



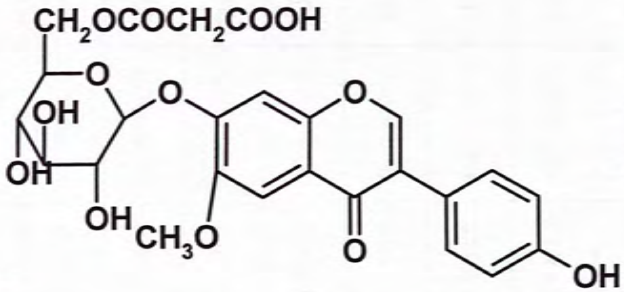
Compound 2. Glycitin



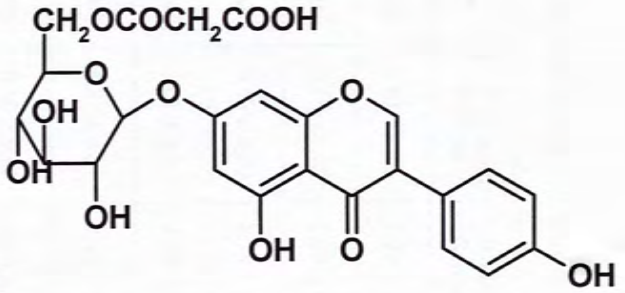
Compound 3. Genistin



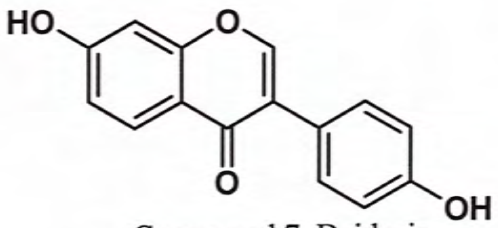
Compound 4. 6"-*O*-Malonyldaidzin



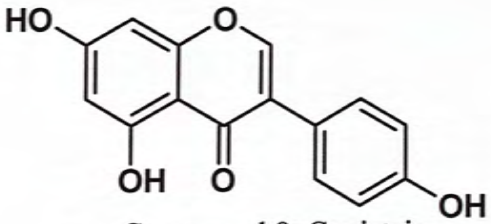
Compound 5. 6"-*O*-Malonylglycitin



Compound 6. 6"-*O*-Malonylgenistin



Compound 7. Daidzein



Compound 8. Genistein

Figure 2.2 Structure of compounds in soybean

Table 2.1 The compositions of isoflavones in some food samples (mg/g).

	Daidzin	Genistin	6''-*O*-Malonyl-Daidzin	6''-*O*-Malonyl-Genistin	Daidzein	Genistein	Total Isoflavones
Hard Tofu	0.91 ± 0.02	1.10 ± 0.02	0.91 ± 0.02	1.24 ± 0.03	0.08 ± 0.00	0.07 ± 0.01	4.22 ± 0.01
Soft Tofu	0.17 ± 0.03	0.23 ± 0.01	0.18 ± 0.02	0.28 ± 0.01	0.04 ± 0.03	0.04 ± 0.01	0.94 ± 0.01
Sweet Bean Curd	0.44 ± 0.01	0.48 ± 0.01	0.19 ± 0.03	0.24 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	1.39 ± 0.01
Soybean	0.25 ± 0.00	0.32 ± 0.01	1.01 ± 0.02	1.38 ± 0.02	0.00 ± 0.00	0.01 ± 0.00	2.97 ± 0.03
Black Turtle Bean	0.56 ± 0.02	0.84 ± 0.01	0.77 ± 0.01	1.13 ± 0.01	0.08 ± 0.00	0.07 ± 0.00	3.45 ± 0.01
Soy Milk	0.92 ± 0.02	1.46 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.01	0.06 ± 0.00	2.51 ± 0.02
Black Turtle Bean Milk	0.87 ± 0.01	1.40 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.01	0.10 ± 0.01	2.48 ± 0.02
Soy Bean Milk (Vitasoy)	0.42 ± 0.02	0.67 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	1.14 ± 0.01
Mated Soy Bean Milk (Vitasoy)	0.34 ± 0.02	0.52 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.93 ± 0.02
Soy Bean Curb Clot	1.00 ± 0.01	1.86 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.00	0.00 ± 0.00	2.99 ± 0.01
Black Bean Sauce	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.02	0.09 ± 0.00	0.24 ± 0.01
Bean Curb	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.00	0.10 ± 0.00	0.16 ± 0.01
Soy Sauce	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.01	0.08 ± 0.01	0.14 ± 0.00
ADZUKI Bean	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Black Eye Bean	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Chick Peas	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Red Bean	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Red Kidney Bean	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Green Bean	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Baked Bean	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data are expressed as mean ± S.D. (n=3)

2.5 Discussion

Soybean, hard tofu, soft tofu, sweet bean curd and black turtle bean contained a mixture of 6"-*O*-malonylglucoside conjugates and β-glucoside conjugates together with the aglycone conjugates (Figure 1, Table 2.1). Soybean and black turtle bean contained predominantly 6"-*O*-malonylglucoside conjugates (Table 2.1). Soymilk, black turtle bean milk, soy bean milk (Vitasoy), mated soya bean milk (Vitasoy) and soybean curb clot, which were prepared by high heat treatment, contained predominantly β-glucoside conjugates but no 6"-*O*-malonylglucoside conjugates were found (Table 2.1). This is in agreement with the previous study by Lori, *et al.* (1998), who demonstrated that hot aqueous extraction, used to produce tofu or soymilk, resulted almost entirely in the formation of β-glucoside conjugates. Black bean sauce, bean curb and soy sauce, prepared by high heat treatment together with fermentation, contained only aglycone conjugates (Table 2.1). ADZUKI bean, black eye bean, chick peas, red bean, red kidney bean, green bean and baked beans, which were not soy based foods, contained no isoflavones (Table 2.1).

This study showed that the glucoside composition of soy foods was determined by the processing conditions. They are easily altered during different treatments. Isoflavones are mainly found in soybeans as well as black turtle bean as their 6"-*O*-malonylglucoside conjugates. Aqueous heating and extraction, used to

produce tofu, soymilk or soybean curb clot, led to the conversion of 6"-*O*-malonylglucoside conjugates to β -glucoside conjugates. The result demonstrated that the 6"-*O*-malonylglucoside conjugates were unstable when they were exposed to heat. Room temperature extraction also led to a loss of 6"-*O*-malonylglucoside conjugates, but at a much slower rate (Coward *et al.*, 1993). Fermentation, used to produce black bean sauce, bean curb or soy sauce, causes conversion of both 6"-*O*-malonylglucoside conjugates and β-glucoside conjugates to their aglycones (Table 2.1). The total isoflavone concentration in the food would not be reduced under normal cooking condition, however, their glucoside composition might be altered (Coward *et al.*, 1993).

The composition of the glucoside conjugates may have significant effects on the bioavailability and pharmacokinetics of the isoflavones. In general, most studies on soy isoflavones *in vitro* or in animal models were carried out by using the aglycones. Whereas in most clinical trial, soy foods were the source of isoflavones that contained almost exclusively glucoside conjugates. The present study showed that the composition of the isoflavone glucoside conjugates varied from one food to another. Furthermore, different treatments or cooking procedures may alter their glucoside conjugates. Because the composition of the isoflavone glucoside conjugates may affect the rate of absorption and possibly the degree of further

metabolism, alterations in the chemistry of isoflavones need to be taken in account when the data from clinical trials are interpreted.

Chapter 3
Hypocholesterolemic Effects of Soymilk in Hamsters

3.1 Introduction

3.1.1 Lipoproteins and their functions

Most of the cholesterol in plasma is transported in 3 major lipoproteins namely: very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) (Superko, 2001). VLDL, one of the major triglyceride carriers in the blood, is synthesized and secreted from the liver. After a series of interactions with the enzyme lipoprotein lipase, the VLDL particles become more dense and relatively cholesterol rich. An intermediate density lipoprotein (IDL) precedes the appearance of LDL, which is normally the greatest source of cholesterol transport among the lipoproteins. The role of LDL is to transport cholesterol from the liver to the peripheral tissues. In contrast to LDL, the HDL synthesized in the liver is the main vehicle for the transfer of cholesterol from plasma and the peripheral tissues back to the liver for catabolism (Eckardstein *et al.,* 1998, 2001; Stein and Stein, 1999; McNamara, 2000).

3.1.2 Risk factors of cardiovascular disease

A major medical disorder of the twenty-century was coronary heart disease (CHD), which was the leading cause of death in many developed countries. Over the past 40 years, epidemiologic studies showed that blood levels of serum total cholesterol (TC) and LDL cholesterol (LDL-C) are directly related to cardiovascular disease (LaRosa *et al.,* 1990; Carleton *et al.,* 1991). Cholesterol accumulation in the artery is accompanied by the development of atherosclerosis (Brown and Goldstein, 1986). It has been demonstrated that high serum cholesterol is a single, independent factor associated with CHD (Rifkind & Lenfant, 1986). However, it is found that high cholesterol level is not an absolute prerequisite for the development of atherosclerosis (Hamilton, 1997). LDL is the major cholesterol carrier in the blood. LDL initiates the sequence of events leading to advanced atherosclerosis. These events include the entering of monocytes into intima and the development of foam cells. However, recent evidence suggests that LDL itself may not be the prime culprit, but it needs to be oxidatively modified before being taken up by the monocyte-derived macrophages (Steinberg, 1997; Parhami *et al.,* 1993; Diaz *et al.,* 1997; Young *et al.,* 2001). As the amounts of LDL cholesterol in the blood increase, the chance of oxidation will increase. Therefore, the amount of LDL cholesterol present in the blood can be used as one of the index for the risk of cardiovascular

disease (Hamilton, 1997; Ballantyne, 1998).

Several prospective studies have found an inverse relationship between HDL cholesterol (HDL-C) concentration and CHD incidence. This is independent of total or LDL cholesterol concentrations (Backer *et al.,* 1998; Boden *et al.,* 2000a, 2000b; Despres *et al.,* 2000). This may be due to the reversal cholesterol transport of HDL from peripheral cells and plasma back to the liver for catabolism (Eckardstein *et al.,* 1998, 2001; Kwiterovich, 1998; Philips *et al.,* 1998). As a result, the effect of reversal cholesterol transport of HDL can prevent the accumulation of cholesterol in plasma and decrease the chance of atherosclerosis. In addition to LDL-C, the ratio of LDL-C to HDL-C or non-HDL-C to HDL-C is also commonly used as an assessment of the risk of cardiovascular diseases.

It is still an open issue that high level of serum triglyceride (TG) is an independent risk factor for CHD (Hulley *et al.*, 1980). Several studies have shown that serum TG is not associated with the risk of CHD independently (Holme *et al.*, 1985). For instance, TG does not appear to be significantly associated with CHD in a multivariate analysis (Rhoads *et al.,* 1978; Holme *et al.,* 1985).

3.1.3 Hamster as an animal model of cholesterol metabolism

Golden Syrian hamster (*Mesocricetus Auratus*) has been used widely as an experimental animal model in studying and estimating the efficacy of hypocholesterolemic agents in humans (Sugiyama *et al.,* 1995; Campos *et al.,* 1998; Terpstra *et al.,* 1998; Trautwein *et al.,* 1998, 1999; Ntanios and Jones, 1999; Schneider *et al.,* 2000). The plasma cholesterol distribution in hamster is similar to that in humans, the major cholesterol carrier in hamster is LDL (Nistor *et al.,* 1987). As determined by electrophoresis in agarose gel, it was found that about 50% of plasma cholesterol in hamsters occurs in the LDL fraction (Nistor *et al.,* 1987). Also hamsters are similar to humans in biliary sterol secretion (Spady and Dietschy, 1985; Bocan and Guyton, 1985). Moreover, intrinsically low rates of hepatic cholesterol synthesis are found in both humans and hamsters. It is because increased hepatic influx of absorbed cholesterol could not be compensated in the species by down-regulation of cholesterol synthesis, but may alter hepatic cholesterol excretion (Berr *et al.*, 1993). Rat is another common laboratory testing animal, however, they lack the cholesteryl ester transfer protein and are resistant to developing atherosclerosis due to high cholesterol feeding, while hamsters are like humans and are susceptible to atherosclerosis (Bok *et al.,* 1999).

3.2 Objective

Soymilk is a favourite and commonly consumed beverage in China. It is now gaining popularity in Hong Kong. Although soybean has been shown to reduce plasma cholesterol in humans (Anthony *et al.,* 1998; Nilausen and Meinertz, 1998; Potter *et al.,* 1998; Wong *et al.,* 1998), the hypocholesterolemic effects of soymilk have not been thoroughly examined. The objective of this experiment was to examine the hypocholesterolemic activity of soymilk in hamsters.

3.3 Materials and Methods

3.3.1 Preparation of soymilk

Most commercial soymilks in the market contain additives, which may have influence on plasma cholesterol in hamsters. Therefore, soymilk was freshly prepared. In brief, 260 g (dry weight) of soybeans were soaked in one liter of water at room temperature for 8 hours. The soaked soybeans were then put into a soymilk-making machine (Tanisho Soyabean Maker TY-800A3, China) together with 1300 mL of 100 ℃ water, producing soymilk with concentration of 0.2g/ mL soybean. The resultant soymilk was diluted to 0.1g/ mL by mixing with additional 1300 mL of 100 ℃ water and then cooled at room temperature.

3.3.2 Animals

Male Golden Syrian hamsters were housed (3- 4 hamsters per cage) in an animal room at 25 ℃ with 12:12-h light-dark cycles. Fresh 0.1% cholesterol diets were given daily. Food intake was measured daily and body weight was recorded twice a week. The hamsters were allowed access to food and fluid *ad libitum.*

3.3.2.1 Experiment one - Hypocholesterolemic effect of soymilk in hamsters

Hamsters (125- 140 g, n=36) were randomly divided into 3 groups. They were

fed a 0.1%-cholesterol diet (Glen Forrest Stockfeeds, Western Australia, Australia). The energy content was listed in Table 3.1. The pellet is in 5 mm diameter. To minimize changes during the storage, the diets were stored at 4 ℃.

The control group was given the distilled water. The second and the third group of hamsters were given soymilk (SM) and cow's milk (CM) as drinking fluids, respectively.

At the end of week 4, all hamsters were sacrificed after overnight fasting. Blood was collected via the abdominal aorta. After clotting, the blood was centrifuged at 1500 g for 10 minutes and serum was collected. The livers, brains, kidneys and hearts were removed, washed with saline, and stored at –80 ℃.

