CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The investigation of the five medicinal plants and Benjakul preparation were based on the uses of these plants by Thai traditional doctors to treat cancer patients and use as adaptogenic drugs. The previous study found that at least four plants were reported to exhibit cytotoxic and anti-tumor activities but no research on Benjakul preparation has been done. Thus, the objectives of this research were to use *in vitro* bioassays to detect cytotoxicity of Benjakul preparation against four types of human cancer cell lines compared with a normal human fibroblast cell line. The isolation and elucidation of active compounds from the preparation were also studied. In addition, the chemical fingerprints of Benjakul preparation were investigated using high performance liquid chromatography for quality control and stability study of this preparation.

The results on cytotoxicity assay revealed that the ethanolic extract of the root of *Plumbago indica* showed the highest cytotoxic activity against COR-L23 (IC_{50} = 3.43 μg/ml) and the second most effective activity against Hela (IC_{50} = 8.71 μg/ml). The ethanolic extract of the fruit of *Piper chaba*, the stem of *Piper interruptum*, the rhizome of *Zingiber officinale* and Benjakul preparation showed the highest cytotoxic activity against COR-L23 with the IC_{50} value of 15.82, 18.40, 7.90 and 19.80 μg/ml, respectively. All extracts exhibited higher selective cytotoxicity on COR-L23 than on Hela, HepG2 and MCF-7, but showed less cytotoxicity to normal cell line (MRC-5). These results indicated that the ethanolic of Benjakul Preparation showed selective cytotoxic activity against lung cancer but little damage on normal lung cells. These results supported the goal of chemotherapy, which is to selectively kill as many cancer cells as possible and cause little or no damage to normal cells, thus promoting the use of this formula to treat cancer patients (Halliwell & Gatteridge, 1988).

Bioassay-guide fractionation was used to isolated pure compounds from Benjakul, and revealed that FC (Chloroform fraction) showed the highest activity against COR-L23 (IC_{50} = 7.3 μg/ml and % yield = 14.03%). Three compounds were isolated from ethanolic extract of Benjakul preparation namely piperine (BENS1),

plumbagin (BENS2) and 6-gingerol (BENS3), all of which possessed the most active cytotoxicity in this study. They were tested for cytotoxic activity against four types of human cancer cell lines (e.g. cervical, liver, breast and lung) and one type of normal cell line (MRC-5). All compounds showed a significant difference in cytotoxic effects between cancer cells and normal cells.

Of these three pure compounds, plumabagin exhibited potent cytotoxic activity against Hela, HepG2, MCF-7 and COR-L23 cells with IC_{50} values of 4.15, 2.61, 2.29 and 2.55 μM, respectively. Moreover, it showed the highest cytotoxicity against MCF-7 breast cancer cell line (IC_{50} = 2.29 μM) and also high selectivity for COR-L23 lung cancer cell line (IC_{50} = 2.55) since it had less cytotoxic effect on MRC-5 normal lung fibroblast cell line (IC_{50} =1.54μM). After the 72 h exposure time, plumbagin was found to exhibit the highest IC_{50} ratio of MRC-5 normal lung cells to COR-L23 lung cancer cells with a 4.5-fold difference, representing its preferential antitumor activity. Further, its cytotoxicity may be only specific to some lung cancer subtypes because a previous report found that plumbagin had only a low cytotoxicity effect on A549 lung adnocarcinoma, one of the three subtypes of non-small cell lung carcinoma (Acharya *et. al.*, 2008). Similar to plumbagin, Benjakul extract showed a 2.5-fold difference in IC_{50} between normal and cancer cells. Because plumbagin was obtained from Benjakul extract in a high percent yield of 4.18% w/w, it can be also used as a marker for chemical analysis of the ethanolic Benjakul extract (Dinda & Chel, 1992).

Piperine is a type of alkaloid which showed the highest percentage of yield of Benjakul preparation (7.81%) and was isolated from *Piper longum* (Wu *et. al.*, 2004) and many species of *Piper*. Piperine showed slightly cytotoxic activity against MCF-7 (IC_{50} = 35.72 μM) and COR-L23 (IC_{50} = 43.44 μM) but less activity against Hela (IC_{50} = 81.12 μM) and HepG2 (IC_{50} = 61.61 μM). It showed selective cytotoxic activity against breast and lung cancer cells with more than 6-fold difference in IC_{50} when compared with normal cells. Thus, piperine should be an active cytotoxic compound of Benjakul preparation. The previous report showed that piperine inhibited the solid tumor development in mice induced with DLA cells and increased the life span of mice bearing Ehrlich ascites carcinoma tumor to 58.8% (Sunila *et. al.*, 2004). Although piperine is a well-defined compound, its cytotoxic

mechanisms on cancer cells especially breast and lung cancer cells have not yet been examined.

