CHAPTER IV

DISCUSSION AND CONCLUSION

Pinocembrin is a major flavanone component of propolis, and also found in the rhizome of fingerroot *(Boesenbergia pandurata)*. Several lines have shown its pharmacological and biochemical functions including *in vitro* antimutagenicity (Trakoontivakorn *et al*., 2001). We have previously demonstrated that pinocembrin had no toxicity in male rat (Charoensin *et al*., inpress). However, the *in vivo* carcinogenic and anticarcinogenic effects of pinocembrin have not previously been investigated. Then, the *in vivo* model is necessary to determine whether administration of pinocembrin is a practical approach to anticarcinogenesis in rat.

From this point of view, we evaluated the mutagenic effect of pinocembrin and its ability to protect against diethylnitrosamine-induced mutagenesis in rat liver, using liver micronucleus formation as an end-point marker. The results showed that the treatment of 1, 10 and 100 mg/kg bw of pinocembrin for 7 days did not induce the number of micronucleated hepatocytes and mitotic index when compared to a control group. The results suggested that pinocembrin did not present mutagenicity in rat liver.

The effect of pinocembrin on xenobiotics metabolizing enzymes has already been studied. Xenobiotics-metabolizing enzymes play important roles in the metabolism and/or detoxification of xenobiotics. The protective effects of such bioactive compounds could be enhanced by modulating the activity of enzyme systems responsible for the metabolic activation or deactivation of chemical carcinogens (Bock *et al*., 1979). In this study, we investigated the effects of 7 days treatment with pinocembrin on phase I and phase II xenobiotics-metabolizing enzymes in rat liver. Pinocembrin significantly increased heme oxygenase activity in 10 and 100 mg/kg bw of pinocembrin treated groups. However, it did not affect the activities of NADPH: cytochrome P450 reductase,

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NADPH: quinone reductase, UDP-glucuronosyltransferase and glutathione-*S*-transferase. Heme oxygenase (HO) is a stress-responsive enzyme widely distributed in many mammalian tissues. It has been considered to be a potential therapeutic target for a number of chemopreventive agents (Prawan *et al*., 2005). In addition, up-regulation of HO-1 inhibited rat and human breast cancer cell proliferation (Hill *et al*., 2005). Thus, the induction of HO activity by pinocembrin may protect against hepatic damage associated with oxidative stress in rats. Moreover, pinocembrin did not affect on the expression of cytochrome P450 superfamily enzymes including CYP1A1, CYP2B1, CYP2C11, CYP2E1, CYP3A2, and NADPH: cytochrome P450 reductase.

As mentioned previously, pinocembrin inhibited activities of cytochrome P450 isozymes involved in carcinogen activation in the *in vitro* model (Siess *et al*., 1995). Some studies of pinocembrin metabolism have reported that it can be metabolized to various forms of flavanone and flavone, including naringenin, pinobanksin and chysin when incubated with rat liver microsomes (Nikolic and Breemen, 2003). Naringenin has been shown to inhibit CYP3A4 protein expression as well as enzyme activity (Liu et al, 2009) whereas chrysin at higher concentrations could also inhibit the same activity (Siess *et al*., 1995). From our investigation, pinocembrin did not affect on the expression of cytochrome P450 isozymes. The effects of flavonoids on enzymes are generally dependent on the concentration of flavonoids present, and the different flavonoids ingested. Due to the low oral bioavailability of many flavonoids, the concentrations achieved in vivo following dietary administration tend to be low, and may not reflect the concentrations tested under in vitro conditions. Effects will also vary with the tissue distribution of enzymes, and with the species used in testing since differences between species in enzyme activities also can be substantial (Moon *et al*, 2006).

Base on these observations, the treatment of pinocembrin in Wistar rat significantly increased the activity of heme oxygenase but did not affect on the activities of other phase II xenobiotic-metabolizing enzymes or the expression of cytochrome P450 enzyme system. Pinocembrin may confer protection against chemical-induced hepatocarcinogenesis. Further studies are necessary to obtain additional evidence on its effect on *in vivo* antimutagenicity and anticarcinogenicity.