3.3.2.2 Experiment two - The effect of fluid cross-over between soymilk and cow's milk on serum cholesterol in hamsters

Hamsters (125- 140 g, n=36) were randomly divided into 3 groups. They were similarly fed a 0.1%-cholesterol diet (Glen Forrest Stockfeeds, Western Australia, Australia). The control group was given the distilled water throughout the entire experiment. The second group was given soymilk for 8 weeks and then switched to cow's milk for additional 4 weeks (S-C group). The third group was given cow's milk for 8 weeks and then switched to soymilk for additional 4 weeks (C-S group).

Blood was collected at week 4 after overnight fasting from the ophthalmic venous plexus. At the end of 12 weeks, all hamsters were sacrificed after overnight fasting. Blood was collected via the abdominal aorta. After clotting, the blood was centrifuged at 1500 g for 10 minutes and serum was collected.

3.3.3 Serum lipid and lipoprotein determinations

Serum TG and TC levels were determined using enzymatic kits (Sigma Chemical, St. Louis, MO, USA). HDL-C was measured after precipitation of LDL and VLDL with phosphotungstic acid and magnesium chloride, using a commercial kit (Sigma).

3.3.4 Determination of cholesterol in the organs

The liver (300 mg), brain (150 mg), kidney (300 mg) and heart (300 mg) were used to determine the cholesterol level. In brief, the tissue sample and 1 mg stigmastanol, as an internal standard, were homogenized in 15 mL chloroform-methanol (2:1, v/v) and 3 mL saline. The chloroform-methanol phase was removed and dried down under a gentle nitrogen steam. After 1 hour mild hydrolysis with 5 mL NaOH in 90% ethanol at 90 ℃, 1 mL of water and 6 mL of cyclohexane were added for extraction of cholesterol. The cyclohexane phase was

evaporated to dryness under nitrogen, and cholesterol was converted to its TMS-ether derivative by a commercial TMS-reagent (dry pyridine-hexamethyldisilazane-trichlorosilane, 9: 3: 1, v/v/v, Sil-A regent, Sigma). After 1 hour at 60 ℃, the mixture was removed under a gentle stream of nitrogen. The TMS-ether derivative was dissolved in 600 μL of hexane, and after centrifugation, the hexane phase was transferred to a vial for gas-liquid chromatograph (GLC) analysis. The TMS-ether derivative was analyzed in a fused silica capillary column (SAC™-5, 30 m x 0.25 mm, i.d.; Supelco, Inc., Bellefonte, PA, USA) in a Shimadzu GC-14B GLC equipped with a flame-ionization detector (Shimadzu). The column temperature was set at 285℃ and maintained for 20 minutes. Helium was used as carrier gas at a head pressure of 22 psi. A typical GLC chromatogram of liver cholesterol is shown in Figure 3.1.

3.3.5 Statistics

Data are expressed as mean ± standard deviation (SD). The group means were statistically analyzed using one-way analysis of variance (ANOVA) and Student's *t*-test on SigmaStat Advisory Statistical Software (SigmaStat version 2.01, SPSS Inc., Chicago, IL, USA).

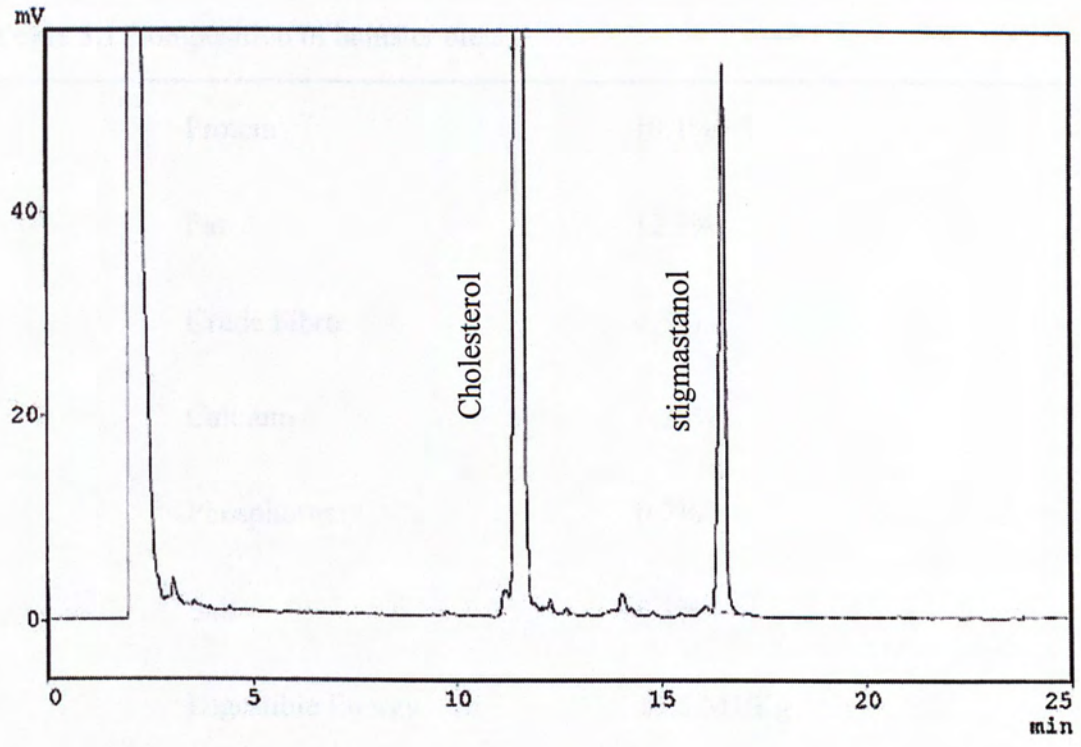


Figure 3.1 Gas liquid chromatographic profile of cholesterol in the liver

Table 3.1 Composition of hamster diets

Protein	19.1%
Fat	12.3%
Crude Fibre	4.5%
Calcium	0.75%
Phosphorus	0.7%
Salt	0.1%
Digestible Energy	15.5 MJ/Kg
Lard	100g/Kg
Cholesterol	1g/Kg

(adapted from Glen Forrest Stockfeeders)

3.4 Results

3.4.1 Experiment one - Hypocholesterolemic effect of soymilk in hamsters

3.4.1.1 Growth and food intake

The body weight gain and food intake of hamsters are shown in Table 3.2. No significant differences in body weight, fluid intake and food intake were observed among the control, SM and CM group.

3.4.1.2 Effect of SM and CM on serum TG, TC and HDL-C

After hamsters were given with different fluids for 4 weeks, their serum TG, TC and HDL-C were measured. There were no significant differences in serum TG and TC between the SM group and the control while a significant increase was observed in CM group (Table 3.3).

A significant increase in HDL-C by 15.9% and 35.2% was observed in the SM group and CM group, respectively, compared with the control group (Table 3.3).

3.4.1.3 Effect of SM and CM on non-HDL-C and ratio of non-HDL-C to HDL-C

Non-HDL-C was defined as a difference between TC and HDL-C. Non-HDL-C levels for the control, SM and CM groups were 63.2 ± 17.2, 42.2 ± 21.3 and 66.0 ± 18.2 mg/ dL, respectively. A significant reduction in non-HDL-C by 33% compared with that in the control was observed in the SM group ($p< 0.05$). In contrast, a significant increase in non-HDL-C by 56% compared with that in the SM

group was seen in the CM group ($p < 0.05$) (Table 3.3). The ratio of non-HDL-C to HDL-C in the SM group was found to be significantly lower than those of the control and the CM groups (Table 3.3).

3.4.1.4 Effect of SM and CM on concentration of hepatic cholesterol

The liver weights of the hamsters were measured immediately after the hamsters were killed. The hepatic cholesterol contents in the control, SM and CM groups were 104.1 ± 16.8, 92.2 ± 16.4 and 63.8 ± 11.3 mg/ g of liver, respectively. It was found that the hepatic cholesterol concentrations were significantly different among the three groups (Figure 3.2).

3.4.1.5 Effect of SM and CM on brain, heart and kidney cholesterol

There was no significant difference in brain, heart and kidney cholesterol content among the three groups (Figure 3.3).

Table 3.2 Effect of soymilk (SM) and cow's milk (CM) on body weight and food intake in hamsters in experiment one

	Control	SM	CM
Initial body wt (g)	131.3 ± 7.1	126.5 ± 5.9	125.7 ± 9.1
Final body wt (g)	161.5 ± 6.6	159.4 ± 8.0	162.1 ± 8.5
Fluid intake (mL/ day)	25.2 ± 5.6	24.2 ± 8.1	27.1 ± 6.3
Food intake (g/ day)	7.2 ± 1.7	6.8 ± 1.4	6.6 ± 1.0

Data are expressed as mean ± S.D., n= 12

Table 3.3 Effect of fluid intake on serum TG, TC, HDL-C, non-HDL-C and the ratio of non-HDL-C to HDL-C

	Control	SM	CM
TG (mg/ dL)	152.0 ± 63.4^{b}	139.7 ± 36.0^{b}	259.6 ± 72.5^{a}
TC (mg/ dL)	140.6 ± 23.5^{b}	131.9 ± 30.3^{b}	170.7 ± 23.6^{a}
HDL-C (mg/ dL)	77.4 ± 15.9^{c}	89.7 ± 19.1^{b}	104.7 ± 17.0^{a}
Non-HDL-C (mg/ dL)	63.2 ± 17.2^{b}	42.2 ± 21.3^{c}	66.0 ± 18.2^{a}
Non-HDL-C/ HDL-C	0.71 ± 0.11^{a}	0.48 ± 0.24^{b}	0.65 ± 0.22^{a}

Data are expressed as mean ± S.D., n= 12

SM: Soymilk group

CM: Cow's milk group

Non-HDL-C = [TC] – [HDL-C]

Means at the same row with different superscripts (a,b,c) differ significantly at $p< 0.05$

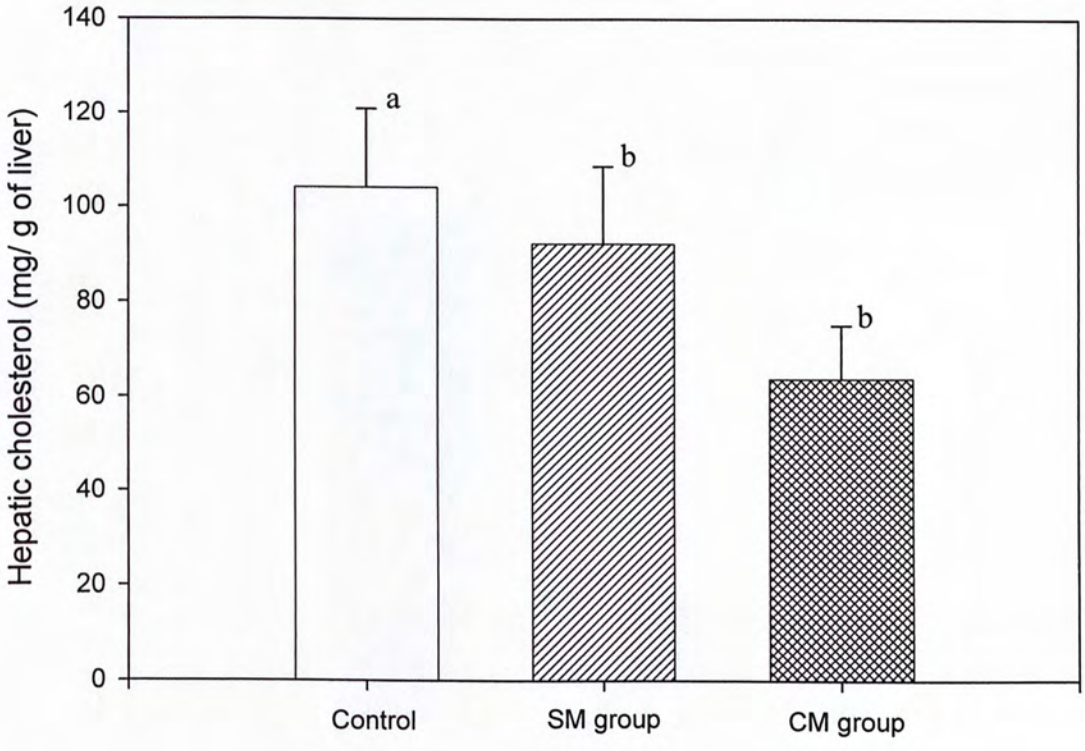


Figure 3.2 Effect of soymilk (SM) and cow's milk (CM) on concentration of hepatic cholesterol in hamsters. Data are expressed as means ± S.D., n= 12. Means with different superscript letter (a, b) differ significantly at $p < 0.01$

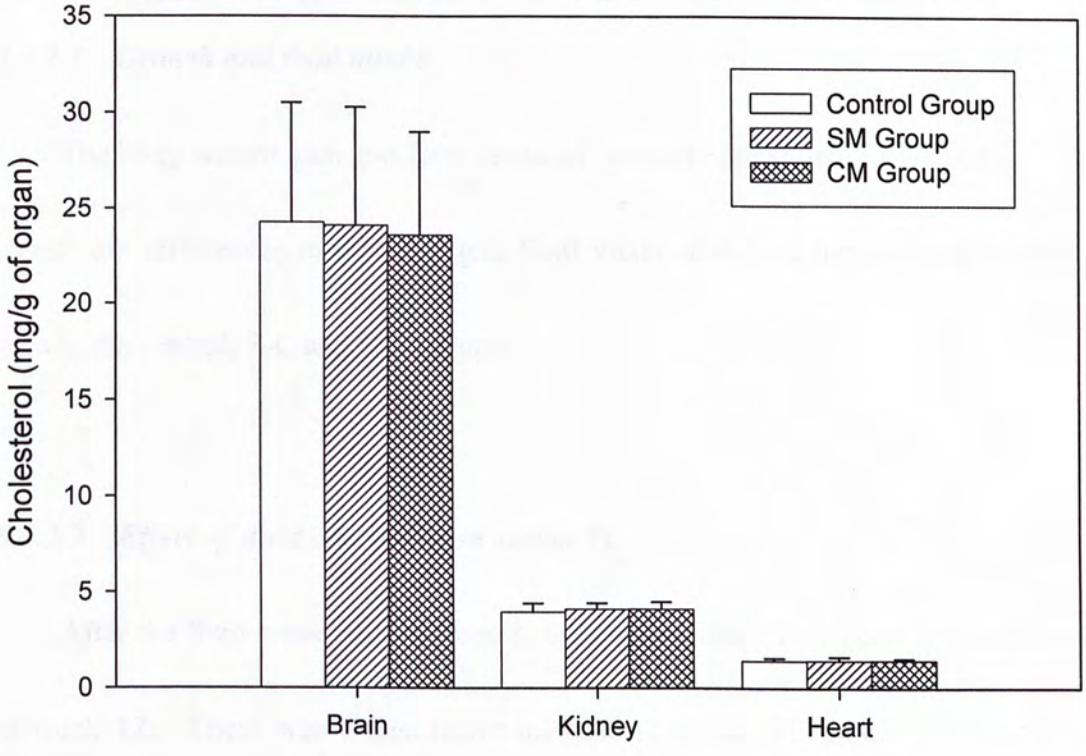


Figure 3.3 Effect of soymilk (SM) and cow's milk (CM) on concentration of tissue cholesterol levels in hamsters. Data are expressed as means ± S.D., n= 12.

