CHAPTER II

LITERATURE REVIEW

In this chapter, the background on the reactive oxygen species, the antioxidant and the example of the antioxidant assays such as the DPPH, ORAC and DCFH-dA assay is reviewed. The glutamate-induced oxidative stress and the causes of the neurodegeneration diseases are also mentioned in this chapter. Finally, the information of *P. mirifica* and the protection of phytoestrogens is provided.

1. Biologically relevant reactive oxygen species (ROS)

1.1 Superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2)

Superoxide formed *in vivo* is largely converted by superoxide dismutase (SOD)-catalysed or nonenzymic dismutation into H_2O_2 (Fridovich, 1989). Some enzymes, e.g. glycollate and xanthine oxidases, produce H_2O_2 directly. Unlike $O_2^{\cdot-}$, H_2O_2 is thought to cross all cell and organelle membranes. Both $O_2^{\cdot-}$ and H_2O_2 can find some molecular targets to which they can do direct damage, but in general, their reactivity is limited.

1.2 Hydroxyl radical

Much of the molecular damage done by $O_2^{\cdot-}$ and H_2O_2 *in vivo* is thought to be due to their conversion into more reactive species. The most important of which is the hydroxyl radical ($OH^{\cdot}$). Formation of $OH^{\cdot}$ *in vivo* occurs by at least four mechanisms: (1) transition metal ion catalysis, especially by iron and copper, (2) background exposure to ionizing radiation, (3) Reaction of $O_2^{\cdot-}$ with $NO^{\cdot}$ to give peroxynitrite

$$O_2^{\cdot-} + NO^{\cdot} \longrightarrow ONOO^{-} \qquad \text{Eq. 1}$$

which is directly cytotoxic and can also decompose at physiological pH to several noxious products, apparently including nitrogen dioxide ($NO_2^{\cdot}$), and some $OH^{\cdot}$ (Beckman et al., 1994, Van der Vilet et al., 1994) . (4) Reaction of HOCl with $O_2^{\cdot-}$

$$HOCl + O_2^{\cdot-} \longrightarrow OH^{\cdot} + O_2 + Cl^{-} \qquad \qquad Eq. 2$$

1.3 Peroxyl radicals

Formation of peroxyl radicals ($RO_2^{\cdot}$) is a key step in lipid peroxidation, but they can also be formed from DNA and proteins (Sevilla et al., 1989) and when thiyl ($RS^{\cdot}$) radicals combine with oxygen (Fahey, 1988).
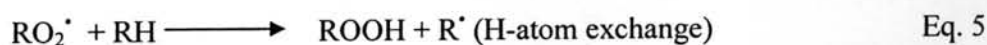
1.4 Singlet oxygen

Oxygen has two singlet states. Singlet oxygen, although not a free radical, is a powerful oxidizing agent, able to attack rapidly several molecules, including polyunsaturated fatty acids. Singlet oxygen can be produced by photosensitization reactions. It is also formed when $ONOO^{-}$ reacts with H_2O_2 (Di Mascio, 1994), and by self-reaction of peroxyl radicals during lipid peroxidation (Wefers, 1987).

2. Antioxidant

A broad definition of an antioxidant is any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of' that substrate (Halliwell, 1990). The term "oxidizable substrate" includes every type of molecule found *in vivo*.

The function of antioxidants is to intercept and react with free radicals at a rate faster than the substrate, and since free radicals are able to attack a variety of targets including lipids, fats, and proteins, it is believed that they are implicated in a number of important degenerative diseases including aging itself (Harman, 1981, Ozawa,

1997, Beckman and Ames, 1998). There are two pathways for oxidation in which antioxidants can play a preventive role. The first is H-atom transfer, illustrated below for the important case of lipid peroxidation:
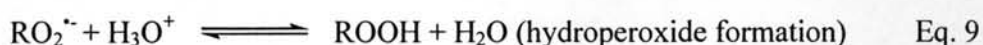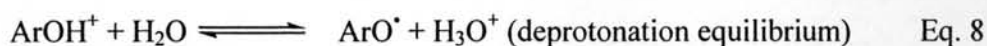
$$RH \longrightarrow R^{\bullet} \quad \text{(initiation)} \qquad \text{Eq. 3}$$

$$R^{\bullet} + O_2 \longrightarrow RO_2^{\bullet} \quad \text{(addition of } O_2) \qquad \text{Eq. 4}$$

$$RO_2^{\bullet} + RH \longrightarrow ROOH + R^{\bullet} \quad \text{(H-atom exchange)} \qquad \text{Eq. 5}$$

Once a free radical $R^{\bullet}$ has been generated, then reactions 4 and 5 form a chain reaction. As the chain cycles through (4) and (5) many lipid molecules (R-H) are converted into lipid hydroperoxide (ROOH), resulting in oxidation and rancidity of fats.

