5.0 Discussion

5.1 Plant sample extraction

In sample extraction, distilled water was selected over organic solvent in order to simulate the traditional method of preparation. This involved boiling of *F. deltoidea* in water for several hours before being consumed. The consumption of this plant as decoction suggested that the active components originated from water extract.

The primary aim of this work was to determine the presence of bioactive peptides with anticancer activity against several cancer cell lines. Ammonium sulfate precipitation was the method of choice for fractionation and precipitation of proteins and/or peptides present in the crude extract. This method was used due to its ability to conserve the structure of precipitated proteins. Moreover, it also has the following advantages: low heat solubilization, low density of saturated solution, prevents protein denaturation and microbial growth and it is a cost effective method (Protein purification handbook).

The precipitated samples were pooled at 30, 60 and 90% saturation based on the protein concentration curve. Different fractions might give rise to different types of proteins or peptides as ammonium sulfate precipitation precipitate the proteins and peptides based on their hydrophilicity. The protein which was higher in their hydrophilicity needed higher percentage of ammonium sulfate in order to be precipitated. Most of the proteins in leaves are hydrophobic as they were precipitated at the lower percentage of ammonium sulfate. For fruits, the graph showed continuous ascending trends indicating that they contained proteins that were more hydrophilic compared to the leaves. The protein concentration curve indicated that SF has less proteins compared to BF.

5.2 Preliminary antioxidative studies

In total phenolic content assay, the total phenolic content of the semi-purified fractions showed a similar trend as compared to the protein content. This may be due to the presence of polar and aromatic amino acids. Lysine consists of hydroxyl group while tyrosine possesses phenolic ring which has the ability to reduce the Folin-Ciocalteu reagent.

DPPH results elucidated that the sample with lower IC_{50} value has better radical scavenging ability. Apart from SF semi purified fractions, all of the IC_{50} value for extracts and fractions were obtained. Ascorbic acid, the positive control, has the lowest IC_{50} value compared to *F. deltoidea's* samples. High IC_{50} value might be due to the breakdown or decomposition of the antioxidants in the crude extract following boiling procedure in the sample preparation. Besides, antioxidants were more organic solvent like due to its polarity. This results in lower antioxidant concentrations in the water extracts. It was also observed that the crude samples have lower IC_{50} value compared to the semi-purified fractions. The fractions which underwent ammonium sulfate precipitation were mostly proteins and less likely to have antioxidative compounds. This might suggest that unlike secondary metabolites; proteins do not have extra electrons that could be donated and stabilized the DPPH radicals.

In lipid peroxidation assay, only five samples responded towards the inhibition of lipid peroxidation. However, these samples were not considered as good candidates due to their relatively high IC_{50} values (> 8 mg/ml) compared to ascorbic acid (1.6 ± 0.01 mg/ml). This result suggested that the samples might not be active in the binding of free ions present. In order to inhibit lipid peroxidation, it is crucial to have a compound which is capable of binding the extra ions (iron or copper) present in the environment.

This might be the result of the boiling step performed during sample extraction. At high temperature, most of the antioxidant which has the said ability could be destroyed through the process.

The initial results showed that high total phenolic content helps in the scavenging of free radicals in DPPH assay. However, this phenomenon was not observed in lipid peroxidation assay. High total phenolic content gave a low IC_{50} value in DPPH assay but not in lipid peroxidation assay. This suggested the specificity of the samples towards different assays or antioxidative pathways or mechanisms.

5.3 MTT assay

The cytotoxicity of plant extracts towards different cell lines were tested on two cancerous and one normal cell lines through MTT assay. The cell lines selected were human cervical carcinoma cell line (Ca Ski), human hepatocellular liver carcinoma cell line (Hep G2) and human liver cell line (Chang Liver). All of the cell lines were epidermal cells. Doxorubicin was chosen as the positive control as it was a known cancer drug and most importantly doxorubicin dissolved easily in water. Prior to the addition of MTT solution to the treated cells, the media and samples were removed to avoid contact between MTT and samples. The presence of reducing agent (free thiol group) in the samples will reduce MTT even without the presence of any living cells (Peng *et al.*, 2005; Shoemaker *et al.*, 2004) giving a false negative result.

