CHAPTER V
DISCUSSION AND CONCLUSION

1. Neuropharmacological Activities of Quercetin With Oral Conventional Delivery System and Quercetin Liposomes via Nasal Administration in Healthy Condition

The present investigation demonstrated that both of quercetin with oral conventional delivery system and nasal administration of quercetin liposomes exerted the neuropharmacological activities in central nervous system of rodents including anti-depression, anxiolytic and cognition which could explain the alteration in animal behavior models such as forced swimming, elevated plus maze, Morris water maze and object recognition tests respectively (Porsolt et al., 1978; Morris, 1981; Ennaceur, Delacour, 1988; Dawson, Tricklebank, 1995).

Forced swimming test (FST) is widely used for screening potential antidepressants (Porsolt et al., 1978). Antidepressants reduce the immobility time in FST. In the present study, our results demonstrated that acute effect of oral administration of quercetin and nasal administration of quercetin liposomes produced the antidepressant-like response in FST. Moreover, our study also showed that the repeated administration of this substance and this strategy were also able to produce an antidepressant-like effect too.

To avoid false positive results in the FST, it is important to rule out the possibility that reductions in immobility time were not a result from psychostimulant effects of the extract. In our study, either acute or repeated treatment with quercetin and quercetin liposomes did not increase spontaneous motor activity at doses that produced an antidepressant-like effect, indicating a specific effect of this substance on behavioral models predictive of antidepressant activity.

Elevated plus maze test (EPM) is one of the most frequently used animal models in behavioral psychopharmacology for screening drugs with potential anxiolytic effects (Wall and Messier, 2000). In general, the reduction or increase in the number of entries and times spent into the open arm induced by a given substance

had been regarded as good indicators of its anxiogenic or anxiolytic effect respectively (Pellow et al., 1985). The results of present study demonstrated that both the administration of quercetin with conventional form and the nasal administration of quercetin liposomes via nasal showed the anxiolytic-like effect in EPM. In our study, rats treated with quercetin at dose of 100 mg/kg BW induced an anxiolytic-like effect after single administration whereas single administration of 200 and 300 mg/kg BW of quercetin failed to show significant changes on these parameters. However, they started to exhibit anxiolytic effect when the treatment duration was further increased to 2, 3 and 4 weeks. This phenomenon might occur via the increasing dose of quercetin might could also increase the ratio of inactive of metabolite of quercetin that could mask the active component which exhibited anxiolytic effect.

No unexpected mortality of any animals occurred after nasal administration of quercetin liposomes used in the present study. As a result, vesicles were considered to be safe at the dosing schedule used. Interestingly, a lower dose and a faster rate were observed with intranasal quercetin liposomes when compared with oral quercetin, conventional administration.

Monoamine oxidase (MAOs) are enzymes which catalyses the oxidative deamination of primary, secondary and tertiary amines (Edmondson et al., 2004). The primary functions of MAOs are the metabolism of exogenous amines and the regulation of neurotransmitter level and intracellular amine stores. Monoamine oxidase A (MAO-A) preferentially deaminates serotonin (5-hydroxytryptamine) and norepinephrine (Billett, 2004). Thus, inhibition of MAO-A may alleviate symptoms of depression (Yamada and Yasuhara, 2004). Besides monoaminergic system, serotonergic system had long been implicated in the etiology of this condition. Decrease in brain concentrations of 5-HT and 5-HT1A (the major metabolite of 5-HT) were commonly observed in animals and in patients experiencing stress and depression, suggesting a dysfunction of serotonergic system (Risch and Nemeroff, 1992; Spreux-Varoquaux et al., 2001; Southwick et al., 2005).

However, monoaminergic system and serotonergic system played the important role not only on antidepressant effect but also on anxiolytic effect (Aan het Rot et al., 2009). Several lines of evidence demonstrated that numerous neurotransmitters including monoamine such as serotonin and norepinephrine and

GABA contributed the important role on anxiety. Previous studies reported that increase serotonin synthesis by the increasing the tryptophan supplement could improve social anxiety (Young, Gauthier, 1981). In addition, the level of norepinephrine and the activity of sympathetic nervous system were prolonged in patients with anxiety disorder (Sullivan et al., 1999). Moreover, most of the available anxiolytic drugs nowadays are still target on the GABAergic system (Ballenger, 1999).

