1.3. Functions in the Plants

The flavonoids are a remarkable group of plant metabolites. No other class of secondary product has been credited with so many — or such diverse — key functions in plant growth and development. Many of these tasks are critical for survival. Others provide a competitive edge to plants that grow under sub-optimal environments. The flavonoids are evidently extremely useful to plants, so that species from all orders of the plant kingdom invest significant amounts of metabolic energy into the production of these compounds (Gould & Lister, 2006).

However, it is difficult to see any underlying structural feature of flavonoids that leads to a specific biological function, although it is entirely possible that there is some subtle feature or features of which we are unaware (Bohm, 1998).

1.3.1. Functions in Plant Growth

1.3.1.1. Attractant for Pollination and Seed Disperal

As mentioned before, flavonoids were found in the beginning as pigments of plants. Some flavonoids are intensely coloured, e.g. the anthocyanins, and provide a wide range of red to blue colours in flowers, fruits and leaves. Others, like the flavones, are essentially colourless and provide the "whiteness" of white flowers and also act as co-pigments to the widespread anthocyanins (Harborne & Baxter, 1999a). For example, the blue flower colour is usually due to the presence in the petals of an anthocyanin based on delphinidin (see figure 1-14). However, most delphinidin glycosides are mauve in colour and the shift to the blue region usually requires the presence of a flavone co-pigment, and occasionally of one or more metal cations (Harborne & Williams, 2000).

One of the best established functions of flavonoid pigments is in the production of flower colour and the provision of colours attractive to plant pollinators. Plants that are insect-pollinated generally have flowers with large, brightly coloured petals, in contrast to most wind-pollinated plants for which flowers are small, dull, and often

apetalous. Pigmentation presumably acts as a signal to attract pollinating insects or birds (Gould & Lister, 2006). Flavonoids can determine which animal vector is attracted to effect pollination. It is clear that bees generally prefer blue and yellow, butterflies pink or white, birds red, and moths white (Harborne *et al.*, 1975).

Flavonoids in fruit probably serve to attract frugivores that assist in seed dispersal. This is especially important for larger plants such as trees, for which seeds need to be transported some critical distance away from the parent to ensure germination (Gould & Lister, 2006).

Besides attracting pollinators, flavonoid pigments also play important roles in pollen germination and plant tube growth, though the mechanism remains unclear.

1.3.1.2. Legume Nodulation and Mycorrhizal Fungi

Flavonoid levels in plants can be affected by their nutritional status. Low nitrate concentrations, for example, induce flavonoid accumulation, which then serve as chemo-attractants to nitrogen-fixing bacteria. Nodulation then restores the nitrogen economy of the plant (Peters *et al.*, 1986; Redmond *et al.*, 1986).

Flavonoids act as signal molecules in the early stage of legume-*Rhizobium* symbiosis. Flavonoids can induce various *nod* genes in soil-borne rhizobia after being released into the soil (Redmond *et al.*, 1986). *Nod* genes are responsible for the synthesis of lipochitin-oligosaccharides (LCOs), also called *Nod* factors, which are species-specific signaling molecules that initiate root nodule development. The *nod* gene inducers so far identified from seeds and seed coats of legumes include flavones, flavonones, isoflavones, flavonols and anthocyanins (Gould & Lister, 2006).

Certain flavonoids also act as inhibitors of *nod* gene expression, often by competitive inhibition. Inhibitors are sometimes strain-specific. The isoflavone daidzein induces *nod* gene expression in soybean nodulates but is an inhibitor in clover and pea nodulates (Peters *et al.*, 1986). The environment may also influence whether compounds act as inducers or inhibitors of *nod* induction.

The capacity for nitrogen fixation via nodulating bacteria is limited to relatively few plant species. In contrast, arbuscular mycorrhizal fungi (AMF) associations with roots occur in about 80% of plant species. The mutualistic association is important for improving the nutritional status of plants in soil where nutrients such as phosphate are limited (Gould & Lister, 2006). As with nodulation, exudates from seeds and seedlings affect plant infection by AMF, and flavonoids are one group of compounds present in such exudates. There are numerous reports of the effects of specific flavonoids on mycorrhizae in a wide range of plant species. However, flavonoids are not always essential signal molecules in mycorrhizal symbioses. Various other phenolic acids can elicit similar effects (Gould & Lister, 2006).

1.3.2. Protective Functions

1.3.2.1. Flavonoids as Ultraviolet Shields

One of the most frequently cited functions for flavonoids is to serve as ultraviolet filters. Ultraviolet radiation is generally classified into three wavelength ranges: UV-A (320nm-390nm), UV-B (280nm-320nm), and UV-C (<280nm), each with a different energy and different ecological significance. Radiation reaching the earth is high in the UV-A region, drops sharply in the UV-B region and drops nearly to zero at 290nm (Bohm, 1998). UV-B is the band of lower wavelength and higher energy, which can penetrate the ozone layer in the stratosphere and hence potentially cause damage to plant life because the absorption spectrum of DNA lies in the range 240-310nm, within the wavelength range of UV-B that reaches the surface of the planet (Bohm, 1998). The flavonoids generally absorb in the 280-320nm region and thus are capable of acting as UV filters, thereby protecting the underlying photosynthetic tissues from damage. A growing body of evidence suggests that plants subjected artificially to UV-B radiation respond by changes in the pathway of flavonoid synthesis (Harborne & Williams, 2000).

The colourless flavonoids are thought to be primarily involved in the protection against UV radiation. Plants often respond to UV light by the activation of flavonoid biosynthetic genes (Gould & Lister, 2006). For the last two decades, the anthocyanins

have been included alongside other flavonoids and the hydroxycinnamic acids as potential UV protectants (Gould & Lister, 2006). Red leaves often reflect significantly less solar UV than do green leaves as a consequence of their higher UV absorbance (Lee & Lowry, 1980). If the energy of the absorbed UV is not transmitted to cellular organelles, then anthocyanins could serve to moderate the damage to DNA, proteins, and membranes in plants that grow naturally in high UV-B environments (Gould & Lister, 2006).

1.3.2.2. Protection from Insect and Mammalian Herbivores

Flavonoids, which include isoflavonoids, anthocyanins, flavones, flavonols, and proanthocyanidins, along with other phenolics, help protect plants from herbivory by both insects and mammals (Harborne, *et al.*, 1975). Although most research has looked at leaves, protective flavonoids have also been found in other plant parts, such as the roots and seed coats. Insecticidal activity of flavonoids is achieved through various mechanisms including effects as feeding deterrents, digestion inhibitors, and direct toxicity (Harborne, 1994). One of the best-known and commercially valuable flavonoid insecticides is the family of rotenoid isoflavonoids, in particular rotenone. Rotenone is potent against a wide range of pests including leaf-chewing beetles, caterpillars, flea beetles, and aphids (Gould & Lister, 2006).

Some flavonoids can also serve as herbivore deterrents for mammals (Bohm, 1998). Higher oligo-meric forms of proanthocyanidins are feeding deterrents, or else they impair digestion due to their ability to precipitate proteins. Some mammals have adapted to a diet containing condensed tannins by the production of proline-rich proteins in the saliva. These proteins have a strong affinity for tannins and bind to them in the mouth so that the hydrogen-bonded complex passes through the stomach without causing any damage (Bohm, 1998). There are examples where the tannin-binding capacity is restricted to condensed tannins and the reaction does not occur with hydrolyzable tannins. Scandinavian and North American moose can feed on twigs and bark from a range of trees and shrubs but they cannot eat tissue of *Rubus* and *Alnus*, which contain both classes of tannin. North American deer can eat more widely as their salivary proteins can bind both types of tannins (Gould & Lister, 2006).

1.3.2.3. Defence against Pathogenic Microbes

The isoflavonoids, which act as effective phytoalexins, can be defined as small molecular weight anti-microbial compounds or biological stress metabolites. They can be constitutive, or else are inducible by wounding or biological attack. The constitutive versus inducible response varies between different species, and can also vary within the plant depending on age or environment (Gould & Lister, 2006). Isoflavonoids are extremely toxic to fungal pathogens. These flavonoids inhibit fungal spore germination, germ tube elongation, and hyphal growth through causing damage to membrane systems (Bohm, 1998). Antimicrobial activity has also been noted in the flavans, flavanones, 3-hydroxyflavanones, and flavonols.

1.3.2.4. Other Protection

Chilling stress has been shown to promote the formation of colourless flavonoids. Cold treatments (and drought stress) caused increases in levels of (–)-epicatechin and hyperoside (quercetin 3-galactoside) in two species of hawthorn, *Crataegus laevigata* and *C. monogyna* (Kirakosyan *et al.*, 2003b). Such treatments also enhanced the antioxidant capacity of the shoot extracts, and this may be the primary function of these cold-inducible flavonoids. Similarly, high temperatures have also been shown to influence flavonoid gene expression (Gould & Lister, 2006).

Additionally, flavonoids also participate in the resistance to high levels of metals in soils. Roots of maize (*Zea mays*) plants that had been exposed to aluminum have been found to exude high levels of phenolic compounds (Gould & Lister, 2006). The observation is consistent with the metal-binding activity of many flavonoids. Certain flavonoids can form complexes with aluminum in the root and condensed tannins possibly bind and detoxify aluminum in the root apices (Harborne, 1994). High concentrations of metals such as copper and aluminum could result in the production of reactive oxygen species. Plants with high flavonoid production may be able to combat this oxidative stress (Gould & Lister, 2006).

