CHAPTER V

DISCUSSION

A wide range of chemicals derived from plant and human-made xenobiotics are reported to have hormonal activity and nowadays there are increasing tendencies to use some natural products like phytoestrogens for hormone replacement therapy. *Pueraria mirifica* (PM) is commonly known in Thai as White Kwao Krua, it has been used as a rejuvenating substance in folk medicine. The enlarged underground tube accumulates phytoestrogens comprising isoflavones such as daidzin, daidzein, genistin, genistein and puerarin (reviewed by Lee et al., 2002). While many studies have been conducted on the effects of phytoestrogens on reproductive system, the study on PM is still limited. Therefore, this study aimed to evaluate the proliferative effects of ethanol-extracted *Pueraria mirifica* root in comparison to daidzein, genistein and 17β-estradiol in normal porcine endometrial cells and RL-95, the human endometrial cancer cells. To evaluate the proliferative and anti-proliferative effects in this study; an *in vitro* assay, the MTT proliferation assay was selected, which the developed color was shown to correlate with number of viable cells.

Additionally, the modulatory effects of PM on the estrogen receptor protein expression were also determined using the semi-quantitaive Western blot analysis in relating to its proliferative effects. The present results showed that PM had no proliferative effect in normal porcine endometrial and RL-95 cell. Indeed, it contained anti- proliferative effects in both cell types when cells were treated with high doses (10^{-6}-10^{-4} M) at 24, 48 and 72 hrs. This effect was likely to be more pronounced in cancer cells than normal cells as the 10^{-6} M was able to reduce cell numbers in RL-95 but not normal cells. This anti-proliferative effect was shown to be responded to PM in a dose-dependent manner at dose 10^{-6}-10^{-4} M. However, it should be noted that at the highest concentration used in this study (10^{-4} M), the anti-proliferative effect may not solely the PM effect but may be in part due to vehicle effect. The vehicle dissolving PM was distilled water, that in order to prepare a desired concentration a volume of vehicle was increased as the limited solubility of PM. Therefore, the osmotic effect had to be accounted for the reduction in cell numbers following treated with 10^{-4} M PM. Additionally, from our observation

during the protein preparation for western blot analysis, we found that although the protein concentration was unlikely to be affected but the immunoblot detecting β-actin, the internal stand was indeed lowered (data not shown).

For other phytoestrogens, daidzein and genistein, we found that both phytoestrogens had no proliferative effect in both normal and cancer endometrial cells. At high dose (10^{-4} M), both daidzein and genistein had anti-proliferative effects on both cell types. This finding was somewhat similar to previous study that the most of isoflavones such as daidzein, daidzin, genistin and genistein at low (10^{-12}-10^{-9} M) had no proliferative effect on Ishikawa cell, human endometrial cancer cells (Kayisli et al., 2002). However, Kayisli and co-workers (2002) reported that genistein at high dose (10^{-8}-10^{-6} M) can increase the endometrial cancer cell, the Ishikawa cell proliferation. Similarly, Power and Thompson (2003) also demonstrated that genistein (10^{-8}-10^{-4} M) promoted cell growth in breast cancer cells, MCF-7 cells. This difference may be explained in part by the cell specific properties, for example the difference in estrogen receptor expression or the intracellular cascades. Interestingly, in the present study, we found that daidzein was likely to inhibit cell proliferation of cancer cells better than that of normal cells; as demonstrated by that the reduction in cell numbers of RL-95 was evident as early as 24 hrs although not significant (77.79 % of control; $P>0.05$) and significant at 48 and 72 hrs (50-60 % of control). On the contrary, the effect on normal cells was significantly different at 72 hr (71.21 % of control; $P<0.05$). This finding suggested that daidzein can inhibit cancer cell growth. For genistein, the data revealed that it equally affected cancer and normal cells since there was no different in time or concentration required to inhibit cell proliferation.

Collectively, as PM used in this study contained miroestrol and puerarin as the main active ingredients (35.7 and 24.4 mg in 100 g powder, respectively) and a small amount of daidzin and genistein (12.8 and 1.4 mg in 100 g powder, respectively), it may be therefore concluded that the anti-proliferative effect of PM was the action of miroestrol and puerarin. This based on the knowledge that daidzin itself has no effect unless it is metabolically changed to daidzein by microbial, which occurred only in gastrointestinal tracts (PARK et al., 2006), and then it is not applicable in *in vitro* study. Moreover, supported further by the genistein effect in this study, the higher concentration of genistein is required in order to produce anti-proliferative effect and this was not occurred in the PM treated-cell due to limited

amount of genistein found in PM. Further supported by Chansakaow and co-workers (2000b), they also found that miroestrol, the main chemical compound in PM, has strong estrogenic effects.

The effects of estrogen (E_2) on the normal endometrial cells showed that there were effects of both concentrations and length of treatment time. At 24 hrs, cells treated with E_2 at high doses (10^{-6}-10^{-4} M) significantly decreased cell proliferation. At 48 hrs, E_2 had a biphasic action in that at the concentrations of 10^{-8} and 10^{-7} M, it inhibited cell growth, but then stimulated at 10^{-6} and 10^{-5} M, and then inhibited at 10^{-4} M. However, this effect was not seen at 72 hrs, only the anti-proliferative effect was evident when cell were treated with E_2 at the highest dose (10^{-4} M). The effect seen in normal porcine endometrial cell at 72 hrs was similar to the effect of E_2 at the highest dose (10^{-4} M) in the endometrial cancer cell, RL-95 at 48 and 72 hrs.