Recently, numerous lines of evidence demonstrated that flavonoids including quercetin could pass blood brain barrier and could exert profound influence on the function of central nervous system (Juergenliemk et al., 2003; Youdim et al., 2004). Various flavonoids could modify the function of various neurotransmitters and gave rise to the antidepressant and anxiolytic effects.

Taken together, I suggested that the mechanism underlying the anxiolytic and anti-depression activity of quercetin with oral conventional delivery system and quercetin liposomes via nasal administration may occur via the inhibition MAO-A, increase the serotonin level and increase in the GABAergic activity respectively.

These results are in accordance with the report of quercetin effect to inhibit MAO-A activity (Chimenti et al., 2006). In addition, the electropharmacogram of adult rats which received quercetin have the same pattern as the well known antidepressant moclobemide, MAO-A inhibitor (Dimpfel W, 2009). Moreover, flavonoids including quercetin could modify the function of GABA receptor (Goutman et al., 2003). Therefore, the anti-depression and anxiolytic effects of quercetin might be associated with the ability to pass blood brain barrier of quercetin and its metabolites and exerted the influence to modify the function of monoamine and GABA as mentioned earlier.

To the best of our knowledge, this is the first investigation that extends available data on nasal administration of quercetin liposomes antidepressant and anxiolytic effects by indicating that the modification of neurotransmitters may be involved in its mechanism of action. Further studies should be carried out to investigate the molecular mechanism of action of this substance, as well as the impact on the central nervous system.

2. Cognitive Enhancer and Neuroprotectant Effect of Quercetin With Oral Conventional Delivery System and Quercetin Liposomes Via Nasal Administration in Healthy Condition

Previous studies had demonstrated that learning and memory appeared to be a complex process requiring the coordination of various brain regions both in hippocampus and cerebral cortex (Knowlton et al., 1995; Riedel et al., 1999; Manns et al., 2003; Birzniece et al., 2006). In addition, many neurotransmitter systems had been recognized to play the crucial roles in learning and memory. Among the various neurotransmitters, acetylcholine had attracted special attention in the spatial memory (Stancampiano et al., 1999). However, acetylcholine especially in the forebrain also played an important role in non-spatial memory via the promotion of proliferation and survival of new neurons in hippocampus (Mohapel et al., 2005). Several lines of experimental evidence had reported that serotonin (5-HT) played a crucial role in learning and memory (McEntee et al., 1991). Reportedly, it had been reported that 5-HT exerted a modulatory effect on memory via interactions with the cholinergic system (Cassel et al., 1995; Robbins, 1997). The serotonergic projection from raphe nuclei to frontal cortex, hippocampus (Murray, 1998) and the distribution of 5-HT1A in these areas (Karten et al., 1999; Bijak et al., 2001; Czyrak et al., 2002; Hayashi et al., 2006; Naumenko et al., 2006) had also demonstrated. Moreover, the 5-HT1A deficit animal showed the impairment of hippocampal-dependent memory (Sarnyai et al., 2000).

Unlike the spatial memory which assessed via Morris water maze test in this study, object recognition test was reported to involve the recognition between familiarities and non-familiarity object. It was reported that recognition memory was based on the recollection of specific information associated with a previous episode and on the assessment of the familiarity of an item which involved the function of medial temporal cortex and prefrontal cortex (Knowlton et al., 1995; Manns et al., 2003). Previous study demonstrated that when the rat was confronted with a novel situation in object recognition test; acetylcholine is markedly released in frontal cortex (Giovannini et al., 1998). In addition, there was a strong evidence that serotonin also played a role on recognition memory process (Riedel et al., 1999; Rubinsztein et al., 2001; Lieben et al., 2004) via both presynaptic and postsynaptic 5-

HT1A. The stimulation of postsynaptic 5-HT1A seemed to induce memory deficit (Carli et al., 1992) whereas the stimulation of presynaptic 5-HT1A leading to the memory facilitation (Cole et al., 1994).

Quercetin, a main flavonoid found in fruits, vegetables, and beverages, was reported to possess antioxidant and cognition (Boots et al., 2008; Reiter et al., 2009). Previous studies confirm that quercetin supplementation improve memory deficit condition induced by reserpine in mice (Naumenko et al., 2006). The results obtained from the current study also confirmed the cognitive enhancing effect of quercetin in both spatial and non spatial memory in different of routes, administered form and species of animal.