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In the antimutagenicity experiments, pinocembrin was investigated under 2 protocols for studying its inhibitory and preventive effects on diethylnitrosamine–induced rat hepatocarcinogenesis. In the inhibitory study, rats treated with 2, 10 and 50 mg/kg bw of pinocembrin showed no significant effect on the number of micronucleus formation induced by diethylnitrosamine. From the results indicated that pinocembrin did not inhibit the micronucleus formation induced by diethylnitrosamine. Furthermore, our result showed 10, 25 and 50 mg/kg bw of pinocembrin did not prevent diethylnitrosamine-induced micronucleus formation even if was administered pinocembrin for a longer durations or lower the concentration of diethylnitrosamine. Although pinocembrin was exhibited a strong antimutagenic activity against mutagenic heterocyclic amines *in vitro* using Ames test (Trakoontivakorn *et al*., 2001). The results of the present investigation clearly showed that pinocembrin did not present an antimutagenic potential on diethylnitrosamine-induced mutagenesis in rat.

To determine the effect of pinocembrin on the promotion stage in diethylnitrosamine-induced hepatocarcinogenesis, we examined the effects of pinocembrin on the formation of GST-P positive foci as an enzyme marker that occurs during hepatocarcinogenesis (Ito *et al*., 1996; Ito *et al*., 2003). If test compounds significantly induce the number of GST-P positive foci higher than untreated controls, this compound may have a carcinogenic potential (Bannasch, 1986). If a test compound significantly reduced GST-P positive foci in the treated animals as compared with the concurrent untreated controls, suggested that this compound have an anticarcinogenic potential in rat. The present studies are of considerable interest because the *in vivo* carcinogenic and anticarcinogenic effects of pinocembrin have not previously been investigated.

In this study, we evaluated the effect of pinocembrin concentrations at 2 and 10 mg/kg bw after and before diethylnitrosamine injections for 10 and 15 weeks, respectively. No adverse effect was observed with regard to general observations, body weights, organ weights and the serum AST, ALT and ALP activities finding. Pinocembrin did not present carcinogenicity in rat. Our results also showed that rat treated with 2 and 10 mg/kg bw of pinocembrin did not decrease the the number of GST-P positive foci when received before or after diethylnitrosamine injection. These

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results suggested that pinocembrin did not inhibit or prevent the promotion stage in diethylnitrosamine-induced rat hepatocarcinogenesis.

The biological activities of chemical compounds depend on the concentration and route of administration. A chemical with an optimal concentration and route of administration as well as high absorption, the chemical can act at its target site (Klaassen, 2008). Therefore, the anticancer activity of pinocembrin may influence on pinocembrin concentration, route of administration, absorption, biotransformation and excretion. From the result, pinocembrin increased the activity of heme oxygenase indicated that this compound could be absorbed. However, the absence of anticarcinogenic activity might be cause from its structure.

Previous studies have been reported the methoxylated flavonoids presented chemopreventive potency higher than unmethylated flavonoids or polyphenols. Mechanistically, it is related to the free hydroxyl groups of the polyphenols, giving rise to rapid intestinal/hepatic conjugation by glucuronidation and/or sulfation and excretion (Walle *et al*., 2007). In flavonoids with a catechol-containing B-ring, there is also extensive O-methylation catalysed by the action of catechol-O-methyltransferase (COMT) in positions 3' and 4' of the B ring (Tripoli *et al*., 2007).

Methylation of dietary flavonoids may not only in a dramatic increase in their hepatic metabolic stability but also in great improvement of their intestinal absorption, both of which should greatly increase their oral bioavailability. Thus, blocking the free hydroxyl groups by methylation removes the influence of the highly efficient conjugation pathways (Wen and Walle, 2006).

In our laboratory, we have previously demonstrated that pinostrobin prevented initiation stage of rat hepatocarcinogenesis (Charoensin, 2008), on the other hand, pinocembrin did not present this activity. Therefore, the chemopreventive efficiency of flavonoids might be strictly correlated to the structure of the compounds. Moreover, the pharmacokinetic study in plasma after intravenous administration of pinocembrin in rat found that the plasma concentration of pinocembrin rapidly declined because of either fast excretion and/or extensive metabolism (Yang *et al*., 2009). From this reason after receiving pinocembrin into the body, it might rapidly conjugate with either glucuronide or sulfate and then excrete from the body. It might be one of the major concerns for the

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biological activity of pinocembrin that did not present anticarcinogenic activity in rat liver.

In conclusion, pinocembrin increased the activity of heme oxygenase but did not affect on the activities of other phase II xenobiotic-metabolizing enzymes as well as the expression of cytochrome P450 enzymes in rat liver. Pinocembrin did not show both mutagenicity and carcinogenicity in rat liver. However, it did not present antimutagenicity and anticarcinogenicity in DEN-induced rat hepatocarcinogenesis.

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