1.4. Flavonoids and Human Health

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans well as valuable components of seasoning, beverages, cosmetics, dyes and medicines. The World Health Organization estimated that ≈ 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components (Anton, 1988; Craig, 1999). This is based on the premise that plants contain natural substances that can promote health and alleviate illness. Flavonoids are one of these beneficial groups.

1.4.1. Epidemiology Studies

Probably the most active area of flavonoid research at the present time is in the possible medicinal contribution that flavonoids make to human health because research interest in flavonoids has grown considerably due to a wealth of evidence suggesting that a flavonoid-rich diet is beneficial for health (Nijveldt *et al.*, 2001). The most direct evidence comes from a number of large epidemiological studies whose results have become known in the last two decades.

1.4.1.1. Flavonoids and Cardivascular Disease

Most, but not all, prospective cohort studies have indicated some degree of inverse association (from borderline to modest) between flavonoid intake and cardiovascular disease (CVD) or stroke. Some studies have also provided more detailed results regarding specific types and sources of flavonoids.

A cross-sectional study by Hertog *et al.* (1995) examined the association between flavonoid intake and 25-year risk of coronary heart disease (CHD) mortality, pooling data from the Seven Countries Study, including cohorts from Europe, Japan and the USA. Data on dietary intake was provided by 12,763 middle-aged men, from which total intake was calculated. Average flavonoid intake was shown to be inversely related with CHD mortality risk. In age-adjusted models, flavonoid intakes accounted for 15% of the variance ($p = 0.048$) in CHD mortality; additional adjustment for

saturated fat intake and smoking resulted in flavonoids explaining 8% of the total variance ($p = 0.01$). Besides, a number of cohort studies found an inverse association between flavonoid intake and cardiovascular disease, which included the Zutphen Elderly Study (Hertog *et al.*, 1993a), the Finnish Mobile Clinic Study (Knekt *et al.*, 1996; Knekt *et al.*, 2002), the Iowa Women's Health Study (Yochum *et al.*, 1999), the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study (Hirvonen *et al.*, 2001a), and the Rotterdam Study (Geleijnse *et al.*, 2002). However, no association was found between flavonoid intake and risk of coronary heart disease in subjects free of disease at baseline in the Health Professionals Follow-up Study (Rimm *et al.*, 1996) and in the Women's Health Study (Sesso *et al.*, 2003). Interestingly, in the Caerphilly Study (Hertog *et al.*, 1997), flavonol intake in 1900 men from Wales was directly associated with the risk of ischemic heart disease and all-cause mortality. The authors suggested that this association could be explained by high tea consumption, which in turn is related to a less healthy life-style and lower social class in the UK, in contrast to other populations in which high tea consumption is associated with a more healthy life-style. The above-mentioned studies are summarized in table 1-1 sorted by date.

1.4.1.2. Flavonoids and Cancer

The epidemiological evidence regarding the cancer-protecting effects of flavonoids is conflicting. Some of the case-control studies have indicated an inverse association between intake of flavonoids and cancer risk (lung cancer (De Stefani *et al.*, 1999a; Le Marchand *et al.*, 2000), upper digestive tract cancer (De Stefani *et al.*, 1999b), and gastric cancer (Garcia-Closas *et al.*, 1999)). Other case-control studies found no associations (lung cancer (Garcia-Closas *et al.*, 1998) and bladder cancer (Garcia *et al.*, 1999)). In 2 cohort studies, no association between intake of flavonoids and risk of several cancer types was present (Hertog *et al.*, 1994; Hertog *et al.*, 1995), but in 2 other cohort studies, an inverse association was shown for lung cancer (Knekt *et al.*, 1997; Hirvonen *et al.*, 2001b).

A much larger study from Finland (Knekt *et al.*, 1997) provides stronger evidence for a protective role of flavonoids against cancer. The incidence of cancer at all sites including lung, stomach, colorectum, pancreas, prostate, urinary organs, nervous system, leukaemia/lymphoma, skin and breast were inversely associated with

Table 1-1 Summary of cohort studies on flavonoids and risk of CHD or stroke

Authors (year)	No. of subjects	Age (yr)	Follow-up period	Study location	Flavonoid sources	Flavonoid components	Flavonoid intake (mg/d)	Outcome RRs (95%CI) & conclusions
Hertog *et al.*, 1993a	805 men	65 – 84	5 yrs	Netherlands	tea; onions; apples	quercetin; kaempferol; myricetin; apigenin; luteolin	12.0 – 41.6	CHD incidence 0.42 (0.20-0.88); CHD death 0.32 (0.15-0.71); +
Keli *et al.*, 1996	552 men	50 – 69	15 yrs	Netherlands	tea; apples	quercetin; kaempferol	18.3 – 28.6	All stroke 0.27 (0.11-0.70); +
Knekt *et al.*, 1996	2,748 men 2,385 women	30 – 69	14 yrs	Finland	apples, onions, berries, etc.	quercetin	2.1 – 5.5	CHD death: men 0.67 (0.44-1.00); women: 0.73 (0.41-1.32); +
Rimm *et al.*, 1996	34,789 men	40 – 75	6 yrs	USA	tea; onions; apples; broccoli	quercetin; kaempferol; myricetin	7.1 – 40	CHD incidence1.08 (0.81-1.43); CHD death 0.63 (0.33-1.20); ±
Hertog *et al.*, 1997	1,900 men	45 – 59	14 yrs	South Wales	tea; onions	quercetin	13.5 – 42.8	IHD incidence 1.1 (0.6-1.6); IHD death 1.6 (0.9-2.9); –
Yochum *et al.*, 1999	34,492 women	55 – 69	10 yrs	USA	tea; apples; broccoli	quercetin; kaempferol; myricetin; luteolin; apigenin	4.0 – 28.6	CHD death 0.62 (0.44-0.87); + stroke 1.02 (0.59-1.79); ±
Hirvonen *et al.*, 2001a	25,372 male smoker	50 – 69	6.1 yrs	Finland	fruits; berries; vegetables; tea; wine	quercetin; kaempferol; myricetin; luteolin; apigenin	3.9 – 17.8	MI incidence 0.77 (0.64-0.93); CHD death 0.89 (0.71-1.11); +
Geleijnse *et al.*, 2002	1,836 men 2,971 women	> 55	5.6 yrs	Netherlands	tea	quercetin; kaempferol; myricetin	16.8 – 40.0	MI incidence 0.57 (0.33-0.98); MI death 0.35 (0.13-0.98); +
Knekt *et al.*, 2002	10,054 men & women	30 – 69	6 yrs	Finland	apples; onions; etc.	quercetin; kaempferol; myricetin; naringenin; hesperetin	4.3 – 39.5	IHD incidence 0.79 (0.63-0.99); +
Sesso *et al.*, 2003	38,445 women	45 – 89	6.9 yrs	USA	tea; broccoli; apples; onions; tofu	quercetin; kaempferol; myricetin; luteolin; apigenin	8.9 – 47.4	CHD incidence 0.88 (0.68-1.14); CHD death 0.80 (0.59-1.09); ±

RR: relative risk; CI: confidence intervals; CHD: coronary heart disease; IHD: ischemic heart disease; MI: myocardial infarction;

+: flavonoid intake inversely associated with the risk; ±: flavonoid intake not associated with the risk; -: flavonoid intake positively associated with the risk

flavonoid intake, and this association was primarily due to the lower rates of lung cancer in the groups with the highest flavonoid intake (relative risk of 0.54 with a 95% confidence interval of 0.34-0.87, in comparison with the lowest quartile). The protection was greatest in individuals who were under 50 years of age (relative risk of 0.33 with a 95% confidence interval of 0.15-0.77) and in non-smokers (relative risk of 0.13 with a 95% confidence interval of 0.03-0.58). Furthermore, this association was found not to be due to other nutrients that have been studied for their ability to prevent cancer, such as vitamin E, vitamin C, or β-carotene.

However, in the Zutphen study (Hertog *et al.*, 1994), which associated the intakes of 5 flavonoids — quercetin, kampferol, myricetin, apigenin, and luteolin — with the incidence or mortality from all causes of cancer or with the mortality from alimentary or respiratory tract cancers by a cohort of 878 men beginning in 1960 for 25 years, flavonoid intake was found not to be associated with all-site cancer outcomes but inversely with the rates of cancer in the alimentary or respiratory tract. In the 7 countries (Hertog *et al.*, 1995) that were included in the Zutphen study it was also found that cancer risk was not associated with flavonoid intake, and similarly in the research on the relation to lung cancer in Spanish women (Garcia-Closas *et al.*, 1998).

Knekt *et al.* (2002) studied the association between the intake of flavonoids and the risk of several chronic diseases in 10,054 participants of the Finnish Mobile Clinic Health Examination Survey. Higher quercetin intakes were associated with lower risk of asthma, lung cancer, type 2 diabetes and ischemic heart disease. The incidence of cerebrovascular disease and asthma was also lower with higher intakes of hesperetin and naringenin.