3.4.2 Experiment two - The effect of fluid cross-over between soymilk and cow's milk on serum cholesterol in hamsters

3.4.2.1 Growth and food intake

The body weight gain and food intake of hamsters are shown in Table 3.4. No significant differences in body weight, fluid intake and food intake were observed among the control, S-C and C-S groups.

3.4.2.2 Effect of fluid cross-over on serum TC

After the fluid cross-over at week 8, their serum total cholesterol was measured at week 12. There was a significant increase in serum TC in the S-C group by 64.2% ($p < 0.01$) and a significant decrease in serum TC in the C-S group by 24.3% ($p < 0.01$) before and after the fluid cross-over (Figure 3.4).

Table 3.4 Effect of fluid intake on body weight and food intake in hamsters in experiment two

	Control	S-C Group	C-S Group
Body wt at week 0 (g)	151.7 ± 13.2	140.0 ± 10.8	138.8 ± 6.1
Body wt at week 8 (g)	155.4 ± 9.2	146.2 ± 11.2	142.9 ± 6.9
Body wt at week 12 (g)	155.0 ± 11.3	152.2 ± 13.2	157.5 ± 10.6
Fluid intake (mL/ day)			
Week 0-8	26.3 ± 8.2	28.6 ± 8.9	29.2 ± 6.8
Week 9-12	25.9 ± 10.2	29.8 ± 9.6	27.5 ± 5.9
Food intake (g/ day)			
Week 0-8	6.8 ± 2.1	6.5 ± 1.5	5.9 ± 2.2
Week 9-12	7.0 ± 1.7	6.2 ± 1.3	6.5 ± 1.9

Data are expressed as mean ± S.D., n= 12

Control: Distilled water

S-C group: Soymilk for 8 weeks followed by cow's milk for 4 weeks.

C-S group: Cow's milk for 8 weeks followed by soymilk for 4 weeks.

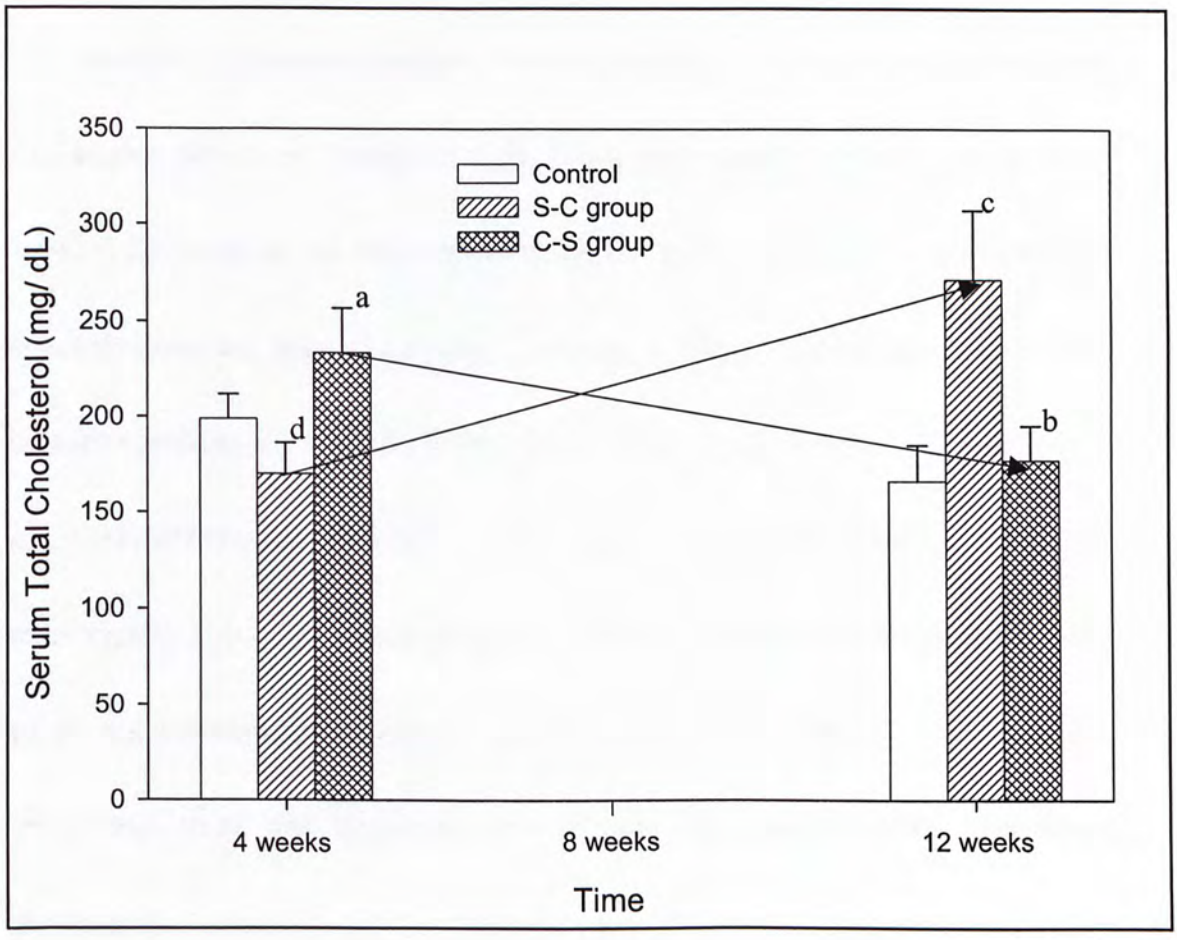


Figure 3.4 Effect of fluid cross-over on serum total cholesterol in hamsters.

Data are expressed as means ± S.D., n= 12.

Control: Distilled water

S-C group: Drank soymilk for 8 weeks followed by cow's milk for additional 4 weeks.

C-S group: Drank cow's milk for 8 weeks followed by soymilk for additional 4 weeks.

Means with different superscript letter (a, b) differ significantly at $p < 0.01$

Means with different superscript letter (c, d) differ significantly at $p < 0.01$

3.5 Discussion

Soymilk is an aqueous extract of whole soybeans. It contains soy protein and isoflavones, which are thought to have an anti-atherogenic effect (Anthony *et al.*, 1996). Evidence for an independent effect of isoflavonoids on blood cholesterol concentrations has been demonstrated in rats, hamsters, nonhuman primates and humans (Anthony *et al.*, 1996; Balmir *et al.*, 1996; Cassidy *et al.*, 1995; Clarkson *et al.*, 1998; Pelletier *et al.*, 1995). It has been reported that genistein is absorbed more rapidly than its glucoside (King *et al.*, 1996). Soymilk contains higher levels of the β-glucosides form rather than aglycones (Lori *et al.*, 1998). The purpose of the present study was to find whether soymilk was effective in lowering blood cholesterol.

The present study demonstrated that drinking soymilk could decrease serum TG and TC compared with drinking cow's milk. Also, drinking soymilk was associated with a significant decrease in the ratio of non-HDL-C to HDL-C compared with the control. In contrast, drinking cow's milk increased serum TG and TC, accompanied with an increase in the ratio of non-HDL-C to HDL-C compared with drinking soymilk. The hypocholesterolemic activity of soymilk compared with cow's milk is also reflected from the observation that serum TC was decreased when the hamsters were switched from cow's milk to soymilk. These findings suggest that drinking

soymilk could cause the redistribution of serum cholesterol. The non-HDL-C to HDL-C ratio is commonly used as a risk factor of cardiovascular disease. The higher the ratio, the greater the risk of cardiovascular diseases. If the data could apply to humans, the decrease in the ratio of non-HDL-C to HDL-C indicated that drinking soymilk might be associated with a decreased risk of cardiovascular diseases.

The mechanisms why soy products can decrease risk of cardiovascular diseases are still under investigation. Potential mechanisms by which soymilk induce lowering of blood cholesterol concentrations include thyroid status, bile acid balance and the estrogenic effects of genistein and daidzein. Some other studies also point out that isoflavones exhibit antioxidant properties and have favorable effects on arterial compliance.

Previous studies showed that supplementation of soy-protein could significantly increase the HDL-C and decreased the ratio of LDL-C to HDL-C, but TC was not significantly reduced in humans (Nilausen and Meinertz, 1998). This is in agreement with the result in the present study, implying that some components present in both soymilk and soybean might be responsible for the observed effect. A previous study showed that supplementation of soy isoflavones in the diet could decrease non-HDL-C and increase HDL-C (Anthony *et al.*, 1998). Since, genistein

can bind to estrogen receptors (Kuiper *et al.,* 1998) to exert estrogenic activity and may share the LDL reducing and HDL increasing effect of human estrogen (Lilley *et al.,* 1998; Westerveld, 1998; Godsland, 2001). Therefore, soy isoflavones may be one of the active ingredients which were responsible for the observed activity.

The present results showed that there was a decrease in the hepatic cholesterol in the SM group. Dietary soy protein has been reported to enhance bile acid excretion in rabbits and rats (Potter, 1996). Although an increase in the serum HDL-C might increase the rate of cholesterol transported form the peripheral parts of the body back to the liver for degradation, the rate of bile acid synthesis by the liver might be higher than the rate of the cholesterol entering into the liver, leading to a decrease in hepatic cholesterol. However, the mechanism by which drinking cow's milk was also associated with a decrease in hepatic cholesterol remains unclear. It is known that saturated fatty acids in cow's milk down-regulate hepatic LDL-receptor, thus elevating serum cholesterol level and lowering hepatic cholesterol content. In fact, serum non-HDL-C in cow's milk group was elevated and hepatic cholesterol was decreased compared with the control and soymilk group.

Chapter 4
Antioxidant Activities of Soybean Isoflavones and Their Glycosides

4.1 Introduction

A major medical disorder nowadays is cardiovascular disease. It is the leading cause of mortality in Western populations (Grundy, 1990; Martin *et al.*, 1986). It is responsible for about half of the deaths in the developed countries and its incidence in developing countries is increasing. The underlying cause of coronary heart disease, stroke and peripheral arterial disease is mainly atherosclerosis. Oxidation of low density lipoprotein (LDL) is implicated in the development of atherosclerosis and dietary antioxidants may provide a useful therapy in the prevention of LDL oxidation and atheroma development. In recent years, there has been a great interest in health effect of dietary antioxidants on coronary heart disease (Parthasarathy *et al.*, 1998).

Atherosclerosis is thought to be a degenerative disease that is an inevitable consequence of aging. The harmful effect of atherosclerosis is mainly on the large and medium-sized arteries. The arterial wall has three layers namely innermost layer, tunica intima, tunica media and tunica adventitia. The intima is normally a thin layer but it becomes greatly thickened when an atherosclerotic lesion forms.

The formation of atherosclerotic lesions may last over a period of years or decades, but their bulk can impede the flow of blood through the arteries. Most heart attacks (myocardial infarctions) are due to the sudden fissuring of an atherosclerotic lesion. These fissures cause blood to come into contact with substances inside the lesions that cause a thrombus and block the flow of blood (Wilcox *et al.,* 1995), leading to a myocardial infarction if the artery supplies the muscles of the heart.

4.1.1 Role of low density lipoprotein oxidation in the development of atherosclerosis

The oxidative theory of atherosclerosis proposes that the oxidized LDL is atherogenic in the arterial wall rather than the LDL itself (Diaz *et al.,* 1997 and Young *et al.,* 2001). LDL enters the arterial wall from the plasma and accumulates in the extracellular subendothelial space of arteries. Through the action of resident vascular cells, the LDL is mildly oxidized to a form known as minimally modified LDL. This minimally modified LDL induces local vascular cells to produce monocyte chemotatic protein, which stimulates monocyte recruitment and differentiation to macrophages in arterial walls (Parhami *et al.,* 1993). The accumulating monocytes and macrophages stimulate further peroxidation of LDL.

In contrast to the uptake of native LDL by the LDL receptor on macrophages, the uptake of oxidized LDL by the scavenger-receptor pathway is not subject to

negative-feedback regulation. The aldehydes formed during LDL oxidation can combine with the epsilon-amino groups of the lysyl residues of apo B-100 to form Schiff bases. When a Schiff base forms, the normal positive charge of the lysyl residue is abolished and the LDL particles acquire a greater negative charge (Steinbrecher, 1987). As a result, the oxidized LDL can no longer be recognized by the native LDL receptor and thus results in massive uptake of cholesterol (from oxidized LDL) by the macrophages (Diaz *et al.,* 1997). The amount of cholesterol in LDL entering the cells is thought to overwhelm the capacity of the macrophages to release it and the cholesterol therefore accumulate inside the cells, converting them into foam cells (Figure 4.1). The oxidized LDL also has direct chemotactic activity for monocytes and stimulates the binding of monocytes to the endothelium. The monocytes will become trapped in the subendothelial space, if they cross the endothelial layer. This is because that the oxidized LDL can inhibit their egress from the arterial wall (Quinn *et al.,* 1987).

In the later lesions, deposition of extracellular cholesterol esters forms a large pool of lipids at the base of the lesions. Then proliferation of smooth muscle cells in the intima will occur and these smooth muscle cells will secrete large amounts of collagen, which adds bulk to the lesions. The macrophages die in the deeper parts of the lesions, mainly at the edge of the lipid pool in advanced lesions. Therefore,

oxidation of LDL is highly related to atherosclerosis.