For the phenolic antioxidant we will use the generic term ArOH, since by definition it contains at least one hydroxy group attached to a benzene ring. The role of the antioxidant ArOH is to interrupt the chain reaction according to

$$RO_2^{\bullet} + ArOH \longrightarrow ROOH + ArO^{\bullet} \qquad \text{Eq. 6}$$
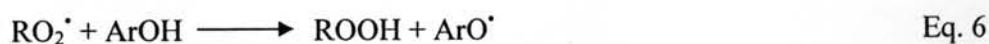
To be effective, $ArO^{\bullet}$ must be a relatively stable free radical, so that it reacts slowly with substrate RH but rapidly with $RO_2^{\bullet}$, hence the term "chain-breaking antioxidant".

Another possible mechanism by which an antioxidant can deactivate a free radical is electron transfer, in which the radical cation is first formed followed by rapid and reversible deprotonation in solution, according to

$$RO_2^{\bullet} + ArOH \longrightarrow RO_2^{-} + ArOH^{+} \quad \text{(electron transfer)} \qquad \text{Eq. 7}$$

$$ArOH^{+} + H_2O \rightleftharpoons ArO^{\bullet} + H_3O^{+} \quad \text{(deprotonation equilibrium)} \qquad \text{Eq. 8}$$

$$RO_2^{\bullet-} + H_3O^{+} \rightleftharpoons ROOH + H_2O \quad \text{(hydroperoxide formation)} \qquad \text{Eq. 9}$$

The net result from above is the same as in the atom-transfer mechanism.

$$RO_2^{\bullet} + ArOH \longrightarrow ROOH + ArO^{\bullet} \qquad \text{Eq. 10}$$
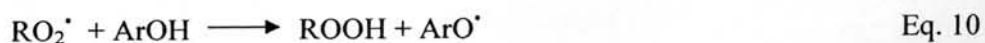
In addition to the two major mechanisms above, in some cases, other factors may also play a role in determining what makes an effective antioxidant, including the presence of bulky groups near the OH group, hydrogen bonding characteristics of the solvent (Valgimigli, Ingold and Lusztyk, 1996) or in a biological context, solubility, and transport to specific tissues. (Burton and Ingold, 1986)

Both the H-atom transfer (HAT) and the single-electron transfer (SET) mechanisms must always occur in parallel, but with different rates. In most cases of phenolic antioxidants, H-atom transfer will be dominant (Wright, Johnson and DilaBio, 2001). It is expected that the SET mechanism will be strongly solvent dependent due to solvent stabilization of the charged species, whereas HAT will be only weakly solvent dependent.

3. Antioxidant assays

There are a variety of methods to assess "total" antioxidative activity (Huang, Ou and Prior, 2005, Prior and Cao, 1999, Prior, Wu and Schaich, 2005). Because multiple reaction characteristics and mechanisms as well as different phase localizations are usually involved, no single assay will accurately reflect all of the radical sources or all antioxidants in a mixed or complex system. A protocol is needed to involves measurement of more than one property because polyphenols have multiple activities, and the dominant activity depends on the medium and substrate of testing.

A primary factor to consider is selecting a suitable method which is related to the mechanism of reaction and its relationship to what might occur in the target

application. For classical antioxidant action, an assay based on HAT mechanism is preferred over a SET reaction mechanism because the peroxyl radical is the predominant free radical found in lipid oxidation in foods and biological systems. However, it may also be important to develop assays using other radical sources such as the hydroxyl, superoxide and peroxynitrite, because these are active in cells and tissues of plants and animal alike, and it is clear that not all antioxidants behave the same toward different radical sources. Among other factors that are important and influence the selection of a good method are biological relevance and endpoint as well as method of quantitation. Oxygen radical absorbance capacity (ORAC) assay representing a HAT reaction mechanism, is considered to be one of the most biologically relevant assays. ORAC assay utilizing area under the curve (AUC) for calculation, thus reflect the different reaction kinetics. From this standpoint, it provides better data than methods using a fixed time point. The Folin-Ciocalteau reagent (FCR) assay is an electron transfer based assay and gives reducing capacity, which has normally been expressed a phenolic content. This assay is a simple, speedy, inexpensive and robust assay that does not require specialized equipment. Another assay mentioned in this dissertation is 2,2-Diphenyl-l-picrylhydrazyl (DPPH) assay which is both HAT and SET mechanism based assays using the stable organic nitrogen radical from 2,2-Diphenyl-l-picrylhydrazyl (DPPH). This assay is simple and rapid to perform, which explains its widespread use in antioxidant screening (Prior, Wu and Schaich, 2005).

