CHAPTER V

DISCUSSION AND CONCLUSION

Extraction of phenolic components from mulberry leaves was performed by using four different solvents: water, 50% aqueous methanol, 100% methanol and 1-butanol. These solvents posses their values of polarity index of 9.0, 7.0, 5.1 and 4.0, respectively which are effective in extracting a wide range of antioxidant phytochemicals (Tsao and Deng, 2004). In literature, the methanol and water extracts of mulberry leaves have been reported to have the yields of 12.35% and 6.50% dry weight, respectively (Arabashahi-Delouee and Urooj, 2007) which are considerable lower than our results (16.28% for 100% methanol extract and 13.10% for hot water extract).

In terms of total polyphenol content, the values of the Folin-Ciocalteu determination were expressed as gram equivalents of either chlorogenic acid, rutin or gallic acid per 100 g dry leaves (% dry weight). The results showed that the four extracts had significantly different values of total polyphenol contents (Table 1). The highest values were obtained from the solvent extraction by 100% methanol followed by 50 % aqueous methanol, 1-butanol and hot water. There extractable values appeared to highest when they were expressed based on chlorogenic acid followed by rutin and gallic acid, with the ratio of 2.4:1.9:1.0, respectively. The ratio should reflect the presence of chlorogenic acid and rutin as major phenolics and gallic acid as a minor one in the mulberry leaves as reported previously (Yan *et al.*, 2004; Chu *et al.*, 2006)

Thus the four different solvent extracts appeared to contained total phenolic content in a wide range which was useful in this comparative study. It has been reported that the methanol and water extracts of mulberry leaves contains 9.32 % and 7.10 % total phenolic, respectively, based on gallic acid equivalent (Arabashahi-Dolouee and Urooj, 2007). Our ethanol extract also showed very similar value (9.2%, gallic acid equivalent) whereas the water extract showed much lower (3.5%) probably due to using different method of water extraction.

Partial characterization of phenolic constituents in each solvent extract of mulberry leaves was conducted in order to compare the phenolic profiles of all the four extracts. This was performed by using a combination of diode array detection (DAD) and positive electrospray ionization mass spectrometry (ESI^+), couple to a HPLC. This HPLC-DAD-MS method allowed accurate structure elucidation of individual phenolics based on their MS and UV characteristics. It can be seen that most of the flavonoids detected were glycosides in which their mass spectra showed both the protonated molecule $[M+H]^+$ and the ion corresponding to the protonated aglycone $[A+H]^+$. The latter is formed by losing of the moieties of glucose, galactose and rhamnose moieties from the glycosides. Thus, based on the structure identification, we concluded that the phenolic constituents in mulberry leaves contained at least two phenolic groups of chlorogenic derivatives and flavonol derivatives (Table 2). Among these, rutin (peak no. 8), isoquercetin (peak no. 9), kaempferol glycosides (peak no. 10, 11) and chlorogenic acid (peak no. 2) appeared to be the major phenolic constituents.

In order to verify the antioxidant capacity, the DPPH assay was chosen because it is one of the widespread methods used to evaluate the antioxidant activity in plant or food extracts and this make the comparison easier with data published in

the literature (Aruoma, 2003; Prior, Wu, and Schaich, 2005). The free radicals scavenging was tested by their ability to scavenge the stable DPPH radical, as previous reported (Sánchez-Moreno1999; Choi *et al.*, 2002; Nehir-El and Karakaya, 2004; Wong, Leong, and Koh, 2006; Tuberco *et al.*, 2007).

