CHAPTER 4

Discussion and conclusion

The spread of malignant cells originating in one site to distant sites is a characteristic of metastasis that obstructs current clinical treatments and thus become a major etiology of cancer patient death. To achieve metastasis, cancer cells require multiple steps, as following; cell adhesion, invasion, proliferation and formation of new vessels (186). To facilitate invasion, a proteolytic disruption of the stromal extracellular matrix is found to be a crucial step in order to carry on with metastasis. Additional antagonists of tumor invasion, which are responsible for the degradation, are MMPs and uPA (82).

Since many evidences have pointed out that MMPs and uPA are overexpressed during that progression. MMP and uPA expression is controlled by multiple extracellular molecules including cytokines (IL-1, IL-6, TNF-α), growth factors and their adjacent cells/ECM interactions. It is of interest to design a new avenue for therapeutic intervention by targeting at the modulation of MMPs and uPA expression and their activities. However, many of MMP inhibitors used in clinical trials have not shown any impressive results. Besides, they have been reported on their toxic side effects at non-physiological dose (187). To search for more clinical effective anti-invasion compounds that have no fewer or no negative side effects becomes the utmost importance. Fortunately, a long history and a widespread usage of natural products for healing purposes have been well documented. It is worth it to lend such a power to natural products derived from plant sources to generate powerful tool to fight cancer cell progression.

Red rice consumption has gradually increased as consumers have become increasingly receptive of issues upon health and well-being. This popularity has a correlation with the publication of many studies that have revealed numerous positive effects of the bioactive constituents provided when red pigmented rice is consumed. In particular, phenolics,

flavonoids, vitamin E analogs and γ-oryzanol, mainly found in red rice extract, showed substantial antioxidative, anti-inflammatory and anti-cancer cell proliferation activities (152, 188).

Presumably, the red rice extract has anti-metastasis, however, there is no concrete evidence to date. This present study was aimed to examine the ability of red rice extract fractions as anti-metastasis and anti-inflammatory agents on human invasive cancer cells. Identifying the bioactive constituent(s) in red rice fraction by the bioguided fractionation was also an objective of this study. All fractions were tested to quantitatively determine the hydrophilic and hydrophobic phytochemicals in red rice fractions by colorimetric and HPLC methods. In addition we investigated the biological activities that diminish cancer cell invasion, ECM proteolytic enzyme secretion and activities in human invasive cells. Their ability to decrease the secretion of inflammatory mediators (nitric oxide; NO), cytokines (IL-1β, IL-6) and tumor necrosis factor-α (TNF-α) in LPS-induced macrophage cells were also examined.

It was found that the red rice fractions were shown to contain almost all secondary metabolites such as phenolics, flavonoids, proanthocyanidin and anthocyanin compounds. The highest content of phenolics, flavonoids, and proanthocyanidin were in CEE followed by water, EtOAc, DCM and Hex fractions. On the other hand, TAC showed the lowest content in all fractions, which was approximately 30% in comparison with black rice extract (data not shown).

Solvent fractionation is frequently used for isolation and extraction of natural phenolic and flavonoid compounds from plants. The amount of compounds present is strongly dependent on the suitable solvent for extraction; water soluble extraction such as ethanol, methanol, water and fat-soluble extraction such as hexane and ethyl acetate. In this study, the phenolic and flavonoid content was increased in according to the polarity of the solvents; the highest amount of total phenolic content corresponded to water and CEE fractions. This result is in agreement with the reports from previous studies, which found that ethylacetate had a higher content of phenolics than the hexane fraction when fractioned with 70% ethanolic extract from the rice bran (189-193).

Proanthocyanidins are a class of polyphenolic and bioflavonoids, the (+)-catechin and (-)-epicatechin oligomers or polymers units of polyhydroxy flavan-3-ol present in berries, pine barks, grape seeds and color races. Previous reports showed that proanthocyanidins are the major water soluble components in red rice extract, with amounts approximately 20 fold higher than that of purple, brown, light brown and white rice (32, 33, 148).

