CHAPTER IV
DISCUSSION AND CONCLUSION

Development of multidrug resistance *MDR* is the main reasons for weak responses in tumor chemotherapeutic treatment. Combination chemotherapy was developed in order to circumvent specific resistance mechanisms, as well as to allow higher overall doses of anti-tumor chemicals by combining agents with different side-effects/organ toxicity. One of the mechanism for the development of multidrug resistance was the increased overexpression of the membrane drug efflux pump (ATP-dependent drug efflux pump) encoded by *mdr1* gene. This protein involved in decreasing intracellular drug accumulation and the therapeutic effects of many chemotherapeutic agents in various *MDR* tumor cell lines.

So far, the screening for P-gp modulators use existing pharmacological agents. As a result, most of these agents have strong pharmacological actions distinct from their activity on the efflux pump. In many cases these actions reduce the overall dose or the duration of that dose which can be given without any significant side effects. The two most commonly used modulators, verapamil and cyclosporin A generally cause the side effects of cardiac abnormalities, circulatory problems, constipation, headache and potent immunosupression, nephrotoxicity, musculoskeletal pain, nausea and vomiting, respectively [57]

The ideal modulators should inhibit the resistance mechanism at a low, and readily physiologically achievable concentration. It should also display certain level of selectivity for cancerous tissue over normal tissue to maintain the protective mechanisms in non-cancerous cells while producing a more toxic effect in the tumor. Therefore, the effects of many plant-derived compounds on P-gp were investigated because plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. Many reports have investigated the effect of several natural products or other phytochemicals from plants which may inhibit Pgp function and reverse *MDR* in cancer prevention and treatment [88,95-97].

The extracts of bitter melon, have been previously reported to have a potent cancer chemopreventive activity [78], the bitter melon extracts exhibited an antimutagenic in animal model studies [98] and non-toxic [77]. The investigation of bitter melon extracts on P-gp function was carried out because of its chemopreventive and non toxic properties. The result obtained from the experiments herein confirmed the inhibition of P-gp mediated drug efflux, that lead to an increase in the intracellular accumulation and cytotoxicity of chemotherapeutic drugs in drug-resistant human cervical carcinoma cell lines.

The experiments were carried out with a vinblastine-resistant KB-V-1 cell line, which express high levels of P-gp on their plasma membrane, compared to drug sensitive KB-3-1 cell line. KB-V-1 cells were selected by subjecting KB-3-1 cells in a step-wise manner of increasing vinblastine concentration [99]. The level of P-gp in drug resistance KB-V-1 cell membranes is about 1% of total plasma membrane protein, while it was not found in the parental drug sensitive KB-3-1 cells [100].

The study of P-gp expression in KB-3-1 and KB-V-1 cell lines by Western blot analysis showed that immunoblottable amount of P-gp significantly expressed in KB-V-1 cells that were maintained in both 0.5 and 1 μg/ml vinblastine. The expression level of P-gp correlated well with the elevated concentration of drug. KB-3-1 cells did not express P-gp at a level detectable by the method used in this experiment (Figure 10). As KB-V-1 cells, which maintained in 0.5 μg/ml vinblastine, have been extensively characterized by MDR phenomenon as well as the function of P-gp. Throughout the study, KB-V-1 and KB-3-1 were used to assess the effect of bitter melon extracts on the function of P-gp.

Effect of bitter melon extracts on MDR phenotype

The effect of bitter melon extracts on MDR phenotype was determined by MTT assay. Non cytotoxicity doses (≥ 80 % survival) of the extracts are used in this experiment. Whole plant extract (15 and 25 μg/ml) significantly increased cytotoxicity of vinblastine in KB-V-1 cell line ($p < 0.05$), while the leaf extract worked significantly at higher concentration (75 μg/ml, see Table 25). The control

KB-3-1 cells which lack P-gp had no changes in MDR phenotype after treating the cells with the whole plant or leaf extracts.

Unlike the leaf, the fruit and tendril extracts have no MDR reversing properties. Combination of active ingredient(s) in whole plant extract may be responsible for synergetic effects on P-gp modulator. This result also indicated that the active compounds that act as P-gp modulation are mainly found in the leaves of bitter melon.

Effect of bitter melon extracts on P-gp-mediated drugs transport

P-gp activity was determined in term of drug accumulation and drug efflux by using drug resistance KB-V-1 and drug sensitive KB-3-1 cells. Whole part and leave extracts (25 to 100 μg/ml) significantly increased the accumulation of ^{3}[H]-vinblastine in KB-V-1 cells in a dose-dependent manner. The accumulation of ^{3}[H]-vinblastine did not change in the presence of fruit and tendril extracts. The effect of whole plant and leaf extracts on ^{3}[H]-vinblastine efflux was evaluated. Both whole plant and leaf extracts at concentration between 25 to 100 μg/ml caused a decrease in the amount of ^{3}[H]-vinblastine efflux in a dose-dependent manner in drug resistance KB-V-1 cell.

