CHAPTER 4

DISCUSSION AND CONCLUSION

Colon cancer is one of the most common cancers in human. Diet is an important risk factor of this disease. It was found that a diet high in red meat and low in fresh fruit and vegetables increases the risk of colon cancer (Chao et al., 2005). Evidence has emerged from various studies that suggest that products derived from plants are useful in the prevention as well as treatment of cancer (Taraphdar et al., 2001). Apoptosis induction is hoped to use as a therapeutic strategy and achieve tumor-selective killing without compromising normal cell function (Kasibhatla and Tseng, 2003). Antioxidant vitamins and natural substances show promise in cancer therapy by their ability to induce apoptosis in cancer cells (Taraphdar et al., 2001; Borek, 2004).

4.1 Content of total phenolics and flavonoid in dried longan extracts and their antioxidant activity

These results showed that total phenolic and flavonoid contents in crude acetone extract of dried seed was higher than dried pulp (Table 3.1). Synthesis of phenolic compound in seed takes place during the maturation. Fruit seeds are known to contain many phenolic compounds capable of protecting them from oxidative damage and defending them against yeast, fungi, virus and bacteria that might inhibit their germination (Soong and Barlow, 2005).

The crude acetone extracts of dried seed and pulp were fractioned by solvent partition using ethyl acetate. The total phenolic and flavonoid contents of EFLS and EFLP were found to be higher than their crude acetone extracts (Table 3.1). The results indicated that ethyl acetate is a good solvent for extraction of phenolic and flavonoid compound from this plant.

As normal metabolic action going on in human body, active oxygen and free radicals are constantly formed. The reactive oxygen species (ROS) include superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$). Accumulation of these ROS can result in oxidative stress that has been related to human diseases such as atherosclerosis, cardiovascular diseases, diabetes, and cancer (Willcox et al., 2004). The ROS damage DNA, RNA, and proteins by chemical reactions. This leads to an increase in mutations and alterations in the functions of important enzymes and proteins. Several experiments have proved that chemical compounds, which create ROS in excess, encourage carcinogenesis (initiation, promotion and neoplasic progression) through genotoxicity (Oliveira et al., 2007). However, the action of active oxygen and free radicals is opposed by a balanced system of antioxidant defences, including antioxidant compound and enzymes (Halliwell and Gutteridge, 1999). Hence the presence of antioxidants is essential for good health. Fruits and vegetables contain many antioxidant compounds, such as carotenoids, thiols, vitamins such as ascorbic acid and tocopherols, flavonoids, and phenolics. Some dietary antioxidants may have potential as adjuvant in cancer therapy by their ability to induce programmed cell death (apoptosis). In addition, antioxidants also show promise in cancer therapy by their palliative action, reducing painful side effects associated with treatment (Borek, 2004). In the present study,

longan extracts were evaluated for their ability to neutralize the stable free radicals such as DPPH radicals. Free radical scavenging activities of plant extracts in decreasing order were EFLS > crude acetone extract of seed > EFLP > crude acetone extract of pulp (Table 3.2). Dried longan pulp extract exhibited very weak scavenging activities. The antioxidant activities of these longan extracts correlated with their phenolic and flavonoid contents. Most antioxidant capacities of longan extracts may be attributable to the polyphenolic compounds in this plant. Seed extracts contain high levels of polyphenolic compounds which are composed of one (or more) aromatic rings bearing one or more hydroxyl groups, thus they are capable of direct free radical scavenging and inactivation. Longan seed has previously been shown to possess potent antioxidant activity which could be ascribed to their phenolic contents (Soong and Barlow, 2004). In addition, the DPPH scavenging activity of EFLS was as strong as ascorbic acid (Table 3.2). These results suggested that longan seed extracts may be developed further as a natural source of free radical scavenging phytochemicals.

4.2 Effect of dried longan extracts on human colon cancer cell lines *in vitro*

Cytototic effect of dried longan extracts was investigated by MTT assay. The results showed that EFLS had antiproliferative property on colon cancer cells (Figure 3.1). EFLS inhibited cell proliferation in HCT-15 and RKO cells while other extracts had less or non-cytotoxic to both kinds of cell (Figure 3.2-3.4). The antiproliferative activity of EFLS may be attributable to its phenolic and flavonoid contents. The IC_{50} values of EFLS extract on HCT-15 and RKO cells were less than those in NIH3T3

cells. This indicated that the EFLS had cytotoxic in colon cancer cell lines more than in normal cell (NIH3T3, a normal mouse fibroblast).