3.1 Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC assay is based upon the early work of Ghiselli et al., 1995 and Glazer, 1990, as developed further by Cao, Alessis and Cutler, 1993. ORAC measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidative activity by H atom transfer (Ou, Hampsch-Woodill and Prior, 2001). In the basic assay, the peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product, which can be quantitated easily by fluorescence. Antioxidant capacity is determined by a decreased rate and amount of product formed over time:

$$R-N=N-R \xrightarrow{O_2} N_2 + 2ROO^\bullet \qquad \text{Eq. 12}$$

$$ROO^\bullet + probe \longrightarrow ROOH + \text{ oxidized probe} \qquad \text{Eq. 13}$$
$$\text{(fluorescent)} \qquad\qquad \text{(loss of fluorescence)}$$

$$ROO^\bullet + AH \longrightarrow ROOH + A^\bullet \qquad \text{Eq. 14}$$

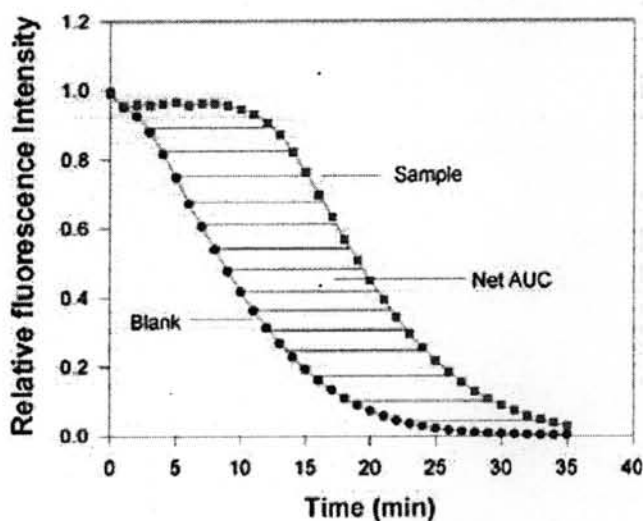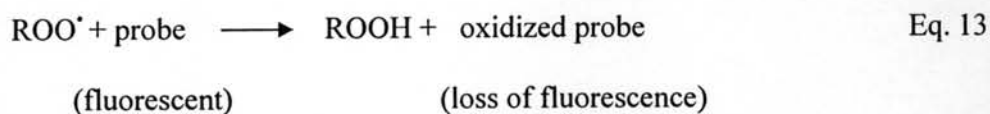$$ROO^\bullet + A^\bullet \xrightarrow{fast} ROOA \qquad \text{Eq. 15}$$

Figure 1 ORAC antioxidative activity of tested sample expressed as the net area under the curve (AUC). From (Prior, Wu and Schaich, 2005)

The fluorescent probes that are currently preferred such as fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one) or dichlorofluorescein (H_2DCF-dA; 2',7'-dichlorodihydrofluorescein diacetate) are stable (Ou et al., 2002). The oxidized products of fluorescent probe induced by peroxyl radicals have been identified by LC-MS, and the reaction mechanism has been verified as a classic HAT mechanism.

Probe reaction with peroxyl radicals is followed by loss of fluorescence over time. Traditional antioxidant analyses followed extension of the lag phase only, but antioxidant effects often extend well beyond early stages of oxidation. To avoid underestimation of antioxidative activity and to account for potential effects of secondary antioxidant products, the ORAC assay follows the reaction for extended periods, for example $\geq$ 30 min. Calculation of protective effects of an antioxidant is from the net integrated areas under the fluorescence decay curves (AUC) as shown in Figure 1 and accounts for lag time, initial rate, and total extent of inhibition in a single value. ORAC values are usually reported as Trolox equivalents.