Free radical scavenging of the tested samples was measured spectrophotometrically with DPPH, which produces a violet solution in methanol. When each mulberry extracts were examined using stable DPPH radicals, It is reduced in the presence of an antioxidant molecules give rise to colorless solution. Previous reports have shown to have antioxidative flavonoids from mulberry leaves (Kim *et al.*, 1999; Zhishen, Mengcheng, and Jianming, 1999; Choi *et al.*, 2002; Arabashahi-Dulouee and Urooj, 2007).In this study, when each mulberry extracts were examined using stable DPPH radicals, all of them exhibited various degree of free radical scavenging. Among those, the 100% methanol extract showed the most scavenging by the lowest value of IC_{50} (356 μg/mL) followed 50% methanol (544 μg/mL), hot water (606 μg/mL) and 1-butanol extracts (743 μg/mL), respectively, and also with a dose dependent manner in DPPH assay. The superoxide radical radical is a highly toxic species which is generated by numerous biological and photochemical reactions. The results show that mulberry leaf extracts contains water-soluble scavengers of superoxide radicals and that these react in a dose-dependent manner. Among the reactive oxygen species, the hydroxyl radical is the most reactive and induces severe damage to the adjacent biomolecules (Haliwell, 2007). Mulberry leaf extracts was found to be a powerful scavenger of the hydroxyl radical. The IC_{50} value amounted range of 396-543 μg/ml. The effect of S Mulberry leaf extracts on non-enzymatic peroxidation of lipids when incubated in the presence of ferrous sulphate is

shown in Table 3. All the extracts of mulberry leaves inhibited lipid peroxidation in a concentration-dependent manner IC_{50} values for the inhibition of lipid peroxidation were range of 442-801 μg/ml

In addition, the antioxidant capacity was expressed as Trolox equivalents per gram of dried leaves since it is more meaningful and descriptive expression. This assay expresses the antioxidant activity as the percentage decrease in the absorbance, and thus providing a direct comparison of the antioxidant capacity with Trolox. A high correlation between TEAC value and the polyphenolic content has been reported (Tsao, and Deng, 2004; Javanmardi, *et al.*, 2003; Ivanova, *et al.*, 2005; Guendez, *et al.*, 2005). The interaction of a potential antioxidant with DPPH depends on its structural conformation and this structural requirement is correlated with the presence of hydroxyl groups on the flavonoids (Cook and Samman, 1996; Burda and Oleszek, 2001; Heim, Tagliaferro and Bobilya, 2002). However, there are a few reports on quantitative antioxidant activity or either specificity the amounts of antioxidants in mulberry leaves. These extracts of mulberry leaves showed the high polyphenolic content and these polyphenols are well recognized for their antioxidative properties. They are considered cancer chemopreventive, because polyphenols can quench or prevent the formation of reactive oxygen and nitrogen species, which play important roles in carcinogenesis (Loo, 2003). Evidence for these mechanisms, however, has been mostly circumstantial and more investigations are needed.

Previous reports have been shown that mulberry leaf extracts was able to inhibit cancer cells in human leukemia cells (HL-60, K-562, and B380 cell lines) and B16 mouse melanoma cells (Kim, Gao, and Kang, 2000; Nam *et al.,* 2002). This is the first reported to the effect of mulberry leaf extracts on HepG2 cells. Human

hepatoma HepG2 is a well-differentiated transformed cell line that meets all biochemical requirements for the present study. This cell line has been widely used in biochemical and nutritional studies because it is considered one of the experimental models that more closely resembles the human hepatocyte in culture (Seow *et al.*, 2001; Mersch *et al.*, 2004; Xu, Duaz, and O Brien, 2004). In the present study, we focused on the effects of mulberry leaves extracts on the growth inhibition of HepG2 hepatoma cells which were determined by Cell Counting Kit-8 (CCK-8). CCK-8 allows convenient assays using Dojindo's tetrazolium salt, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-Methoxy PMS. CCK-8 solution which is added directly to the cells is a sensitive nonradioactive colorimetric assay for determining the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium. The amount of formazan produced is directly proportional to the number of living cells.

Our results showed that the extracts of mulberry leaves exhibited a strong growth inhibitory affect in HepG2 cells. The examination of the differential cell viability toward human hepatoma (HepG2) and normal human liver cells was also tested as a result shown in Figure 11. It can be seen that dose ranging from 0-500 μg/mL, the viability of normal liver cells decreased with an increase of dosage. At IC_{50} concentration of normal liver cell was higher 8.3 fold than IC_{50} concentration of HepG2 cells. These results showed that mulberry extract posed good cytotoxicity against hepatoma HepG2 cells, but showed less sensitivity to normal liver cells base

on IC_{50} value. It is possible that the greater sensitivity of the malignant cells may reflect in part the difference in the growth rates of malignant and non-malignant cells. The more pronounced cytotoxicity of mulberry extracts to hepatoma cells than to normal cells suggests a potential for developing cancer preventive photochemical.