Cytotoxic effect of EFLS on colon cancer may be involved with its antioxidant property because phenolic antioxidants can be both pro-oxidative and antioxidative depending on the cell type, dose and/or time of treatment. It was found that pro-oxidant action of plant-derived phenolics may be an important mechanism for their anticancer and apoptosis-inducing properties (Galati and O'Brien, 2004). However, mechanism of cytotoxicity of these phenolic antioxidants should be investigated by measurement of free radicals generation in the cells and use of animal models.

To identify whether the process of cell death induced by EFLS in HCT-15 and RKO was apoptotic type, the most recognized marker of apoptosis was demonstrated by DNA fragmentation analysis (Zhang and Ming, 2000). At exposure to 100 µg/ml of EFLS for 48 h, fragmented DNA was clearly observed in HCT-15 and RKO cells (Figure 3.5). DNA fragmentation is a relatively late event in apoptosis. These results suggested that EFLS induced colon cancer cell death via the apoptosis mechanism.

Caspase is a group of cysteine proteases. These enzymes are produced as inactive zymogens and are activated during apoptosis. The biological function of caspases is to amplify the apoptotic signals and to specifically proteolyse their target proteins. Caspases are divided into 2 groups in process of apoptosis include initiator caspases (caspase-2, 8, 9, 10) and effector caspases (caspase-3, 6, 7). Initiator caspases play a role in initiating the apoptosis pathway. Initiator caspases transmit apoptotic signals by promoting the cleavage and activation of the effector caspases. When activated, Effector caspases in turn cleave numerous cytoplasmatic or nuclear substrates which mark many of the morphologic features of apoptotic cell death

(Sellers and Fisher, 1999; Strasser et al., 2000). The present study showed that caspase-3, one of the effector caspases, is activated in HCT-15 and RKO cells after incubation with EFLS (Figure 3.7). Caspase-3 plays important role in both chromosomal DNA fragmentation and chromatin condensation during apoptosis (Liu et al., 1998). The increase in caspase-3 activity emphasized the ability of EFLS to induce apoptosis in colon cancer cells.

Bcl-2 family proteins play an important role in the regulation of the mitochondrial pathway. These proteins are broadly characterized into pro-apoptotic protiens that include Bax, Bak, Bik, Bad and Bid, and anti-apoptotic proteins such as Bcl-2 and Bcl-xL, based on their ability to either suppress or induce the release of cytochrome c. Several Bcl-2 proteins have been reported to homodimerize and heterodimerize through the specific conserved domains to modulate cell death signals (Finnegan et al., 2001). Upon apoptosis induction, pro-apoptotic Bcl-2 proteins in the cytosol such as Bax translocate to the outer mitochondrial membrane where the anti-apoptotic proteins such as Bcl-2 are located, Bcl-2-Bax heterodimer formation evokes a survival signal for the cells but free Bax protein can induce apoptosis. The interaction between pro- and anti- apoptotic proteins leads to change in the mitochondrial membrane potential and cytochrome c release. Altered expression of Bcl-2 family proteins has been reported in a variety of human cancers and has been linked to treatment response (Fulda and Debatin, 2006). It is widely accepted that ratio of Bax/Bcl-2 is a critical determinant of cells for undergoing apoptosis (Sellers and Fisher, 1999). The present study, the effect of EFLS on expression of Bax and Bcl-2 protein was investigated by Western blot analysis. The result showed that EFLS was able to decrease expression of Bcl-2 in HCT-15 cells and increase

expression of Bax in both HCT-15 and RKO cells (Figure 3.8). These findings suggest that apoptosis induction in EFLS-treated HCT-15 and RKO cells could be associated with the activation of the mitochondrial pathway through an increase in the Bax to Bcl-2 ratio.

The phosphoinositide-3 kinase (PI3K)/Akt pathway is a potent mediator of cell survival signals and this pathway can be activated by various growth factors. Its downstream Akt have been implicated in the inhibition apoptosis and the promotion of cell proliferation. Further evidence has shown that Akt, a downstream kinase of PI3K, is also involved in malignant transformation. Akt substrates which involve in apoptosis pathway such as Bad and caspase-9 (Chang et al., 2003). Bad, a pro-apoptotic protein, can binds and inhibits anti-apoptotic Bcl-2 molecule when Bad is not phosphorylated. Akt can modulate Bad activity by phosphorylation. Phosphorylated Bad then dissociates from Bcl-2 to form a complex with the 14-3-3 adaptor protein. Dissociation of Bad from Bcl-2 is associated with cell survival. Another notable substrate of Akt is the death protease caspase-9. Phosphorylation of caspase-9 decreases apoptosis by directly inhibiting the protease activity. Thus, strategies to block the activity of Akt would ideally lead to the inhibition of proliferation and induction of apoptosis. In the present study, the expression of p-Akt (activated form) was decreased after exposure to EFLS in both HCT-15 and RKO cells. The results suggested that EFLS acted only in the phosphorylation of Akt but was not involve in the level of Akt (Figure 3.8).