The ORAC assay provides a controllable source of peroxyl radicals that model reactions of antioxidants with lipids in both food and physiological systems, and it can be adapted to detect both hydrophilic and hydrophobic antioxidants by altering the radical source and solvent (Ou et al., 2002, Huang et al, 2002). The reaction has been determined to be a HAT mechanism. The principles of the ORAC method can be adapted to utilize other radical sources. However, because the ORAC reaction is temperature sensitive, close temperature control throughout the plate is essential. Incubation of the reaction buffer at 37 °C prior to the peroxyl radical such as (2, 2-azobis-2-amidinopropane-dihydrochloride) AAPH being dissolved decreased the

intra-assay variability (Prior et al., 2003). This is not unique to the ORAC assay, but will be true for any assay that is highly temperature sensitive that uses microplates and microplate readers in the assay. Fluorescent markers, although sensitive, require detection by fluorometers, which may not be routinely available in analytical laboratories, although this instrument is used routinely in many cell culture laboratories. The long analysis time (~1 h) has also been a major criticism, but this limitation has been partially overcome by development of high-throughput assays (Huang et al., 2002).

3.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

A method widely used to predict the ability of flavonoids is based on the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams, Cuvelier and Berset, 1995, Sanchez-Moreno, Larrauri and Saura-Calixto, 1999) DPPH method has been applied to the phenolic compounds commonly present in wine. The spectrophotometric technique employs the DDPH free radical, which shows a characteristic UV–vis spectrum with a maximum of absorbance close to 515 nm in methanol. The addition of an antioxidant results on a decrease of absorbance proportional to the concentration and antioxidative activity of the compound itself (Brand-Williams, Curvelier and Berset, 1995). This method presents the advantage of using a stable and commercially available free radical and has been extensively applied on the study of antioxidative activity of food items, such as olive oil, fruits, juices and wines (Da Porto et al., 2000, Gorinstein et al., 2003, Llorach et al., 2003). This method is easy to perform, highly reproducible and comparable with other methods such as ABTS, reduction of superoxide anion and inhibition of lipid peroxidation (Gil et al., 2000, Lu and Fu, 2000).

Nitrogen centered radicals such as DPPH radical react with phenols (ArOH) via two different mechanisms: (1) a direct abstraction of phenol H-atom (HAT reactions) and (2) an electron transfer process from ArOH or its phenoxide anion (ArO−) to DPPH radical (SET reactions) (Foti, Daquino and Geraci, 2004).The contribution of one or the other pathway depends on the nature of the solvent and/or the redox potentials of the species involved. Generally in apolar solvents the HAT mechanism is predominant, but in polar solvents such as methanol or ethanol, capable of forming strong hydrogen bonds with the ArOH molecules, the SET mechanism becomes important. Polyphenolic compounds usually exhibit different behaviors towards DPPH free radical, both in terms of capacity and rate of scavenging.

4. Apoptosis

Apoptosis was originally defined as a distinct mode of cell death on the basis of a series of characteristic ultrastructural features according to the following sequence of events: nuclear and cytoplasmic condensation, cell fragmentation and phagocytosis (Kerr, Wyllie and Currie, 1972). Two principle pathways for apoptosis initiation exists. One pathway that is termed the extrinsic pathway is mediated by death receptors. The second pathway, which is referred to as the intrinsic, or possibly more accurate, Bcl-2-controlled pathway is consequently controlled by members of the Bcl-2 family. Both pathways have the same outcome; the activation of cascade of proteolytic enzymes and members of the caspase family which is central to the apoptotic process.

Central to the intrinsic pathway is the formation of an intracellular caspase-9-activating complex, the apoptosome. A key player in regulating apoptosome

formation is the mitochondrion. The apoptosome forms after cytochrome c is released from mitochondria which its membrane integrity regulated by proteins of the Bcl-2 family. This protein family can be divided into three groups based on their domain architecture. It consists of anti-apoptotic members such as Bcl-2 or Bcl-x_L which are associated with the mitochondrial outer membrane, serving to maintain mitochondrial integrity. The second group acts as sentinels over various cellular organelles and processes. The third group of Bcl-2 family members is the large pro-apoptotic members such as Bax, Bad or Bak. These proteins associate with the outer mitochondrial membrane during apoptosis, breaching its integrity (Sprick and Walczak, 2004).

5. Oxidative stress

In aerobic cells molecular oxygen (O_2) is used as the terminal electron acceptor in oxidative phosphorylation. These cells must be capable of dealing with the toxic side-effects of O_2 and its reactive derivatives (Fridovich, 1978). Reactive oxygen species (ROS) include both oxygen free radicals and other compounds such as hydrogen peroxide.

