CHAPTER IV

DISCUSSION AND CONCLUSION

Ma-kiang (*Cleistocalyx nervosum* var. *paniala*) is the plant in same family (Myrtacae) as Luk-waa (*Syzygium cumini*). Surprisingly, there is no report on biological activity of *C. nervosum* especially *in vivo* study, though *S. cumini* was known to possess anti-hyperglycemic and antioxidant effects (Banerjee *et al.*, 2005 and Sharma *et al.*, 2006).

The aqueous extract of *C. nervosum* used in this study composed of 181.16±0.59 mg gallic acid equivalences per 100 gram fresh weight of total phenolic compounds and 54.86±3.45 mg catechin equivalences per 100 gram fresh weight of total flavonoids. Patthamakanokporn and colleagues have reported that total phenolic compounds of 50% ethanolic extract of this plant was 269.7±3.0 mg gallic acid equivalences per 100 gram fresh weight (Patthamakanokporn *et al.*, 2008). Anyhow, it was found that total phenolic content depends on method of extraction. This plant extract contained lower total flavonoid content than the total phenolic content because flavonoids are one member of phenolic substances (Pietta, 2000). The main anthocyanins in this extract were cyanidin-3, 5-diglucoside, cyanidin-3-glucoside and cyanidin-5-glucoside.

In this study, DPPH radical scavenging assay and deoxyribose assay were performed to evaluate the antioxidant capacity and mechanism of antioxidant activity of the *C. nervosum* extract. The DPPH radical scavenging assay is the most common method used for assessment of the antioxidant properties of natural products. The results revealed that the DPPH radical scavenging activity of *C. nervosum* might be attributed to the hydrogen donating ability of phenolics and flavonoids. The antioxidant activity of several polyphenols as inhibitors of hydroxyl radical formation and lipid peroxidation has been correlated with iron-chelating properties (Grinberg *et al.*, 1997). Furthermore, deoxyribose assay was performed to assess hydroxyl radical

scavenging and iron chelating activities. In this study, aqueous extract in the presence of EDTA scavenged hydroxyl radical presenting in the free solution and thus protect the degradation of deoxyribose to thiobarbituric acid reactive material. It was further observed that the extract also chelated ferric ion in the absence of EDTA and made it unavailable to detector molecule to impair the formation of hydroxyl radicals at a particular site. The mechanism of antioxidant activity of *C. nervosum* extract might be involved in scavenging free radical and/or chelating iron generating Fenton reaction. From the results obtained, it might conclude that the phenolic compounds found in aqueous extract of *C. nervosum* take a part on antioxidant capacity.

In acute toxicity study, *C. nervosum* extract 5000 mg/kg caused neither visible signs of toxicity nor mortality. Generally, the reduction in body weight gain and internal organ weights is a simple and sensitive index of toxicity after exposure to toxic substance (Raza *et al.*, 2002 and Teo *et al.*, 2002). In the present study, gross examination of internal organs of all rats revealed no detectable abnormalities.

In subacute toxicity study, 100 and 500 mg/kg bw of an aqueous extract of *C. nervosum* did not produce any marked changes of general behavior or other physiological activities in both male and female rats. All animals survived throughout the end of an experiment and did not present gross pathological alteration in the internal organs such as organ swelling, atrophy or hypertrophy. The relative heart and stomach weights of male rats given 100 mg/kg bw of *C. nervosum* extract were significantly changed when compared to that of the control group but they did not exhibit any gross morphological lesions. Hematological parameters such as hematocrit, hemoglobin concentration, platelets, and white blood cells of treated male rats did not show any significant differences from those of control male rats. In the present study, no significant differences in blood chemical parameters of both genders were detected. Nevertheless, serum triglyceride level in female rat given 100 mg/kg bw of aqueous extract was significantly decreased as compared to that of control group but anyhow this triglyceride value lay within the normal limits (Pimainog *et al.*, 2003). The previous study found anthocyanins decreased lipid accumulation in the liver, including a significant decrease in liver triacylglycerol concentration (Prior and Wu, 2006). Many previous studies have shown that anthocyanins might prevent

cardiovascular disease (Hollman *et al.*, 1996; Ghiselli *et al.*, 1998 and Muth *et al.*, 2000). This result suggested that *C. nervosum* extract containing anthocyanins might decrease triglyceride in serum. In addition, serum creatinine level in female rat given 500 mg/kg bw of aqueous extract was significantly increased as compared to that of the control group. But this value is in the normal limits (Pimainog *et al.*, 2003). In summary, the aqueous extract of *C. nervosum* is fairly non-toxic since it did not produce acute and subacute toxicities in rats.

Furthermore, antioxidant activity of *C. nervosum* extract was confirmed in male rat. The present study demonstrated that the level of hepatic MDA significantly increased in rats given 100 mg/kg bw of *C. nervosum* extract. The aqueous extract of *C. nervosum* did not affect total GSH content, GR, GPx and CAT activities. At dosage of 500 mg/kg bw, the extract significantly increased heme oxygenase activity and decreased hepatic TBARs formation. *C. nervosum* extract at low dose enhance oxidative stress in rat liver, on the other hand, its high dosage reduce oxidative stress by enhancement of non-glutathione pathway such as HO. Most free radical scavengers act in oxidation-reduction reactions that are reversible. Phenolic photochemicals can act both as antioxidants and pro-oxidants depending on their structures and the conditions. The pro-oxidant activity is thought to be directly proportional to the total number of hydroxyl groups (Lee and Lee, 2006). The results obtained from the present study suggested that *C. nervosum* extract might exhibit biphasic effect on antioxidant activity. The results showed that the activity of heme oxygenase in rats administrating *C. nervosum* extract for 4 weeks significantly enhanced whereas the other antioxidant markers did not change. The reasons that *C. nervosum* extract presented mild antioxidant activity might be due to the shortage of dose administration and duration of treatment.