To examine whether the cytotoxicity of the mulberry leaf extracts was associated with an arrest in the cell cycle progression of this human HCC cells, further studies were carried out.

Based on the fact that the unbalanced control proliferation or cell cycle progression and apoptosis are the main characteristics of cancer cells, compounds that inhibit cell cycle or induce apoptosis are good candidates as cancer chemopreventive agents (Hartwell and Kastan, 1994; Fischer, Glover, Lane, 2004). In this study, the steps of after fixation and permeabilisation, cells were stained with propidium iodide (PI) and assayed for their cell cycle distribution by flow cytometric analysis. The organic extracts of the mulberry leaves all showed their effects on the arrest of HepG2 cells in the G2/M phase of cell cycle, but no changes in hot water extract treated cells. It is excluded by viable cells but can penetrate cell membranes of dying or dead cells.

Apoptosis is a physiological process that plays a functioned role during embryogenesis, tissue homeostasis control of tissue integrity, tumor regression and immune development (Tamm, *et al.*, 2001; Sun, *et al.*, 2004). On receiving specific signals, a number of distinctive biochemical and morphological changes occur in the cell. A family of proteins knows as caspases are activated in the early apoptosis. Subsequently, active caspases specifically process various substrates that are implicated in apoptosis (Lavrik, Golks, and Krammer, 2005). Activation of caspases can be detected using fluorochrome labeled inhibitors of caspases (FLICA™). The

reagent associated a fluoromethyl ketone (FMK) moiety, which can react covalently with a cysteine, with a caspase specific amino acid sequence. The recognition sequence is valine-alanine-aspartic acid (VAD). A carboxyfluorescein group (FAM) is attached as reporter. The FLICA reagent is though to interact with the enzymatic reactive center of an activated caspase via the recognition sequence, and then to attach covalently through FMK moiety. The Vybrant FAM Poly Caspases Assay Kit provides FAM-VAD-FAM FLICA reagent, a generic probe for the detection of most caspases (including caspases-1, -3, -4, -5, -6, -7, -8, and -9). In the present study, apoptosis assay was combined with a PI stain, this assay have been used as previously (Smolewski *et al.*, 2002; Pozarowski *et al.*, 2003; Valero *et al.*, 2005; Wlodkowic, Skommer and Pelkonen, 2007). The mulberry leaf extracts of organic solvents, 100% methanol, 50% aqueous methanol and 1-butanol could induce apoptosis in HepG2 cells. These suggested that the organic extracts could induce caspases activity in the treated HepG2 cells. Previous reports have demonstrated that mulberry leaves were able to inhibit cell proliferation to induce a significant cell cycle arrest in G2/M phase and to induce apoptosis in leukemia cell lines (Kim, et al., 2000; Nam, et al., 2002; Takuya and Yukikazu, 2002). These results are in agreement with this study.

The mulberry leaf extracts were also evaluated for their inhibitory effect on the catalytic activities on human DNA topoisomerase IIα. This assay used the DNA relaxation assay, which is known as in vitro test to select chemopreventive agents (Cho *et al.*, 2000). The results showed that the mulberry leaf extracts inhibit catalytic activity of topoisomerase IIα in a dose dependent manner. The inhibition of the topoisomease IIα activity may contribute to the chemopreventive activity of mulberry

leaves extracts, making them promising candidates for the investigation of chemopreventive agents.