Free radicals are highly reactive atoms or molecules containing one or more unpaired electrons (Halliwell and Gutteridge, 1985). As a consequence of normal aerobic respiration, mitochondria consume O_2, reducing it by sequential steps to produce H_2O. Inevitable by-products of this process are superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). *In vivo* they serve both beneficial and deleterious functions. Oxygen radicals can attack proteins, deoxynucleic acids, and lipid membranes, thereby disrupting cellular functions and

integrity. Normally, in cells there is equilibrium between anti-oxidant defense mechanisms and factors that promote free-radical formation (Halliwell, 1992). Any imbalance in this equilibrium favoring free radical formation is defined as a state of oxidative stress.

6. Oxidative stress and neurodegeneration

There is a large amount of evidence indicating that oxidative stress plays a crucial role in aging as well as in neurodegenerative, cerebrovascular and cardiovascular diseases (Mariani et al., 2005).

The brain contains large amounts of polyunsaturated fatty acids (PUFA), which are particularly vulnerable to free radical attack, because the double bonds within membranes allow easy removal of hydrogen atoms by reactive oxygen species such as OH$^{•}$ (Haliwell and Gutteridge, 1985). Additionally, the central nervous system also appears to be particularly vulnerable to oxidative stress. Neuronal membranes contain a high proportion of free radical-susceptible PUFA, thus making them more susceptible to peroxidative damage.

Under aerobic conditions, lipid peroxidation continues as conjugated dienes combine with O_2 to form additional organic peroxy radicals. Peroxy radicals abstract hydrogen from adjacent fatty acid chains, thereby propagating the lipid peroxidation process. The carbon radical formed in the PUFA undergoes molecular rearrangement to form more stable conjugated dienes, which can cross-link fatty acids within cellular membranes. Furthermore, peroxyl radicals can combine with an abstracted hydrogen atom to form lipid hydroperoxides which, in the presence of Fe^{2+}, decompose to alkoxy radicals and aldehydes. Thus the action can initiate a chain

reaction that generates numerous toxic reactants that rigidify membranes by cross-linking, disrupt membrane integrity and damage membrane proteins (Coyle and Puttfarcken, 1993).

In Alzheimer's disease (AD), a "two-hit" hypothesis has been postulated for which, although either oxidative stress or abnormalities in mitotic signaling can independently serve as initiators, both processes are necessary to propagate disease pathogenesis (Zhu et al., 2004). The exact biochemical mechanism of the pathogenesis of AD is still unknown, but much attention is given to the role of the massive loss of the neurotransmitter acetylcholine (necessary for cognition and memory) and to the possible implication of oxidative stress in its development. Excitotoxicity and oxidative stress-induced triggering of degenerative signaling appears to play an important role (Longo and Massa, 2004). Oxidizing conditions cause protein cross-linking and aggregation of Aß peptides (Dyrks et al., 1993) and also contribute to aggregation of tau (Troncaso et al, 1993) and other cytoskeletal proteins (Bellomo and Mirabelli, 1992).

Increased levels of a specific marker of *in vivo* lipid peroxidation were found to be significantly elevated in cerebrospinal fluid, plasma and urine of mild cognitive impairment (MCI) subjects compared with controls (Pratico et al., 2002), suggesting that lipid peroxidation may be an early event in the pathogenesis of the disease. MCI is a condition in which memory or other cognitive abilities are slightly abnormal but coexist with normal function in the activities of daily living, normal general cognitive function, and absence of dementia (Petersen et al., 2001). MCI and AD subjects showed lower means of non enzymatic antioxidants Vitamin A, Vitamin C, Vitamin E, uric acid and of the carotenoids. In addition, patients with MCI and AD showed

similarly lower activities of plasma and erythrocyte superoxide dismutase (SOD) as well as of plasma glutathione peroxidase as compared to controls, suggesting that subjects developing MCI and subsequently AD may have an antioxidant enzymatic activity inadequate to counteract the hyperproduction of free radicals during a recently established condition of oxidative stress (Rinaldi et al., 2003).

Parkinson's disease (PD), as a result of neurodegeneration occurring in the substantia nigra and in the striatum and of dopamine depletion, is clinically characterized by bradykinesia, postural instability, gait difficulty and tremor. The mechanisms of cell death in PD have not yet been fully elucidated, but increased oxidative stress, increased lipid peroxidation in the PD brain, abnormal mitochondrial function and excitotoxicity are perhaps among the most important initiators or mediators of neuronal damage (Mariani et al., 2005).

