CHAPTER 4

DISCUSSION AND CONCLUSION

Glutinous purple rice (*Oryza sativa* L.var. *indica*) variety Kum Doisaket contains purple pigment in the hull and pericarp as a unique characteristic. Our previous antimutagenicity study on various extracts of glutinous purple rice variety Kum Doisaket has shown that the methanol extract of glutinous purple rice seed and dichloromethane extract of glutinous purple rice hull presented the strongest antimutagenicity in Salmonella mutation assay. Furthermore, the acidified methanol extract of glutinous purple rice hull showed both strong antimutagenicity and mutagenicity in Ames test (Punvittayagul *et al*., 2011). Therefore, these extracts were selected to evaluate their clastogenicity and anticlastogenicity in animal model.

The acidified methanol and dichloromethane extracts of glutinous purple rice hull and the methanol extract of glutinous purple rice seed did not produce any signs of toxicity or mortality in the rats according OECD guideline TG425. The administration of glutinous purple rice extracts for 28 days did not induce the number of micronucleated hepatocytes, micronuclei and binucleated hepatocytes. Thus, all of extracts were safe and non-clastogenic in rat liver.

Although our previous data showed the acidified methanol extract of glutinous purple rice hull presented mutagenicity in Ames test, it did not present mutagenicity in rat liver using micronucleus assay. These results agree with previous reports in which some flavonoids are mutagenic in Salmonella mutation assay but not in in vivo mutagenicity tests. Quercetin rutinoside was non-mutagenic but its metabolite, quercitin, was mutagenic in Ames test (Rueff *et al*., 1990). However, quercetin presented neither mutagenicity nor carcinogenicity (Okamoto, 2005). Thus, the measurement of quercetin content in acidified methanol extract of glutinous purple rice hull should be further evaluated.

The acidified methanol and dichloromethane extracts of glutinous purple rice hull and the methanol extract of glutinous purple rice seed significantly modulated number of micronuclei in rat liver initiated by diethylnitrosamine (DEN), a hepatocarcinogen. The acidified methanol extract of glutinous purple rice hull presented the strongest anticlastogenicity in this model. The contents of phenolic compounds in the methanol extract of seed and the acidified methanol extract of hull were no difference but the the seed methanol extract presented higher amounts of total flavonoids and anthocyanins when compared to those of the hull acidified methanol extract. These data could indicate that flavonoids and anthocyanins might not be active compounds in the acidified methanol extract of purple rice hull. Due to the strongest anticlastogenicity and the highest percent yield of acidified methanol extract of glutinous purple rice hull when compared to the other glutinous purple rice extracts, the possible inhibitory mechanism involving xenobiotic metabolizing enzymes of this extract on the diethylnitrosamine induced mutagenesis was further investigated.

Diethylnitrosamine is metabolized by CYP2E1 and spontaneously broken down to form reactive alkyldiazonium ions. These electrophiles commonly conjugate with nucleophile molecules by phase 2 enzymes, preventing them from forming DNA adducts (Sheweita and Tilmi sany, 2003). The CYP2E1 protein expression was significantly induced in the hepatocyte of diethylnitrosamine-treated rats, but it was modulated by the acidified metanol extract of glutinous purple rice hull. The acidified methanol extract of glutinous purple rice hull significantly increased phase 2 enzymes activities including glutathione *S*-transferases, NADP(H) quinoneoxidoreductase and UDP-glucuronyltransferase. Glutathione *S*-transferases alpha expression was also enhanced by acidified methanol extract of glutinous purple rice hull in DEN-initiated rats. It might be suggested that the acidified methanol extract of glutinous purple rice might act as a monofunctional enzyme inducer in diethylnitrosamine induced the initiation stage of hepatocarcinogenesis. The possible inhibitory mechanism of acidified methanol extract of glutinous purple rice hull on DEN induced micronuclei formation in rat liver was displayed in Figure 4-1.

