CHAPTER V

DISCUSSION AND CONCLUSION

This study intended to investigate the potential cytotoxic effect on cancer cells of compounds from *Micromelum hirsutum*. Active compounds were extracted from the branches and the leaves of this plant by methanol (BM and LM), dichloromethane (BD and BH) and hexane (BH and LH), respectively. Both methanol extracts did not contain active compounds that have cytotoxic effect on Ramos cells. They did not change cell viability of the cancer cells by resazurin assay. BD, BH, LD and LH were cytotoxic to Ramos cells in a concentration dependent manner with their IC50 values 41.88, 84.27, 11.71 and 50.94 µg/ml, respectively. These differences in the IC50 values may imply that each extract contains difference active compounds that exhibit cytotoxic effect to the cancer cells. It is interesting that these extract have significantly higher toxicities at the different degree on Ramos cells than on normal cells like human PBMCs in this study. The results make these extract to be the good candidates for anticancer agents.

When the four extracts were investigated for Ramos cell apoptotic induction activities, all of them induced Ramos cell death mainly by apoptosis after 12 h of exposure. BD and BH induced total Ramos cell death and Ramos cell apoptosis in a concentration dependent manner at 12 h of exposure. Etoposide which was used as the positive control in this study induced cell death mainly by late apoptosis plus necrosis after 12 h of treatment. However, LD and LH induced total cell death and apoptosis in a concentration-independent pattern but in a time-dependent fashion. The concentration independent apoptotic effects of LD (6.25-25 µg/ml) and LH (25-100 µg/ml) are the drawback for their potential anticancer candidates. These extract were not further investigated in this study.

The degree of caspase dependence is varying among apoptotic stimuli **[110-112]**. Both BD and BH induced Ramos cell death almost completely dependent on caspase activation. Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK) is a potent, cell permeable pan caspase inhibitor. It irreversibly binds to the catalytic site of several caspases and inhibits the enzyme activities. Z-VAD-FMK almost completely inhibited apoptotic effects of BD and BH at all concentrations of the extracts used in the study. This pan caspase inhibitor cannot be used to identify whether it was the intrinsic or the extrinsic pathway that involved in the caspase activation by the extracts.

The caspase activation of the extracts possibly did not happen by the extrinsic pathway of apoptosis. Inhibition of Fas-Fas ligand interaction by using an anti-Fas ligand antibody did not inhibit apoptotic effects of the extracts. Many clinically used anticancer drugs mainly induce cancer cell apoptosis via the intrinsic or mitochondria dependent pathway **[112]**. The mitochondrial pathway of apoptosis is regulated by proteins in the Bcl-2 family. These proteins are classified as anti-apoptotic Bcl-2 proteins, e.g., Bcl-2 and Bcl-XL, which prevent apoptosis and pro-apoptotic Bcl-2 proteins, e.g., Bax and Bak, which promote apoptosis. Anti-apoptotic proteins play role on preserving the membrane integrity of the mitochondria to prevent the release of several apoptotic promoting factors such as cytochrome c from these organelles. Pro-apoptotic Bcl-2 proteins, which are selectively expressed in abundance during apoptotic process, induce the release of these apoptotic promoting factors from the mitochondria. The expressions of these Bcl-2 proteins are partly regulated by p53 protein. This study demonstrated that BD and BH induced Ramos cell apoptosis via the mitochondrial/ intrinsic pathway. BD significantly decreased the mRNA expression of anti-apoptotic Bcl-2 and Bcl-XL and increased the mRNA expression of pro-apoptotic Bax. BH also demonstrated similar effects on the mRNA expression of these Bcl-2 proteins but the

results were not statistically significant. Bcl-2 forms heterodimer with BAX in different molar ratio. This ratio is critical for cells in several tissues to undergo or evade from apoptotic process **[113]**. A high Bcl-2 to Bax ratio favors cell survival while a low ratio provokes apoptosis. Down-regulation of Bcl-2 expression may lead to a decrease in Bcl-2 to Bax ratio and increase free BAX. These free BAX molecules can translocate to mitochondrial membrane to form homodimer Bax to trigger the caspase activation by alteration of mitochondrial membrane permeability. This results in the release of apoptosis promoting factors into the cytoplasm. It has have shown that each cell type is protected from apoptosis by at least one member of anti-apoptotic Bcl-2 proteins. It was suggested that Bcl-XL may play a more important role than BCL-2 in protecting B-cells from apoptosis. The overexpression of Bcl-XL in immature mouse B-cells, WEH1-231, protects these cells from apoptosis induced by many stimuli **[114-115]**. It cannot rule out by these studies that BD may have effect on either translational or post-translational levels. It is known that the activity of Bcl-2 family proteins is controlled at transcriptional, translational and post-translational levels. Their protein levels and the protein–protein interactions among these proteins also play critical roles in controlling their activity. It has been reported that the phosphorylation of Bcl-XL might inhibit its anti-apoptotic function **[116]**. The effect of BD and BH on proteins levels and phosphorylation of Bcl-2 family members is needed to be elucidated. The p53 is a tumor suppressor protein that is activated by DNA damage, oxidative stress, cellular calcium overload and other cellular stresses. It promotes apoptosis through the direct activation of pro-apoptotic Bax and the BH3-only proteins PUMA and Noxa gene expression. BD and BH had no effect on the mRNA expressions of p53.