The Hex fraction showed the highest amount of γ-tocotrienol, γ-tocopherol and γ-oryzanol than all other fractions. The concentration of γ-tocotrienol in Hex fraction was 1.4-2.65 times highest than that of EtOAc and CEE, respectively. In Hex fraction, γ-tocotrienol and γ-tocopherol are the major components and the content of total tocotrienols was 14 times higher than that of total tocopherols. This result was similar to previous study that reported by Fasahat *et al*., 2012 (194). It is considered that the structure of tocotrienol, tocopherol, and γ-oryzanol which contained mostly hydrophobic moieties resulting in it being freely soluble in non-polar solvent such as hexane, diethyl ether, or ethyl acetate. These compounds also could be found in CEE and water fraction due to the hydroxyl group of the chroman rings of tocols and the polar molecule in γ-oryzanol such as triterpene alcohol or sterols and fururic acid. The polar substituent's of these compounds make it easily dissolved in ethanol and water (190, 195, 196).

The first part of this study focused on the question of whether the CEE, Hex, DCM, EtOAc and water fractions could inhibit human cancer cells invasion. Human breast cancer cells (MDA-MB-231) and human fibrosarcoma cells (HT-1080) were used as a classic experimental model. We reported for the first time that γ-oryzanol at 50 μg/ml was able to reduce MDA-MB-231 cell invasion, which corresponded to the finding that Hex and DCM fractions can help to reduce cancer cell invasion. Moreover, the results confirmed that γ-tocotrienols at 10 μg/ml can reduce the MDA-MB-231 cell invasion. This discovery was consistent with the report of Liu *et al*., 2010 which found that γ-tocotrienol can down regulate MMP-2 and MMP-9 expression, thus it can be able to inhibit the SGC-7901 gastric adenocarcinoma invasion (159). Furthermore, several studies have reported that γ-tocotrienol exhibited anti-tumor and inhibitory effects against angiogenesis and metastasis as well as a potent agent to induce apoptosis (197, 198). This

data collectively acknowledges the inhibitory effect of γ-tocotrienol and γ-oryzanol in the Hex and DCM fractions on the cancer cell invasion.

Catechins were the major phenolic found in the EtOAc and DCM fractions. It is especially the EtOAc fraction that contained high amounts of protocatechuic acid as well as contents of vanillic acid, ferulic acid and coumaric acid. Although the other phenolic compounds in the EtOAc fraction could not reduce cancer cell invasion, our report, on the other hand, demonstrated that vanillic acid and ferulic acid at 50 μg/ml could effectively reduce MDA-MB-231 cell invasion. A similar finding was also reported in many studies on the anti-metastasis property of vanillic acid and ferulic acid (199, 200). However, the tested concentration of the vanillic acid and ferulic acid at 1 μg/ml from EtOAc fraction was not sufficient enough to promote anti-invasion process.

It was found that cancer cell invasion was reduced when treated cell lines with CEE and water fractions, which highly contained proanthocyanidin. Our result was resembled to the previous report by Sun *et al.*, 2011 stated that proanthocyanidins derived from various kinds of medicinal plants, including grape seeds, have anti-metastasis effect against cancer cells (201). Thus, the presence of proanthocyanidins in CEE and water fractions was partly contributed to the reduction of cancer cell invasion process.

The breakdown of ECM components in the tumor environment allowed the invasion of tumor cells to secondary sites. The main proteolytic enzymes that are involved in the processes are MMPs. Although many enzymes are related in this process, it shows that MMP-2 and MMP-9 act as an important function in cell invasion because both enzymes degrade type IV collagen, a main component of the ECM (16). For the secretion assay revealed that the CEE, Hex and water fractions significantly decreased the secretion of both MMP-2 and MMP-9 from HT 1080 cells more than that of DCM, while EtOAc fraction showed slightly effect on the secretion. On the other hand Hex fraction could inhibit MMP-9 secretion from MDA-MB-231cells more than the CEE, DCM and water fractions, whereas EtOAc slightly inhibit the enzyme secretion. For the ECM degradation enzyme activities assay, water fraction significantly decreased the activities of MMP-2 and MMP-9 secreted from HT-1080 cells in a concentration-dependent manner followed