Stroke is the main cause of disability and mortality in Western countries. It has also been estimated that up to 30% of all ischemic strokes will eventually undergo hemorrhagic transformation. Brain ischemia, and especially the condition of ischemia and reperfusion occurring after stroke, has been shown to be associated with free radical-mediated reactions potentially leading to neuronal death (Alexandrova et al., 2004). Several sources of free radicals have been proposed, including inflammatory cells, xanthineoxidase, cyclooxygenase, and mitochondria (Piantadosi and Zhang, 1996). Additionally, the large increases in glutamate and aspartate that accompany ischemia may contribute to free radical generation by excitotoxic mechanisms (Morimoto et al., 1996, Yang et al., 1996).

7. Glutamate and neurodegeneration

Although multiple factors can precipitate oxidative stress in cells, the neurotransmitter glutamate is the major effector of this process in brain, primarily through activation of its ionotropic receptors. Considerable circumstantial evidence is convincing that oxidative stress represents as important pathway, initiated in part by that leads to neuronal degeneration in a manner consistent with the course and pathology of several degenerative disorders of the brain (Coyle and Puttfarcken, 1993). Dysfunction of glutamate mechanisms or evidence of oxidative stress have been implicated in an increasing number of conditions involving acute damage to brain tissue, including stroke, hypoxia-reperfusion, trauma, and epilepsy (Siesjo, 1992). Systemic administration of either glutamate or aspartate caused degeneration of the neural retina in neonatal mice (Lucas and Newhouse, 1957). Glutamate-induced neurotoxicity resulting from oxidative stress is mediated by a cystine transporter to which glutamate binds (Bannai and Kitamura, 1980). Elevated glutamate in the culture medium caused a degeneration of the neuron-like cells after approximately 8 hours of continuous exposure (Beal et al., 1988). The cytotoxic potency of glutamate inversely correlated with concentration of cystine in the culture medium. The resulting cystine deprivation causes a progressive decline in cellular glutathione; as the lowest point is reached, oxidants accumulate intrcellularly. Oka and coworkers have demonstrated that elevated extracellular concentration of glutamate markedly stimulated the efflux of intracellular cystine by a carrier-mediated process, which results in glutathione depletion (Oka et al., 1993).

8. Glutamate and HT-22

Two distinct pathways of glutamate-induced cell death have been identified thus far. The first one, the excitotoxic pathway, relies on high concentrations of glutamate generate excitotoxicity via superactivation of neuronal glutamate receptors. This form of neuronal cell death, that is accompanied by a Ca^{2+} influx through ionotropic receptors, has been proposed to occur in acute neurodegenerative conditions such as epileptic seizures (Mattson, 2000) The second one, the oxidative pathway, involves the breakdown of the glutamate/cystine antiporter with concomitant reduction in glutathione synthesis (Ha and Park, 2006).

The immortalized mouse hippocampal cell line HT-22 cells, which phenotypically resemble neuronal precursor cells have been a useful model for studying the mechanism of oxidative glutamate toxicity (Davis and Maher, 1994). These cells lack functional ionotropic glutamate receptors (Maher and Davis, 1996), thus excluding excitotoxicity as a cause for glutamate triggered cell death. Additionally, glutamate receptor antagonists did not have any effects to the glutamate-induced cell death of HT-22 cells (Davis and Maher, 1994).

Glutamate induces oxidative stress by inhibiting the uptake of cystine into the cells via the cystine/glutamate transport system. Cystine is required for the synthesis of glutathione, the major intracellular antioxidant. Receptor-independent generation of oxidative stress by glutamate is termed oxidative glutamate toxicity (Tan, Schubert and Maher, 2001). Following exposure to glutamate, reduced intracellular concentrations of glutathione can be measured. The reduced levels are due to an inhibition of cystine uptake, via the cystine/glutamate antiporter system, by glutamate. The resulting loss of the cytosolic antioxidant, glutathione, effects the elevation of

intracellular reactive oxygen species (ROS), which are neurotoxic (Li, Maher and Schubert, 1998, Murphy et al., 1989). Glutamate-induced oxidative injury and death of neuronal cells have also been attributed to increased calcium (Ca^{2+}) influx, which can activate the neuronal nitric oxide synthase and, thereby, increase production of reactive nitrogen species.

9. Polyphenol and flavonoid and their antioxidative activities

It is well-known that diets rich in fruit and vegetables are protective against cardiovascular disease and certain forms of cancer, (Block, 1992, Block and Langseth, 1994) and perhaps against other diseases also. These protective effects have been attributed, in large part, to the antioxidants present including the antioxidant nutrients vitamin C and t-carotene, but also the minor carotenoids, and plant phenolics such as the flavonoids and phenylpropanoids may also have a significant role. The polyphenolic components of higher plants may act as antioxidants or as agents of other mechanisms contributing to anticarcinogenic or cardioprotective action. The flavonoids constitute a large class of compounds, ubiquitous in plants, containing a number of phenolic hydroxyl groups attached to ring structures, conferring the antioxidative activity.