Our results showed that single administration of quercetin at dose of 300 mg/kg BW enhanced the retention time and could maintain this action throughout the experimental period in Morris water maze test. However, the effect of single administration of quercetin at all dosage range used in this study failed to show the significant change of total exploration toward novel object assessed at 30 minutes, 6 and 24 hours after substance administration in object recognition test. Nevertheless, rats given quercetin (300 mg/kg) showed the significant increase in total exploration time after 7 days of treatment. Furthermore, the increase treatment duration to 28 days, quercetin at all dosage range used in this study significantly enhanced total exploration time all assessment schedules. On the other hand, single and repetitive administration of quercetin liposomes administered via nasal route could improve both types of memory by decrease escape latency but increased retention time and total exploration time of novel object in Morris water maze and object recognition test respectively.

Therefore, taken all data together, the possible explanation for these changes might be related to the effect of quercetin on various regulators including the GABA, acetylcholine and monoamine system.

However, the results obtained from the first part of this study failed to show both dose dependent and exposure time dependent. The possible explanation might occur because the relationship between the quercetin concentration and the observed parameters were not the simple relationship. It was reported that quercetin was easily metabolized after absorption (Manach et al., 2004). Therefore the substances which

were responsible for the central nervous system activity might be not only the quercetin itself but also its metabolites. Therefore the accumulation of active metabolites and their clearance should be considering also contributed the important roles in this case. However, further investigation was still required to elucidate this issue.

Numerous studies reported that many antioxidants could exert cognitive enhancing effect in age-related memory deficit condition. In this study, vitamin C, a very well known antioxidant, failed to show the cognitive enhancing effect in both spatial and non-spatial memory. The discrepancy might occur because the difference in age of rat, variation from the environment or season.

The evidence stems from data of several authors demonstrated that learning and memory were tightly associated with the function of various brain areas, which in turn depended on the density of neurons particularly cholinergic neurons (Katzman, 1986; Mori et al., 1997) Therefore, this study also determined the neuroprotective effect of oral administration of quercetin and nasal administration of quercetin liposomes both in hippocampus and cerebral cortex in normal condition.

Based on the data obtained from the first part of this study, it was found that quercetin administered orally at a dose of 300 mg/kg BW was the most effective dose to produce the cognitive enhancing effect therefore; this dose was selected for further study on the neuroprotective effect both in cerebral cortex and hippocampus. Previous study demonstrated that the neurogenesis could occur throughout adulthood especially in hippocampus and subventricular zone of lateral ventricle (Hagg, 2005). Numerous factors had been reported to be regulators of the adult neurogenesis. They also included various neurotransmitters such as dopamine, acetylcholine, serotonin, glutamate and norepinephrine. All the mentioned neurotransmitters could stimulate the proliferation process (Nacher et al., 2001; Kulkami et al., 2002; Banasr et al., 2004; Baker et al., 2005; Mohapel et al., 2005). Recent findings also proposed that the new neuron occurring from the neurogenesis also contributed the important role on learning and memory (Bruel-Jungerman et al., 2007; Kitabatake et al., 2007).

This study revealed that the oral administration of quercetin and nasal administration of quercetin liposomes could increase the density of survival neurons and cholinergic neurons both in all areas of cerebral cortex and hippocampus of

normal rats. These findings were corresponding to the plasticity of adult brain neurogenesis (Hagg, 2005). Thus, the possible explanation for these changes might be related to the effect of quercetin on various regulators including the GABA and monoamine system. Moreover, the data obtained from this study also clearly demonstrated that querctin could inhibit acetylcholinesterase (AChE) which in turn might increase the available acetylcholine.