by CEE, Hex and DCM fractions, whereas EtOAc provided less effective. For the activity of MMP-9 secreted from MDA-MB-231 cells, CEE fraction significantly reduced MMP-9 activity in a dose-dependent manner followed by Hex, water and DCM fractions, EtOAc was less active. In addition, the collagenase activity was significantly inhibited by CEE fraction followed by water and Hex fractions, while DCM was much less active. These results can be suggested that the cancer cell invasion process could be reduced by the action of active ingredients in CEE, Hex, DCM and water fractions, at least partly inhibiting the secretion and activity of the enzymes involving the ECM degradation.

Inflammation is a response mechanism of organism to non-self compounds. The process occurs due to the action of many cells including macrophages, fibroblasts and endothelial cells. The production of nitric oxide and the increment of the IL-1, IL-6 and TNF-α, the proinflammatory cytokines, become such important mediators in the macrophage-mediated inflammation process. The overproduction of this free radical and cytokines regulate the process involving inflammation which is relevant to the pathogenesis such as carcinogenesis and cancer metastasis (61). This study found that CEE, HEX, DCM and water fractions, accept EtOAc effectively inhibits the production of NO, IL-1β, and IL-6 at high concentrations in LPS-induced RAW264.7 cells, but not TNF-α. This finding suggests that CEE, HEX, DCM and water fractions possess useful anti-inflammatory activity especially concerning pro-inflammatory cytokine and they reduced the production of endogenous free radical, nitric oxide.

In the first conclusion, this study demonstrates that the two hydrophobic fractions, the Hex and DCM, extracted from red rice potentially exhibited anti-invasion activity *via* the inhibition of MMPs secretion and activities. On the other hand, these fraction could reduce inflammation via inhibiting the production of NO and IL-1 and IL-6 inflammatory cytokines induced by LPS. These inhibitory activities could be attributed to the high content of γ-tocotrienol and γ-oryzanol. Moreover, the anti-invasion and anti-inflammation activities of hydrophillic fractions including CEE and water fractions may be partly due to the effect of the proanthocyanidins and γ-oryzanol. It might be suggest that the γ-tocotrienol and γ-oryzanol and proanthocyanidins in red rice possess anti-

invasion and anti-inflammation potential against cancer cells and it could be used as a daily food supplement for treating cancer patients.

The finding let us to further determine whether proanthocyanidin included in water fractions could reduce the invasion in human breast cancer cells by decreased MMP-2,-9 activity and secretion. The second study was carried out to isolate the proanthocyanidin rich fraction from water fraction of red rice (PRFR) and examine whether PRFR could inhibit breast cancer cells invasion and migration. For understanding the molecular mechanism, we tested the effects of PRFR on ECM degradation-associated proteins, as following: uPA, uPAR, PAI-1, MMP-9, MT1-MMP, adhesive molecule ICAM-1, cytokine IL-6, and transcription NF-κB.

An identification of proanthocyanidin type in PRFR found that proanthocyanidin contained in PRFR after acid hydrolysis were catechins, epicatechin, gallocatechin and epigallocatechin. These data showed that the proanthocyanidin type in PRFR might be procyanidin (catechin and/or epicatechin) and prodelphinidin (epigallocatechin and/or gallocatechin). This result was similar to the study of Bordiga *et al* (150), which found that four flavan-3-ol monomers were the component in red rice. Procyanidin B-1 (epicatechin-(4β to 8)-catechin) with the degree of polymerization 4.2 was identified as the major type of proanthocyanidin in red rice (184). While, minimal amount of prodelphinidin was also observed in the agreement with the data from Min *et al* (147). This discovery is similar to previous studies that approximately 1-38 monomers of procyanidin were present in red-hull rice extracts. It has been reported that oligomer of 5-8 mers (40%) were mainly proanthocyanidins in IITA119 red rice, while 29% was polymers (degree of polymerization (DP) >10 (167). In contrast, procyanidin type of proanthocyanidins was 60% of oligomer with DP4 in grape seed (202). By using Sephadex LH20 column chromatography, the proanthocyanidin in PRFR fraction was appeared at the same fraction as that of the grape seed extracts. This could be suggested that proanthocyanidin from both PRFR and grape seed extracts were available in oligomeric forms containing 60% DP. Hence, almost of the proanthocyanidins in the PRFR may be oligomers with the same DP as found in the grape seed extracts. We then proposed the possible structure in Figure 14.1