Anticancer drugs are classified as cell cycle specific and cell cycle nonspecific agents. The cell cycle specific drugs act on selective phase of the cell cycle. They commonly act on S phase by inhibiting DNA synthesis and M phase by inhibit mitotic

spindle polymerization or depolymerization. These drugs can change the pattern of the cell cycle in vitro. An example of the cell cycle specific drugs is etoposide which inhibits topoisomerase II and arrests the cell cycle at late S and G2 phases in vitro. It causes the cell accumulation in the G2/M phase. The cell cycle nonspecific drugs act independently on any phase of the cell cycle. These drugs do not change the pattern of the cell cycle in vitro. The effects of BD and BH on the cell cycle were also investigated in this study. BD may contain cytotoxic compounds that act as cell cycle specific agent. This extract changed the pattern of the cell cycle. The BD-treated Ramos cells accumulated at S and G2/M phases. It has been demonstrated that that carbazole alkaloids and coumarins which are bioactive constituents of plants in genus *Micromelum* **[93, 90]** exerted their cytotoxic effects by disturbing cell cycle in human lung carcinoma cell lines **[8]**. BH did not change the cell cycle pattern of Ramos cells. This extract may have different cytotoxc compounds from BD.

Since BD had effect on the cell cycle pattern, its effects on the mRNA expression of cyclins and cyclin-dependent kinase inhibitors (CKIs) that regulate the cell cycle were also investigated. BD profoundly reduced the mRNA expression of cyclin D1 in Ramos cells when compared to the untreated Ramos cells. Cyclin-D1 is known to activate cyclin-dependent kinases (Cdk4 and Cdk6) that regulate early G1 phase **[117]**. BD slightly decreased the mRNA expression of cyclin E which activates Cdk2 that triggers the S phases. It did not have effect on cyclin A which activates Cdk1 and Cdk2 to complete the S phase. It also had no effect on cyclin B which activates Cdk1 which is responsible for mitosis. It is possible that BD may induce Ramos cell cycle arrest at G1 phase which positively regulated by cyclin D-CDK4 or cyclin D-Cdk6 as well as cyclin E-Cdk2. However, BD also reduced the expression of p21 and p53 which negatively regulate the cell cycle. P21 is a cyclin-dependent kinase inhibitor (CKI) that negatively controls the G1/S transition by tightly binding and inhibiting cyclin D-CDK4, cyclin E-CDK2, and cyclin A-CDK2 complexes **[118]**, which regulates in G1 phase , G1/S phase transition and S phase of the cell cycle, respectively **[119]**. P21 is also positively

regulated by p53 which is an essential transcription factor for cell cycle arrest. The decrease of p53 expression correlated to p21 expression. It has been reported that agents that inhibit G1 phase can down regulate cyclin D1 by up regulating both p21 and p53 expression. **[120]** It is possible that the down regulation of both p53 and p21 expression may be the consequence of the decrease of cyclin D1 and cyclin E expression. The effect of BD on the expression of p21 and p53 should be further investigated to explain the contradiction outcome in this study.

In conclusion, the results suggest that *Micromelum hirsutum* contain active compounds that exhibit cytotoxic effect against Ramos cells with much less cytotoxicity to normal human PBMCs, The dichloromethane and the hexane extracts from the branches and the leaved of this plant induce cancer cell death mainly by apoptosis which is physiologically harmless to neighbor cells. The dichloromethane and the hexane extract from the branches of *M. hirsutum* induced caspase-dependent cancer cell apoptosis via the intrinsic pathway by down regulate the expression of anti-apoptotic Bcl-2 and Bcl-XL and up regulate the expression of pro-apoptotic Bax. These extracts had different effect on the cell cycle. The dichloromethane extract may arrest the cell cycle of Ramos cells at G1 phase while the hexane extract did not arrest the cell cycle.

The results from these studies suggest that BD and BH may be developed as an agent for the management of lymphoma cancer. Especially, BD suggested two interesting characters of chemotherapeutic agent which including apoptotic induction and disturbing cell cycle. Eventhough, more in depth mechanism involved the cell cycle arrest in BD-treated Ramos cells are also need to elucidate for exactly explain their effect on cell cycle arresting. In addition, further studies are needed to explore the above mentioned possibilities.

Conclusion flow chart