The chemical properties of polyphenols in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers predicts their antioxidative activity. For a polyphenol to be defined as an antioxidant it must satisfy two basic conditions: first, when present in low concentration relative to the substrate to be oxidized it can delay, retard, or prevent the autoxidation or free radical-mediated oxidation; ~ second, the resulting radical formed after scavenging must be stable--

through intramolecular hydrogen bonding on further oxidation (Shahidi and Wanasundara, 1992).

The phenoxyl radical formed by reaction of a phenolic antioxidant with a lipid radical is stabilized by delocalization of unpaired electrons around the aromatic ring. The o-dihydroxy substitution in the B ring is important for stabilizing the resulting free radical form. The conclusion drawn was that the three criteria for effective radical scavenging are: (1) the o-dihydroxy structure in the B ring, which confers higher stability to the radical form and participates in electron delocalization; (2) the 2,3 double bond in conjugation with a 4-oxo function in the C ring is responsible for electron delocalization from the B ring--the antioxidant potency is related to structure in terms of electron delocalization of the aromatic nucleus. Where these compounds react with free radicals, the phenoxyl radicals produced are stabilized by the resonance effect of the aromatic nucleus; (3) the 3- and 5-OH groups with 4-oxo function in A and C rings are required for maximum radical scavenging potential.

Overall, the reduction potentials of flavonoid radicals are lower than those of alkylperoxyl and superoxide radicals; thus, flavonoids may inactivate these damaging oxyl species and prevent the deleterious consequences of their reaction (Rice-Evans, Miller and Pagangal, 1996).

10. Phytoestrogen and neuroprotection

Phytoestrogens have been proposed to have many health benefits such as isoflavone protection against breast cancer (Lof and Weiderpass, 2006), antioxidant protection against oxidatively-induced DNA damage (Sierens et al., 2001) and protection of neuronal cells against oxidative stress (Sonee et al., 2004). They are

abundant in fruits, vegetables, legumes, wholegrains and especially flaxseed, clover and soy products. Empirical data and epidemiologic research indicate that the incidence of hormone-dependent diseases is reduced in countries with a high dietary content of phytoestrogens (Greenstein et al., 1996). Thus, modern clinical and molecular biological research has increasingly focused on plant-derived phytoestrogens such as isoflavones, lignans, dihydrochalcones, and coumestans. The most widely studied phytoestrogenic compounds are isoflavones found in soy, such as genistein and daidzein (Kronenberg and Fugh-Berman, 2002). The anticancer effects of phytoestrogens appear to be associated with several possible mechanisms including their ability to inhibit tyrosine kinase(s), growth factors, DNA topoisomerase, steroidogenic enzymes and to act as antioxidant and antiangiogenic agents (Lephart, Setchell and Lund, 2005).

Phytoestrogens are non-steroidal, diphenolic structures that have similar chemical and structural properties to that of estrogens. Hundreds of molecules fall under this classification and there are three main groups of phytoestrogens: (1) isoflavones (derived principally from soybeans), (2) lignans (found in flaxseed), and (3) coumestans (derived from sprouting plants like alfalfa). The major isoflavones include: genistein, daidzein and equol. Isoflavones are estrogen mimics that bind estrogen receptors and this characteristic apparently plays a role in their health promoting effects. In conclusion, phytoestrogens act like natural selective estrogen receptor modulators (SERMs) at various tissue sites throughout the body (Hol et al., 1997, Kuiper et al., 1998) Particularly, there is evidence that phytoestrogens act as estrogen agonists at some tissue-specific targets, whereas, in others they display antagonistic characteristics (Halbreich and Kahn, 2000).