In addition, proanthocyanidins are found in vast variety of plants. Their biological properties are also varied as a result of greatly structural diversity. For example, the proanthocyanidins that found in pine bark are in oligomeric form, containing mainly procyanidin type B as well as a minute amount of prodelphinidin. The oligomers are available in various chain lengths from dimer to pentadecamer (203).

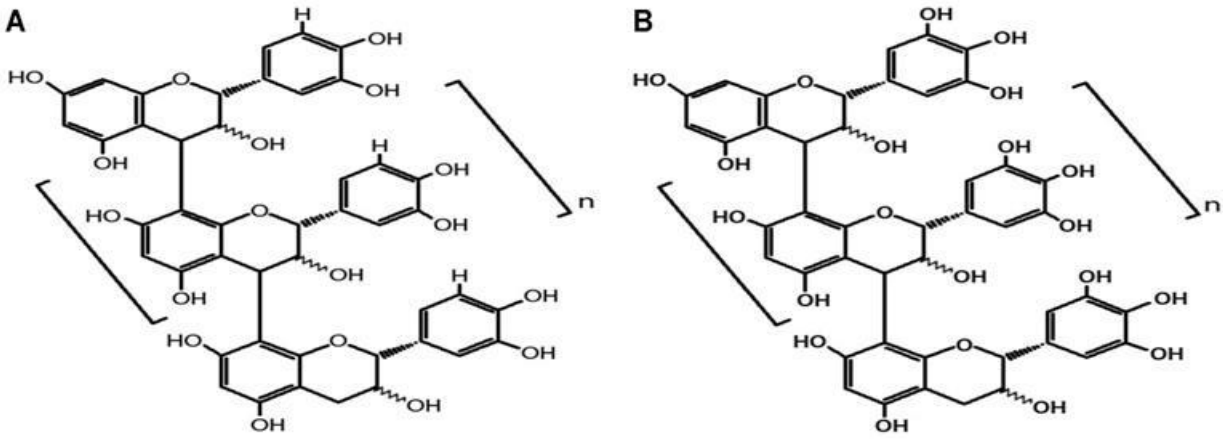

Figure 4.1 The proposed structure of procyanidins (A) and prodelphinidins (B) in PRFR from red rice

Whereas the major type of proanthocyanidin in lingonberry is type A, presenting mainly in a pentamer and heptamer (204). The degree of polymerization is important factor affecting the bioavailability of proanthocyanidins. In particular, the shorter degree of polymerization (monomer to trimers) can be directly absorbed in the small intestine, while the oligomer and polymer require further modification through either acidic conditions in the stomach or the conditions provided by microorganism in the colon (205). It is obvious that the proanthocyanidins in both intact and decomposed form can be absorbed throughout the digestive tract and indeed exert beneficial health effects.

The biological functions of proanthocyanidins from red rice have been widely studied for their anti-oxidant and anti-inflammation as well as their anticancer activities (152). Nevertheless, the regulation of cancer cell metastasis mechanism in the presence of red rice proanthocyanidins remains unknown. Therefore, we aimed to understand the molecular mechanisms of red rice proanthocyanidins on breast cancer cells metastases. Interestingly, the results showed that the inhibition of MDA-MB-231 cell invasion and

migration was successfully using low concentration (0-20 μg/ml) of PRFR with the IC50 value 10.22±0.45 and 10.60±0.59 μg/ml, respectively. This concentration range was in well agreement as reported in the grape seed extract (206). Additionally, this study demonstrated that the invasion of MDA-MB-231 cells was reduced by proanthrocyanidin from grape seed extract with a lower concentration when compared to the concentration of evening primrose extract (IC50 ~60 μM) (164). It has been shown that evening primrose proanthrocyanidin is mainly presented in either form of dimer or trimer (207). Whereas PRFR is predominantly found in oligomers form and its structure is likely similar to proanthrocyanidin in the grape seed extract.