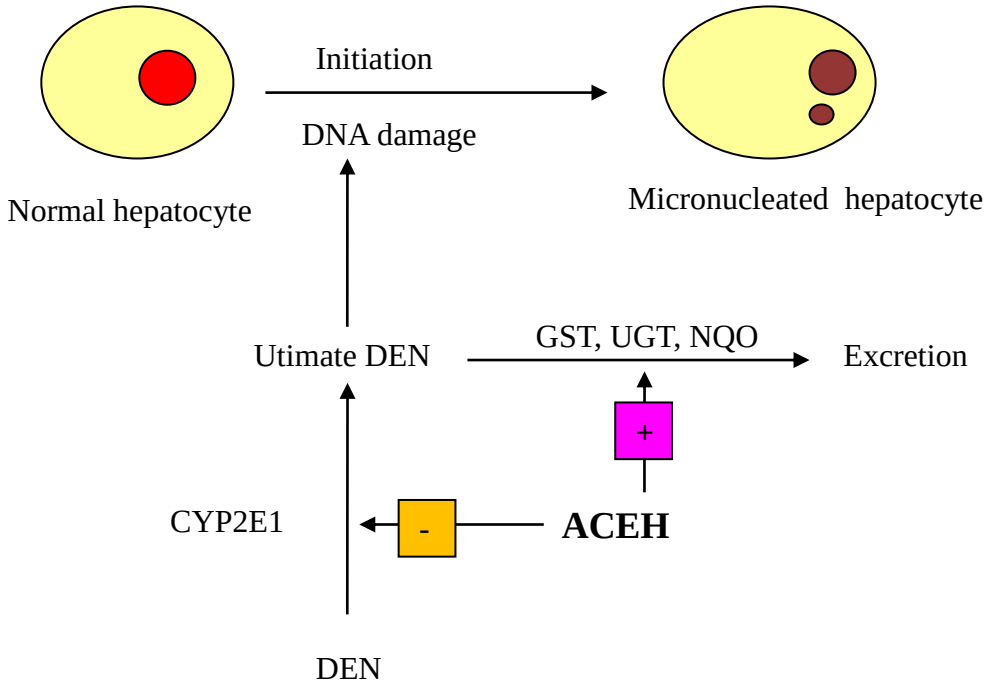

Figure 4-1 The possible inhibitory mechanism of acidified methanol extract of glutinous purple rice hull (ACEH) on diethylnitrosamine (DEN) induced micronucleated hepatocytes

Plant phenolic acids possess the variety of biological activities including anticarcinogenic potential. The rice hull is a rich source of phenolic acids when compared to the other parts of rice. Vanillic and *p*-coumaric acids were the major phenolic acids in rice hull. Ferulic, *p*-hydroxybenzoic, protocatechuic and chlorogenic acids were minor constituents in the hull. (Butsat and Siriamornpun, 2010). Recently, there is no report on the effect of vanillic and *p*-coumaric acids on xenobiotic-metabolizing enzymes. Protocatechuic acid modulated the activities of cytochrome P450 and some phase II enzymes in mouse liver and kidney (Krajka-Kuzniak *et al*., 2005). It suppressed the activities of some cytochrome P450 but elevated the activity and expression of glutathione *S*-transferases (Krajka-Kuzniak *et al.*, 2008). Protocatechuic acid might be considered a monofunctional inducer in liver

The acidified methanol extract of glutinous purple rice hull also contained flavonoids which play a crucial role in cancer prevention. Flavonoids can inhibit metabolic activation of the procarcinogens to their ultimate electrophilic species, or their subsequent interaction with DNA via stimulated the detoxification of

carcinogens, leading to their secretion from the body (Surh, 2003). One of the major mechanisms of protection against carcinogenesis, mutagenesis, and other forms of toxicity mediated by carcinogens is the induction of enzymes involved in their metabolism, particularly phase 2 enzymes such as glutathione *S*-transferases (GSTs), UDP-glucuronyl transferases, and quinone reductases (Kwak et al., 2001). Anthocynins found in acidified methanol extract of glutinous purple rice hull might act as an enzyme inducer. They induced activity of NAD(P)H quinone oxidoreductase and glutathione-related enzymes expression (Shih *et al*., 2007). Furthermore, anthocyanins rich fraction extract from black sorghum was a strong inducer of the activity of NAD(P)H quinone oxidoreductase (Yang *et al*., 2009).