The finding showing that the mulberry leaf extracts can cause cell cycle G2/M arrest and induce apoptosis in HepG2 cells, are relatively new in terms of the mechanisms underlying the effect of mulberry leaf extracts. In order to examine on the cell cycle regulatory molecules involved in the cell cycle progression and topoisomerase IIα, cyclin-dependent kinase inhibitors (Ckis) which play a key role in controlling cell cycle progression were examined (Hartwell and Kastan, 1994). We tested only the effect of the 100% methanol extract because it showed the highest total phenolic content, antioxidant capacity and the most a potent effective to HepG2 cells. As shown in Figure 13, the treatment of the 100% methanol extract on HepG2 cells resulted in a strong decreased topoisomerase IIα activity and an increase in the protein level of $p27^{Kip1}$ but interestingly, did not affect the expression of $p21^{Cip1/Waf1}$. The results suggested that the up-regulated of $p27^{Kip1}$ might contribute to mulberry extract's blockade effect on cell cycle progression at G2/M phase. Similarly, some studies have also shown that $p27^{Kip1}$ is an important regulator of cell cycle progression through G2/M phase and induction apoptosis (Rivard *et al.*,1999; Hsieh et al., 2006). It has been reported that $p27^{Kip1}$ is the major inhibitor in complex of cyclinB1 and Cdc2 (Niculescu *et al.*, 1998). In normal cells Cdc2 activity is regulated by the formation of a complex with cyclin B and by its phosphorylation. It is also reported that the formation of Cdc2-cyclin B complex is required for G2/M transition and for the cell enter mitosis (Loloyd *et al.*, 1999). Some studies have also shown that $p27^{Kip1}$ is an important regulator of cell cycle progression though G2/M phase and induction of apoptosis. In liver cancer show $p27^{Kip1}$ protein levels emerges as a

statistically significant predictor of survival and tumor behavior. It has been suggested that $p27^{Kip1}$ loss occurs early in the carcinogenesis process, with dysplastic epithelium having decreased expression. The more aggressive, metastasizing cancers tend to lack $p27^{Kip1}$ expression as well. The subcellular localization of $p27^{Kip1}$ (cytoplasmic versus nuclear) are as also being of prognostic value (Lloyd, 1999; Chetty, 2003). $p21^{Cip1/Waf1}$ is functionally similar to $p27^{Kip1}$, it plays a lesser role in tumor suppression (Philipp-Staheli *et al.,* 2004). Philipp *et al.* (1999) suggests that the cell cycle regulation function of $p21^{Cip1/Waf1}$ dose not play a major or direct role in tumor suppression but its impact on differentiation may play a more significant role.

For topoisomerase IIα, levels of topoisomerase IIα mRNA peak in late S and G_2/M phase several fold over the amount detected in G_1 phase. There high levels are consistent with idea that topoisomerase IIα is required mainly during the final stage of DNA replication to facilitate chromosome untangling, condensation and mitotic segregation (Cortes, Pastor, and Mateos, 2003). Thus topoisomerase IIα is an important checkpoint of G2/ M phase in cell cycle (Downes *et al.,* 1994; Clarke, 2000). Inhibition of topoisomease IIα by mulberry leave extracts is sufficient the results to arrest cells in G2 and early M phases (Skladanowki *et al*., 2005)

In conclusion, we have found that various solvent extracts from mulberry leaves tea contain high polyphenolic content and antioxidant activity. Methanol appears to be the most efficient solvent that give the highest of polyphenolic compounds and also exhibited the strongest antioxidant capacity because of the most effective effect on molecular targets in HepG2 cells. The water extract, on the other hand, did not alter cell cycle progression and apoptosis and a little affect to inhibit cell proliferation. It can be explained by the presence of chlorogenic acid as mainly

compound in the extract since it known to have no prominent effects on the cell death rate and apoptosis in HepG2 cells (Ramos *et al.*, 2005; Garnado-Serrano *et al.*, 2007). However, it has been known that chlorogenic acid has a little modulating effect on colon cancer cells viability, apoptosis and DNA synthesis (Zheng *et al.*, 2002)

The data obtained from the organic extracts show clearly that they contain bioactive substances against to HepG2 cells. The data are also consistent with the conclusion the extracts are cytotoxic to the HepG2 cells. The resulting in cell death is mediated through the induction of $p27^{Kip1}$ and inhibits topoisomerase IIα which affect their cell cycle by G2/M arrest and apoptotic induction. These actions are likely to be the results of the presence of the mainly constituents in the organic extracts of rutin, isoquercetin, kaempferol 3-rhanopyranosyl-glucopyranoside, quercetin 3-(6-malonylglucoside), astragalin and kaempferol 3-(6-malonylglucoside) as flavonols glycosides, that have quercetin and kaempferol as aglycone.