4.1.2 LDL oxidation

LDL can be oxidatively modified in a cell-free system by transition metals such as iron and copper and by all the major cells of the arterial wall such as endothelial cells, smooth muscle cells, and monocyte-macrophages (Jialal & Devaraj, 1996). The mechanisms by which these cells oxidize LDL are poorly understood. Involvement of superoxide (Steinbrecher *et al.,* 1988; Hiramatsu *et al.,* 1987) and lipoxygenase (Parthasarathy *et al.,* 1989; Rankin *et al.,* 1991) have been proposed. Another proposed mechanism is that cells might oxidize LDL by releasing the thiol-containing amino acid cysteine (Heinecke, 1987). Oxidation of cysteine leads to form cystine in the extracellular space, producing sulf- or oxygen centered free radicals that may attack LDL. When cysteine is oxidized to cystine, iron (III) may be reduced to iron (II) while copper (II) is converted to copper (I). These transition metal ions in their lower valency states may oxidize LDL better than in their higher valency states (Heinicke *et al.,* 1998).

Whatever the mechanism is, oxidation of LDL will involve the abstraction by an unidentified free radical of a hydrogen atom from a methylene (CH_2) group of a polyunsaturated fatty acid (PUFA). Molecular rearrangement of the resulting

unstable carbon radical results in a more stable configuration, a conjugated diene. The conjugated diene reacts very quickly with molecular oxygen, and the peroxyl radical thus forms a crucial intermediate. The PUFA peroxyl radical in LDL may abstract a hydrogen atom from an adjacent PUFA to form a hydroperoxide and another lipid radical, a reaction which results in chain propagation. The fragments of lipid hydroperoxides will eventually break down into shorter-chain aldehydes and other products, including malondialdehyde (MDA) and 4-hydroxynonenal (Figure 4.2) (Young *et al.*, 2001)

4.1.3 Thiobarbituric acid reactive substances (TBARS) as an index of LDL oxidation

Measurement of TBARS formation is a most frequently used test as an index of lipid peroxidation. A breakdown product of lipid peroxidation, malondialdehyde (MDA), is formed when lipid hydroperoxide is decomposed during LDL oxidation (Figure 4.2). These MDA molecules react with thiobarbituric acid (TBA) in acidic condition to form a pink chromagen, which has an absorption maximum at 532 nm. The amount of these chromagen can be quantified by spectrophotometrically or fluorometrically (Ohkawa *et al.*, 1978). This method is sensitive to determine the degree of lipid peroxidation.

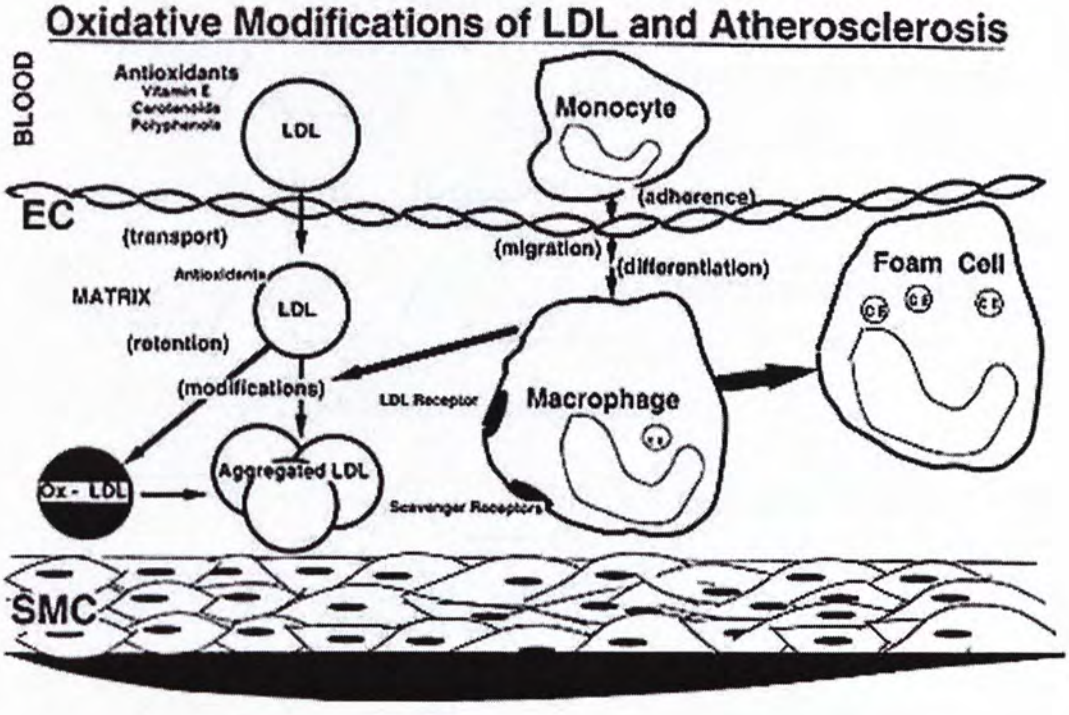


Figure 4.1 Macrophage-mediated oxidation and aggregation of LDL, and formation of foam cells. Native LDL particles migrated into the extracellular subendothelial space and then oxidized by the adjacent cells. The oxidized LDL was taken up by the macrophages. Cholesterol esters in oxidized LDL enter the macrophages and overwhelm their capacity to release them. These cholesterol esters therefore accumulate inside the cells, converting them into foam cells (Adapted from Aviram *et al.*, 1998).

CE: Cholesterol ester
SMC: Smooth muscle cell
Ox-LDL: Oxidized LDL

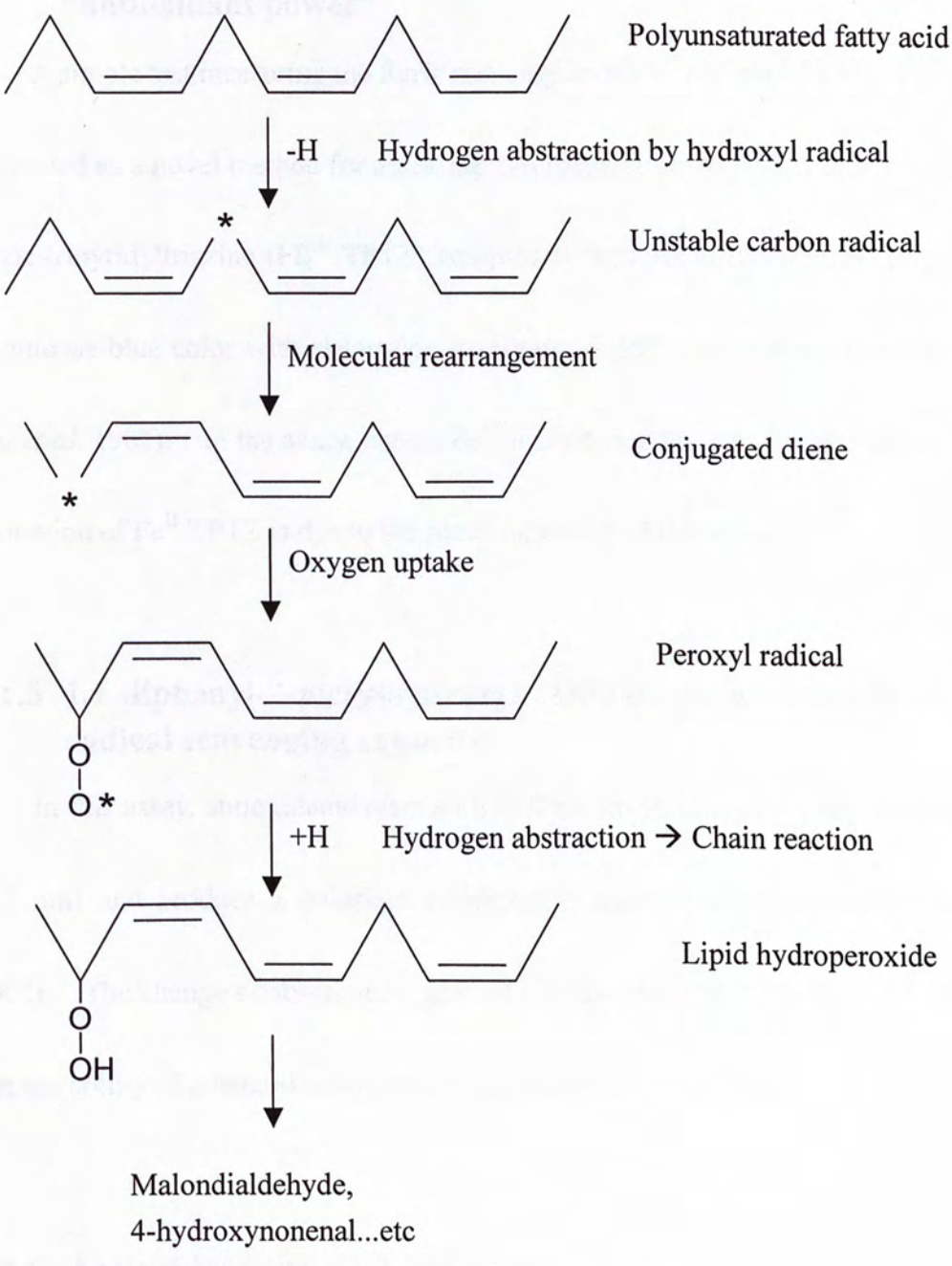


Figure 4.2 Basic reaction sequence of lipid peroxidation (Adapted from Young, 2001 with some modifications)

4.1.4 The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power"

A simple test measuring the ferric reducing ability of plasma, the FRAP assay, is presented as a novel method for assessing "antioxidant power". At low pH, when a ferric-tripyridyltriazine (FE^{III}-TPTZ) complex is reduced to the ferrous (Fe^{II}) form, an intense blue color with absorption maximum at 593 nm develops (Benzie 1996; Liu *et al.* 1982). In the assay, excess Fe^{III} is used, and the rate-limiting factor, color formation of Fe^{II}-TPTZ is due to the reducing ability of the sample.

4.1.5 1,1-diphenyl-2-picrylhydrazyl (DPPH) as a measure of free radical scavenging capacity

In this assay, antioxidants react with DPPH (which gives a strong absorption at 517 nm) and produce a colorless 1,1-diphenyl-2-picrylhydrazyl (Saracoglu *et al.* 2002). The change of absorbance produced in this reaction has been widely used to test the ability of a natural antioxidant to scavenge the free radical.

4.1.6 Antioxidant and LDL oxidation

The major lipid-soluble antioxidant present in LDL is vitamin E (α-tocopherol). It is a chain-breaking antioxidant and traps peroxyl free radicals. Enrichment of vitamin E has been shown to retard LDL oxidation and inhibit the proliferation of smooth muscle cells (Chan, 1998). A cross-sectional study of 16 European

populations also showed a significant inverse correlation between serum vitamin E concentrations and coronary heart disease (CHD) mortality (Grey *et al.*, 1991).

Some natural antioxidants, for example, flavonoids may protect vitamin E in LDL, either by scavenging free radicals themselves or by regenerating vitamin E from its radical form (Zhu *et al.,* 1999). Some studies showed that a mixture of flavonoid, α-tocopherol and ascorbic acid might inhibit LDL oxidation synergistically (Negre *et al.,* 1995; Hwang *et al.,* 2000).

4.2 Objective

The objective of the present study was to examine the antioxidative potency of individual soybean isoflavones and their glycosides by using Cu^{2+}- mediated human LDL oxidation, FRAP and DPPH as three different assay models.

4.3 Materials and Methods

4.3.1 Preparation of samples

Seven individual soybean isoflavones and their glycosides, including genistein, genistin, 6"-*O*-malonylglycitin, 6"-*O*-malonylgenistin, daidzein, daidzin and glycitin, were extracted and purified by the method described in part ***2.3.1***. All of them were obtained with purity higher than 95%. Authentic standards for flavone and epicatechin (EC) were obtained from Sigma Chemical Company (St. Louis, MO, USA); α-tocopherol was obtained from ICN Biomedicals Inc. (USA).

4.3.2 Isolation of LDL from human serum

Human serum was collected from healthy subjects at the Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong. To prevent lipoprotein modification, EDTA and NaN_3 were added to the serum collected (final concentrations of EDTA and NaN_3 were 0.1% and 0.05%, respectively). LDL was isolated from the serum according to the method described previously (Havel *et al.*, 1995). In order to prevent LDL from oxidation, the centrifuge tubes containing serum were flushed with nitrogen gas. Firstly, the serum was centrifuged at 1500 g for 15 minutes to remove cells and cell debris. NaCl-KBr solution (dissolve 153 g NaCl, 354 g KBr and 100 μg EDTA in one liter of water, 1.33 g/ mL) was then added

to increase the density to 1.019. The serum was re-centrifuged at 160,000 g for 20 hours at 4 ℃. After the removal of the top layer containing chylomicron and very low-density lipoprotein (VLDL), the density of remaining serum fractions was increased to 1.064 and re-centrifuged at 160,000 g for an additional 24 hours at 4 ℃. The top LDL fraction was collected and then flushed with nitrogen and stored at –70 ℃. The protein content of isolated LDL was determined using Lowry's method (Lowry *et al.*, 1951).

4.3.3 LDL oxidation

The stock LDL fraction (7.43 mg protein/ mL) was dialyzed against 100 volumes of the degassed dialysis solution (pH = 7.4) containing 0.01 M sodium phosphate, 0.9 % NaCl, 10 μM EDTA and 0.05% NaN_3 in dark at 4 ℃ for 24 hours. The dialysis solution was changed four times. Oxidation of LDL was conducted as previously described by Puhl *et al.* (1994). LDL protein (100 μg) was incubated in a mixture containing 5 μM $CuSO_4$ at 37 ℃ for up to 24 hours. The oxidation of LDL was then stopped by addition of 25 μL of 1.0% EDTA and cooled at 4 ℃.

4.3.4 TBARS assay

The degree of LDL oxidation was monitored by measuring the production of

TBARS as previously described (Buege & Aust, 1978). After the reaction was stopped by addition of EDTA at 4 ℃, 2 mL of 0.67% thiobarbituric acid and 15% trichloroacetic acid in 0.1 N HCl solution were added to the LDL-incubated tube. The incubation mixture was then heated at 95 ℃ for 1 hour, cooled on ice, and centrifuged at 1000 g for 20 minutes. The formation of TBARS was determined by measuring the absorbance at 532 nm. Calibration was done with a MDA standard solution prepared from tetramethoxylpropane. The extent of LDL oxidation was expressed as nmol MDA/ mg LDL protein.