The neurobehavioral effects of phytoestrogens have been reviewed. Evidence showing that phytoestrogens can reach the brain after intraperitoneal injection of genistein and daidzein to adult Sprague–Dawley rats (Gamache et al., 1996, Setchell, 1998). In that study, rapid appearance of both isoflavones was noted from the greatly increased isoflavone levels in brain tissue. Phytoestrogens, like endogenous estrogens, can clearly enter the lipophilic environment of the brain, and do so rather quickly. daidzein and genistein levels in the medial basal hypothalamus (MBH) were approximately eightfold higher in phyto-rich fed animals vs. Phyto-free. In a more recent study where improved methodologies enabled the detection of the aglycones (daidzein and genistein) plus the metabolite, equol, MBH results were confirmed along with the finding that the phytoestrogen content of the cerebellum of Phyto-rich fed animals was approximately ninefold higher than in animals fed the Phyto-free diet (Lephart et al., 2000, Lund et al., 2001). Soy phytoestrogens regulate choline acetyltransferase, nerve growth factor and brain-derived neurotrophic factor in brain areas such as the frontal cortex and hippocampus of female rats (Pan, Anthony and Clarkson, 1999). Subsequently dietary phytoestrogens attenuates tau protein phosphorylations associated with Alzheimer's disease, suggesting a neuroprotective effect of phytoestrogens (Kim et al., 2000). Moreover, Linford and Dorsa or Zhao et al. showed evidence that isoflavones displayed neuroprotective effects in primary cortical or hippocampal cultures, respectively (Linford and Dorsa, 2002, Zhao, Chen and Brinton, 2002).

The isoflavone, genistein at low micromolar concentrations in various brain tissues has been found to prevent glutamate-induced apoptosis (Kajta et al., 2007). Several studies suggest that estrogen receptors may be involved in flavonoid-mediated
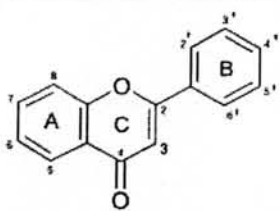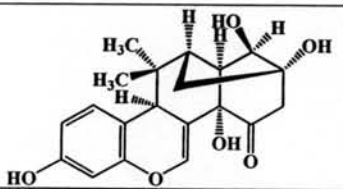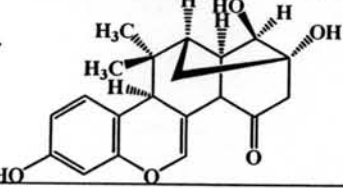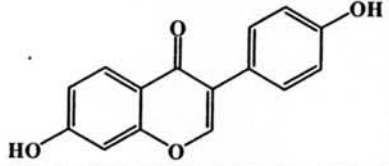
neuroprotection (Kajta et al., 2007, Linford and Dorsa, 2002, Bang et al., 2004). However, whether or not the protective effects of estrogenic compound are dependent on estrogen receptor signaling is still unclear (Zhu et al., 2007). It has been suggested that some of the beneficial effect of isoflavones might be, at least in part, mediated by their antioxidative activity (Wu and Chan, 2007, Zeng, Chen and Zhao, 2004).

11. *Pueraria candollei* var. *mirifica*

Pueraria candollei var. *mirifica* (*P. miriifica*) or Kwao Kruae Kao has long been used as a rejuvenating and anti-aging supplement in Thailand and Mynmar (Cain, 1960). The consumption of *P. mirifica* has been believed to improve the human physical appearances such as re-growing hair, promoting black hair, improving flexibility of the body and sexual performance, enlarging breast, recovering smooth skin and prolonging life (Kasemsanta and Suvatabandhu and Airy, 1952). *P. mirifica* was found to alleviate menopausal symptoms in women (Muangman and Cherdshewasart, 2001) and inhibit the bone loss in long and axial bones, in sex hormone-deficient male rats (Urasopon et al., 2007). Many phytoestrogens including daidzein, genistein, coumestrol and miroestrol (Chansakaow et al., 2000a) have been isolated from the ethyl acetate extract of *P. mirifica*'s roots. Furthermore, the new phytoestrogen, deoxymiroestrol, has been identified and investigated for its estrogenic activity. Deoxymiroestrol exhibited the strongest estrogenic activity. After aerial oxidation, it was readily converted into miroestrol which is also a unique potent phytoestrogen (Chansakaow et al., 2000b). From their estrogenic and/or antioxidative activity, *P. mirifica* extract should have the therapeutic benefit for neurodegenerative diseases. Howerver, there have been no report of the levels of antioxidative activity of

P. mirifica extracts. Therefore, we investigated the antioxidative activity of extracts by various assays. Furthermore, we examined the neuroprotection potential of *P. mirifica* extracts and whether the estrogen receptor pathway is involved. The studies were conducted as a model of oxidative stress-induced cell death, glutamate-induced cell death in HT-22 mouse hippocampal neuronal cells.

Table 1 Molecular structures of flavone nucleus and some phytoestrogens found in *P. mirifica* extract

Flavone nucleus	
Miroestrol	
Deoxymiroestrol	
Daidzein	
Genistein	