Flavonoids are mostly present as the form of glycosides in which one or more sugar groups is bound to phenolic group by glycosidic linkage (Robards *et al.*, 1999; Cheynier, 2005). In the past, it was strongly believed that flavonoids glycosides could not be absorbed after oral ingestion but hydrolyzed to their aglycones by the β-glucosidase in bacterial flora in the lower part of the intestine. Intact flavonoid glycosides are hardly absorbed from the small intestine because and sugar moieties into the structure increases their hydrophilic properties (Griffiths and Barrow, 1972; Bokkenheuser *et al.*, 1987). In 1995 Hollman et al., proposed that flavonoid glycosides can be absorbed intact, presumably via the sodium-dependent glucose transporter SGLT1. Many data supports that hydrolysis of flavonoid glycoside can occur in the small intestine by glycosidase in the food, oral cavity, or the broad-

specific enterocyte β-glucosidase, bacteria, gastrointestinal mucosa (Andlauer *et al.*, 2001; Mutaro and Tarao, 2003; Carbonaro and Grant, 2005; Matsumoto, *et al.*, 2005; Walle *et al.,* 2005). Recent studies suggest that quercetin-3-glucoside is better absorbed that quercetin aglycone, rutin, or quercetin-3-rhamnoside. Rutin is much less bioavailability than quercetin aglycone (Morand *et al.*, 2000). The bioavailability flavonoid glycosides from the diet depend on a variety of sugar group attached to their phenolic group. In addition, glucose-bound glycosides are likely to be much more absorbable than other sugar bounds (Arts *et al.*, 2004).

In this study, quercetin and kaempferol are the two most commonly aglycones found in the mulberry leaves. A few studies have evaluated the inhibitory effects of phenolic compounds on HepG2 cell growth. Ramos *et al.* (2005) studied the effects of pure polyphenols (quercetin, chlorogenic acid, and epicathechin) and fruit extracts (strawberry and plum) on HepG2 cell population growth and apoptosis. They found that quercetin was the most active with an IC_{50} of 87 μM (about 26 μg/mL). Quercetin can induce apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI-3-kinase/Art and ERK pathways (Granado-Serrano *et al.*, 2006). This studied Kaempferol has also been induced apoptosis in cancer cells which related to its ability to change the expression of caspase-3 (Leung *et al.*, 2007). Quercetin and kaempherol have been also reported that they are topoisomerase II catalytic inhibitors (Rao *et al.*, 1995; Cho *et al.*, 2000; Cantero *et al.*, 2006; Bandele and Osheroff, 2007). According to Rao *et al.* (1995) quercetin and kaempferol were the two potent chemopreventive agents with effective inhibition of catalytic activity on mammalian topoisomerase II (IC_{50} of 6.9 μg/mL for quercetin and 8.1 μg/mL for kaempferol) but rutin which as glycoside of quercetin lack topoisomerase inhibitory activity.

From this study, these flavonoids can interfere with multiple cell-signaling pathways and can be used either in their natural form for the prevention and perhaps in their pure form for the therapy, where large doses may be needed. While these agents are pharmacologically safe in most situations, one of the concerns commonly expressed is the lack bioavailability should not be evaluated in the same manner as synthetic compounds. Most modern medicines currently available for treating cancer are very expensive, toxic, and less effective in treating the disease. Thus one must investigate further in detail the compounds derived from natural sources, described traditionally, for the prevention and treatment of cancer and disease. More clinical trials are also needed to validate the usefulness of these compounds either alone or combination with existing therapy.

In summary, our study suggests that the mulberry extracts obtained from organic solvents can inhibit HepG2 hepatoma cell population growth at G2/M phase through (i) inhibiting topoisomerase IIα, (ii) induced apoptosis processes, and (iii) induced the expression of $p27^{Kip1}$. According to these results, mulberry leaves extract appears to exert its chemopreventive properties to reduce liver cancer.