CHAPTER V

DISCUSSION

In vitro IC_{50} and CC_{50} study showed that 12 of 13 medicinal plants had anti-HBsAg activity, except for the extract from *Litchi chinensis* which had IC_{50} over 2000 μg/ml. The lowest IC_{50} was found in crude extract of *Saussurea lappa* (42.18 μg/ml). Followed by *Phyllanthus amarus*, *Derris scandens*, *Rhinacanthus nasutus*, *Santalum album*, *Duranta repens*, *Houttuynia cordata*, *Caesalpinia sappan*, *Homalomena aromatica*, *Loranthus pentandrus*, and *Phyllanthus emblica*. Zheng and Zhang (1990) reported that *Caesalpinia sappan*, *Gossypium herbaceum*, and *Litchi chinensis* had anti-HBsAg activity (Zheng and Zhang, 1990) but our study found anti-HBsAg activity in *Caesalpinia sappan* and *Gossypium herbaceum* not in *Litchi chinensis*. This difference maybe occur from the difference in part of use of medicinal plants or method. Chen et al. (1995) reported that *Saussurea lappa* showed anti-HBsAg activity against Hep3B cells (human hepatoma cells that produce and secrete HBsAg) and Yeh et al. (1993) reported that *Phyllanthus amarus* had anti-HBsAg activity against HepA2 cells (human hepatoma cells that produce and secrete HBsAg and HBeAg). *Phyllanthus amarus* and *Saussurea lappa* also showed anti-HBsAg activity against PLC/PRF/5 cells. The highest SI was found in *Saussurea lappa* (13.21), *Phyllanthus amarus* (4.67), and *Rhinacanthus nasutus* (3.85), respectively. The CC_{50} of *Litchi chinensis*, *Loranthus pentandrus*, and *Phyllanthus emblica* was over 2000 μg/ml. The IC_{50} of *Loranthus pentandrus* and *Phyllanthus emblica* was 486.74, and 555.26 μg/ml, respectively. So the SI of *Loranthus pentandrus* and *Phyllanthus emblica* should over 4.11 and 3.6, respectively, where as the other plants exhibited relatively low SI. These results showed that ethanol extract of *Loranthus pentandrus* and *Phyllanthus emblica* might have lower cytotoxicity than other ethanol extracts in this experiment.

Comparison of IC_{50} and SI between these medicinal plants showed that *Saussurea lappa* and *Phyllanthus amarus* were the most interesting plants for further study but the active ingredients with anti-HBsAg activity were already elucidated by

Huang et al. (2003) and Chen et al. (1995), respectively. Thus, *Derris scandens*, *Rhinacanthus nasutus*, and *Santalum album* extracts were further purified by extraction with hexane, chloroform, ethyl acetate and butanol.

The butanol fraction from *Derris scandens*, hexane fraction from *Rhinacanthus nasutus*, and hexane fraction from *Santalum album* had the lowest IC_{50} and the highest SI in each plant. The IC_{50} of these fractions were lower than their ethanol extracts where as SI were both lower and higher. There were many reports on the IC_{50} and SI of the compounds which exhibited *in vitro* anti-HBV acitivity. For example, robustaflavone, a naturally occurring biflavonoid isolated from *Rhus succedanea*, was found to be a potent inhibitor of HBV replication in 2.2.15 cells (HBV-transfected human hepatoma cells), with an IC_{50} of 0.25 μM (CC_{50}/IC_{50} = 1348) , and an IC_{90} of 2.2 μM (CC_{50}/IC_{90} = 153). (Zembower et al., 1998). FIAU [1-(2'-deoxy-2'-fluoro-1-beta-D-arabinofuranosyl-5-iodo)]inhibited viral replication in a human hepatoblastoma cell line with an IC_{50} of 0.90 μM, and displayed a CC_{50} of 344.3 μM, as determined using the MTT assay. The selectivity index of FIAU (CC_{50}/ IC_{50}) was 382.6 (Staschke et al., 1994). IC_{50} in 2.2.15 cells of BMS-200475,a carbocyclic 2'-deoxyguanosine analog, and lamivudine were 3.75 nM and 116.26 nM, respectively (Innamio et al., 1997). Costunolide and dehydrocostus lactone, derived from *Saussurea lappa*, suppressed the HBsAg produced by Hep3B cells with IC_{50} of 1.0 and 2.0 μM, respectively (Chen et al., 1995). Furomollugin and mollugin, naphthohydroquinones, were isolated from root of *Rubia cordifolia* could suppressed the secretion of HBsAg with IC_{50} of 2.0 μg/ml in human Hep3B cells (Ho et al., 1996).