Although, both oral and nasal administration of quercetin could produce the same cognitive enhancement and neuprotective effect. However, it was noticeable that the dose of quercetin required via nasal administration was very much lower and rapid onset of cognitive enhancing effect than that of quercetin with oral conventional delivery system. In this respect, quercetin could be delivered to the brain even though it was administered in nondissolved form in a liquid that was not a good candidate for delivery of this hydrophobic compound (Cho et al., 2006; Paulke et al., 2006). Some of the suspended particles of quercetin might not be absorbed after all. Our present study suggests that the enhanced delivery of quercetin in the form of liposomes to the brain could effectively reduce the dose, which would also reduce the potential of toxicity of the substance (Metodiewa et al., 1999; Lee et al., 2003) and increase bioavailability (Jamal et al., 2000). Moreover, it was also provided numerous benefits especially the direct nose-to-brain delivery, bypassing the blood brain barrier (Wu et al., 2008) and avoid the first pass metabolism (Graf et al., 2006). Therefore, this raise possibility that nasal administration of quercetin liposomes may be serving as the potential novel strategy to protect against Alzheimer's disease.

3. Neuroprotective Effect of Quercetin With Oral Conventional Delivery System and Quercetin Liposomes Via nasal Administration Against Alzheimer's Disease

It had been reported that one of the most prominent neurochemical changes in Alzheimer's disease brain was a reduced concentration of acetylcholine in the hippocampus and neocortex, caused by degeneration of cholinergic neurons (Jarrard et al., 1984; Kristensen, 1990, Perry et al., 1999). Therefore, in order to mimic the cholinergic deficit and neurodegeneration in Alzheimer's disease, a selective cholinotoxin or ethylcholine aziridinium (AF64A) had been applied as the tool to

induce the neurodegeneration of the cholinergic system and resulting in memory deficit (Hortnagl, 1994; Hanin, 1996; McDonald and Overmier, 1998). AF64A could increase oxidative stress in both cerebral cortex and hippocampus, the areas contribution important role on learning leading to the neurodegeneration in the mentioned areas resulting in learning and memory deficit (Chrobak et al., 1988; Gulyaeva et al., 1996) as those observed in Alzheimer's disease (Sultana et al., 2009). Based on the correspondence changes of behavior and neuropathology induced by AF64A and Alzheimer's disease, this study used AF64A to develop animal model of Alzheimer's disease. The data of the present study provided direct evidence that the cognitive deficit condition induced by AF64A mimicked the cognitive deficit condition as those presented in Alzheimer's disease. It was founded that AF64A could induce memory deficit at least via two pathways. The first pathway appeared to occur via the increasing acetylcholinesterase (AChE) which in turn resulted in decrease available acetylcholine (ACh) leading to memory impairment. The other pathway appeared to occur via the reduction of scavenging enzymes activities including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), while enhanced the level of malondialdehyde (MDA), which is an indicative of lipid peroxidation. Extensive evidence previously reported that the increase lipid peroxidation gave rise to the loss of membrane integrity resulted in neurons degeneration and memory impairment (Halliwell, Gutteridge, 1984). These findings were in good concordance with the previous study as mentioned earlier.

Oxidative stress is a major factor implicated in the degeneration of cholinergic neurons and neuronal damage in Alzheimer's disease (Olanow, 1993). Recently, the potential compounds targeting on the oxidative homeostasis had been tested in order to develop the new therapeutic strategy against Alzheimer's disease. Accumulating lines of evidence demonstrated that several antioxidants including both direct antioxidants and indirect antioxidants could improve cognitive function (Behl, Moosmann, 2002). Numerous polyphenol compounds were shown to protect against neurodegeneration and improve memory impairment (Rossi et al., 2008; Singh et al., 2008).

Quercetin, a natural flavonoid, is a strong antioxidant and radical scavenger (Choi, 1996; Chen et al., 1998). It has been reported that quercetin possesses

neuroprotective effect. In *in vitro* study with PC 12 cell line, it showed inhibitory effect against cell damage (Gelinas, Martinoli, 2002). Moreover, quercetin attenuated memory deficit and neurotoxicity induced by D-galactose (Lu et al., 2006). It also protected against neurotoxicity induced by NMDA in *in vivo* model (Silva et al., 2008).

The present study on the neuroprotective effect of quercetin either administered via oral or nasal route could improve neurons and cholinergic neurons density both in cerebral cortex and hippocampus. It was found that the neuron density which decreased by AF64A was reversed to normal condition. However, this improvement might occur as a result of the attenuation of neurodegeneration or the neurogenesis. To clarify this issue, further researches are still required.