4.3.5 FRAP assay

The FRAP assay was performed according to the procedures described by Benzie *et al.* (1996) with some modifications. In brief, the reagents used included 300 mmol/ L acetate buffer, pH 3.6 [3.1 g sodium acetate and 16 mL of acetic acid per liter of buffer solution]; 10 mmol/ L TPTZ (2, 4, 6,-tripyridyl-s-triazine, Sigma, Switzerland) in 40 mmol/ L HCl (BDH, England) and 20 mmol/ L $FeCl_3 \cdot 6H_2O$ (Pancreac, Spain). Working FRAP reagent was prepared freshly by mixing acetate buffer, TPTZ solution and ferric solution in the 10: 1: 1 ratio. Standard Aqueous solution of known Fe^{II} concentration, in the range of 100-5000 μmol/ L ($FeSO_4 \cdot 7H_2O$, Riedel de Haen, Germany), were used for calibration.

Freshly prepared FRAP reagent (1000 μL) was warmed to 37 ℃ and a reagent blank reading was taken at 593 nm; 20μL of sample was then added. Absorbance readings were monitored immediately using UV spectrophotometer (Shimadzu, UV-1601, Tokoy, Japan) up to 10 min. An absorbance change becomes constant before 8 min and the reaction is completed by 8 min after sample-reagent mixing, so 8 min readings were selected for calculation of FRAP values. The change in absorbance between the 8 min reading and the blank reading was calculated for each sample according to the Fe^{II} standard solution. Samples and standard solutions were always analyzed in duplicates in a "forward-then-reverse" ordering in order to eliminate the experimental error.

4.3.6 DPPH assay

The DPPH assay for free radical scavenging effect was adapted from Blois (1958) with a slight modification. Briefly, 0.3 mL test samples and 0.2 mL methanol were mixed with 2.5 mL of 75 μ M DPPH in a cuvette. After shaking, the mixture was incubated for 90 min in darkness at room temperature and then absorbances were measured at 517 nm using an UV spectrophotometer (Shimadzu, UV-1601, Tokoy, Japan). The difference in absorbance between a test sample and a control (methanol) was taken. This activity is given as % DPPH radical-scavenging.

Two positive controls were executed with α-tocopherol (ICN Biomedicals Inc., USA) and epicatechin (Sigma Chemical Company, USA).

4.3.7 Statistics

Results were expressed as mean ± standard deviation (S.D.). Student's t-test and one-way analysis of variance (ANOVA) were used for statistical evaluation of differences between groups (SigmaStat version 2.01, SigmaStat Advisory Statistical Software, MO, USA).

4.4 Results

4.4.1 Effects of seven individual soybean isoflavones and their glycosides on LDL oxidation

Effects of soybean isoflavones and their glycosides on production of TBARS was examined by incubating human LDL in the presence of 5 μM $CuSO_4$ as an oxidation initiator. As shown in Figure 4.3, the control LDL was significantly oxidized within 3 hours. Flavone at the concentrations of 12 μM demonstrated no or little protection to LDL from Cu^{2+} -mediated oxidation (Figure 4.3). On the other hand, the positive control LDL in the presence of 12 μM epicatechin (EC), a major antioxidant present in tea drinks, did not oxidize until 18 hours incubation. (Figure 4.3). Seven soybean isoflavones and their glycosides at a concentration of 12 μM showed different potency on the protection to LDL from Cu^{2+} -mediated oxidation (Figure 4.3). The LDL was oxidized completely within 5 hours in the presence of glycitin and diadzin, within 9 hours in the presence of daidzein, within 11 hours in the presence of both 6"-*O*-malonylgenistin and 6"-*O*-malonylglycitin and within 17 hours in the presence of genistin and genistein (Figure 4.3).

4.4.2 The antioxidant power of individual soybean isoflavones and their glycosides in the FRAP assay

The order of the FRAP values was epicatechin > genistin > daidzin > 6"-*O*-malonylgenistin > 6"-*O*-malonylglycitin > glycitin > genistein > daidzein >

flavone. The FRAP values of these isoflavones and their glycosides showed a dose-dependent manner at concentrations ranged from 4 μM to 12 μM.

4.4.3 Activity of individual soybean isoflavones and their glycosides as radical scavenging antioxidants

The radical scavenging activity of the isolated isoflavones and their glycosides from soybeans, epicatechin, α-tocopherol and flavone was estimated by their reactivity with DPPH. The addition of 12 μM of each compound to DPPH caused a rapid decrease in absorbance at 517 nm. Except for the two positive controls, epicatechin and α-tocopherol, genistin showed the highest DPPH radical-scavenging activity followed by daidzin (Figure 4.5). All the other compounds demonstrated similar DPPH radical-scavenging activity.

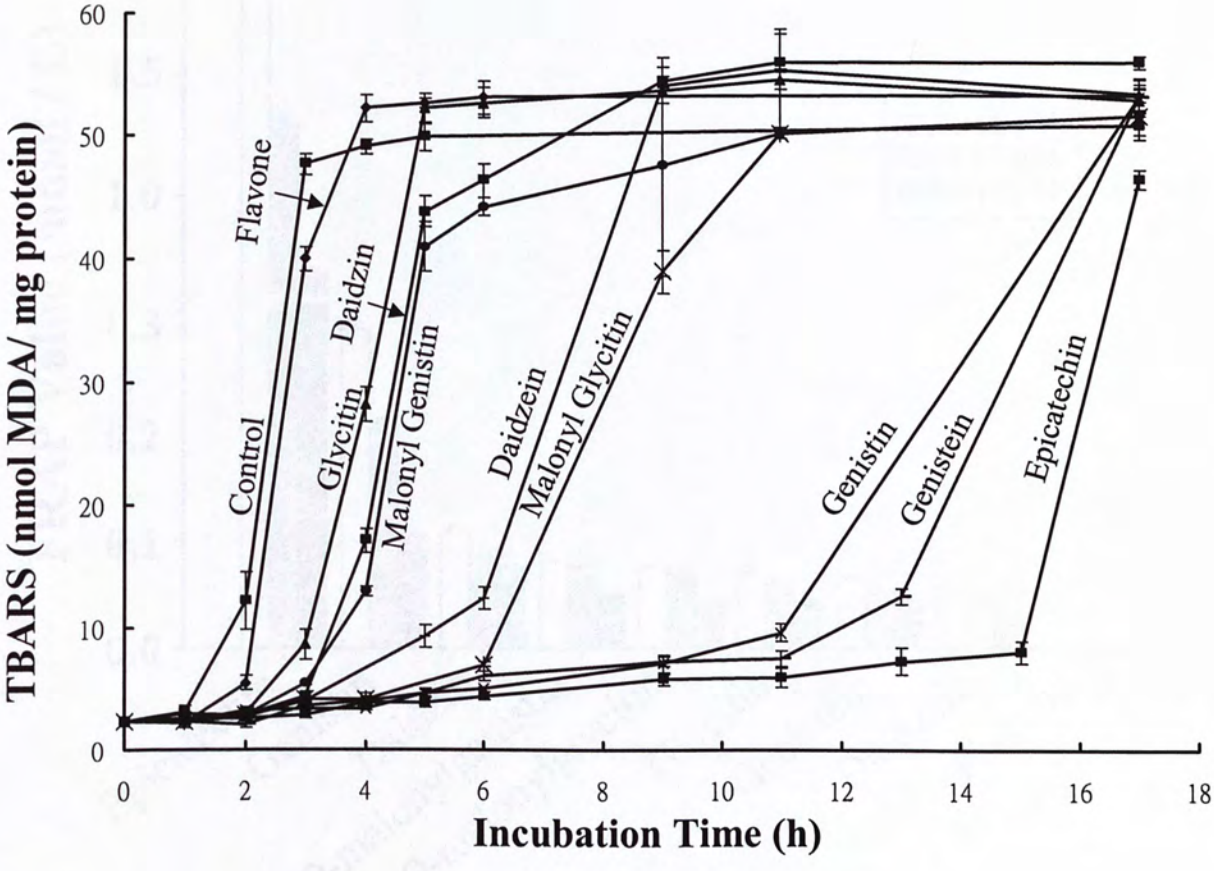


Figure 4.3 Inhibitory effects of epicatechin, flavone, isoflavones and their glycosides isolated from soybeans on the production of thiobarbituric acid reactive substances (TBARS) in Cu^{2+}-mediated oxidation of human LDL. Data are expressed as mean ± S.D. of n= 3.

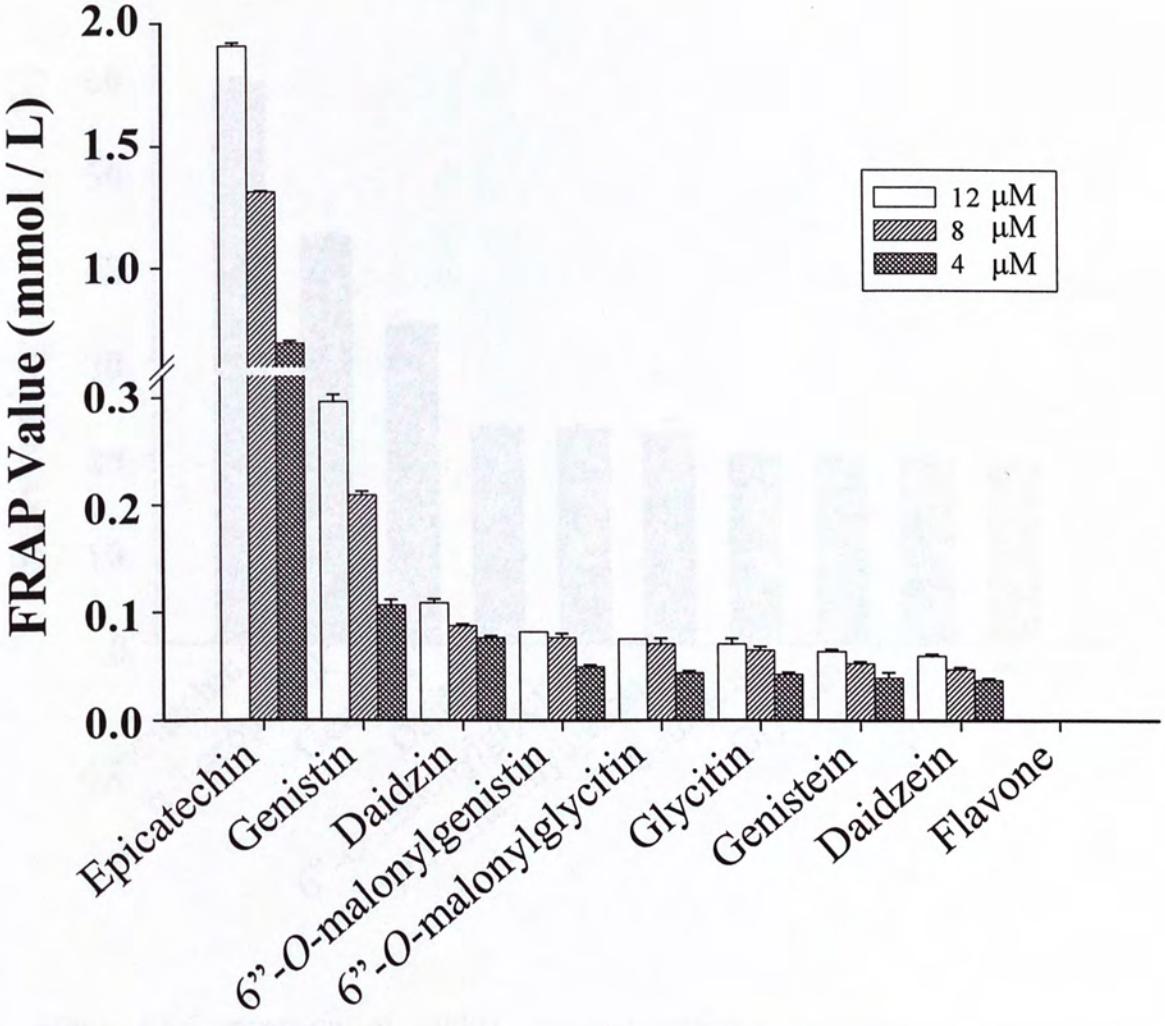


Figure 4.4 FRAP values of epicatechin, flavone, isoflavones and their glycosides isolated from soybeans. Data are expressed as mean ± S.D. of n= 3.

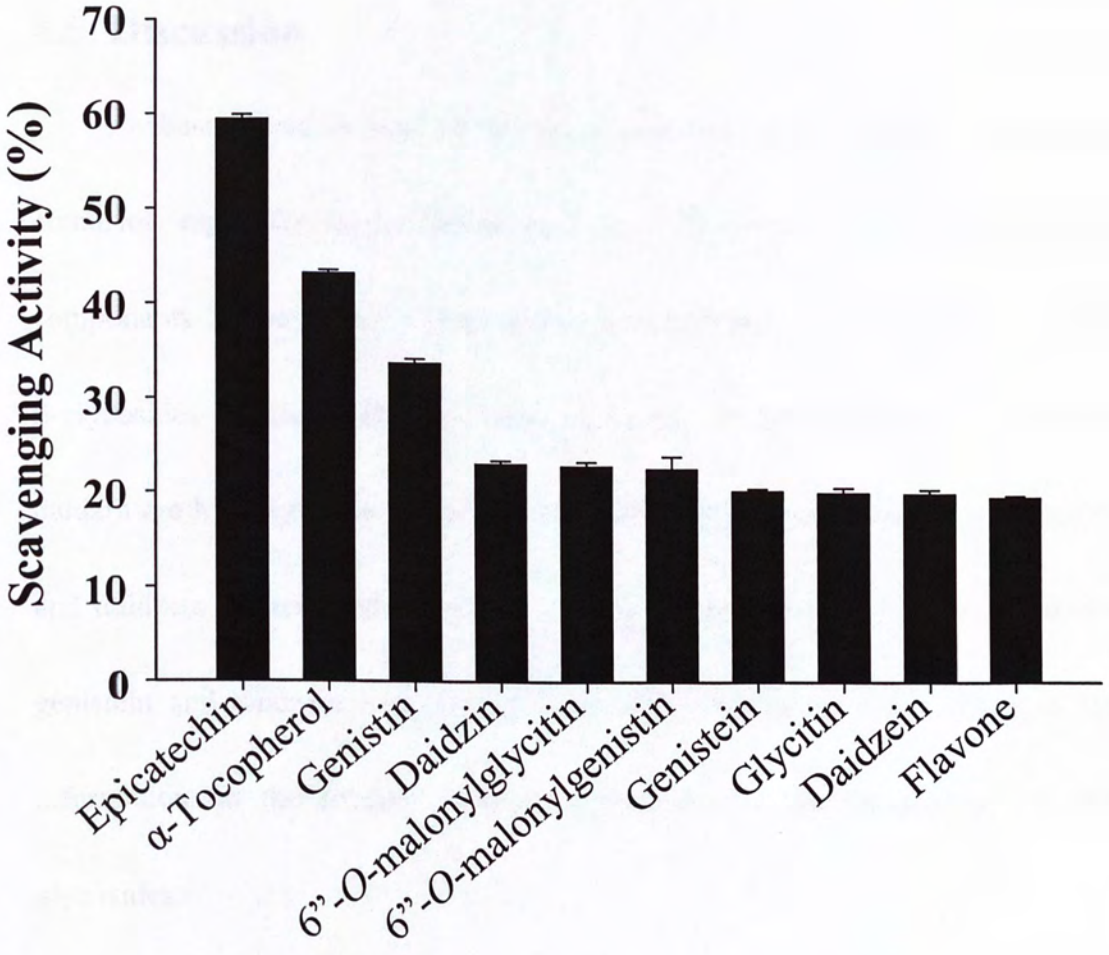


Figure 4.5 Comparison of DPPH radical-scavenging activities of epicatechin, α-tocopherol, flavone, isoflavones and their glycosides isolated from soybeans. Data are expressed as mean ± S.D. of n= 3.