It has been suggested that drug efflux from cells is mediated by the activity of transport protein (P-gp) directly and was also the central to the active pump model for study P-gp transporters [31,101,102]. In order to compare the effect of whole plant and leaf extracts on ^{3}[H]-vinblastine efflux in KB-V-1 cells, the cells were treated with 50 and 100 μg/ml of whole plant or leaf extracts for 30 min in an efflux period. The leaf extract was found to decrease ^{3}[H]-vinblastine efflux more strongly than the whole plant extract at the same concentration. Again, this finding confirmed the presence of active P-gp modulator(s) in the leaves of bitter melon.

Both whole plant and leaf extracts had modulating effect on drug accumulation and efflux in KB-V-1 cells but not in KB-3-1 cells. It may be due to the differences of P-gp efflux pump in both cell lines. KB-V-1 cells showed the overexpression of MDR-1 gene, which is known as a major factor in changing intracellular drug concentration. It also showed a high resistance to vinblastine when compared to KB-3-1 cells. Most of the drugs enter the cells by passive diffusion

through the plasma membrane, cell changes in drug efflux ability can be related to the changes in the transmembrane transport across a P-gp pump revealing the differences between some drug sensitive (lack of P-gp pump) and drug resistance cells (high P-gp level) [31].

Ikegawa *et.al.*, 2002 found that flavonoid derivatives increased the uptake of [^{3}H]-vincristine by human myelogenous leukemia (K562) cells and adriamycin-resistant human myelogenous leukemia (K562/ADM) cells. They thought that these flavonoid derivatives possess antitumor promoter activity and also may become candidates of effective multidrug resistance-reversing agents in cancer chemotherapy [103]. The increase of intracellular ^{3}[H]-vinblastine accumulation in KB-3-1 at high dose of whole plant extract of bitter melon can be the mechanism of antitumor property activity of bitter melon and on the other hand its can show the MDR reversing agents in KB-V-1 cells.

Phytochemical test of bitter melon extracts

In order to identify and classify the phytochemical compounds in the bitter melon extracts, phytochemical tests were implemented for this study. The extracts from whole plants, leaves, fruits and tendrils of bitter melon were tested for anthraquinone glycosides, cardenolides, saponins, alkaloids, tannins, polyphenols and flavonoids. No anthraquinone glycosides, cardenolides, tannins and polyphenols was found in all parts of bitter melon extracts but alkaloids and saponins were found in whole plant, leaf and fruit extracts. Octacosane, 1-triacontanol, 7-stigmasten-3β-ol, 7,25-stigmastadien-3β-ol, 5,25-stigmastadien-3β-ol glucoside, phytosphingosine, momordicine I, II, III were isolated and identified in leaves of bitter melon [69]. The alkaloids and saponins found in bitter melon could be the posible candidate of MDR reversing agent or these compounds may interrupt the P-gp activity and pure compounds should be tested in KB-V-1 cells in comparison to KB-3-1 cells.

Among the flavonoids tested, flavanone and flavonol were found in the extracts and the positive test of aurone, chalcone, flavone and xanthone were found in only in whole plant and leaf extracts of bitter melon. The bioflavonoids especially flavones, flavonols and chalcones have been found to be an effective P-gp inhibitor

[103]. This result implies that the active compound(s) that cause the decrease in P-gp activity may be the flavonoids; aurone, chalcone, flavone and xanthone.

Possible mechanism of P-gp modulation by bitter melon

As shown in this study, the whole plant and leaf extracts increased the intracellular concentration of vinbltastine, probably through its interaction with P-gp and from phytochemical study some alkaloids or flavonoids may play an important role on modulation of P-gp activity. In previous study, plant alkaloids are used in both modern and traditional medicine, e.g. vinblastine, vincristine and taxol are prescribed as anticancer drugs and bisbenzylisoquinoline alkaloid that isolate from natural plant (berberine), can reverse multidrug resistance by increasing the intracellular drug accumulation through inhibiting the activity of P-gp [104,105].

Recently flavonoids and their derivative compounds from fruits and vegetables have been reported to have anticarcinogenetic properties. These compounds could stimulate the P-gp mediated efflux of 7,12-dimethylbenzanthracene (DMBA) in MDR human breast cancer MCF-7 cells [106]. On the other hand Mitsunaga *et al.*, 2000 found that aglycones, chrysin, flavone, hesperetin and naringenin significantly increased the uptake of ^{3}[H]-vincristine. These findings indicate that the aglycone-type bioflavonoids modify the blood–brain barrier transport of ^{3}[H]-vincristine. It was speculated that the increased uptake of ^{3}[H]-vincristine was due to the inhibition of P-gp by high concentrations of bioflavonoids rather than glycosides (hesperidin, naringin and rutin) [107].