Although medicinal plant extracts in our study were not showed IC_{50} and SI as good as robustaflavone, lamivudine, costunolide, dehydrocostus lactone and FIAU, but they were pure compounds and experiments did not performed in PLC/PRF/5 cells so we cannot directly compare with these results. In addition, the report about IC_{50} and SI of crude extract from medicinal plant was very rare.

The butanol extract of *Derris scandens* was partially purified with column chormatography. Fraction 29-35, 36-55, and Me had anti-HBsAg activity with IC_{50} of 48.36, 84.53, and 54.51 μg/ml, and SI of 1.88, 1.40, and 2.72, respectively. Fraction 56-

64 had IC_{50} over CC_{50}. Fraction Me was the most interesting fraction because this fraction showed the lowest IC_{50} and the highest SI. Fraction 28 seemed to express good anti-HBsAg activity but its CC_{50} was not determined because of very low yield. Chromatogram from TLC (Fig. 15b) showed one obvious spot in fraction 28 at Rf of 0.82. Both fraction 29-35 and 36-55 gave spots at Rf of 0.82 and 0.75, respectively. Thus, these two spots may possess an anti-HBsAg activity. Fraction 56-64 showed obvious spot at Rf of 0.66 (Fig. 15b) and their IC_{50} were more than their CC_{50}. Fraction Me spotted at Rf of 0.66 and at starting line. So anti-HBsAg of fraction Me should come from the spot at starting line.

The hexane extract of *Rhinacanthus nasutus* was partially purified with column chromatography. Fraction Me5-Me8 yielded the lowest IC_{50} and the highest SI. Fraction Me5-Me8 was further purified with gel filtration chromatography. Fraction 17-18 was the most interesting fraction with IC_{50} of 3.13 μg/ml and SI of 4.51. These fraction showed lower IC_{50} and higher SI compared with their ethanol extract (IC_{50} = 140.48, SI = 3.85). Chromatograms from TLC of fraction 13-16 and 17 – 18 were nearly similar. However, obvious different spot was luminous spot at Rf of 0.80 (detected with 365 nm ultraviolet) and spot at Rf of 0.69 (detected with 10% sulphuric acid) which found in fraction 13-16 but not found in fraction 17-18. Absence of these two spot in fraction 17-18 may cause decreasing of IC_{50}. There were reports about pure compounds in *Rhinacanthus nasutus* and at least 17 rhinacanthin compounds were isolated from *Rhinacanthus nasutus*. Fifteen compounds were napthoquinones and two compounds were lignans (Anna et al., 1996) (Tian et al., 1998) (Tian et al., 1998). The compounds, isolated from aerial root, were identified as two lignans, rhinacanthin-E, and rhinacanthin-F. They could exhibit anti-influenza virus activity but could not inhibit herpes simplex virus type2 (HSV-2) (Michael et al., 1997). Two naphthoquinones, rhinacanthin-C and rhinacanthin-D, exhibited inhibitory activity against cytomegalovirus (CMV) with EC_{50} values of 0.02 and 0.22 μg/ml, respectively (Anna et al., 1996). These indicated that many of rhinacanthin compounds could be found in *Rhinacanthus nasutus* and some of them exhibited antiviral activity. Thus, it was possible that rhinacanthin compounds might be responsible for *in vitro* anti-HBsAg in our study. In addition, we observed a red band

color while hexane extract of *Rhinacanthus nasutus* moved through column chromatography and while fraction Me5-Me8 moved through gel chromatography. The solution of fraction 17-18 which exhibited the lowest IC_{50} was also red-brown color. Napthoquinones such as rhinacanthin-H and rhinacanthin-I were red-brown oil. Rhinacanthin-J and -M were orange oil. Rhinacanthin-K and –L were red oil (Tian et al., 1998). Rhinacanthin-N and –Q were orange needles (Tian et al., 1998). Thus, it was possible that napthoquinone compounds might be responsible for *in vitro* anti-HBsAg in our study. However, an amount of fraction 17-18 of *Rhinacanthus nasutus*, fraction 28, and fraction Me of *Derris scandens* was very low and inadequate to perform more purification or tested for activity. Extraction of medicinal plants should performed with more than 2 kg (dry weight) of medicinal plants to obtained large quantity of extracts.

In our investigation, we partially purified butanol extract of *Derris scandens* and hexane extract of *Rhinacanthus nasutus* but the active ingredients for anti-HBsAg activity were not identified. Thus, further studies should be emphasized on the identification of the compounds which exhibited anti-HBsAg activity and on their chemical structure.