4.5 Discussion

Soy-based food is one of the most widely consumed foods in the Asian countries, especially in the Mainland China. Isoflavones are one of the major components in soybean. They occur predominantly as biologically inactive ß-glycosides (Setchell, 1998). After ingestion, the glycosides of genistin and daidzin are hydrolyzed by bacteria to release the bioactive phytoestrogens genistein and daidzein (Kurzer and Xu, 1997). Many studies have demonstrated that both genistein and daidzein have strong antioxidant activity *in vitro*. There is little information on the relative antioxidant activity of other isoflavones and their glycosides.

Oxidative stress in the body can damage the macro-molecules like DNA, proteins and lipids, and might be involved in atherosclerosis (Holvoet *et al.,* 1998; Heinecke, 1998), cancer (Keum *et al.,* 2000; Mukhtar *et al.,* 2000; Steele *et al.*, 2000) and chronic inflammation (Halliwell, 1994). In atherosclerosis, for example, oxidation of low-density lipoproteins is thought to play an important role (Hamilton, 1997; Young *et al.,* 2001). It has been hypothesised that the flavonoid antioxidants may protect tissue against damage caused by free radicals (Aviram *et al.* 1998).

The present study examined the protective effect of soybean isoflavones and their glycosides against the Cu^{2+}-mediated LDL oxidation. It clearly demonstrated

that the individual soybean isoflavones and their glycosides, especially the genistein and genistin, could inhibit the LDL-oxidation. This result is in agreement with pervious studies conducted by Hodgson *et al.* (1996); Rifici *et al.* (1994); Tsai & Chait (1995), who have demonstrated antioxidant effect of soybean isoflavones in copper or cell-mediated oxidation of LDL and serum.

Generalizing the data from the Cu^{2+}-mediated LDL oxidation test, the antioxidative activities of individual soybean isoflavones tested were in the order of genistein $\geq$ genistin > 6"-*O*-malonylglycitin $\geq$ 6"-*O*-malonylgenistin > daidzein > daidzin $\geq$ glycitin. The different protective effects may be due to the different number and location of the aromatic hydroxyl groups (Chen *et al.,* 1996). However, their inhibitory effects on LDL-oxidation were not as strong as that of EC, which has been shown to be potent antioxidant present in the tea drinks.

Vitamin E (α- tocopherol) is a major lipid soluble antioxidant present in LDL. Depletion of endogenous vitamin E would lead to LDL oxidation (Diaz *et al.,* 1997). Regeneration of vitamin E by the secondary antioxidant can inhibit LDL oxidation and decrease the rate of atherosclerosis (Hamilton, 1997). Soybean isoflavones and their glycosides may function as a primary antioxidant by directly reducing the formation of free radicals mediated by Cu^{2+}. They may spare and maintain the level of vitamin E and thus delay the onset of lipid peroxidation.

A previous study by Rice-Evans *et al* (1996) suggested that hydroxyl groups are necessary for isoflavones to have antioxidant activity. In the present study, genistein showed the strongest antioxidant activity among the individual isoflavones and their glycosides. One possibility is that the C-4 carbonyl group and the double bond between C-2 and C-3 of the C ring exert anti-peroxidative activity (Figure 1.1) (Cook and Samman, 1996). One may assume that isoflavone molecules are positioned at the surface of the LDL particle in such a way that the fatty acyl residues are interspersed with the fatty acyl residues of phospholipid monolayer. The ring structure with the unsaturated C-ring on the lipoprotein surface facing the aqueous phase would be anchored to the lipid layer by the fatty acyl carbon chains. For other isoflavones and their glycosides, the smaller number of hydroxyl group or the bulky molecule attached on the A-ring results in lesser potency (Figure 2.2).

For the ferric reducing ability of plasma (FRAP) assay, the results demonstrated that individual soybean isoflavones and their glycosides had potent antioxidant properties *in vitro*. They could significantly reduce the ferric-tripyridyltriazine (FE^{III}-TPTZ) complex to the ferrous (Fe^{II}) form in a dose-dependent manner. Generalizing the data from the FRAP assay, the "antioxidant power" of individual soybean isoflavones tested were in the order of genistin > daidzin > 6"-*O*-malonylgenistin > 6"-*O*-malonylglycitin > glycitin > genistein > daidzein.

The result was slightly different from that of the Cu^{2+}-mediated LDL oxidation test above. This may be due to the "steric effects" of the hydroxyl groups that may behave differently in the media of Cu^{2+}-mediated LDL oxidation and the FRAP assay.

Although antioxidant activity of isoflavones has been reported in several studies by measuring indirect parameters such as oxidative damage to lipids, proteins and DNA (Harper *et al.,* 1999; Ruiz *et al.,* 1999), their direct scavenging activity against a variety of reactive oxygen and nitrogen species is largely unknown. In the present study, the radical-scavenging activities of soybean isoflavones and their glycosides were also determined using the DPPH assay. The assay showed similar results with the FRAP assay. The two positive controls, epicatechin and α-tocopherol, had the highest radical-scavenging activity. Among the soybean isoflavones, genistin gave the greatest radical-scavenging activity, followed by daidzin, 6"-*O*-malonylgenistin and 6"-*O*-malonylglycitin. Genistein, glycitin and daidzein showed the lowest radical-scavenging activity. This result is in agreement with that of Sato *et al.* (1992) and Mitchell *et al.* (1998), who demonstrated genistein and daidzein did not strongly scavenge DPPH or galvinoxyl radicals.

By comparing the results in DPPH assay with those of the FRAP assay, some soybean isoflavones, such as daidzin, 6"-*O*-malonylgenistin and

6"-*O*-malonylglycitin, showed different "antioxidant power" in the two assays. In addition, genistin showed relatively higher DPPH radical-scavenging activity, but it demonstrated a lower antioxidant activity in the Cu^{2+}-mediated LDL oxidation. These results may suggest that DPPH radical-scavenging activity can be used as an indication of potential antioxidant activity, but it may not always correlate linearly with other assays. The activity of putative antioxidants has been attributed to various mechanisms namely prevention of decomposition of peroxides, prevention of hydrogen abstraction, and radical scavenging (Diplock, 1997).

It is concluded that individual soybean isoflavones and their glycosides possess antioxidant activities. However, their antioxidant capacity was much weaker compared with that of epicatechin and α-tocopherol.

Chapter 5
Hypocholesterolemic Effects of Soybean Isoflavones in Ovariectomized Golden Syrian Hamsters

5.1 Introduction

5.1.1 Coronary heart disease in women

Coronary heart disease in women is often diagnosed later and treated less aggressively than that in men. One of three women aged 65 or older and 1 of 9 women aged 45-64 will develop clinical evidence of CHD (Wenger, 1995). Yet many women and some physicians still do not recognize CHD as a serious health risk to women. Women and men share many of the same risk factors for CHD, but the degree of risk often differs. The pattern of risks may also vary with age. A man's risk of heart attack typically begins to increase at age 40-45. A woman's risk starts to rise sharply about 10 years later – after menopause at about age 50-55. Women ultimately catch up with men, as the CHD risk continues to rise among women in their 70s and 80s. Women tend to be 10-15 years older than men when they first presentation with symptomatic CHD, and this may partly account for their high death rates.

5.1.2 Menopause as a risk factor in CHD

Menopause, whether natural or surgical, is the CHD risk factor unique to women. In natural menopause, a woman's estrogen level decreases over the course of several years, and, therefore, her risk of CHD begins to increase somewhat even before she actually stops menstruating. Estrogen may protect against CHD through its effects on serum cholesterol levels, clotting factors, the vasculature, and endothelial cells and possibly myocardial function. Estrogen increases HDL cholesterol level and decreases LDL cholesterol level, lowers fibrinogen level, is a weak antioxidant, and has some calcium channel blocking activity. Estrogen is also a vasodilator and can cause even atherosclerotic arteries to dilate in response to appropriate stimuli by normalizing endothelial function. As a result, menopause is associated with elevations of circulating TC and LDL-C concentrations, placing postmenopausal women at great risk for CHD (Bruschi *et al.*, 1996; Fukami *et al.*, 1995; Goldstein and Stampfer, 1995; Sullivan, 1996). These changes are a consequence of reductions in the level of circulating estrogen, which is the basis for estrogen replacement therapy (ERT). ERT reduces the risk of CHD in part through the modulation of serum cholesterol (Godsland *et al.*, 1987; Knight and Eden, 1996). However, ERT and other cholesterol-lowering pharmacological agents may be accompanied by some side effects (U.S. Department of Health and Human Services

1993) and therefore are recommended only for women without known contraindications. Moreover, many women chose not to comply with a recommendation for ERT because of safety concerns (Kessel, 1998). Therefore, other means that present no side effects for the treatment of postmenopausal hypercholesterolemia are preferred.

5.1.3 Dietary soy in treatment of postmenopausal hyper-cholesterolemia

The potential benefits of dietary soy in the prevention and treatment of chronic diseases, namely heart disease and cancer, have long been known. Soy protein, isoflavones, phospholipid, and phytate have been investigated as components responsible for the anti-atherogenic effect of soy, although the mechanism was not completely established (Sugano *et al.*, 1993; Anthony *et al.*, 1996; Knuiman *et al.*, 1989; Jariwalla *et al.*, 1990).

The cloning and description of a novel estrogen receptor, ER-β (Kuiper *et al.*, 1996), has made it possible to understand the selective effects of structurally closely related estrogenic substances. The primary soy-derived isoflavones, genistein and diadzein, have weak estrogenic activity, and their functions as both estrogen agonists and antagonists *in vitro* have been reported (Mathieson & Kitts, 1980). They both bind to ERs, a finding probably explained by their structural similarity with estrogen.

According to Kuiper *et al.* (1998), the binding affinity of genistein to the recently discovered ER-β was about 20 times greater than to ER-α. Compared with estradiol, the binding affinity of genistein for ER-α was 4%, and for ER-β was 87% (Kuiper *et al.,* 1998). Compared with estradiol, the binding affinity of daidzein for ER-α and ER-β was 0.1 and 0.5%, respectively (Kuiper *et al.,* 1998).

5.2 Objective

The hypocholesterolemic activity of soymilk was previously demonstrated in hamsters. The objective of the present study was carried out further to examine the hypocholesterolemic activity of isoflavones in soymilk and soybean extract by using the ovariectomized Golden Syrian hamsters as an animal model.

5.3 Materials and Methods

5.3.1 Preparation of soymilk

Soymilk was prepared as previously described in Chapter 3 (3.3.1) with some modification. In brief, 260 g (dry weight) of soybeans were soaked in 1000 mL of water at room temperature for 8 hours. The soaked soybeans were then put into a soymilk-making machine (Tanisho Soyabean Maker TY-800A3, China) together with 1300 mL of 100 ℃ water, producing soymilk with concentration of 0.2g/ mL soybean. The soymilk was concentrated to 1.0-1.5 mg total isoflavones/ mL using a freeze-drier. The isoflavones content were determined as previously described in Chapter 2 (2.3.2.3).

5.3.2 Preparation of soybean extract

Soybean extract was prepared as previously described in Chapter (2.3.1.1) with some modification. In brief, dried soybean powders (3 kg) were extracted with 18 liters of 70% ethanol three times at 60 ℃. Ethanol was evaporated under vacuum in a rotary evaporator. The extract was then dissolved in 2 liters of distilled water and partitioned with chloroform in a ratio of 1:1 for three times. The chloroform was removed and the remaining aqueous solution was freeze-dried and re-dissolved into 100 mL of water. The isoflavones were determined as previously described in

Chapter 2 (2.3.2.3).

5.3.3 Animals

Twenty-four (120 – 130 g) female Golden Syrian hamsters were housed (3- 4 hamsters per cage) in an animal room at 25 ℃ with 12:12-h light-dark cycles. The animals were given free access to a stock diet (Glen Forrest Stockfeeds, Western Australia, Australia) for 10 days. All the hamsters were ovariectomized (Chonan *et al.,* 1995) and waited for seven-day recovery from the operation before the experiment started. Blood (0.5 mL) was collected before and after the operation via the ophthalmic venous plexus.

The ovariectomized hamsters were randomly divided into three groups. Six of them were assigned to the control (CTL), nine were assigned to soymilk group (OVX-SM) and nine were assigned to soybean extract group (OVX-SE). They were given free access to 0.1% cholesterol diet (Glen Forrest Stockfeeds, Western Australia, Australia). The OVX-SM group was given a single oral dose of soymilk (equivalent to 30 μg isoflavones/ g body weight/ day). The OVX-SE group was given a single oral dose of soybean extract (equivalent to 30 μg isoflavones/ g body weight), while the CTL was given a single oral dose of 2 mL of distilled water everyday. The body weight was recorded once a week, and food consumption was

recorded every 2 or 3 days. After 4 weeks of the experimental diets, feces were collected for 7 days.

At the end of 4 weeks, all the hamsters were killed after overnight fasting. Blood was collected via the abdominal aorta. After clotting, the blood was centrifuged at 1500 g for 10 minutes and serum was collected. The liver, heart and kidney were also removed, washed with saline, and stored at –80 °C.

5.3.4 Serum lipid determinations

Serum TG, TC and HDL-C levels were determined as previously described in Chapter 3 (3.3.3).

5.3.5 Determination of tissue cholesterol content

The cholesterol content of animal tissues including liver, heart and kidney were measured as previously described in Chapter 3 (3.3.4).