Diets in Japan and China are associated with lower rates of cancers than in Europe and the USA, such as those of breast cancer, prostate cancer and colon cancer (Birt *et al.*, 2001; Le Marchand *et al.*, 2002) and this is often attributed to the high soy food consumption in eastern countries. However, eastern diets are different from western diets in many more ways and soy food has many components with anti-cancer properties other than isoflavones (Greenwald, 2004). Evidence to associate flavonoid consumption with a reduced risk of cancer from epidemiological studies is not as strong as that regarding CVD, although protection remains a possibility.

1.4.1.3. Flavonoids and Sex Hormone-Dependent Disease

The estrogenic activity of isoflavones may influence some sex-hormone dependent diseases. The consumption of isoflavones could be effective at reducing menopausal symptoms and for this reason isoflavone supplements are used as a natural remedy by many women instead of hormone replacement therapy (Carusi, 2000). The supplementation of soy isoflavones has been reported to prevent osteoporosis (Anderson & Garner, 1997), improve menopausal symptoms (Brzezinski *et al.*, 1997; Messina, 2002), and lower cholesterol levels (Lichtenstein, 1998). Additionally, isoflavones might have beneficial effects on bone health (Ma *et al.*, 2008), although there have been mixed positive and negative outcomes from a number of epidemiological studies (Setchell & Lydeking-Olsen, 2003).

1.4.2. Antioxidative Activities

The most important and best-described property of flavonoids is their capacity to act as antioxidants. The flavones and catechins seem to be the most powerful flavonoids for protecting the body against reactive oxygen species (Nijveldt *et al.*, 2001).

Body cells and tissues are continuously threatened by the damage caused by free radicals and reactive oxygen species, which are produced during normal oxygen metabolism or are induced by exogenous damage (Halliwell & Gutteridge, 2007). The mechanisms and the sequence of events by which free radicals interfere with cellular functions are not fully understood, but one of the most important events seems to be lipid peroxidation, which results in cellular membrane damage. This cellular damage causes a shift in the net charge of the cell, changing the osmotic pressure, leading to swelling and eventually cell death. Free radicals can attract various inflammatory mediators, contributing to a general inflammatory response and tissue damage.

To protect themselves from reactive oxygen species, living organisms have developed several effective mechanisms. The antioxidant defence mechanisms of the body include not only enzymes such as superoxide dismutase, catalase, and glutatione peroxidase, but also non-enzymatic counterparts such as glutathione, ascorbic acid, and

α-tocopherol (Halliwell, 1995). The increased production of reactive oxygen species during injury results in consumption and depletion of the endogenous scavenging compounds. Flavonoids may have an additive effect to the endogenous scavenging compounds.

Flavonoids can exercise their antioxidant activity in different ways, for example: scavenging radical $OH^{\bullet}$ (hydroxyl), O_2, $^1O_2$, $O_{2-}^{\bullet}$ (superoxide); anti-lipoperoxidation ($R^{\bullet}$; $ROO^{\bullet}$; $RO^{\bullet}$); and activities of metal chelation (Bombardelli & Morazzoni, 1993). Flavonoids can interfere with several different free radical-producing systems, and can also increase the function of the endogenous antioxidants (Tripoli *et al.*, 2007).

1.4.2.1. Direct Radical Scavenging

Flavonoids can prevent injury caused by free radicals in various ways, one of which is the direct scavenging of free radicals. Flavonoids are oxidized by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical before causing damage to cells and tissues. The most common reactive part of the flavonoids is the hydroxyl group, which has high reactivity. The theory (Korkina & Afanas'Ev, 1996) can be expressed as:

$$\text{Flavonoids (OH)} + R^{\bullet} \longrightarrow \text{flavonoids } (O^{\bullet}) + RH$$

where $R^{\bullet}$ is a free radical and $O^{\bullet}$ is an oxygen free radical. For instance, epicatechin and rutin are powerful radical scavengers (Hanasaki *et al.*, 1994). By scavenging radicals, flavonoids can inhibit low density lipoprotein (LDL) oxidation *in vitro*. This action protects the LDL particles and, theoretically at least, may prevent atherosclerosis (Aviram & Fuhrman, 2003).

1.4.2.2. Nitric Oxide

Nitric oxide is produced by several different types of cells, including endothelial cells and macrophages. The early release of nitric oxide through the activity of constitutive nitric oxide synthase is important in maintaining the dilation of blood vessels, but the much higher concentrations of nitric oxide produced by inducible nitric oxide synthase in macrophages can result in irreversible oxidative damage to the cell

membrane (Shoskes, 1998). Flavonoids can scavenge free radicals by acting as antioxidants, and resulting in less damage. Therefore, it has been speculated that nitric oxide scavenging plays a role in the therapeutic effects of flavonoids. Silibin is a flavonoid that has been reported to inhibit nitric oxide in a dose-dependent manner (Dehmlow *et al.*, 1996).

1.4.2.3. Leukocyte Immobilization

The immobilization and firm adhesion of leukocytes to the endothelial wall is major mechanism responsible for the formation of oxygen-derived free radicals, but also for the release of cytotoxic oxidants and inflammatory mediators and further activation of the complement system. Under normal conditions, leukocytes move freely along the endothelial wall. During ischemia and inflammation, various mainly endothelium-derived mediators and complement factors may cause adhesion of the leukocytes to the endothelial wall, thereby immobilizing them and stimulating de-granulation of the neutrophil. As a result, oxidants and inflammatory mediators are released, resulting in injury to tissues. Oral administration of a purified micronized flavonoid fraction was reported to decrease the number of immobilized leukocytes during reperfusion (Friesenecker *et al.*, 1994), which may be related to the decrease in total serum complement and is a protective mechanism against inflammation-like conditions associated with, for example, reperfusion injury (Friesenecker *et al.*, 1995). The inhibitory effect of some flavonoids on mast cell de-granulation was shown to be due to modulation of the receptor-directed Ca^{2+} channels in the plasma membrane (Bennett *et al.*, 1981).

1.4.2.4. Xanthine Oxidase

The xanthine oxidase pathway has been implicated as an important route in the oxidative injury to tissues, especially after ischemia-reperfusion (Shoskes, 1998). Xanthine oxidase is a source of oxygen free radicals because it reacts with molecular oxygen, thereby releasing superoxide free radicals. Some flavonoids, including hesperetin, theaflavin-3, 3′-digallate, quercetin, rutin and silibin, inhibit xanthine oxidase activity, thereby resulting in decreased oxidative injury (Chang *et al.*, 1993; Dew *et al.*, 2005). Cos *et al*. (1998) carried out a study on structure-function

relationships in which luteolin (3',4',5,7-tetrahydroxyflavone) (see figure 1-6) was reported to be the most potent inhibitor of xanthine oxidase.

1.4.2.5. Chelation

Lipid peroxidation results when reactive oxygen species are in the presence of iron. Specific flavonoids are known to chelate iron, thereby removing a causal factor for the development of free radicals (Korkina & Afanas'Ev, 1996; Hider *et al.*, 2001). Quercetin in particular is known for its iron-chelating and iron-stabilizing properties.

1.4.2.6. Structure-Function Relationships

Flavonoids do not react specifically with a single species and so a number of different evaluation methods have been developed which makes comparison of the various studies difficult (Harborne & Williams, 2000). Often an overall antioxidant effect has been measured.

Flavonoids are powerful antioxidants against free radicals, because they act as "radical-scavengers". This activity is attributed to their hydrogen-donating ability (Burda & Oleszek, 2001; Majo *et al.*, 2005). According to kinetic studies of aroxyl radical formation and decomposition reactions, the antioxidant capacity of a flavonoid is linked to its particular chemical structure. Three structural groups are important for the antioxidant capacity (Dugas *et al.*, 2000; Heim *et al.*, 2002): (A) the ortho-dihydroxy (catechol) structure in the B-ring; (B) the 2, 3-double bond; (C) the presence of both 3- and 5- hydroxyl groups. The flavonoid antioxidant capacity is linked to a combination of these chemical and structural elements, including glycoside presence or absence (glycosides or aglycones) and the presence of free hydroxyls or the number and position of hydroxyls eventually esterified (Rice-Evans *et al.*, 1996; Bors *et al.*, 2001).

The absence of the hydroxyl group at position 3 in flavanones and flavones decreases their antioxidant ability, as does the absence of the catechol structure in the B-ring (Sichel *et al.*, 1991). However, the double bond at 2, 3 makes the structure more reactive, for this reason apigenin is a moderate antioxidant compound, while

naringenin has no activity against the superoxide ion. Butein (figure 1-17) and other 3,4-dihydroxychalcones are more active than analogous flavones because of their ability to achieve greater electron delocalisation (Dziedzic & Hudson, 1983). Similarly, isoflavones are often more active than flavones because of the stabilising effects of the 4-carbonyl and 5-hydroxyl in the former. In the antioxidant action of *o*-dihydroxyflavonoids metal chelation is an important factor. Moreover, it has been reported that the corresponding 3-*O*-glucosides are more active than are their aglycones (Tripoli *et al.*, 2007).