The ECM degradation enzymes secretion and activity assays revealed that the MMP-9 secretion as well as its activity could be reduced by the action of PRFR. Furthermore, the degradation of DQ-gelatin by collagenase was strongly reduced in the present of PRFR. This result was similar to previous findings which have demonstrated resembling result in that the proanthocyanidins extracted from Japanese quince fruit and blueberry were able to decrease MMP-2 and MMP-9 activities (208, 209) .

Many reports have been published which intensely investigate whether breast cancer cell invasion and metastasis can be inhibited by targeting the expression levels of uPA/uPAR system (8, 210). It was found that PRFR at a low concentration could inhibit the uPA secretion and uPAR expression. PAI-1 is a multifunctional protein and conflicting results regarding its relationship between PAI-1 levels and cancer cell metastasis have been reported as its functions seem to be context and concentration dependent. The high expression of PAI-1 in serum has been reported in several studies that suggested its poor prognostic biomarker for ovarian, gastric and breast cancer patients (211, 212). PAI-1 and uPAR can compete for binding to vitronectin, and its receptor, integrin, resulting in enhanced cell motility (213). The expression of PAI-1 has a positive correlation with the invasiveness of MDA-MB-231 human breast cancer cells (214). Our results showed that the level of uPAR and PAI-1 was significantly reduced when MDA-MB-231 cells were treated with PRFR, which might lead to decreased of cell invasion. Furthermore, the results are compatible with previous study that stated proanthrocyanidin from grape seed extract inhibited the expression of uPA, uPAR and PAI-1 (215). Besides, PRFR

powerfully decreased expression of MT1-MMP, an activator of MMP-2 and MMP-9, in MDA-MB-231 cells.

Cancer cells attach to the endothelial membrane, which is a crucial step for cell metastasis and several adhesive molecules are involved in this process including adhesion molecules such as ICAM-1, VCAM-1 and E-selectin. ICAM-1 has been shown to have higher levels in tumor patients excluding breast cancer patients (216, 217). We demonstrated that PRFR decreased ICAM-1 expression levels in the MDA-MB-231 cells, which is in line with previous studies that proanthocyanidin from grape seeds inhibited ICAM-1 expression in sclerosis patient (218). It could be suggested that PRFR implicated in tumor cell invasion mediated by the reduction of ECM degradation proteins expression and activities and adhesive protein expression.

In breast cancer, the expression of IL-6 influences cell growth, proliferation, differentiation and angiogenesis by altered the immune system. The upregulation of IL-6 also implicated in the ability of a tumor cell to metastasize through the adhesion and invasion properties (219). It has been revealed that IL-6 activates STAT-3 and MAPK oncogenic signaling pathways. Additionally, STAT3 can promote MAD-MB-231 cell invasion upon IL-6 stimulation. Notably, inhibition of IL-6 reduced tumor development, angiogenesis and breast cancer patient metastasis (220). This study demonstrated that PRFR reduced IL-6 expression in MDA-MB-231 cells which suggested that PRFR could potentially inhibit cytokines expression that in turn, inhibits cell migration and invasion.