F. deltoidea leaves and BF samples do not show any cytotoxic effects even in a concentration of 0.5 mg/ml gave us an indication that the samples were safe to be consumed. Only SF fractions showed IC_{50} values in the assay. Even though the IC_{50} values for SF semi-purified fractions were much higher compared to positive control,

the results were still acceptable. This could be explained by the complexity level of the two samples. Doxorubicin was a single compound while semi-purified fractions were a mixture of a few components. Furthermore, the semi purified fractions contains mostly protein and protein bound compounds. Considering that they normally possess higher molecular mass compared to doxorubicin; there will be significant difference in the number of molecules for a particular mass. This will contribute to the apparently higher IC_{50} value which is expressed in µg/ml. Further studies need to be conducted to evaluate the suitability of SF fractions as potential anticancer agent by further purification of the samples.

5.4 Endogenous antioxidants

Endogenous antioxidants, catalase, glutathione peroxidase and superoxide dismutase were tested with different concentration of treatments. Unlike catalase, glutathione peroxidase activities and SOD inhibition levels were generally decreased upon treatment. On the other hand, treatment with doxorubicin decreased all the three endogenous enzymes in Hep G2 and Chang Liver cells but not in Ca Ski cells.

Catalase concentrations were not significantly changed upon treatments in Hep G2 and Ca Ski cells but the catalase levels were shown to be higher compared to Chang Liver cells. Chang Liver cells has a catalase level which is less than 0.25 µg/ml upon treatment compared to the other two cell lines. This phenomenon occurred as Chang Liver cells were considered as normal cell. Normal cells usually do not produce stress-related compound. Therefore, catalase levels were low compared to cancerous cells. Apart from the difference of the cell lines, the difference of catalase concentration might be due to the complexity of the samples. In the crude and semi-purified samples, there were more than one type of compound or molecule present. This might give a

different effect towards the cells upon treatment as some of the compounds in the samples might contribute to the increase of the catalase concentration. Besides that, high endogenous oxidative stress will lower sensitivity of cells towards treatments (Glorieux *et al.*, 2011; Yamamoto *et al.*, 2003). Thus, even though it was shown to inhibit the growth of cancer cells, the amount needed were high in SF samples.

The changes of GPx and SOD activities might due to the reasons similar as catalase in which the purity of the samples might affect the results of antioxidative enzymes. Apart from that, antioxidative enzymes were important in the promotion of growth and survival of cells. In the research done by Nakano *et al.* showed that Mn-SOD protects cells against radiation effects in treatments (Nakano *et al.*, 1996). Thus, lower level of SOD suggested that decrease of GPx activity and SOD level in the cancer cells following treatment suggested an inhibition or halting of cell proliferation. The decrease of GPx and SOD activities in Hep G2 cells suggested that SF treatments have the potential to inhibit the cancer cell growth. However in Ca Ski cells, only SF30 and SF60 treatments lowered the SOD levels.

5.5 DNA fragmentation

DNA fragmentation into oligonucleosomes with intervals of 180-200bp is a characteristic phenomenon in apoptotic cell death. This is due to the presence of DNA fragmentation factor (DFF) which acts as the apoptotic endonuclease. During apoptosis, DFF45 which act as the inhibitor of DFF40 will be dissociated from the DFF by the cleavage of activated caspase 3. Free or activated DFF40 which possess intrinsic nuclease activity will be able to cleave chromosomal DNA at the internucleosomal site (Zhang and Ming, 2000).