DEN is a classical hepatocarcinogen known to induce cancer in experimental animals (Sreepriya and Bali, 2005). PB acts as a tumor promoter or a non-genotoxic carcinogen when administered by subsequent to an initiating carcinogen like DEN to amplify number of initiated cells. The combination of DEN and PB has been used as a model not only to study carcinogenicity but also anticarcinogenicity of several agents in experimental animals. In the preliminary study, we determined the effect of

DEN concentration on the early stages of hepatocarcinogenesis in male Wistar rats when combined the treatment with PB using GST-P positive foci, preneoplastic lesions in the rat liver as the end point marker. It was found the number of GST-P positive foci was increased in rats injected by 100 mg/kg bw of DEN for either 2 or 3 times, when compared to that of the control. The carcinogenic potency of triple DEN injection was approximately 4-folds of the double injection. Furthermore, the level of MDA in serum and liver was also increased in DEN treated groups. Lipid peroxidation is observed as one of the basic mechanisms of tissue damage caused by free radicals (Esterbauer *et al.*, 1991). Administration of DEN has been reported to generate lipid peroxidation products in general (Nakae *et al.*, 1997 and Thirunavukkarasu *et al.*, 2002) and PB enhanced the formation of the activated oxygen species in the preneoplastic nodules in rat liver (Scholz *et al.*, 1990 and Jeyabal *et al.*, 2005). This strong action might lead by the imbalance between production of free radicals and reduction of antioxidants.

The effect of *C. nervosum* containing antioxidant activity on oxidative stress induced early stage of hepatocarcinogenesis in rats was investigated using the triple DEN injection and PB administration, Protocol I. The correlations between TBARs formation and induction of preneoplastic lesions in rat hepatocarcinogenesis have indicated the role of lipid peroxidation in carcinogenesis (Sanchez-Perez *et al.*, 2005). The co-administration of DEN and PB caused increased lipid peroxidation in rat. The results showed that the liver and serum GSH contents and GPx activity were decreased, while GR and CAT activities were increased in positive control group. These finding indicated the alterations in antioxidant enzymes under conditions of oxidative stress (Szatrowski and Nathan, 1991). Co-administration of DEN and PB induced oxidative stress leading to an increment of number of GST-P positive foci. The *C. nervosum* extract did not affect the number of GST-P positive foci, preneoplastic lesions in liver induced by triple DEN injection and PB administration. It might be partly due to either strong carcinogenic potency of chemicals in this model or low antioxidant capacity of *C. nervosum*. Interestingly, the administration of silymarin, a known antioxidant, significantly increased in the number of GST-P positive foci when compared to a positive control. Silymarin has been known to

protect rodent liver against hepatotoxicity induced by ethanol (Valenzuela et al., 1985), carbon tetrachloride (Favari and Perez-Alvarez, 1997; Dvorak et al., 2003), cisplatin (Mansour et al., 2006), D-galactosamine (Chrungoo et al., 1997), and acetaminophen (Muriel et al., 1992; Nencini et al., 2007) but no publication about its effect on DEN and PB induced rat hepatocarcinogenesis has been reported. The structure of silymarin is a flavonoid complex. The antioxidant activity of flavonoids is depending on their concentration. Although flavonoids are strong antioxidants, numerous studies have also indicated pro-oxidant effects and the same structure-activity relationships seem to be applicable to pro-oxidant effects. The relative antioxidant/pro-oxidant activity of flavonoids depends on concentration, time of administration and redox potentials (Min and Ebeler, 2008). Flavonoids have been shown to modulate selectively the activities of the P450 1A and 2B dependent monooxygenases and the conjugating enzymes (Siess *et al.*, 1989). In this study, PB is metabolized by CYP 2B1 leading to ROS production. Silymarin might induce CYP 2B1 leading to increase oxidative stress which was a possible cause for the enhancement of GST-P positive foci number.

The further protocol which reduced genotoxic potency and increased *C. nervosum* concentration was designed. The effect of *C. nervosum* containing antioxidant activity on oxidative stress induced early stage of hepatocarcinogenesis in rats was investigated using the double DEN injection and PB administration, Protocol II. Although the amount of DEN was reduced in this protocol, oxidative stress in rat was still occurred. In the present study, the results showed that the number of GST-P positive foci decreased in the liver of rats treated with 1000 mg/kg bw of the aqueous extract. The *C. nervosum* extract reduced the level of MDA in serum and liver of rat receiving DEN and PB. It also modulated total glutathione level and the activities of glutathione peroxidase, catalase and heme oxygenase. The decreased serum and hepatic lipid peroxidation and increased activities of antioxidant enzymes were related to the decreased number of GST-P positive foci in rats pre-treated with 1000 mg/kg bw *C. nervosum*. The chemopreventive activity of *C. nervosum* may be caused from its antioxidant activities.

In conclusion, *C. nervosum* containing anthocyanins demonstrated cancer chemopreventive effect on chemicals induced the early stages of rat hepatocarcinogenesis. The possible chemopreventive mechanism may be due to either enhancement of the antioxidant status or reduction of oxidative stress in the liver of carcinogens induced rats.