Many types of cancers are tightly related to various signaling molecules and thus to signaling pathway particularly in controlling the gene expression involving in tumor metastasis. For example, the breast cancer that is exposed to a constitutive activation of NF-κB induces inflammation and cell metastasis. The signaling pathway of NF-κB is known to control the genes related in the tumor metastasis process. It is now well known that the NF-κB signaling pathway regulates genes expression that is implicated in tumor metastasis as well as uPA, uPAR, MMP-9, MT1-MMP, ICAM-1, and IL-6 (221). Interestingly, the present study revealed that PRFR was able to inhibit the NF-κB DNA binding activity in MDA-MB-231 treated cells. This results implied a closely relationship

among all genes involving in tumor metastasis process, including MMP-9, MT1-MMP, uPA, uPAR, IL-6 and ICAM-1. This data are also in agreement with recent report demonstrating the inhibitory effect of grape seed proanthrocyanidin on NF-κB-DNA binding activity in prostate cancer cells (222).

In conclusion, the NF-κB-DNA binding capacity was markedly decreased in the MDA-MB-231 cells treated with PRFR. On this account, we then proposed the inhibitory mechanism of PRFR on cancer cell metastasis that occurred through the down-regulation of MMP, uPA, IL-6 and ICAM-1 (Figure 4.2). Furthermore, it may provide valuable information on their involvement into the prevention of cancer metastasis which may be useful for therapeutic approaches.

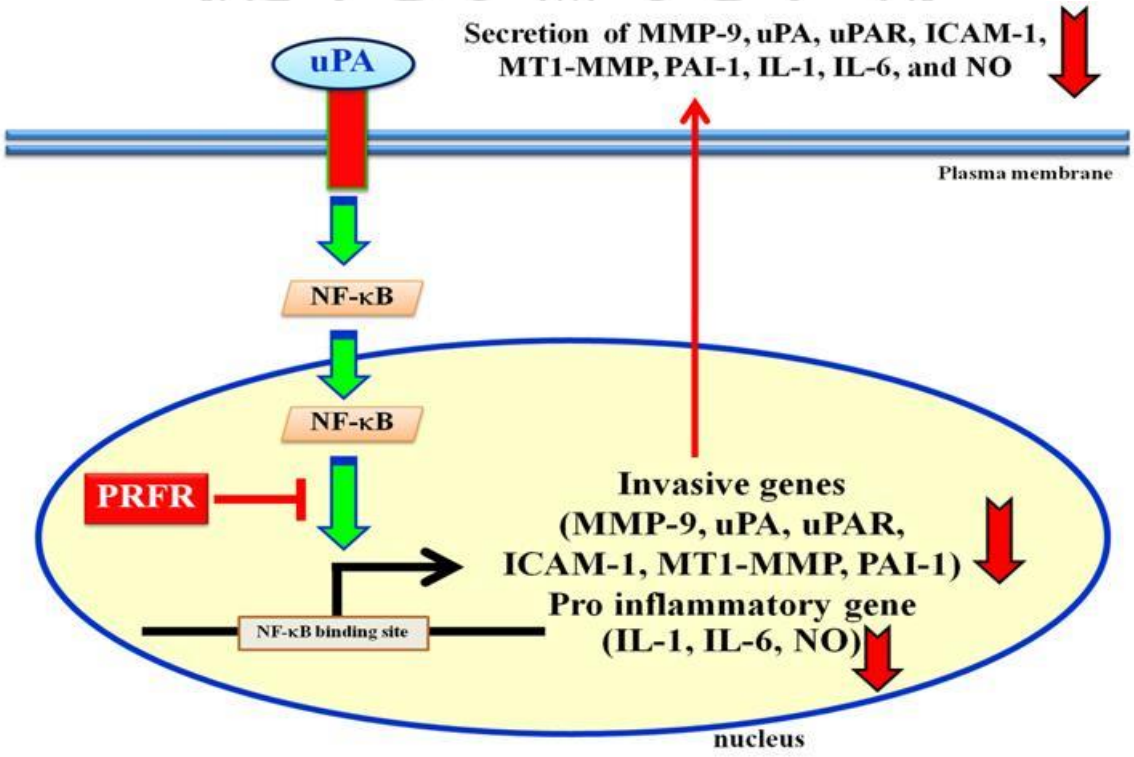

Figure 4.2 Proposed mechanism of PRFR to inhibit MDA-MB-231 cells invasion