1.4.3. Enzyme Inhibition

Compared with research on the antioxidant capacities of flavonoids, there has been less research on other beneficial effects of flavonoids. The major effects of flavonoids (e.g., anti-allergic effects) may be the result of radical scavenging (Halliwell, 2000). Another possible mechanism by which flavonoids act is through interaction with various enzyme systems. Furthermore, some effects may be a result of a combination of radical scavenging and an interaction with enzyme functions (Nijveldt *et al.*, 2001).

As mentioned before, some flavonoids inhibit the enzyme xanthine oxidase, which catalyses the oxidation of xanthine and hypoxanthine to uric acid. Another feature of flavonoids is a reduction in the release of peroxidase. This reduction inhibits the production of reactive oxygen species by neutrophils by interfering with α1-anti-trypsin activation. A progressive inactivation of proteolytic enzymes was described in neutrophils (Middleton & Kandaswami, 1992).

Another effect of flavonoids on enzyme systems is the inhibition of the metabolism of arachidonic acid, which gives flavonoids anti-inflammatory and anti-thrombogenic properties (Ferrandiz & Alcaraz, 1991; Boots *et al.*, 2008). The release of arachidonic acid is a starting point for a general inflammatory response. Neutrophils containing lipoxygenase create chemotactic compounds from arachidonic acid. They also provoke the release of cytokines (Ferriola *et al.*, 1989).

A number of structure-activity studies shown that a C2, 3-double bond, a C4-keto group and a 3, 4, 5-trihydroxy B-ring are significant features of those flavonoids showing strong inhibition of enzymes (Hodnick *et al.*, 1994; Cos *et al.*, 1998).

1.4.4. Health Benefits

Flavonoids have extensive biological activities that promote human health and help reduce the risk of disease. There are many hypotheses about the flavonoids and diseases (figure 1-19), which need further study and confirmations.

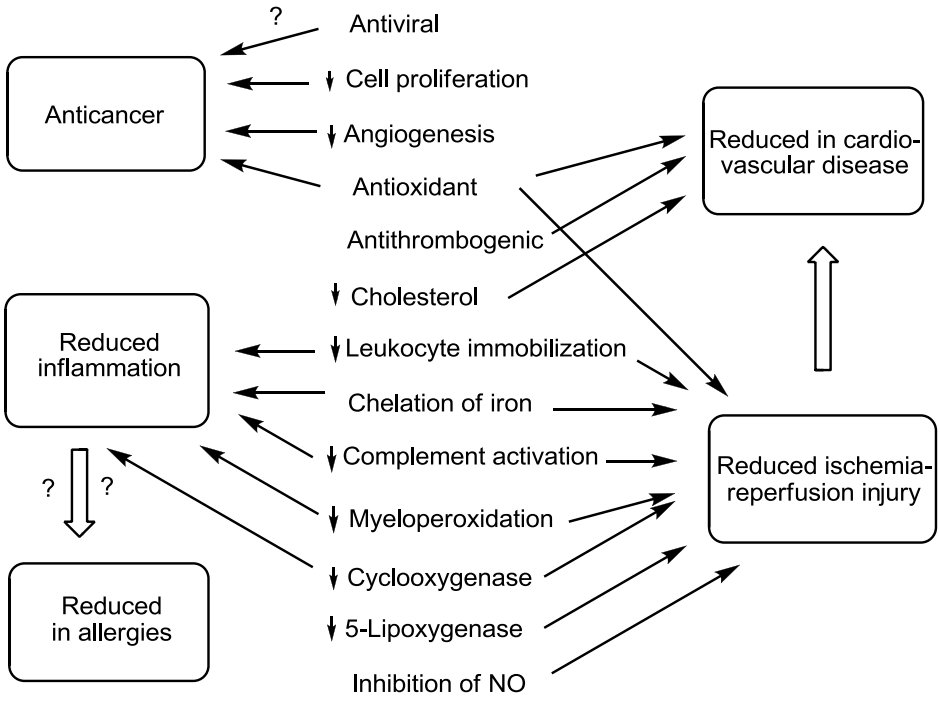

Figure 1-19 Hypotheses of the links between the working mechanisms of flavonoids and their effects on disease (Nijveldt *et al.*, 2001)

1.4.4.1. Flavonoids and Cardiovascular Disease

Coronary heart disease (CHD) is usually associated with high cholesterol levels in blood. CHD has been a serious health problem affecting populations in many developed parts of the world. High levels of LDL cholesterol in plasma may play a role in the initiation of atherosclerotic plaque. Physicochemical and biological properties of LDL can be modified through enzymatic modification such as lipases and oxygenases or non-enzymatic modifications such as glycosylation, proteoglycans

and immune complexes (Avirma, 1993). In atherosclerosis, lipid peroxidation plays an important role during the initiation and propagation steps of this disease. Serum cholesterol levels may not necessarily be a cause of the problem as manifested in the French Paradox, but oxidation of cholesterol, especially LDL (Avirma, 1993) could be the problem. Piotrowski *et al.* (1990) found high levels of cholesterols and lipid per-oxidation in tissues and phospholipids in aortic tissues obtained from people with coronary heart diseases. Atherosclerotic plaque could form as a result of lipid oxidation, especially of LDLs in plasma.

LDL peroxidation can be affected by several factors such as presence of copper ions, antioxidant content of cells (both enzymatic and non-enzymatic), and 'the composition and location of polyunsaturated fatty acids' of LDL (Aviram & Fuhrman, 2003). Extrinsic factors like antioxidant concentration in blood and other tissues may play an important role in the reduction of coronary heart disease risks. Flavonoids are able to block the oxidation of LDL by acting as antioxidants and may be responsible for their cardio-protective effect (Manthey & Guthrie, 2002).

1.4.4.2. Flavonoids and Cancer

Cancer is defined by the American Cancer Society (ACS, 2001) as "a group of diseases characterized by uncontrolled growth and spread of cells". The malfunction of genes, which control cell growth and division, may induce cancer. Cancer can be induced by many factors such as lifestyle, environmental and genetic factors.

Flavonoids are especially promising candidates for cancer prevention (Adlercreutz, 1995; Hollman & Katan, 1997; Whitten & Naftolin, 1998; Galati & O'Brien, 2004; Lof & Weiderpass, 2006). Flavonoids have been considered to be chemo-preventive or anti-cancer agents due to their antioxidative activities and inhibiting enzymes feature (Potter, 1997; Magee & Rowland, 2004; Yilmaz & Toledo, 2004; Tripoli *et al.*, 2007). There is a huge amount of research focused on the medicinal potency of flavonoids against cancer. Flavonoids can be potentially involved in each stage of the tumour process, which include DNA damage (induction), tumour development (promotion), and tumour invasion (proliferation) (Manthey *et al.*, 2001; Tripoli *et al.*, 2007). Considerable attention has been paid to their abilities to inhibit the cell cycle,

cell proliferation, and oxidative stress, and to induce detoxification enzymes, apoptosis, and the immune system (Adlercreutz, 1995; Messina & Bennink, 1998; Birt *et al.*, 2001).

1.4.4.3. Flavonoids with Estrogenic Activities

The main group of flavonoids that are well known to possess estrogenic activities are the isoflavones, such as daidzein and genistein. But the isoflavones are not the only flavonoid sub-class with estrogenic activities. By using an estrogen receptor-dependent transcriptional response assay, Miksicek (1993) reported that commonly occurring flavonoids also had estrogenic activity, and the order of estrogenicity was genistein > kampherol > naringenin > apigenin > daidzein > biochanin A > formononetin > luteolin > fisetin > catechin/taxifolin > hesperetin (Miksicek, 1995).

Isoflavones and flavonoids may be estrogenic or anti-estrogenic, depending amongst other things on the level of natural estrogens. Isoflavonoids and/or flavonoids might counteract endogenous estrogens through competitive binding to estrogen receptors, thus the estrogen level can be adjusted (Birt *et al.*, 2001).

The possible role of isoflavonoids in the prevention of cancer and in particular hormone-dependent cancers such as breast and prostate cancer is currently extensively investigated (Adlercreutz, 2002a; Adlercreutz, 2002b; Cornwell *et al.*, 2004; Dixon, 2004; Greenwald, 2004; Magee & Rowland, 2004; Holzbeierlein *et al.*, 2005). In addition, consumption of soy foods rich in isoflavones has been weakly associated with reduced colon cancer (Adlercreutz, 2002b; Guo *et al.*. 2004), although the mechanism has not been fully elucidated. Postmenopausal women may well benefit in terms of protection against heart disease and osteoporosis from estrogen replacement therapeutics strategies that utilize isoflavonoids. Older men may also benefit from protection against problems such as prostate cancer and cardiovascular disease (Wiseman, 2006).