The cell cycle is a complex and highly ordered process that takes place in a cell leading to its duplication and transmission of genetic information from one cell generation to the next. During the process, DNA must be accurately replicated and

identical chromosomal copies distributed to two daughter cells. The regulation of the cell cycle must ensure that the events in each phase are complete before moving to the next. Some phytochemicals have been found to have anti-tumor activity by induction of apoptosis and/or cell cycle arrest. In the present study, the cell cycle analysis using PI showed that EFLS arrested cell cycle at S phase in both HCT-15 and RKO cells (Figure 3.10-3.11). The growth inhibition of HCT-15 and RKO cells by the EFLS extract could be partially explained by its induction of cell cycle arrest. EFLS caused the arrest of cell cycle in S phase in HCT-15 and RKO cells that may be because of changes in expression of proteins and cell signaling involved in cell cycle, or DNA failed to replicate after exposure to the extract, so they become arrested until the damage is repaired (Lodish et al., 1999). Prolonged disruption of the cell cycle results ultimately in death that has been proposed to occur via programmed cell death apoptosis.

The p53 tumor suppressor gene plays an important role in preventing cancer development, by arresting or killing potential tumor cells. Mutations within the p53 gene, leading to the loss of p53 activity, are found in about half of all human cancers (Vousden, 2002). The p53 acts as a transcription factor in the control of G_1 arrest and apoptosis. It reduces *Rb* phosphorylation and induces a stop at the G_1-S checkpoint to allow cells to undergo DNA repair or Bax/Bcl-2-mediated apoptosis when the damage is irreversible (Tsao et al., 2004). HCT-15 is p53 mutant cell and RKO is p53 wild-type cell. In the present study, The EFLS extract markedly inhibited cell growth of both HCT-15 and RKO. As an exposure time (24 h) of the EFLS extract is sufficient to effectively induced S phase cycle arrest in HCT-15 cells. HCT-15 cells seem to be more susceptible to the toxic effect of the EFLS extract when compared with RKO

cells (S phase arrest at 48 h after EFLS exposure). The other explanation could be attributed to the time RKO cells spend in different phases of the cell cycle significantly longer than in HCT-15 cells. RKO and HCT-15 grow in culture with doubling time of about 24 h (Bozko et al., 2002) and 20 h (Dexter et al., 1982), respectively. In addition, within 24 h of exposure of RKO cells to the EFLS extract, p53 may be involved in transient G_1 phase cycle arrest.

In addition, Sub G_1 peak, which means the appearance of apoptotic cells, was observed after 48 h exposure in both RKO and HCT-15 cells (Figure 3.11). These results showed that EFLS can induce apoptosis in both p53 wild-type and p53 mutant cells. Thus, p53 may not be an important target in the induction of apoptosis by EFLS.

To gain insight into the cell cycle signaling pathway involved in the EFLS extract action in HCT-15 and RKO cells, this study examined the PI3K/Akt pathway. The PI3K/Akt pathway has been shown to not only play a role in prevention of apoptosis, but also regulates cell cycle progression. Many studies have indicated that deregulated PI3K/Akt contributes to tumorigenesis (Sellers and Fisher, 1999). Further evidences have suggested that the PI3K/Akt pathway can induce cell cycle progression (including DNA synthesis, Cdk activation, and prevention of cell cycle arrest) by modulating various target proteins such as $p21^{Cip1}$, $p27^{Kip}$ and GSK-3 (Chang et al., 2003). Inhibition of this pathway is a promising approach for novel chemotherapeutic agent. The present study suggested that the decrease in the amount of p-Akt after EFLS treatment (Figure 3.8) may play important roles in cell cycle arrest in both HCT-15 and RKO cells.

In conclusion, this thesis has increased the general knowledge concerning the anticancer effect of longan extracts. EFLS, which contains high phenolic and flavonoid content and has strong antioxidant activity, can inhibit cell proliferation by cell cycle arrest and led to the induction of apoptosis. EFLS-induced cell cycle arrest and apoptosis in colon cancer cell lines may be due to inactivation of Akt. The results imply that apoptosis induced by EFLS may be mediated by the Bax and Bcl-2 pathways in HCT-15 and RKO cells and then induced the effector protease, caspase-3 to finalize the apoptosis event. It is however not known in the detail of active compounds in EFLS which may have potential in cancer chemoprevention and therapy. Therefore, the active compound in EFLS should be further investigated and the detailed mechanisms of cell cycle arrest, apoptosis and other biological activities of this longan extract should be clarified.