5.3.6 Extraction of neutral and acidic sterols from fecal samples

The fecal neutral and acidic sterols were determined according to the method described by Czubayko *et al.* (1991) with some modifications. The fecal samples were first dried in a lyophilizer and then grounded into powder. Stigmasterol (0.5

mg in 1 mL of chloroform) was added into a tube as an internal standard for total neutral sterols. The tube was dried down under a gentle stream of nitrogen. Then 300 mg of grounded fecal sample and 0.5 mg hyodeoxycholic acid (0.5 mg in 2 Ml 1 N NaOH) as an internal standard for total acidic sterols were added. The samples were then subjected to alkaline hydrolysis with 8 mL 1 N NaOH in 90% ethanol at 90 ℃ for 1 hour followed by cooling down to room temperature. Then 1 mL of distilled water and 8 mL of cyclohexane were added to extract total neutral sterols. After centrifugation, the upper cyclohexane phase and the lower aqueous phase were separated, and the total neutral and acidic sterols were quantified as described below.

5.3.6.1 Determination of neutral sterols

The cyclohexane phase was evaporated to dryness under a gently stream of nitrogen. The neutral sterols were converted to their TMS-ether derivatives using TMS-reagent (dry pyridine-hexamethyldisilazane-trichlorosilane, 9: 3: 1, v/v/v, Sil-A regent, Sigma) at 60 ℃ for 1 hour. The mixture was then dried down under nitrogen stream and the TMS-derivatives of neutral sterols were dissolved in 400 μL of hexane. After centrifugation, the hexane layer was transferred to a vial for GLC analysis.

5.3.6.2 Determination of acidic sterols

For determination of acidic sterols, 1 mL of 10 N NaOH was added to the lower aqueous phase. The mixture was heated for 3 hours at 120 ℃. Distilled water (3 mL) was added to the mixture and cooled at room temperature. The mixture was acidified with 1 mL of 25% HCl. And the acidic sterols were extracted with 7 mL diethyl ether for two times. The ether phases were pooled and then dried down under a stream of nitrogen. Methylation of the acidic sterols was performed by adding 2 mL of methanol, 2 mL of dimethoxypropane and 40 μL of concentrated HCl. The mixture was mixed thoroughly and allowed to stand at room temperature for overnight. The solvents were then dried down under a stream of nitrogen. TMS-reagent was added and heated at 60 ℃ for one hour to convert the acidic sterols into their TMS-ether derivatives. The mixture was dried down under a stream of nitrogen and the TMS-derivatives were dissolved in 300 μL of hexane. After centrifugation, the hexane layer was transferred to a vial for GLC analysis.

5.3.6.3 GLC analysis of neutral and acidic sterols

The analysis of fecal neutral and acidic sterols was carried out in a GLC equipped with a fused silica capillary column as described in Chapter 3 (3.3.4). For the neutral sterols, the column temperature was set at 285 ℃ and maintained for 30

min. For the acidic sterols, the column temperature was programmed from 230 ℃ to 280 ℃ at a rate of 1 ℃/ min. Helium was used as a carrier gas at a head pressure of 22 psi in both neutral and acidic sterol analysis. Typical chromatograms of neutral and acidic sterols were shown in Figures 5.1 and 5.2, respectively.

5.3.7 Statistics

Data are expressed as mean ± standard deviation (SD). The group means were statistically analyzed using one-way analysis of variance (ANOVA) and Student's *t*-test on SigmaStat Advisory Statistical Software (SigmaStat version 2.01, SPSS Inc., Chicago, IL, USA).

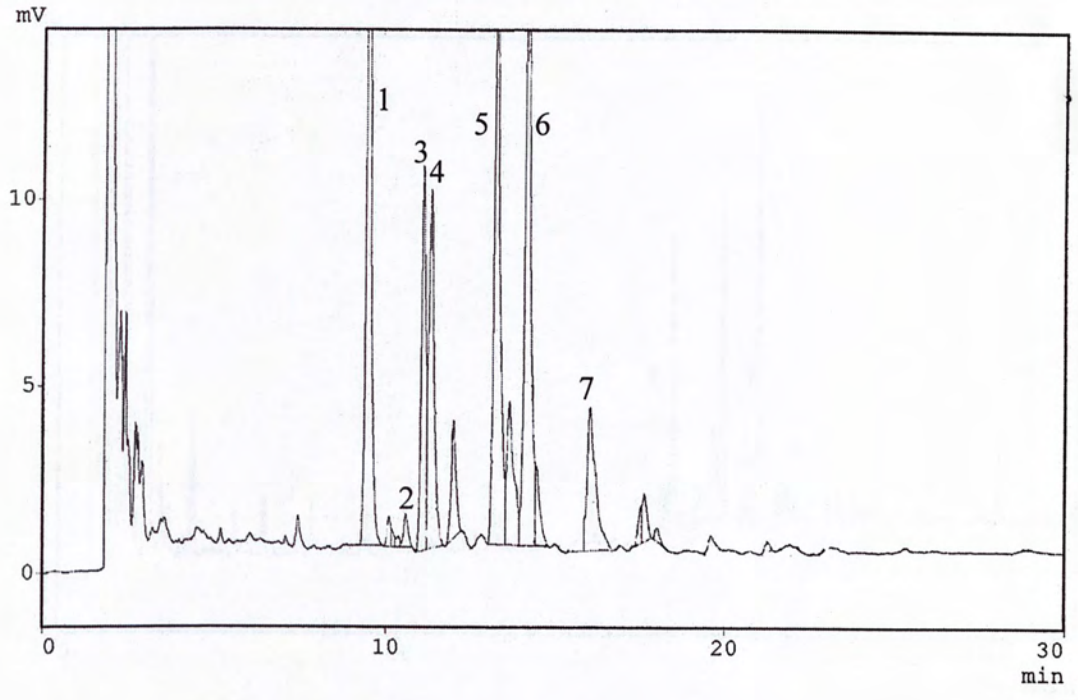


Figure 5.1 Gas liquid chromatographic profile of neutral sterols in feces. Identification of peaks: 1, coprostanol; 2, coprostanone; 3, cholesterol; 4, dihydrocholesterol; 5, campersterol; 6, stigmasterol (internal standard) and 7, β-sitosterol

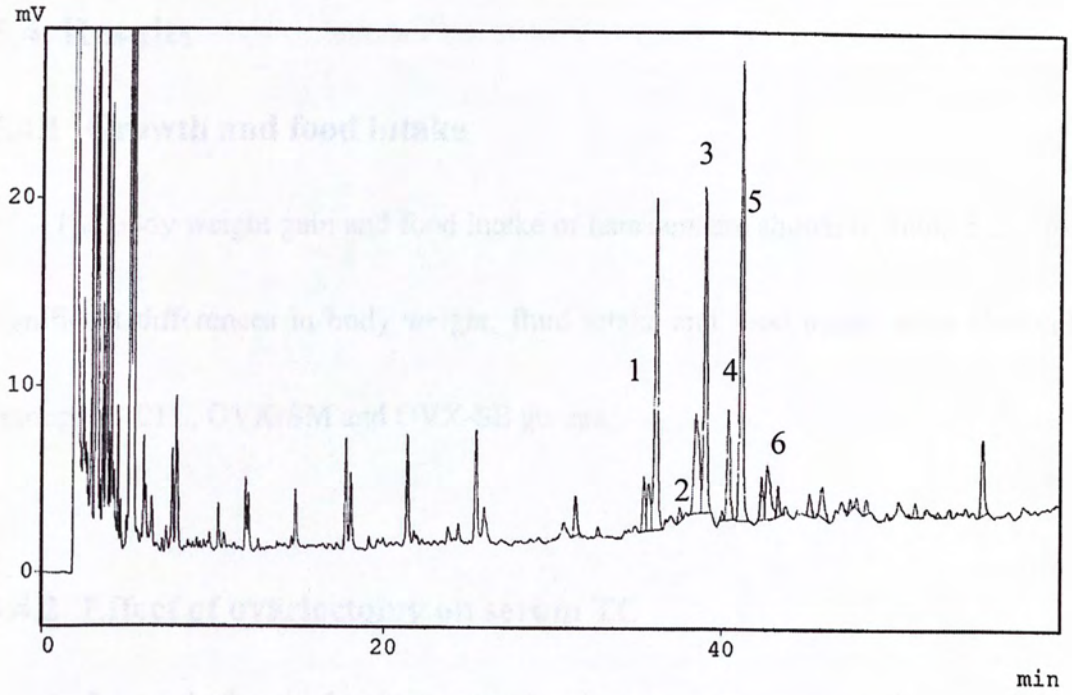


Figure 5.2 Gas liquid chromatographic profile of acidic sterols in feces. Identification of peaks: 1, lithocholic acid; 2, deoxycholic acid; 3, chenodeoxcholic acid; 4, cholic acid; 5, hyodeoxycholic acid (internal standard) and 6, ursodecholic acid.

5.4 Results

5.4.1 Growth and food intake

The body weight gain and food intake of hamsters are shown in Table 5.1. No significant differences in body weight, fluid intake and food intake were observed among the CTL, OVX-SM and OVX-SE groups.

5.4.2 Effect of ovariectomy on serum TC

Before and after the hamsters were ovariectomized, their total cholesterol was measured. The result showed that ovariectomization significantly elevated serum TC level (Table 5.2). However, no significant differences were observed among the CTL, OVX-SM and OVX-SE groups before SM and SE were given (Table 5.2).

5.4.3 Effect of soymilk and soybean extract on serum TC, TG and HDL-C

Significant reduction in serum TC was observed in the OVX-SM and OVX-SE compared with the value of the CTL group ($P<0.01$) (Table 5.3). To be specific, the serum TC in the OVX-SM and OVX-SE groups were 18% and 17% lower than that in the CTL hamsters, respectively. For the serum TG, the OVX-SM and OVX-SE were 6% and 13% lower than that in the CTL hamsters, respectively but the differences were not statistically significant. Soymilk or soybean extract had no

significant effect on the serum HDL-C level (Table 5.3)

5.4.4 Effect of soymilk and soybean extract on non-HDL-C and ratio of non-HDL-C to HDL-C

Non-HDL-C was defined as a difference between TC and HDL-C. Non-HDL-C levels for the CTL, OVX-SM and OVX-SE were 109.9 ± 17.0, 81.8 ± 9.4, 78.6 ± 19.2 mg/ dL, respectively. A significant reduction in non-HDL-C by 26% and 28% compared with that in the CTL group was observed in the OVX-SM and OVX-SE hamsters ($p<0.01$), respectively (Table 5.3). The ratio of non-HDL-C to HDL-C in the OVX-SE was found to be significantly lower than that of the CTL (Table 5.3).

5.4.5 Effect of soymilk and soybean extract on concentration of hepatic cholesterol

The livers were weighted immediately after the hamsters were scarified. The hepatic cholesterol in the CTL, OVX-SM and OVX-SE was 27.3 ± 4.9, 24.5 ± 4.9 and 27.3 ± 3.4 mg/ g of liver, respectively. There was no significantly different among the three groups (Figure 5.3).

5.4.6 Effect of soymilk and soybean extract on heart and kidney cholesterol

There was no significant difference in heart and kidney cholesterol content among the three groups (Figure 5.4).

Table 5.1 Changes in body weight and food intake

	CTL (n=6)	OVX-SM (n=9)	OVX-SE (n=9)
Initial body wt (g)	125.0 ± 12.6	123.0 ± 9.8	124.7 ± 11.4
Final body wt (g)	151.7 ± 9.2	153.3 ± 10.9	150.6 ± 12.9
Food intake (g/ day)	7.6 ± 1.7	7.5 ± 1.3	7.7 ± 2.1

Data are expressed as mean ± S.D.; Number in parentheses indicates the number of hamsters used.

Table 5.2 Changes in serum total cholesterol (TC) before and after the hamsters were ovariectomized (mg/ dL)

	CTL (n=6)	OVX-SM (n=9)	OVX-SE (n=9)
Before	97.5 ± 2.6	95.6 ± 1.8	95.9 ± 2.4
After	142.7 ± 6.5*	147.6 ± 4.5*	143.8 ± 5.4*

Data are expressed as mean ± S.D.; Number in parentheses indicate the number of hamsters used.

* differs significantly from the value before ovariectomization ($P<0.01$).

Table 5.3 Effects of soymilk (OVX-SM) and soybean extract (OVX-SE) on levels of serum triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), non-HDL-C and the ratio of non-HDL-C to HDL-C

	CTL (n=6)	OVX-SM (n=9)	OVX-SE (n=9)
TG (mg/dL)	234.3 ± 83.0	219.7 ± 78.0	204.7 ± 69.9
TC (mg/dL)	225.3 ±7.7^{a}	184.6 ± 26.8^{b}	186.4 ± 32.0^{b}
HDL-C (mg/dL)	115.5 ± 15.7	102.7 ± 18.6	107.8 ± 16.2
Non-HDL-C (mg/dL)	109.9 ± 17.0^{a}	81.8 ± 9.4^{b}	78.6 ± 19.2^{b}
Non-HDL-C/ HDL-C	0.98 ± 0.29^{a}	0.81 ± 0.08^{ab}	0.73 ± 0.14^{b}

Data are expressed as mean ± S.D.

Non-HDL-C = (TC) – (HDL-C)

Means at the same row with different superscripts (a, b) differ significantly ($p< 0.01$)

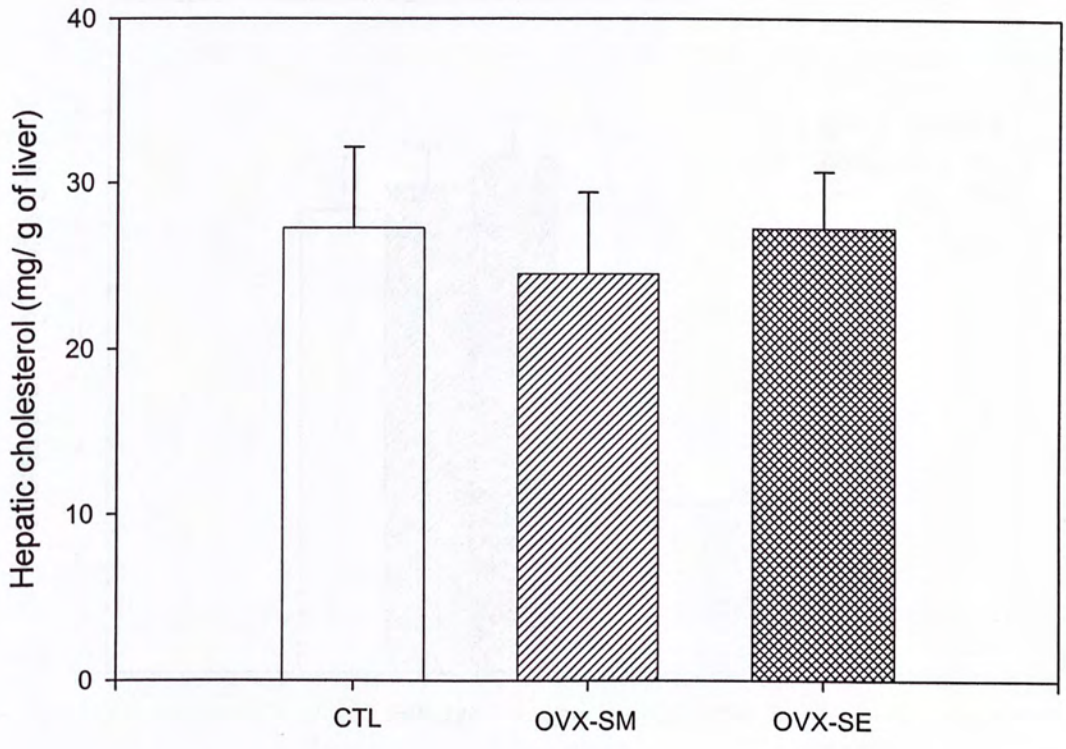


Figure 5.3 Effects of soymilk (OVX-SM) and soybean extract (OVX-SE) on concentration of hepatic cholesterol in hamsters. Data are expressed as means ± S.D.