1.5. Bioavailability

Bioavailability is a difficult concept, with several definitions. Bioavailability seeks to quantify the exposure of a target cell/tissue/organ to a bioactive form of a food-derived substance (Manach *et al.*, 2004). The notion of bioavailability integrates several variables, such as intestinal absorption, excretion of glucuronides toward the intestinal lumen, metabolism by the micro-flora, intestinal and hepatic metabolism, plasma kinetics, the nature of circulating metabolites, binding to albumin, cellular uptake, intracellular metabolism, accumulation in tissues, and biliary and urinary excretion (Manach *et al.*, 2004). Absorption, distribution, metabolism and excretion are physiological steps that determine the bioavailability of a component, which is important to fully evaluate their potential beneficial role in human health (Hollman, 2000). Understanding the bioavailability of flavonoids is critical and must always be considered (Holst & Williamson, 2004). The common pharmacokinetic parameters regarding flavonoid bioavailability include: $C_{max,}$ the highest concentration; T_{max}, time to reach C_{max}; $T_{1/2Eli}$, elimination half-life, etc. Table 1-2 shows pharmacokinetic parameters of some individual flavonoids.

1.5.1. Dietary Intake

Flavonoids are universally distributed in higher plants and many lower plant groups, while in mammals, flavonoids occur only through dietary intake of plant-based foods (Markham & Bloor, 1998). However, an estimation of the total flavonoid intake is difficult, because only limited data on contents of foods are available. On the other hand, in comparison with that of macronutrients, the beneficial intake range of flavonoids, being a micronutrient, is relatively narrow and there is a possibility of both adverse effects at higher doses and no effect at very low doses (Holst & Williamson, 2004).

In 1976, Kuhnau calculated that in the United States, dietary intake of flavonoid glycosides was 1g/day which is equivalent to an aglycones intake of 650mg/day, which has been frequently quoted although these figures are thought to be over-estimated due to poorly detailed conditions. For example, the flavonoid contents used

by Kuhnau were too high, not only because they were obtained with methods now considered obsolete, but also because sometimes flavonoid contents of non-edible parts were included (Hollman & Arts, 2000). Later, Hertog *et al.* (1993b) estimated the average intake of flavonols and flavones in the Netherlands was 23mg/day, of which the flavonol quercetin contributed 16mg/day, kaempferol 3.9mg/day and myricetin 1.4mg/day. In Denmark, the average intake of flanonols, flavones and flavanones was estimated to be 26mg/day (Dragsted *et al.*, 1996). This study also suggested that, in addition to these commonly determined flavonoids, intake of other flavonoids included 40-50mg/day catechins from tea and 6-60mg/day anthocyanins from berries, red cabbage and red wine. In Spain the total consumption of catechins and proanthocyanidin has been estimated at 18-31 mg/day (Manach *et al.*, 2004). From the UK National Food Survey database, the average daily intake of flavones and flavonols was 30mg/day with beverage (especially tea) providing 82% of the total (Ibe & Shepherd, 1995). Consumption of soya in the Asian countries is 10-35g/day, which is equivalent to a mean intake of 25-40mg isoflavones/day, with a maximum intake of 100mg/day (Kimira *et al.*, 1998; Adlercreutz *et al.*, 1991b; Coward *et al.*, 1993). Isoflavone intake is low in the UK, with average daily isoflavone intakes less than 1mg although soya-consumers' average daily intakes were higher (8.6mg in women and 7.5mg in men). But these data may be under-estimated due to soya being added to so many commercial products. Bread, bread rolls, vegetable dishes and milks made the highest contribution to isoflavone intake (Mulligan *et al.*, 2007).

Most estimates of flavonoid dietary intake range from 10-100mg/day, depending on the population studied and the technique used (table 1-1). However, it has to be noted that only part of the flavonoid sub-groups have been calculated in these studies with the flavonols and flavones most popular. Hence, these may lead to the under-estimation of flavonoid contribution to the diet. So, some authors agreed to enhance the estimate dietary intake of total flavonoids to be several hundreds of milligram per day (Hollman & Katan, 1999; Werne, 2000).

Flavonoid intake varies from country to country, reflecting cultural and dietary habits. Tea, onions and apples appear to be the most important contributors to dietary intakes of specific flavonols and flavones in North America and north European populations. Other studies have shown that red wine or soya products may be the main contributors

to the intakes of flavonoids in countries where they are widely consumed (Werne, 2000). Better estimates of flavonoid intake need improved methodology.

Table 1-2 Pharmacokinetic parameters of flavonoids

Flavonoids	T_{max} (hr)	C_{max} (μM)	Urinary excretion (%)	Elimination half-life (hr)
Daidzin	6.3 ± 0.6	1.92 ± 0.25	42.3 ± 3.0	5.3 ± 0.8
Daidzein	4.9 ± 1.0	1.57 ± 0.52	27.5	8.5 ± 0.8
Genistin	6.5 ± 0.6	1.84 ± 0.27	15.6 ± 1.8	7.8 ± 0.7
Genstein	4.1 ± 0.6	2.56 ± 1.00	8.6	7.1 ± 0.3
Glycitin	5.0	1.88 ± 0.38	42.9 ± 12.0	8.9
Hesperidin	5.5 ± 0.1	0.46 ± 0.21	8.6 ± 4.0	2.2
Naringin	5.0 ± 0.2	0.50 ± 0.33	8.8 ± 3.17	2.1 ± 0.4
Quercetin glycosides	1.1 ± 0.3	1.46 ± 0.45	2.5 ± 1.2	17.9 ± 2.2
Rutin	6.5 ± 0.7	0.20 ±0.06	0.7 ± 0.3	19.9 ± 8.1
EC	1.8 ± 0.1	0.40 ± 0.09	18.5 ± 5.7	2.5 ± 0.4
EGC	1.4 ± 0.1	1.10 ± 0.40	11.1 ± 3.5	2.3 ± 0.2
EGCG	2.3 ± 0.2	0.12 ± 0.03	0.06 ± 0.03	3.5 ± 0.3
Anthocyanins	1.5 ± 0.4	0.03 ± 0.02	0.4 ± 0.3	
Proanthocyanidin	2.0	0.02 ± 0.01		

Adapted from (Manach *et al.*, 2005). All data were converted to 50mg intake.

1.5.2. Absorption and Metabolism

Flavonoids are believed to possess potential health benefits due to their bioactivities. However, most of the studies regarding their bioactivities are based on the flavonoid aglycones, while in nature, virtually all flavonoids exist as the glycoside form, i.e. with a sugar moiety, except for the catechins. In the conjugated form, the biological activity may be greatly changed. So the big question is which form is absorbed: aglycone, glycoside, or both. The aglycones of flavonoids are mostly hydrophobic which enables them to diffuse across the intestinal membranes by passive diffusion. Conjugation makes the compound much more hydrophilic and thus less able to be absorbed across membranes by diffusion (Day *et al.*, 2000b; Foti *et al.*, 2006). As

mentioned previously, flavonoids occur predominantly as glycoside derivatives in food. Studies showed that processing methods such as canning, frying and boiling did not result in the deglycosylation of the flavonoids, though some might be lost through leaching (Crozier *et al.*, 1997; Price *et al.*, 1997; Price *et al.*, 1998a; Price *et al.*, 1998b). These results suggest that the glycosides are the compounds that are consumed. However, in food that has undergone microbial fermentation, such as red wine, black tea, tempeh and miso, the aglycone isomers are the major form present (Wang & Murphy, 1994b; McDonald *et al.*, 1998; Chun *et al.*, 2008).

In the past two decades, there has been an increase in the number of studies on flavonoid absorption, metabolism, and excretion both *in vivo* and *in vitro* predominantly focused on flavonols — represented by quercetin, flavanols — represented by catechins, and isoflavones — represented by daizdein and genistein. But the data are limited and the results are contradictory (Erlund, 2004; Karakaya, 2004; Williamson & Manach, 2005; Nielsena &Williamson, 2007).

The study of flavonol absorption in humans has been carried out through providing subjects with various sources of flavonols, e.g. flavonol-rich food, flavonol aglycone capsules and other encapsulated flavonol glycosides. In the study conducted by Hollman *et al.* (1995), the absorption of various forms of quercetin in human volunteers was determined. To avoid bacterial degradation in the colon, healthy ileostomy subjects were used. The results showed that absorption of quercetin was 52% for quercetin glycosides provided by an onion-rich meal, 24% for quercetin aglycone, and 17% for rutin supplements. They concluded that humans could absorb significant amounts of quercetin in both aglycone and glycoside form, with higher efficiency in the absorption of the conjugated form. However, in a similar study conducted by Walle *et al*. (2000) with healthy ileostomy subjects, quercetin glycosides were not detected in the ileostomy fluid. Instead, substantial amounts of the aglycone were detected. They suggested that the quercetin glycosides were hydrolyzed to quercetin in the small intestine by β-glycosidases, and the quercetin was absorbed.

In general, for quercetin, the bioavailability differs among food sources, depending on the type of glycosides they contain (Manach *et al.*, 2005). For example, quercetin

from onions are absorbed more efficiently than those from apples, tea or quercetin glycoside supplements.

The exact mechanisms of quercetin absorption in humans are not yet known. In a report by Day *et al*. (1998), a cytosolic β-glycosidase present in cell-free extracts of human small intestine was shown to be capable of hydrolyzing various flavonoid and isoflavone glycosides, with high affinity for quercetin-4'-glucoside. The enzyme complex, lactase phlorizin hydrolase (LPH) present in the brush-border membrane of the small intestine (Leese & Semenza, 1973; Day *et al.,* 2000b) was speculated to be responsible for the hydrolysis of quercetin-3-glucoside observed in the small intestine cell-free extract, releasing the aglycone into the intestine.