The DNA fragmentation results indicated that SF crude and fractions might caused cell death via apoptosis in Hep G2 and Ca Ski cells. It was affected by the concentration of the treatment given. Comparison between crude and semi-purified fractions demonstrated that lower concentration of semi-purified fractions gave more significant change as compared to crude sample. This might be due to the components present in the crude sample and semi-purified fractions. At the same concentration, the amounts of active components in semi-purified samples were higher compared to crude samples. On the other hand, doxorubicin treatment was shown to have the strongest ability to fragment DNA compared to other samples. Besides, Chang Liver cells showed little or no DNA fragmentation upon SF samples treatments. This indicates that the treatments do not caused much effect towards normal cells.

5.6 Gel Image Analysis

After the gels were analyzed with ImageMaster Platinum version 7.0 software, spots with expressions that were deregulated by more than two folds and with a $p<0.001$ were considered as significant. Doxorubicin which served as the positive control gave the greatest changes following treatments. Among the three SF semi-purified samples, SF60 demonstrated the most change in all cell lines. While for SF30 and SF90, the results were more prominent in HepG2 cells but not in Ca Ski and Chang Liver cells. Upon treatment with the semi-purified fractions, the deregulated proteins were highly different as compared to doxorubicin. Identification of the deregulated proteins might give us an idea regarding the changes in the cellular system.

5.7 Identification of protein by MALDI and validation by RT-PCR

Based on the identified proteins, it was shown that deregulated proteins were different upon treatment with doxorubicin and SF semi-purified fractions. In Hep G2 cells

doxorubicin and SF fractions treatments caused the deregulation of apolipoprotein A-I precursor and cathepsin D precursor. Apolipoprotein A-I precursor was increased in all treated samples while cathepsin D was only increased in SF semi purified fractions treated samples. Apolipoprotein A-I precursor may increase the production of apolipoprotein A-I which participates in the reverse transport of cholesterol from tissues to the liver for excretion (Gudheti *et al.*, 2003). On the other hand, cathepsin D precursor might induce the synthesis of cathepsin D. Cathepsin D; a kind of acid protease that is active in intracellular protein breakdown has been used as a biomarker in cancer. The protein was claimed to play a role in apoptosis induction. However, the actual function of this protein has yet to be determined (Minarowska *et al.*, 2007). Real time RT-PCR validation showed that Cathepsin D precursor was down-regulated following doxorubicin and all SF fractions treatments. The difference in both gene and protein may be due to the retention of the protein in the cells following treatment as they have not been eliminated.

Heat shock proteins function as a chaperone that can be either a pro- or antiapoptotic molecule. Heat shock protein HSP-90 beta is a proapototic molecule while heat shock cognate 71 kDa protein and stress-70 protein are antiapoptotic molecules. Deregulation of these proteins play roles in activation or inhibition of apoptosis (Kiang and Tsokos, 1998; Solary *et al.*, 2002). Hep G2 cells treated with SF30 fraction and doxorubicin showed deregulation of these proteins. Expression dynamics were confirmed by RT-PCR. In Chang Liver cells, only heat shock cognate 71 kDa protein and stress-70 protein were up-regulated upon SF60 treatment. Only the gene expression of stress-70 protein was confirmed by RT-PCR. Protein DJ1, also a chaperone was shown to be down-regulated in doxorubicin treated Chang Liver cells. It is important in combating oxidative stress and promotes cell growth (Kahle *et al.*, 2009). The expression dynamic

of this protein was confirmed by RT-PCR. The results in Chang Liver cells showed that SF60 treatment was not as harmful as doxorubicin.

Apart from that, there were different types of cell cycle proteins being deregulated following treatments. For Hep G2 cells, protein N-myc downstream-regulated gene 1 protein (Protein NDRG1) that was up-regulated upon SF30 treatment may have a role in the inhibitory of cell growth (Yan *et al.*, 2008). The RNA expression of this gene was similar as the protein level. Proliferating cell nuclear antigen (PCNA) which is important in DNA synthesis and repair (Naryzhny *et al.*, 2010) were down-regulated following SF60, SF90 and doxorubicin treatment. Down-regulation of PCNA could be an indication that the proliferation of the cancer cells was being affected. RT-PCR results indicated that the gene was expressed lower in all treated cells compared to untreated cells. Besides PCNA, ubiquitin-conjugating enzyme E2-25 kDa which may suppress apoptosis at high levels was down-regulated following doxorubicin treatment in both RNA and protein level. Other than that, RuvB-like 1 which is essential in cell proliferation was up-regulated following doxorubicin treatment (Ikura *et al.*, 2000). However, RT-PCR showed the opposite result. This might be due to the protein which participate in cell division was not yet been degraded following treatment.

