CHAPTER 4
DISCUSSIONS

Doxorubicin and daunorubicin are widely used in clinical oncology, essentially in the treatment of acute leukemia, Hodgkin's or non-Hodgkin's lymphomas, and also in some solid cancers (breast, ovarian, or endometrial cancers). Cytotoxicity mediated by doxorubicin and daunorubicin is generally thought to be the result of drug-induced damage to DNA via quinone-generated redox activity, intercalation-induced distortion of the double helix, or stabilization of the cleavage complex formed between DNA and topoisomerase II . Up to date, more than 4,000 molecules derivatives of doxorubicin and daunorubicin were synthesized and studied for their anticancer activity. Among these anthracycline derivatives, pirarubicin, a doxorubicin derivative was reported to be a candidate as anticancer drug (Monneret, 2001). In this study, the efficacy of doxorubicin, daunorubicin and pirarubicin to inhibit erythomyelogeneous leukemic K562 and K562/adr cell growth was investigated. The three anthracyclines possess variation in molecular parameters as indicated in table 1-2. The doxorubicin and daunorubicin have the same amino sugar with pKa ($-N^+ H_3/NH_2$) equal to 8.4 at 37 C^o but have a very different lipophilicity (log P). Under the similar experimental condition, the same percentage of neutral and positively charged form will be found. Using daunorubicin as reference, log P of doxorubicin, pirarubicin can be empirically calculated which is equal to -1.67 and -1.06, respectively. There is the fact that an efficacy of a drug depends on its concentration at intracellular targets. This is governed by parameters of drug transports into cells which are predominantly influenced by log P. These include the mean influx coefficient (k_+), the mean efflux coefficient and the affinity to its intracellular targets. By using the same cell line; K562 and K562/adr cell; the mean influx coefficients (k_+) were previously reported which were 0.1×10^{-10}, 2×10^{-10} and 35×10^{-10} s^{-1} for doxorubicin, daunorubicin and pirarubicin, respectively (Mankhetkorn, 1996, 1998).

For K562, non-P-gp overexpressing cells; at steady-state, the free cytosolic concentration of the three anthracyclines should be similarly found due to the absence of P-gp pump. This is strongly confirmed by the same efficacy of K562 cells; IC_{50} are 23.0 ± 2.0, 23.5 ± 2.5 and 22.5 ± 2.0 nM for doxorubicin, daunorubicin and pirarubicin, respectively. It should be noted that, in drug-sensitive cells, the molecular parameters of drugs does not significantly play an important role on the cytotoxicity. By contrast, the three anthracyclines, by using MDR, K562/adr cells, show a lesser degree of cytotoxicity than that of K562 cells with the RF values of 7, 10 and 15 for pirarubicin, daunorubicin and doxorubicin, respectively. The different efficacies among these drugs should be associated to an overexpression of P-gp. The protein membrane P-gp is responsible for pumping intracellular drugs out of the cell, resulting in lowering free cytosolic drug concentration, in particular at intracellular drug targets. This inevitably leads to a decrease of the drug efficacy.

It has been well established that some classes of small molecules such as verapamil, a Ca^{2+}-channel blocker could inhibit the function of P-gp as well as restore the intracellular concentration of anthracycline and enhance its cytotoxicity (Mankhetkorn, Teodori, & Garnier-Suillerot, 2001). This study showed that verapamil inhibited the function of P-gp and enhanced the cytotoxicity induced by doxorubicin, daunorubicin and pirarubicin; with 2 μM verapamil by 80%, 50% and 60%, respectively. Unfortunately, verapamil is very high cardio toxicity which decreases in potential use at clinical level. One of the strategies to overcome MDR phenomena is to find molecules which can inhibit the function of P-gp without any serious side effects. Recently, plant polyphenols are renewed of interest as P-gp inhibitors. Many research groups reported that bioflavonoid such as genistein (Castro et al., 1997) kaempferol and flavopiridol (Hooijberg et al., 1997) inhibited the function of P-gp.

For many years, we (PCMCB team) for the purpose of reversing MDR phenotype have researched molecules originally found in Thai plants that possess such an activity against human cancer cell lines. Our research group has purified and studied the bioactivities of mamoa (*Antidesma thwaitesianum. Muell.Arg*) extracts such as antioxidant and anticancer activities. Mamoa crude extract was fractionated using low pressure column chromatography. It is composed of 15 fractions. The

purity of the fractions was checked by using TLC prior to further analysis by using mass spectrometer as indicated in figure 3-2. The chemical compositions of *Antidesma* wood including *Antidesma membranaceum* (Buske et al., 1997), *Antidesma euphorbiaceae* (Bringmann et al., 2000) were reported; the abundant molecules were found to be antidesmones. All constituents include crude extract were tested for their cytotoxicity against K562 and K562/adr cells. Crude extract was more cytotoxic than pure compounds (1 to 15). Moreover, the crude extract affected the experimental models almost similar to flavonoids in K562, K562/adr cell lines. It is the first time by this study demonstrating that antidesmone, subgroups of polyphenols isolated from *Antidesma thwaitesianum Muell.Arg* or Mamoa (a plant used in Thai traditional medicine) potentially exhibited cytotoxicity in cancer K562 and its MDR cell line.

It is clearly shown that compounds 4, 5, 6, 7, and 8 (their chemical structures shown in figure 3-3) inhibited P-gp-mediated efflux of pirarubicin from K562/adr cells. The concentrations of compound 4, 5, 6, 7 and 8 required to inhibit by 50% ($\alpha_{0.5}$) the function of P-gp-mediated is shown in table 3-3. The direct interaction of compound 4, 5, 6, 7, and 8 with P-gp in living cells using photolabeling technique was performed by using EDP 86. It was demonstrated that it is possible to photolabel verapamil on P-gp in living MDR cells without a deleterious effect using its photoactivable analogs such as EDP82, 86, 87 and 88 (Mankhetkorn et al., 1999). The ratio of mean P-gp-mediated efflux pirarubicin after photolabeling with (k_a^*) and without (k_a^0) the verapamil analog was studied by the authors. In fact, the ratio of k_a^*/k_a^0 is a very good indicator for monitoring the direct interaction of molecules with P-gp, in terms of protection photolabeling of these photoactivable verapamil analogs on P-gp. The results of this study showed that the maximum photolabeling were obtained with 5 μM EDP 86 yielded k_a^*/k_a^0 about 0.8-0.9, that signifies 10 - 20 % of remain P-gp function. The specific fixation of EDP 86 was checked by photo-irradiated MDR cells in the presence of 5 μM EDP 86 and 5 μM verapamil, yields an increase in remain P-gp function by 45%. All studied compounds can protect the fixation of EDP 86 on P-gp at the very high concentration compared with quercetin, apigenin, kaempferol and eriodictyol.

These let us hypothesize that the compounds should probably enhance cytotoxicity induced by anthracyclines in the combined treatment using anthracycline and the compounds. Co-treatment using pirarubicin and daunorubicin with the compounds 4, 5, 6, 7 and 8 were performed. All compounds did not affect IC_{50} of pirarubicin and daunorubicin in K562/adr cells. This is probable that the compounds 4, 5, 6, 7 and 8 inhibit P-gp function only in the short term but do not restore intracellular anthracycline concentration in the long term. However, the mechanism (s) of molecules to inhibit cell growth and MDR reversing action should be studied in more details.

Overall results demonstrated that *Antidesma* tree is composed of various compounds which possess pharmaceutical interests such as anticancer especially for overcoming MDR phenotype and antioxidants.