A study by Gee *et al.* (1998) speculated that the transport of the flavonoid glucosides through the intestine wall was through the sodium-glucose transporter, where the flavonoid conjugates might be carried into the small-gut enterocyte via an active transport mechanism. Extensive hydrolysis of the flavonoid glycosides passing through the small intestine unchanged takes place in the colon by the intestinal microflora, through the secretion of esterases or glucosidases (Spencer *et al.,* 2003). However, the liberated flavonoid aglycone is also subject to extensive degradation by the enzymes.

For catechins, the bioavailability has been studied mainly after ingestion of cocoa or tea although they are present in many fruits and in red wine. In contrast to other flavonoids, catechins exist in nature as the aglycone form and are well-absorbed (Donovan & Waterhouse, 2003). One significant characteristic of catechin bioavailability is that the absorption and elimination are much quicker (Benzie *et al.*, 1999) compared with quercetin (see table 1-2).

Isoflavones are provided by soybean-derived products in natural styles, or as supplements such as extracts from red clover or kudzu. Soy isoflavones have been the main targets of studies. They can be present as aglycones or glycosides, depending on the soy preparation. However, contradictory results for soy isoflavone bioavailability have been obtained (Manach *et al.*, 2005). Some authors investigated the differences in bioavailability between aglycones and glycosides by using pure compounds rather than food preparations. Setchell *et al.* (2001) and Rüfer *et al.* (2008) found greater

bioavailability of glucosides. Izumi *et al.* (2000) found greater bioavailability of aglycones, while Zubik & Meydani (2003) found no significant differences in the absorption efficiency for aglycones and glycosides. Some researchers investigated the differences in bioavailability between aglycones and glycosides by using natural-style soy products. Kano *et al.* (2006) found the amount of isoflavones absorbed was higher from aglycone-rich fermented soymilk than from glycoside-rich non-fermented soymilk. Okabe *et al.* (2011) reported the soy aglycones were absorbed faster and in greater amounts than glycosides in soy beans. Some other researchers found no differences between aglycone and glycoside absorption in soy drinks (Richelle *et al.*, 2002; Tsangalis *et al.*, 2005; Maskarinec *et al.*, 2008).

A significant characteristic of isoflavone bioavailability is the production of equol. Equol is an intestinal bacterial metabolite that has been shown to be more estrogenic than its precursor daidzein. Equol producers may gain more benefits from soy consumption than do non-producers (Setchell *et al.*, 2002a; Ko *et al.*, 2010). There is great inter-individual variability in the capacity to produce equol dependent on race and dietary habits (Lampe *et al.*, 1998; Ko *et al.*, 2010). Only 30-40% of the western population are "equol producers" (Frankenfeld *et al.*, 2004; Cassidy *et al.*, 2006; Wiseman, 2006), while the proportion of "equol producers" in Asia may reach 50% or higher (Morton *et al.*, 2002; Song *et al.*, 2006; Ko *et al.*, 2010).

Comprehensive accounts of the metabolism of the flavonoids are given by several authors such as Aherne & O'Brien (2002), Heinonen *et al.* (2002), Day & Williamson (2003) and Walle (2004). In brief, the absorbed flavonoids may be bound to albumin and transported to the liver via the portal vein. The liver, which is the chief organ involved in flavonoid metabolism, secretes biotransformation enzymes that act upon the absorbed flavonoids and their absorbed colonic metabolites. Flavonoids and their derivatives may undergo reactions such as hydroxylation, methylation, and reductions. The diversity of flavonoid structures means that elucidating clear pathways of metabolism is difficult. Conjugation reactions with glucuronic acid, sulfate, or glucuronic acid and sulfate, seem to be the most common types of metabolic pathways for the flavonoids (Day *et al.*, 2004). As discussed above, flavonoid metabolism may also depend on the intestinal flora present in the colon. The glycosidases, glucuronidases and sulfatases secreted by the micro-flora hydrolyse

flavonoid conjugates, removing their sugar moieties, glucuronic acids and sulfates, liberating the aglycone and phenolic acids which in turn may be absorbed from the colon. It also may involve splitting of the heterocyclic oxygen-containing ring, and the degradation products include a variety of phenyl carboxylic acids that are excreted in the urine (Day & Williamson, 1999). Figure 1-20 shows the routes of dietary flavonoids and their metabolites in humans.

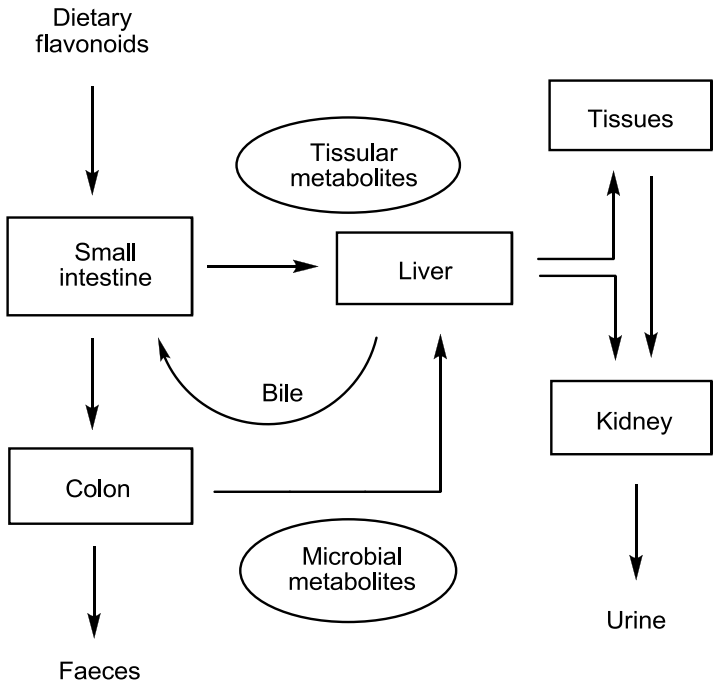

Figure 1-20 Routes for dietary flavonoids and their metabolites in humans (Scalbert *et al.*, 2002)

1.5.3. Determination of Isoflavone Metabolites in Biofluids

Determining isoflavone metabolites in human fluids, including urine and plasma, is very different to the methods of determining isoflavones in plant materials. For example, methods for the hydrolysis of plant glycosides are not easily adapted for the hydrolysis of plasma metabolites. Furthermore, in most studies dealing with flavonoid bioavailability, the plasma has been analysed after total hydrolysis by enzymes or acid and these procedures do not determine the nature of the circulating forms (Morand *et al.*, 2001).

Additionally, human fluid samples normally need to be concentrated due to the low levels of detected components while concentration is not always necessary for detecting isoflavone in natural isoflavone-rich materials, and as a result, detection limits become a most important aspect in developing analysis methods for human fluid samples. Besides, the qualitative aspects of methods are also emphasised since there may be low levels of new unknown metabolites without available purified standards. For detecting isoflavones in natural materials, generally all types of isoflavones need to be determined, either polar compounds or non-polar compounds, while for detecting isoflavone metabolites in human fluid samples, most methods focused on the less polar compounds because the hydrophilic sugar moiety has been removed by hydrolysis.

Isoflavones were first quantified in human urine by gas chromatography (Adlercreutz *et al.*, 1982; Joannou *et al.*, 1995). Then a C18 reversed-phase HPLC method was developed (Lundh *et al.*, 1988) by using UV detection of isoflavone aglycones after enzymatic hydrolysis of major isoflavone metabolites by glucuronidase and sulfatase. The method had 73-91% urine recovery, and 92-105% plasma recovery for equol and daidzein, respectively, and could detect 0.4ng daidzein and 13ng equol/ml blood and 130ng daidzein and 4000ng equol/ml urine. Franke & Custer (1994) also developed a reversed-phase HPLC method for analysis of isoflavone metabolites, after enzymatic hydrolysis, using flavone as an internal standard. Their reversed-phase solid-phase extraction method showed isoflavone recoveries of about 100%, with the UV detection limit of 1.3-2.4ng/ml of daidzein and genistein, 151-201ng/ml of equol and ODMA, respectively. Gamache & Acworth (1998) reported plasma, urine and tissue isoflavone analysis with reversed-phase HPLC and coulometric detection, giving limits of detection of 1-2ng/ml, and recoveries of 85-95%, with intra-assay precision of 2-4% relative standard deviations (RSD). This method also involved an extraction method of incubation with glucuronidase and sulfatase followed by mixing with ethanol, centrifugation, drying of supernatant and re-dissolution in aqueous methanol, providing greater sensitivity for detection of equol.

Additionally, metabolites of isoflavones were identified in human urine and plasma by GC-MS (Adlercreutz *et al.*, 1991a; Kelly *et al.*, 1993; Hutchins *et al.*, 1995; Joannou *et al.*, 1995; Heinonen *et al.*, 1999; Setchell *et al.*, 2003). Dihydrodaidzein,

dihydrogenistein, 6-hydroxy-ODMA and *cis*-4-equol were detected. Isotope dilution liquid chromatography electrospray (LC-ES) MS and MS-MS have been used to detect tissue (and serum) genistein in rats fed diets of varying isoflavone contents (Chang *et al.*, 2000). Limits of detection of genistein, measured after de-conjugation with glucuronidase and sulfatase, were 10-20pg/mg tissue for LC-ES-MS and 3-8pg/mg for MS-MS. Recovery of genistein from tissues ranged between 40-78%, and precision of analysis was 1-9% RSD.