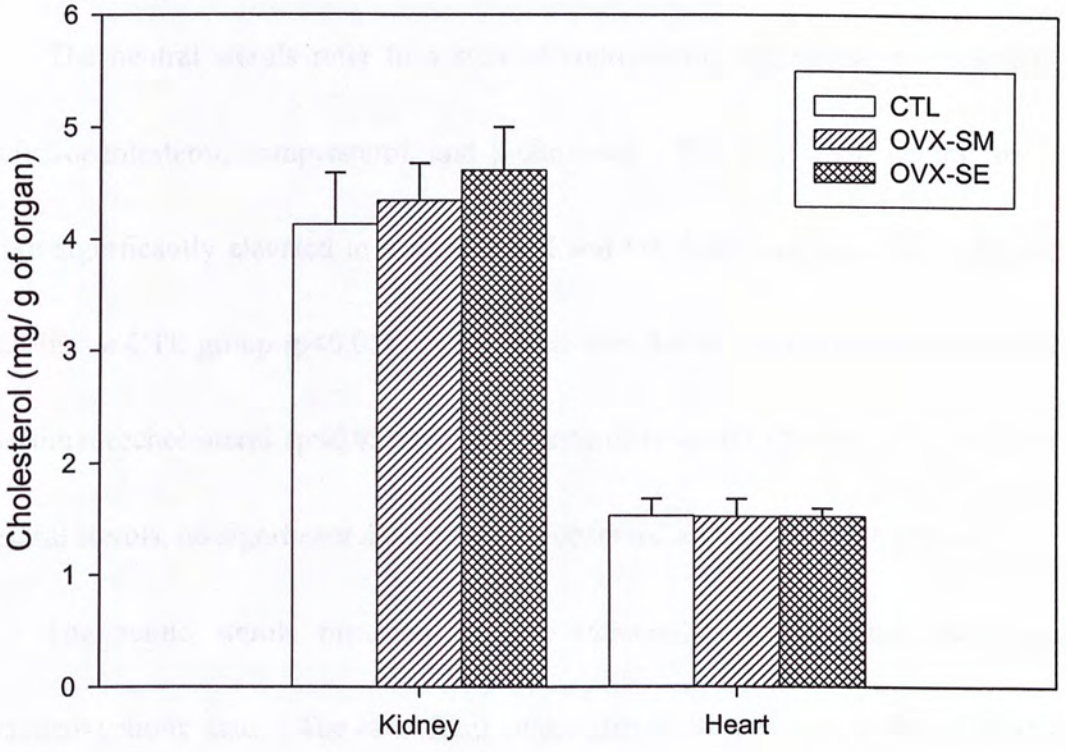


Figure 5.4 Effects of soymilk (OVX-SM) and soybean extract (OVX-SE) on concentration of tissue cholesterol levels in hamsters. Data are expressed as means ± S.D.

5.4.7 Effect of soymilk and soybean extract on fecal neutral and acidic sterols

The neutral sterols refer to a sum of coprostanol, coprostanone, cholesterol, dihydrocholesterol, campersterol, and β-sitosterol. The total fecal neutral sterols were significantly elevated in the OVX-SM and OVX-SE hamsters compared with that in the CTL group ($p<0.05$). This result was due to the significant increase in the dihydrocholesterol ($p<0.05$) and campersterol ($p<0.01$) (Table 5.4). For other neutral sterols, no significant difference was observed among the three groups.

The acidic sterols measured include lithocholic, deoxycholic, cholic and ursodeoxycholic acid. The total fecal acidic sterols were significantly elevated in the OVX-SM and OVX-SE hamsters compared with that in the CTL group ($p<0.01$) (Table 5.5). Among the individual acidic sterols, only lithocholic acid ($p<0.05$), cholic acid ($p<0.05$) and ursodeoxycholic acid ($p<0.05$) were found to be significantly increased in the feces in the OVX-SM and OVX-SE groups (Table 5.4). No significant difference was observed in the other acidic sterols among the three groups.

Table 5.3 Effects of soymilk (OVX-SM) and soybean extract (OVX-SE) on concentration of fecal neutral sterols (mg/ hamster/ day).

	CTL (n=6)	OVX-SM (n=9)	OVX-SE (n=9)
Coprostanol	1.09 ± 0.16	1.14 ± 0.16	1.44 ± 0.14
Cholesterol	0.30 ±0.05	0.31 ± 0.01	0.33 ± 0.08
Dihydrocholesterol	0.79 ± 0.01^{c}	0.84 ± 0.03	0.90 ± 0.05^{a}
Campesterol	1.90 ± 0.08^{b}	2.35 ± 0.03^{a}	2.68 ± 0.04^{a}
β-sitosterol	0.67 ± 0.04	0.66 ± 0.04	0.65 ± 0.12
Total neutral sterols	4.75 ± 0.10^{c}	5.30 ± 0.17^{a}	6.01 ± 0.36^{a}

Data are expressed as mean ± S.D., n=3.
Means at the same row with different superscripts (a, b) differ significantly ($p< 0.01$)
Means at the same row with different superscripts (a, c) differ significantly ($p< 0.05$)

Table 5.4 Effects of soymilk (OVX-SM) and soybean extract (OVX-SE) on concentration of fecal acidic sterols (mg/ hamster/ day).

	CTL (n=6)	OVX-SM (n=9)	OVX-SE (n=9)
Lithocholic acid	0.30 ± 0.06^{c}	0.67 ± 0.09^{a}	0.44 ± 0.05^{b}
Deoxycholic acid	0.01 ± 0.02	0.00 ± 0.00	0.03 ± 0.02
Chenodeoxycholic acid	0.17 ± 0.10	0.15 ± 0.13	0.28 ± 0.03
Cholic acid	0.12 ± 0.06^{c}	0.30 ± 0.04^{a}	0.28 ± 0.01^{a}
Ursodeoxycholic acid	0.40 ± 0.06^{c}	0.55 ± 0.05^{a}	0.55 ± 0.09^{a}
Total acidic sterols	1.00 ± 0.02^{b}	1.67 ± 0.22^{a}	1.59 ± 0.09^{a}

Data are expressed as mean ± S.D., n=3.

Means at the same row with different superscripts (a, b) differ significantly ($p < 0.01$)

Means at the same row with different superscripts (a, c) differ significantly ($p < 0.05$)

5.5 Discussion

Ovariectomization induced an increase in the serum cholesterol level in hamsters. The objective of this study was to investigate whether soymilk or soybean extract are effective in preventing the increase of blood cholesterol caused by ovariectomization. The present study demonstrated that oral doses of soymilk or soybean extract had a favorable effect on the serum cholesterol level. Soymilk or soybean extract could significant decrease serum TC and non-HDL-C in the ovariectomized hamsters, leading to a decrease in the ratio of non-HDL-C to HDL-C compared with the ovariectomized control. Also, supplementation of soymilk or soybean extract was associated with a decrease in serum TG although the difference among three groups was insignificant in the present study.

There are multiple mechanisms by which the isoflavones in soymilk or soybean extract provide such a beneficial effect. The isoflavones are structurally similar to estrogen and bind to the estrogen receptor, so it is biologically plausible that they protect against atherosclerosis development as estrogen agonists. This mechanism may be via alterations in liver cholesterol metabolism (Sirtori *et al.,* 1984)), resulting in the improvement of plasma lipids. A second potential mechanism is that genistein has antioxidant property, which has been proved in the previous experiment described elsewhere in the thesis.

Estrogen injection into ovariectomized rats reduced serum cholesterol level (Sato *et al.*, 1996). A similar result was observed in the present study, suggesting that the hypocholesterolemic effect of soymilk or soybean extract was at least in part due to the estrogenic activity of the soybean isoflavones.

Two recent studies examining isoflavone intake in humans have reported a lack of significant effect on plasma lipids and lipoproteins (Hodgson *et al.,* 1998; Nestel *et al.,* 1997). Results from these studies suggest that isoflavones alone do not improve plasma lipid and lipoprotein variables or impart the same CHD health benefits as intact soy protein. However, the present study demonstrated that both the OVX-SM and OVX-SE groups could improve plasma lipid and lipoprotein profiles. This study is in agreement with Kathryn *et al.* (1999), who found that the isoflavone extract of soy protein had a lipid-lowering effect. Therefore, caution must be exercised in extrapolating the results from animals to humans.

The previous studies showed that estrogen increased HDL-C level and decreased LDL-C level in humans (Bruschi *et al.*, 1996; Goldstein and Stampfer, 1995). This was in contrast to the present study in which both the HDL-C and LDL-C were decreased. This discrepancy may be due to the difference in cholesterol metabolism between ovariectomized hamsters and postmenopause women. The present study clearly demonstrated that soymilk and soybean extract

could lower the ratio of LDL-C to HDL-C. This ratio is commonly used as a risk factor in assessment of cardiovascular disease. The higher the ratio, the greater the risk of cardiovascular diseases.

The mechanisms by which soymilk and soybean extract lowered the ratio of non-HDL-C to HDL-C remained unexplored. One of the possible mechanisms may be associated with increased fecal excretion of total neutral and acidic sterols as observed in the present study. The result is in agreement with that reported by Potter (1996), who demonstrated that the dietary soy protein can enhance bile acid excretion in rabbits and rats. A decrease in intestinal cholesterol absorption has also been suggested as possible mechanism for the lipid-lowering effects of soy protein and/or soybean isoflavones (Nagata *et al.,* 1982; Sugano *et al.,* 1988).

Soymilk or soybean extract may also increase in LDL receptor mRNA in mononuclear cells (Baum *et al.,* 1998) and an increase in LDL receptor activity (Lovati *et al.,* 1987; Sirtori *et al.,* 1984). The increase in LDL receptor activity is very evident, following soy protein intake, in mononuclear cells isolated both from patients with familial hypercholesterolemia (Lovati *et al.,* 1987) and also from postmenopausal women with moderate cholesterol intake. Recent data suggest that soy protein subunits, particularly 7S, one of the major storage proteins of soybean, directly activate LDL receptors in the human liver, thus providing a novel

mechanism of plasma cholesterol reduction different from currently available hypolipidemic drugs (Brooks and Morr, 1992).

The present study in ovariectomized hamster, although not directly applicable to humans, may have some implications for postmanopause women who often consume soymilk and soya products. All data presented here suggest that drinking soymilk is beneficial in maintaining one's blood cholesterol healthy.

Chapter 6
Conclusion

Soybean is one of the major food sources in China. It is usually made and consumed in the form of bean curd and soymilk. Recently, many studies have been carried out to examine the health benefits of soybean. It is believed that isoflavones and their glycosides are the active components that are responsible for these health benefits of soybean. In the present study, seven isoflavones and their glycosides were isolated and purified from soybean, namely genistein, genistin, 6"-*O*-malonylglycitin, 6"-*O*-malonylgenistin, daidzein, daidzin and glycitin.

The ratio of non-HDL-C to HDL-C is commonly used as a risk factor of cardiovascular diseases. The higher the ratio, the greater the risk of cardiovascular diseases. By using hamster as an animal model, soymilk (SM) supplementation as a beverage could significantly lowered the ratio of non-HDL-C to HDL-C in hamsters fed a high cholesterol diet. In contrast, drinking cow's milk (CM) significantly increased the ratio. The hypolipidemic activity of soymilk compared with cow's milk was also reflected from the observation that serum TC was decreased when the hamsters were switched from cow's milk to soymilk. If the data could apply to humans, the decrease in the ratio of non-HDL-C to HDL-C indicated that drinking soymilk might be associated with a decreased risk of cardiovascular diseases.

Soymilk and soybean extract also had a favorable effect on the serum cholesterol level in ovariectomized hamsters. The soymilk and soybean extract could significant decrease serum TC and non-HDL-C, leading to a decrease in the ratio of non-HDL-C to HDL-C in ovariectomized hamsters fed a high cholesterol diet. Also, consumption of soymilk and soybean extract was associated with a decrease of serum TG in these hamsters. However, there was no effect on the concentration of cholesterol in the liver and other issues in both the OVX-SM and OVX-SE hamsters compared with the control group. These results strongly suggest that the hypocholesterolemic activity of soymilk and soybean extract is at least in part related to their estrogenic activity of the soybean isoflavones.

The seven individual soybean isoflavones and their glycosides showed weaker antioxidant activity in Cu^{2+}-mediated oxidation of human low-density lipoprotein (LDL) *in vitro* compared with a major tea antioxidant, epicatechin. A similar result was also shown in the FRAP assay. For the DPPH assay, the seven soybean isoflavones and their glycosides were less effective in scavenging free radicals than α-tocopherol. The present results clearly demonstrated that individual soybean isoflavones and their glycosides possessed antioxidant activity, but, their antioxidant potency was much weaker compared with that of epicatechin and α-tocopherol.

In conclusion, soybean is rich in isoflavones and their glycosides. The

hypocholesterolemic activity of soymilk, together with its weak antioxidant against LDL-oxidation, suggests that soymilk is a potential healthy beverage in prevention of CHD. Soymilk may serve as an alternative to treat the hyperlipidemia, especially for those patients whose cholesterol level is marginally high not to warrant the prescription of cholesterol-lowering drugs.

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