At low dose, 100 mg/kg bw, of acidified methanol extract of glutinous purple rice hull significantly induced the activities of some xenobiotic metabolizing enzymes including cytochrome P450 reductase, NADP(H) quinone oxidoreductase, glutathione S-transferases and heme oxygenase but its higher doses did not affect on xenobiotic metabolizing enzymes. Talalay and his college found that phase 2 enzymes were transcriptionally induced by low concentrations of a wide variety of chemical agents (Talalay *et al*., 1995). Breinholt and his group studied the effect of various concentrations of lycopene ranging from 0.001 to 0.1 g/kg bw. per day to female rats for 2 weeks. Only at doses between 0.005 and 0.05 g/kg b.w. per day could alter the drug-metabolizing capacity and antioxidant status (Breinholt *et al*., 2000). The effect of four concentrations of quercetin (5, 10, 20, and 40 μM) on phase 1 and phase 2 enzyme activities in the SW-480 human colon carcinoma cell line found that the lowest dose slightly increased glutathione *S*-transferases and quinone reductase activites, while the higher doses systematically decreased their activities (Buck, 2001)

Flavonoids at low concentrations activated MAPK pathway leading to activation of Nrf2 and ARE with subsequent induction of phase II and other defensive genes which protect cells against toxic insults thereby enhancing cell survival, a beneficial homeostatic response. Higher concentrations of flavonoids activated the caspase pathways, leading to apoptosis, a potential cytotoxic effect if it occurred in normal cells (Kong *et al*., 2001). Anthocyanin rich fraction extract from blueberries modulated a hormesis response on apoptosis and phase 2 enzymes. Caspase-3 activity and DNA fracmentation were increased by anthocyanins rich fraction extract from

blueberries in dose-dependtdent manner. The activity of NADP(H) quinoneoxidoreductase and glutathione *S*-transferase decreased gradually when treated with increased concentrations of anthocyanin rich fraction extract from blueberries (Srivastava *et al*., 2007). Previous study has descibed the term of hormetic response, several phytochemical has a stimulatory effect at low doses, but is toxic at high doses. Hormesis can be initiated by exposure to various environmental stressors including ingestion of phytochemicals. Such exposures typically result in mild cellular stress involving free radical production, ion fluxes and increased energy demand. As a result, adaptive stress response pathways are activated leading to the synthesis of proteins that protect cells against more severe stress. Examples of stress resistance proteins include antioxidants, protein chaperones, growth factors, and proteins involved in the regulation of energy metabolism and cellular calcium homeostasis (Son *et al*., 2008). Electrophile substances from the metabolism of flavonoids are also inducer of phase 2 transcription response oxidative stress (He and Ma, 2009) that complementary with the result in this research. All concentrations of acidified methanol extract of glutinous purple rice hull extract slightly increased glutathione S-transferase alpha expression whereas no statistical significance was observed.

Many of flavonoids present biphasic effects. Medium and high doses of acidified methanol extract of glutinous purple rice hull acted as a monofunctional enzyme inducer in diethylnitrosamine induced mutagenesis and carcinogenesis in rat. Low dose of the extract was a bifunctional enzyme inducer in normal conditions. Previous study found chalcone exhibited high cytotoxicity against neuroblastoma cell lines by activating a pathway involving caspases 9. Chalcone had no a detrimental effect on normal cerebellar granule cells at the same concentrations tested (Nishimura *et al*., 2007). Prenethyl isothiocyanated (PEITC) also sensitized cancer cell lines to apoptosis by inhibiting the NF-kB and EGFR pathway, the signalling for survival and proliferation. The blockade of NF-κB and/or EGFR signaling by these compounds would sensitize tumor cells to die, but not the normal cells. Conversely, these compounds would redox-dependently affect the Nrf2-mediated cellular detoxifying/antioxidant defense enzymes (Nair *et al*., 2007).

In conclusion, the glutinous purple rice extracts presented anticlastogenic potential. The acidified methanol extract of glutinous rice hull demonstrated the strongest anticlastogenic activity in rat liver. Its possible inhibitory mechanisms were partly due to either induction of detoxifying enzymes or inhibition of metabolizing enzymes. This study suggested glutinous purple rice may be an alternative source of cancer chemopreventive agents.