A more rapid method for analysis of isoflavones in human urine was developed, employing time-resolved fluoroimmunoassay (TR-FIA) (Uehara *et al.*, 2000; L'Homme *et al.*, 2002; Brouwers *et al.*, 2003; de Pascual-Teresa *et al.*, 2006). Antibodies were prepared in rabbits to carboxymethyl derivatives of daidzein and genistein coupled to bovine serum albumin. Europium-labelled isoflavones were used to compete with the native isoflavones for binding to the anti-isoflavone antisera. Urine samples were pretreated with glucuronidase and sulfatase, and only 20-200µl of sample was needed. The urine samples were applied to wells coated with anti-isoflavone antisera, tracer added, and then a fluorescence enhancement solution added before reading the plates with a fluorescence detector. The method had a sensitivity of about 0.1ng/ml, with intra-assay RSD of about 2-5% and inter-assay RSD of 2-10%. The method showed good correlation with standard GC-MS methods (Adlercreutz *et al.*, 1991b), and permits more rapid screening of large populations for urinary isoflavone contents. ELISA, a quick analytical method suitable for large numbers of samples, originally developed for determining isoflavone contents in natural products, was applied to determination of isoflavone metabolites in human urine and plasma, and satisfactory results have been reported (Mathey *et al.*, 2006; Vergne *et al.*, 2007).

For those interested in developing isoflavone analytical capabilities, an HPLC method with coulometric detection is probably the method of choice, being less expensive and easier to institute than the GC-MS or immunofluorescence methods and being more sensitive than HPLC-UV methods of similar cost. For very large numbers of samples, and for greatest sensitivity, the immunofluorescence method would be best for the major soy isoflavones, genistein and daidzein. For sensitivity to equol, GC-MS is somewhat better than HPLC-coulometric detection.

1.5.4. Factors Affecting Isoflavone Metabolism

In human studies, differences between individual subjects may cause huge inter-individual variation, reflecting the considerable variation of results. Other factors also need to be considered as they may affect isoflavone metabolism.

1.5.4.1. Metabolic Response

There is considerable individual variability in metabolic response to a known dose of isoflavone-rich food. Cassidy *et al.* (1994) showed that a daily intake of 45mg/day conjugated isoflavones, fed as 60g/day texturized vegetable protein (TVP) over a 1 month period, resulted in a 1000-fold increase in isoflavone metabolite excretion. Total urinary isoflavone excretion levels ranged from 1 to 17μg/day during the controlled diet period, while levels increased to between 0.4 and 8 mg/day on the TVP diet. Urinary equol excretion varied and only two of the six subjects excreted substantial levels of equol. Setchell *et al.* (1984) showed that following ingestion of 40g/day soy meal over 5 days, four of six subjects excreted equol in urine (3-7mg/day). By investigating a relatively larger population, Frankenfeld *et al.* (2004) and Song *et al.* (2006) found that the prevalence of the equol-producer phenotype was higher (46/92=51% vs. 80/222=36%; P = 0.015) and the ODMA-producer phenotype was lower (76/91=84% vs. 204/222=92%, P = 0.03) in Korean American females than in Caucasian American females. This inability to produce equol following a soy challenge may be due to the absence from the gut flora of the bacterial enzymes responsible for the conversion of isoflavone precursor daidzein to equol, or due to transit time variation, with rapid transit effectively preventing such metabolism. These results also infer individual variability in metabolic capacity and/or differentially active metabolic pathways (Kelly *et al.*, 1993). Equol was considered to be the "physiologically active" metabolite; however, the results of these studies of premenopausal women infer that the biological effects observed are not dependent on the conversion of the aglycones to equol (Cassidy *et al.*, 1994), and suggest that the aglycones or other unidentified metabolites are also biologically active *in vivo*. While Jou *et al.* (2008) confirmed that isoflavone supplementation improved menopausal symptoms only in women with the ability to produce equol.

1.5.4.2. Excretion and Absorption

In the human studies conducted to date, usually approximately 20-30% of the total ingested isoflavone has been accounted for by urinary excretion of aglycones and known metabolites. In a carefully controlled dietary intervention study, only between 2 and 13% of the daily dose (45mg total isoflavone) was recovered in urine (Cassidy *et al*., 1994), while Xu *et al*. (1994) reported average urinary recovery of daidzein and genistein to be 21% and 9% respectively, irrespective of the dose given (0.7, 1.3, 2.0mg isoflavone/kg body weight, fed as soya milk). In a study investigating if the background diet affected short-term bioavailability in women, Xu *et al.* (2000) reported that 48hours urine recovery of daidzein and genistein were 26-27% and 18-20% respectively.

King & Bursill (1998) studied the pharmacokinetics and urinary excretion patterns of the soy isoflavones daidzein and genistein in six healthy men fed soybean flour-based meals. They found that the rate of urinary excretion of daidzein was greater than that of genistein throughout the postmeal period, with mean recoveries of 62±6% and 22±4% ($P < 0.001$) for daidzein and genistein, respectively. Lu & Anderson (1998) determined recoveries of conjugates of genistein, daidzein, and equol as 24%, 66%, and 28% of the amounts ingested in women, respectively, and 15%, 47%, and 15%, respectively, of those in men. Vergne *et al*. (2007) also reported similar recoveries with 65% for daidzein and 33.2% for genistein. de Pascual-Teresa *et al*. (2006) reported urine recovery of total isoflavone was 33-34%, although daidzein recovery (30%) was significantly lower than that of genistein (56%), which was in contrast to other reports. Only 1-2% of isoflavones were excreted in faeces (Xu *et al.*, 1994; Tew *et al.*, 1996; Watanabe *et al.*, 1998; Xu *et al.*, 2000).

Plasma concentrations of isoflavones in the range from 50 to 800ng/ml have been reported in adults after consumption of 50mg isoflavones per day (Setchell & Cassidy, 1999; Setchell *et al.*, 2001; de Pascual-Teresa *et al*., 2006). These values are similar to the plasma concentrations of the Japanese consuming their traditional diet (Morton *et al.*, 2002; Kano *et al.*, 2006; Okabe *et al.*, 2011). Overall, when soy is consumed on a regular basis, plasma isoflavone levels far exceed normal plasma estradiol levels, which generally range between 40 and 80pg/ml. The average daily dietary intake of

isoflavones in Western populations is typically negligible (<1mg/day). The rapidly changing eating habits in Japan and China now make it difficult to generalise accurately about the intake of isoflavones in these countries where soy has been traditionally a staple food since this may vary between urban and rural areas and with other lifestyle factors (Hendrich, 2002).

1.5.4.3. Other Factors

Some other factors have also been identified affecting excretion/absorption of isoflavones. In a study of dietary influences on equol excretion, it was suggested that dietary fibre, or other components of a high-fibre diet, may promote the growth and/or activity of bacteria responsible for equol production in the colon in women (Lampe *et al.*, 1998). The difference between the range of equol in a 24hr urine sample produced by excretors (2,134-20,301nmol/day) and non-excretors (21-233nmol/day) was significant. This study adds further weight to the *in vitro* findings which showed that a high-carbohydrate environment enhanced the conversion of isoflavone precursors to their respective metabolites. Tew *et al.* (1996) reported that a diet high in wheat fibre decreased genistein concentration both in plasma and urine, while daidzein was not affected. Xu *et al.* (2000) reported that bioavailability was not affected by background diet or food sources.

Other factors which influence the concentrations of isoflavones in human fluids are addressed the effects of age, gender, food matrix and chemical composition on the absorption and metabolism of this class of phytoestrogens (Lu & Anderson, 1998; Cassidy *et al.*, 2000; Cassidy *et al.*, 2006; de Pascual-Teresa *et al.*, 2006; Larkin *et al.*, 2007; Franke *et al*., 2008a; Franke *et al*., 2008b; Ko *et al*., 2010). Interestingly, by soy-challenging 410 individuals from 112 families in USA, Frankenfled *et al.* (2004) observed a positive association of greater education with being an equol producer ($p=0.01$), this may be categorised as due to lifestyle.

1.5.5. Intra-individual Differences

Isoflavones are naturally occurring plant chemicals belonging to the "phytoestrogen" class, currently heralded as offering potential alternative therapies for a range of

hormone-dependent conditions (Setchell & Cassidy, 1999; Cornwell *et al.*, 2004; McCue & Shetty, 2004). Phytoestrogens, including isoflavones, lignans, stilbenes, and coumestans, bear a structural resemblance to 17β-estradiol (alternatively expressed as estradiol, oestradiol, or oestradiol-17β sometimes), which is the dominant form of estrogen in the body (Cornwell *et al.*, 2004), and thus can also competitively bind to estrogen receptors, exert estrogenic or anti-estrogenic activities, and interfere with mechanisms controlled by the hormone (Hwang *et al.*, 2006; McCarty, 2006). The structural comparison of one isoflavone, equol, and 17β-estradiol is shown in the figure 1-21. The important functional groups for their estrogenicity including a pair of hydroxyl groups separated by a similar distance and the presence of a phenolic ring which is a prerequisite for binding to the estrogen receptors (Wiseman, 2006). However, small differences in structures of the individual phytoestrogens can dramatically alter estrogenicity (Cassidy *et al.*, 2000; Lin *et al.*, 2008), which can explain the estrogenic variability of different isoflavones.