The brain plasticity plays an important role not only in normal condition but also in Alzheimer's condition induced by AF64A. It was found that quercetin administered via oral route could increase the activities of scavenger enzymes including SOD, CAT and GPx in cognitive deficit condition induced by AF64A or in the animal model of Alzheimer's disease. The data obtained from this study were also in agreement with the previous data which demonstrated that quercetin could exert the neuroprotection mediated via the antioxidant effect. Thus, I suggested that the neuroprotective effect of quercetin in this study occurred partly via its influence to increase the scavenger enzymes and decrease in MDA, resulting in the improvement of oxidative stress homeostasis.

The results of this study was the first document revealed that prophylactic treatment with nasal administration of quercetin liposomes significantly reversed the impact of oxidative alterations (MDA, SOD, CAT and GPx) seen in Alzheimer's condition induced by AF64A; this shows the antioxidant potential of quercetin liposomes via nasal administration.

Currently, the treatments for AD are AChE inhibitors, which increase the availability of ACh at cholinergic synapses (Kang et al., 2005). Thus, the effect of quercetin on acetylcholine could not be excluded. Therefore, this study was designed to determine AChE enzyme activity, the key enzyme inactivating ACh, which had been used as index to indicate ACh turnover rate both in cerebral cortex and hippocampus. Our results showed that a cholinesterase inhibitor, donepezil

hydrochloride, which used as positive control could improve both spatial memory in Morris water maze and non-spatial memory in object recognition test. I suggested that this drug exerted its cognitive enhancing effect via the increase in ACh level in the brain areas responsible for spatial and non-spatial memory particularly in hippocampus and prefrontal cortex. Quercetin, also showed the attenuation effect on the increase AChE level induced by AF64A both in cerebral cortex and hippocampus. This indicated that this substance could enhance an available ACh and the function of cholinergic system. In addition, previous study also demonstrated that quercetin might also exert its influence to increase brain plasticity via the modification of various regulators of neurogenesis such as monoamine system by inhibited the MAO activity.

Nasal administration of quercetin liposomes could significantly produce the cognitive enhancement and neuroprotective effect activity at a very much low concentration Moreover, it could produce the higher magnitude change on the AChE inhibition at lower concentration compared to that of oral administration. The possible explanation might be related with the direct transfer of substances from the olfactory mucosa along the olfactory pathway to the CNS induced by intranasal administration (Mathison et al., 1998; Illum, 2000) by bypassing the BBB, which prevented some CNS-active drugs from reaching the brain (Behl et al., 1998). In addition, it could also decrease the influence of the first pass metabolism (Krauze et al., 2006). However, the main proposed pathway of quercetin delivery via nasal administration was different. It was likely to permeate through the subarachnoid space through the olfactory epithelium and found in the CSF later, because the liposomes behaved as semilipophilic particles. Therefore, quercetin liposome could rapidly absorb into the CSF. However, further studies about the precise changes underlying these effects are still required.

4. Conclusion

Quercetin, a flavonoid commonly found in vegetables and fruits, provided numerous health beneficial effects including anxiolytic, antidepressant, neuroprotective and cognitive enhancing effects. The benefit could occur both in normal healthy condition and in cognitive deficit condition. The mechanism how quercetin enhanced cognitive performance and neuroprotective effect under normal condition might occur

partly via the modification the function of various neurotransmitter systems including acetylcholine, GABA, monoamine system such as dopamine and serotonin leading to the increase in the density of neurons and cholinergic neurons both in cerebral cortex and hippocampus. In addition, it could also attenuate the cognitive deficit and exhibited neuroprotection in animal model of Alzheimer's disease. The possible mechanism might partly relate to the increase the cholinergic function, decrease lipid peroxidation product, which consequently resulted in the improvement of the spontaneous behavior and cognitive performance and enhancement of brain inherent antioxidant capacity. Moreover, quercetin could modify the function of various neurotransmitter systems including monoamine and GABA and its influence on the brain plasticity via the increase various neurotransmitters such as acetylcholine and serotonin which also serve as the important regulator of neurogenesis in cerebral cortex and hippocampus. Hence, nasal administration of quercetin liposomes might provide beneficial effect to improve learning and memory deficits possibly by inhibiting the oxidative stress and by enhancing the level of scavenging enzymes, and finally permitted a rational for implicating as novel therapeutic strategies to protect against Alzheimer's disease. However, more pharmacokinetic and pharmacodynamic studies are needed to clarify the significance of olfactory transfer of quercetin liposomes following nasal administration.