Until now, Ikegawa *et al.*, 2000 have been searching for novel P-gp inhibitors from daily foods and beverages, and found that methoxyflavones such as nobiletin, tangeretin and 3,3',4',5,6,7,8-heptamethoxyflavone from grapefruit juice and orange juice can increase the intracellular accumulation of vincristine in adriamycin-resistant human myelogenous leukemia (K562/ADM) cells [108]. However, it is still unclear whether these flavonoid derivatives inhibit P-gp function. If so, the dual effects (i.e. P-gp inhibition and antitumor promoter effect) of these flavonoid derivatives may act synergistically in cancer chemotherapy.

In previous study using a purified recombinant protein corresponding to a cytosolic nucleotide-binding domain of P-gp, Boumendjel *et al.*, 2001 found that flavonoids were efficient P-gp inhibitors, by binding to cytosolic sites which partly overlapped the ATP-binding site and the modulators interacting region. From the studies of structure–activity relationships, flavones are more efficient than isoflavones, while flavonols and chalcones are even more active. Moreover, it was found that the hydroxyl groups at positions 3 and 5 in flavanone and flavonol are essential for high-affinity binding to P-gp in multidrug resistance cell lines. In a recent study conducted with chalcones, the presence of a hydrophobic substituent at C_4 on the B-ring (Figure 22) considerably enhanced the binding affinity with P-gp [109,110].

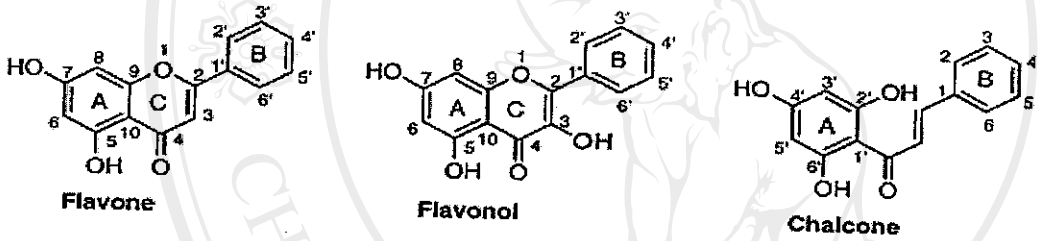

Figure 22. Chemical structure of flavone, flavonol and chalcone

From their studies of *Momordica charantia* Linn., Scartezzini and Speroni., 2000 reported the principal compounds from leaves as shown in Table 10. The phytochemical composition of the plant confirmed to potential use in traditional medicine

Octacosane ($C_{28}H_{58}$, plant wax alkanediols), one of the component in bitter melon leaves, acts as a marker for the determination of diet intake, food selection and digestibility of protein [111]. 1-triacontanol ($C_{30}H_{61}OH$) a natural long chain primary alcohol, constituent of cuticular wax has been known to be a potent plant growth promoting substance [112]. Phytosphingosines ($C_{18}H_{39}NO_3$) are the building blocks of sphingolipids which are the important membrane constituents, the role of sphingosine derivatives in human cells have been essential in cell communications and regulation of cell growth [113]. Lastly, momordicine, a triterpene glucoside is a major antifeedant in bitter melon ground leaves [70,71].

This study suggested that the active compound(s) in whole plant and leaf extracts may interfere the ATPase activity or interact with the drug binding site of P-gp molecule that could lead to an increased drug retention in KB-V-1 drug resistance cells. Thus, further investigation on the possible mechanism of the active compound(s) present in the leaves extract are photoaffinity labelling of P-gp with ^{3}[H]-azidopine to describe whether the compound(s) can bind with drug binding site of P-gp molecule.

However, the modulating effects of these active compound(s) in bitter melon extracts on P-gp molecule have not yet been determined. Additional detailed work is needed to further understand how the compound(s) react directly or indirectly on the P-gp molecule. The isolation, purification and identification of active component(s) in bitter melon leaves extract will help to clarify the P-gp inhibition and its mechanism of action. Besides being easy to get, the plant extract is relatively nontoxic and inexpensive.

Although the result of this study has pointed out that the extract from bitter melon leaves is an effective inhibitor of P-gp activity *in vitro*. More investigation is needed to confirm that bitter melon extract has potential as an effective and safe 'chemosensitizer' for treating cancers expressing P-gp mediated MDR in animal model. In conclusion, this work suggestes that some component(s) of the extracts are useful biochemical modulator and may elevate P-gp efficiency in this drug resistance model.