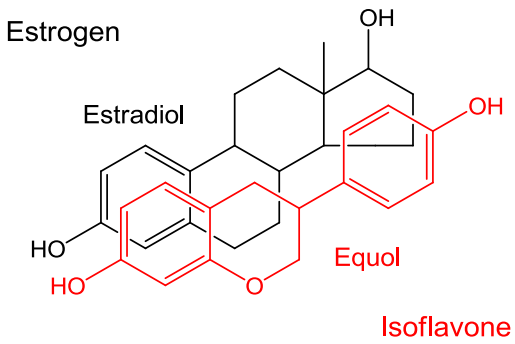

Figure 1-21 Comparison of the structure of isoflavone metabolite equol with that of estradiol showing the striking similarity in planar spatial arrangement of the two molecules (Setchell & Cassidy, 1999)

Basically, the human hormone system changes during different physiological periods. Logically, being a phytoestrogen, isoflavone may react with this system differently, leading to differences of bioavailability at different physiological stages, for example, during the postmenopausal period (Richelle *et al.*, 2002; Cornwell *et al.*, 2004; Mathey *et al.*, 2006). However, information regarding the intra-individual variation cannot be found in the literature, thus leaving this area an unconfirmed assumption and the health benefits poorly estimated. Human intervention studies usually aim to test as large a population as possible, in order to calculate average values for

pharmacokinetic parameters. However, just in doing so, inter-individual variation is easily ignored, in some studies only average values have been calculated, while the intra-individual variation has been submerged completely, although the effect of physiological stages may provide useful information in understanding and estimating isoflavone absorption, metabolism and health benefits.

To investigate the intra-individual variation, a suitable subject needs to be studied many times in order to reduce the inter-individual variation. Specially prepared food(s) with a well-designed protocol, mean that the factors affecting bioavailability can then be tested. This may take a long time. Only if the factors affecting bioavailability of isoflavone are more clearly understood, may it be possible to advise consumers on how to attain maximum health benefits from isoflavone-rich sources.

Additionally, in order to distinguish the differences caused by affecting factors, a more sensitive, rapid analysis method needs to be developed to determine the metabolites of isoflavones at trace level, especially for detecting equol and genistein because they may be eluted together by some chromatographic methods.

Equol is a special isoflavone converted from its precursor daidzein by gut microflora in the body and has attracted attention in research regarding soy isoflavones because it has been reported as possessing strong estrogenic activity (Saitoh *et al.*, 2004; Hwang *et al.*, 2006; Jou *et al.*, 2008). The ability or inability of persons to produce equol has been reported to remain the same for at least several years (Karr *et al.*, 1997; Setchell *et al.*, 2002a), since the composition of the intestinal flora plays a major role causing this variability, and the flora remains fairly stable over short time scales (Hur *et al.*, 2000; Manach *et al.*, 2004). Mathey *et al.* (2006) fed isoflavone-enriched foods (100 mg/day) to 27 postmenopausal French women for 30 days and 12 postmenopausal French women for 60 days, and found there were no changes in their equol-production ability, although their subjects had a higher equol-producer ratio (nearly 60%) than normally reported in Western populations. However, Lu & Anderson (1998) studied 40 American subjects ingesting soy milk daily and two weeks later 3 women developed the ability to produce large amounts of equol during mid-menstrual cycle. A recent study carried out in Taiwan by Ko *et al.* (2010) showed that 8 of 20 non-producers were induced to become equol producers by ingestion of soymilk

weekly for 16 weeks. These observations also manifest the importance of inter- and intra-individual difference in human metabolism.

1.6. Enzymatic Hydrolysis of Flavonoid Glycosides

Metabolism by the gut micro-flora is another factor that is important in influencing the disposition of chemicals in the gut. Interactions with the micro-flora will lead to conversion of the flavonoid glycoside to the aglycone. The cleavage of the sugar moiety of the flavonoid glycoside by the micro-flora may be through the activity of the enzyme β-D-glycosidase (β-D-glycoside glucohydrolase, EC 3.2.1.21) (Setchell *et al.*, 2002b; Ismail & Hayes, 2005).

β-D-Glycosidase catalyses the hydrolysis of glycosidic linkages in aryl and alkyl β-D-glycosides as well as glycosides containing only carbohydrates (Woodward & Wiseman, 1982; Esen, 1993; Yan *et al.*, 1998). This enzyme occurs widely in plants, animals, fungi, and bacteria. In plants, β-D-glycosides are involved in different key metabolic events and growth-related responses, such as defence against pathogens through cyanogenesis and activation of conjugates of plant growth regulators by hydrolyzing β-glucosidic bonds (Esen, 1993). In humans, three native β-glucosidase enzymes have been identified (Hays *et al.*, 1996; Tamura *et al.*, 2008). Two of them are membrane bound β-glucosidases, LPH (lactase phloridzin hydrolase), which exists in the brush-border membrane of the small intestine (Leese & Semenza, 1973), and the glucocerebrosidase, which is a lysosomal enzyme. LPH is responsible for the hydrolysis of lactose, and a deficiency in this enzyme causes lactose intolerance. Glucocerebrosidase is responsible for the hydrolysis of glucoceramide from endogenous membrane glycolipids, and a deficiency in this enzyme is associated with Gaucher disease. The third β-glucosidase is a cytosolic broad-specificity β-glucosidase found in abundance in the liver, kidney and the small intestine (Daniels *et al.*, 1981; Glew *et al.*, 1993; Cygan *et al.*, 1997). This enzyme does not have a defined role or deficiency associated with it. Nevertheless, Day *et al.* (1998) showed that the cytosolic β-glucosidase present in cell-free extracts of human small intestine and liver was capable of hydrolyzing various flavonoid and isoflavonoid glycosides.

Although the mechanisms of β-D-glucoside catalysis have not yet been fully understood (Rye & Withers, 2000; Vocadlo & Davies, 2008), the reaction exhibits potential applications in enzyme therapy as well as food biotechnology. For example, human acid β-D-glucosidase has potential in the development of therapeutic and diagnostic procedures, particularly for the treatment of Gaucher's disease. β-D-Glucosidases from some fungi and bacteria are targets for genetic engineering for specific applications such as biomass conversion (Esen, 1993). β-D-Glucosidases of different origins exhibit variation in their properties such as molecular weight, substrate specificity, pH and temperature optimum and sensitivity (Esen, 1993). Large amounts of research have been carried out to purify and characterize these β-D-glucosidases from a variety of sources. The properties and function of the enzymes from various species of fungus and bacteria are most widely covered. A detailed account of the biochemical nature, properties and applications of fungal and bacteria β-D-glucosidases are recorded in the review by Woodward & Wiseman (1982) and Bhatia *et al*. (2002).

In fruit and vegetables, β-D-glucosidase plays a significant role in the release of a wide variety of volatile compounds from their glycosidic precursors, for example, release and activate plant hormones from their storage forms occur as β-glucosides. This indicates research interesting in its potential applications in certain food and beverage industries other than its functions in plants. Sweet almond is a readily commercial source of β-D-glucosidase. Various research groups have carried out characterisation and functionality studies on the β-D-glucosidase activity from a variety of fruits and vegetables such as cranberry (Zheng & Shetty, 2000), green bean (Dignum *et al.*, 2004), onions (Tsushida & Suzuki, 1996) and strawberry (Orruño *et al.*, 2001).

One purpose of this study is to find food sources of suitable β-D-glucosidases that can be used to generate novel food with altered (de-glycosylated) bioactive compounds and to use the food in absorption studies. Given the contradictory results in the literature (for example, Setchell *et al*., 2001; Tsangalis *et al*., 2005; Kano *et al*., 2006; Maskarinec *et al*., 2008; Rüfer *et al*., 2008; Okabe *et al*., 2011) concerning the relative efficiencies of absorption of flavonoid glycosides and aglycones, such novel foods would be essential in understanding the true situation. (It would, of course, also be

necessary to exert greater control over other dietary compounds in such experiments.) The lack of suitable experimental foods has hindered experimentation in this area.

1.7. Aims of the Project

The differential absorption and metabolism of different flavonoid forms may have significent impact on health benefit. Dietary flavonoids are normally found as conjugated glycosides, except in fermented foods, meaning that such foods are of considerable research interest.

Firstly, the aims of chapters 3 and chapter 4 were to identify and characterise a new source of the enzyme β-D-glucosidase which could hydrolyse flavonoid glycoside efficiently, be ideally sourced from food waste, and which could be used to develop a novel food.

The aim of chapter 5 was to investigate the isoflavone contents and pattern of different dietary sources, obtain information for isoflavone supplementation in the human diet, and to identify an isoflavone source for human study.

Finally, the aims of chapter 6 were to develop a novel, natural-style food by combining the selected enzyme source (chapter 3 & 4) and selected isoflavone source (chapter 5).