Structural proteins such as actin, cytokeratin and vimentin were also being deregulated. Actin is important in the survival of the cells (Gourlay and Ayscough, 2005) while cytokeratin is a caspase-cleaved cytokeratin which increase during apoptosis (Yagmur *et al.*, 2007). In Hep G2 cells, actin was up-regulated following SF60 treatment while cytokeratin-18 was up-regulated following doxorubicin treatment. RT-PCR however, showed an opposite profile. Besides that, actin was shown to be down-regulated following treatment with SF60, SF90 and doxorubicin in Ca Ski cells. RT-PCR results

showed that the gene was down-regulated following doxorubicin treatment but up-regulated following SF60 and SF90 treatments. Doxorubicin treatment up-regulate the expression of cytokeratin-17 and cytokeratin-18 in Chang Liver cells. The expression of cytokeratin-17 was confirmed by RT-PCR. On the other hand, vimentin, a type of filament which is important in cell division (Ivaska *et al.*, 2007) was up-regulated following SF60 and doxorubicin treatments in Chang Liver cells. Following both treatments, RT-PCR result showed that the gene was expressed in the opposite way.

Antioxidative proteins such as peroxiredoxin-6 and thioredoxin-like protein were both down-regulated following doxorubicin treatment in Ca Ski cells. RT-PCR further confirmed the results by showing similar regulation. This suggested that doxorubicin down-regulated the expression of antioxidative enzymes that are important in cell growth and survival thus causing cell death (Powis *et al.*, 2000). Meanwhile, Chang Liver cells which were treated with SF90 fraction showed down-regulation of thioredoxin-like protein 2 and thioredoxin reductase 1. The RNA and protein level were expressed in a similar way again suggesting that the fraction may inhibit the cell growth.

Deregulation of cell signaling protein was also detected following treatment. Anamorsin, a cytokine induced apoptosis inhibitor was up-regulated following SF60 treatment in Hep G2 cells. A high level of anamorsin may inhibit apoptosis. Besides that, ferritin light chain, a storage protein was down-regulated in Hep G2 cells following SF90 treatment. Ferritin light chain helps to store iron which will induce oxidative damage at high concentration. This gave us an idea that the cell death might be related to oxidative damage (Lee *et al.*, 2009). Apart from that, an inhibitory protein, leukocyte elastase inhibitor was up-regulated in Ca Ski cells following SF30 treatment. The protein

regulates the activity of cathepsin G, chymase and chymotrypsin. Besides, it also functions as precursor of pro-apoptotic molecule L-DNase II (Lepr être *et al.*, 2009).

The results of protein deregulation and gene expression did not always tally with each other. This scenario could be due to several reasons. Firstly, the mRNA transcripted may be degraded or was not fully translated. This will lead to the low level of translated proteins. Secondly, post-translational modification gives rise to the formation of active proteins. Besides that, even when protein translation is completed, protein degradation will cause the protein expression level to be different from the mRNA level. However, if the protein and mRNA expression profiles were similar, it can be said that the expression dynamics observed is an actual reflection of the situation in the cells.

Proteins which act as chaperone, cell cycle protein, structural protein and antioxidative protein are important in cell growth. Treatments with SF fractions and doxorubicin regulate some of the genes and proteins in similar pattern. This suggests that the fractions may be adopting the same mechanisms as doxorubicin in inducing cell death. As SF fractions consist of many components, further purification of the samples might give use a clearer idea in the actual pathway or mechanism in halting the growth of cancer cells upon treatment.