The differences in cell proliferation in response to each substance and cell type could be explained in part by the differences in the expression of estrogen receptor (i.e. types and ratio). As it has been shown that E_2 can regulate estrogen target genes in a cell-specific manner and it causes different effects on different types of cell in the uterus (Weihua et al., 2000). The *in vitro* studies have shown that effects of E_2 are depend on the nature of ligand and response elements on DNA which the activated receptors interact; and the estrogen receptor subtypes, ER-α and ER-β can cause opposite effects on gene transcription (Hall and McDonnell, 1999; Paech et al., 1997). It also suggested that ER-β can act as a transdominant repressor on ER-α transcriptional activity at subsaturating concentrations of E_2 (Hall and McDonnell, 1999). In addition, it is believed that the inhibitory effects of ER-β on ER-α function are due to the formation of heterodimers between ER-β and ER-α, and then in turn regulate ER functions (Cowley et al., 1997; Pettersson et al., 1997). It is then interesting to see whether the expression of estrogen receptors in this study is responsible for the different effects in regulating cell proliferation of normal and cancer cells.

The Western blot analysis revealed that there was a different in the ER receptor protein level between these two cells. In the untreated cells, both cell types expressed both estrogen receptor subtypes, ER-α and ER-β. However, the RL-95, human endometrial cancer cells expressed higher level of ER-β than ER-α ratio (16.80) whereas normal porcine endometrial cells express a lower ER-β to ER-α ratio

(0.31). The aberration of ER ratio could be accounted for the cancerous property of RL-95 as previous reports in other cancer cell types (Konduri and Schwarz, 2007; Power and Thompson, 2003). Further, the different in basal ER expressions may also responsible for the different response of cells following E_2 exposure. In addition, it is well accepted that E_2 is preferred to bind to ER-α than ER-β (reviewed by Pettersson and Gustafsson, 2001). Therefore, it may be that in the normal endometrial cell dominantly expressed ER-α, when exposed to lower concentration of E_2, it can induce cell proliferation through the effect of ER-α. On the other hand, the increasing in E_2 concentration may also bind ER-β and then inhibit cell growth causing biphasic effect seen in this study. In the RL-95, we found that E_2 (10^{-4} M) significantly decreased cell proliferation and this may be the effect of ER-β which dominantly expressed in this cells. Although we could not postulate the exact mechanism for this finding, similar result was shown previously in the breast cancer cells, MCF-7 cells. Power and Thompson (2003) demonstrated that E_2 (10^{-4} M) can significantly decrease MCF-7 cells proliferation, and likewise the ER-β expression was also higher in this cell.

Owing to high ER-β expression in RL-95 and previously reports about the binding efficiency of phytoestrogen of ER-β (reviewed by Terreaux et al., 2003), it is likely that daidzein and/or PM inhibit cell growth through the action of ER-β. Since the ratio of ER receptor has been proposed as a prognosticator of cell proliferation (Power and Thompson, 2003), we would like to see whether following exposure to differed substances would cause any effect on ER in relation to cell proliferation. The cells were then treated with selected concentrations that either affected or unaffected cell proliferation, the cells were exposed to substances for 72 hrs under the consideration that the maximal response was achieved. In the porcine endometrial cells, treated with PM (10^{-9} M) cause a reduction in ER-α and ER-β expressions with no effect on the ratio. The higher concentration of PM (10^{-6} M) had no effect on ERs level but increase the ratio of ER-β/ER-α to 1.32. At these concentrations, the PM had no effect on cell proliferation. Although we have shown earlier that at higher concentrations, the anti-proliferation was observed; however, the effect was unable to rule out from the osmotic effect, it was then excluded from further study. In the RL-95, PM (10^{-9} M) had no effect on either ERs or the ER ratio, although the ratio was decreased compared to vehicle control (3.99 vs. 16.80). This concentration of PM had no effect on cell proliferation. However, the western blot analysis revealed that ER-α expression was increased in RL-95 exposed to PM (10^{-6}

M) for 72 hrs, and the ratio of ER-β to ER-α was then decreased (1.26 vs. 16.80). This concentration was also able to decrease cell proliferation as measured with MTT assay. Notably, the ratio of 1.26 was somewhat fallen into the ratio of normal endometrial cell of 1.32. It is of interesting to see that other chemicals affecting cell proliferation would yield similar results.

Unfortunately, the results of the estrogen receptor protein expression both in normal porcine endometrial cells and RL-95 cells when treated with daidzein, genistein or E_2 cannot be used for comparison. These were caused by that upon treated with vehicles (i.e. DMSO and ethanol), the vehicles themselves significantly increased the expression of ER-β/ER-α ratio compared with H_2O vehicle in both cell types. Therefore, the relationship between cell proliferation and protein expressions cannot be done accurately.

However, phytoestrogens deserve a special mentioning when discussing ER-β-mediated cell growth phenomena. Phytoestrogens including genistein and daidzein have been shown to be ER-β-specific agonists for transcriptional activity, in contrast to for instance E_2. Genistein and daidzein were found to have the strongest ER-β binding affinity out of several plant-derived estrogens (Kuiper et al., 1998). One also needs to consider that other cancer-promoting mechanisms may also be important targets for ER-regulated activities, such as VEGF induction (Bausero et al., 2000) or coupling with EGF receptor signaling (Curtis et al., 1996).

In conclusion, the present study showed that endometrial cancer cells had a different property than that of normal endometrial cells, in that the estrogen receptor subtypes were differentially expressed. The aberrant ratio of ER-α and ER-β was likely to responsible for this cancerous property. The ethanol extracted PM was promising to be used as an alternative in clinical trial because: 1) PM had no effect on normal cell proliferation but inhibit cancer cell proliferation. 2) PM can induce/modulate ERs expression which has been proposed as a prognosticator of cell proliferation. Miroestrol, the main active ingredient with/ without puerarin was a strong candidate responsible for this effect of PM, and therefore further studies should be done.