6-Gingerol was isolated from *Zingiber officinale* (Kim *et. al*., 2008), which is one of the five components of Benjakul Preparation. It showed selective cytotoxic against breast and liver cancer cells (IC_{50} = 33.3 and 49.9µM, respectively). This result is consistent with the other previous report in which 6-Gingerol were cytotoxic against human cancer cells despite no report on its cytotoxicity against MCF-7, COR-L23, HepG2 and Hela (Kim *et. al*., 2008). Because 6-Gingerol showed a distinct difference in effect between cancer and normal cells, its possible cytotoxic mechanisms as an isolated compound should be investigated in parallel with the study on those mechanisms of Benjakul extract.

The results revealed that Benjakul preparation possessed high cytotoxic activity against lung cancer cells. Its ethanolic extract should be promoted for industry. Piperine and plumbagin should be used as markers for standardization of chemical and biological fingerprints because piperine was present in high amount as well as plumbagin showed the highest cytotoxic activity.

A reverse-phase high performance liquid chromatographic (RP-HPLC) procedure was used for studying chemical fingerprint of the ethanolic extract of Benjakul preparation. The method was validated and showed good linearity, precision, accuracy and recovery. The calibration curves were linear over the ranges of 50-400 µg/ml for piperine and 25-200 µg/ml for plumbagin, respectively with r^2>0.999. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.80 and 2.66 µg/ml for piperine and 0.22 and 0.75 µg/ml for plumbagin, respectively. The precision of the HPLC method for determining piperine and plumbagin, confirmed by both intra- and inter-day analysis, were higher than 93%. All the coefficient variations for piperine and plumbagin were less than 2%. The accuracy of the method for piperine and plumbagin were studied by spiking standard piperine and plumbagin into the ethanolic extract of Benjakul preparation. The percentage recoveries for piperine and plumbagin were found to be ranging from 93.68 to 100.92%, with 0.20-1.36% of coefficient variations. These results demonstrated that the proposed method has good precision and accuracy.

The stability of the ethanolic extracts of Benjakul preparation was evaluated under 45±2°C with 75±5% RH as an accelerated condition by determining contents of piperine and plumbagin using HPLC methods. The results of stability testing showed that the amount of piperine was slightly reduced from 47.61 mg/g (100%) at day 0 to 45.03 mg/g (84.91%) at day 120 but plumbagin was more quickly reduced. At day 0, the amount of plumbagin was 2.46 mg/g (100%) and reduced to 0.71 mg/g (25.74%) after day 120, indicating that plumbagin was unstable. These results illustrated that the amount of plumbagin was significantly reduced under high temperature due to its low melting point of 78-79° C. Therefore, plumbagin can be evaporated more easily than piperine. In the field of herbal drugs (HD), their preparations (HDP) and herbal medicinal products (HMP), if the constituents with therapeutic activity are not known, a limit of 91% of the initial assay value content has recently been accepted (Xie *et. al.,* 2007). The stability results indicated that the extract could be stored for at least two years without loss of activity, which met the standards of The Institute of Medical Sciences, Ministry of Public health of Thailand (Ungpaiboon *et. al.,* 2005). However, the ethanolic extract of Benjakul should be also tested for cytotoxic activity because the content of ingredients may change with time but biological activity may not change. Thus, the stability study in which Benjakul extract was tested for its stability by determining its cytotoxic activity should be performed in the future. This result suggested that the best storage condition for the ethanolic extract of Benjakul preparation are a super freezer (-70°C) to store for a long time.

In summary, Benjakul as a Thai traditional medicine which was normally used to be adaptogen for cancer treatment showed selective cytotoxic against lung and breast cancer. Three compounds were isolated from the ethanolic extract of Benjakul preparation namely plumbagin, piperine, and 6–gingerol, all of which also showed selective cytotoxic activity against cancer cells. Therefore, the present study supports the use of Benjakul to treat cancer by Thai traditional doctors.

Because of its high cytotoxic activity, the ethanolic extract of Benjakul preparation should be further developed to strengthen its cytotoxic effects for cancer treatment or prevention. The molecular mechanism of isolated compounds from Benjakul extract *in vitro* model should be investigated. However the ethanolic extract

of Benjakul preparation should be studied extensively in animal models for antitumor-promoting, immunomodulatory and chronic toxic effects because there have been no reports on these aspects in animal models. Also, the stability of the ethanolic extract of Benjakul preparation in long term condition should be evaluated by determining marker contents and cytotoxic activity as well. Preformulation study of this extract should be performed before the development of any pharmaceutical or health products in future.