CHAPTER V
DISCUSSION AND CONCLUSION

5.1 Preventive activities of *P. mirifica* and *B. superba* in DMBA-induced rat carcinoma

Regarding the preventive activity of *P. mirifica* pre-treatment to DMBA-induced rats, it was found that *P. mirifica* at the doses of 10, 100, 1000 mg/kg BW had an inhibitory effect on the latency period of mammary tumor development with 2-3 weeks delayed period. The significant preventive action on mammary tumor was clearly seen in the treatment with the highest dose (1,000 mg/kgBW) of *P. mirifica*, with the reduction amount of 27.0% of the tumor incidence. The number of developed tumor was 32.20% reduced in the rats pre-treated with 1,000 mg/kgBW *P. mirifica*. The multiplicity of tumor at the end of the experiment was 46.87% reduced. Nevertheless, there was no change in the localization of the first occurred tumor. At the end of the experiment, the mammary tumor was found was mainly localized at the 2nd and 3rd mammary gland in the control group. Histopathology analysis revealed that the rats pre-treated with 1,000 mg/kgBW exhibited no papillary pattern of the tumor tissue as did found in the control group. It is thus a clearly demonstrated that the plant could significantly reduce the severity of the induced tumor.

The result of ER-analysis by immunohistochemistry revealed that the plant had effect on the ERα amount. The ERα was 72.19% reduced and the ERβ was 44.52% reduced as compared with the control group at the treatment of 1,000 mg/kgBW. It was found that the ERα/ERβ ratio was shift from 0.84 (33.37/39.75) to 0.42 (9.28/22.05). The result is similar to that of soy treatment in induced breast cancer rats (Gallo et, al. 2002). The reduction in ERα/ERβ should be a sign of protective effect to breast cancer as it had been shown that the developed breast cancer exhibited higher ratio of ERα/ERβ (Gustafsson et., al. 1999). It was also found that loss of ER-β expression could be one cause of breast cancer (Lazennec et al., 2001). The high level of ER-β was also associated with poor differentiation of breast cancer (Park et al., 2003).

Rats receiving the highest concentration of *P. mirifica* not only developed a lower mammary tumor number, tumor size and mass per animal than other treated

groups but also the control group. Thus the highest dose of phytoestrogen in *P. mirifica* showed protective effect as did by other phytoestrogens (Hilakivi-Clarke et al., 1999[a]; Barnes et al., 1994; Constantinou, Mehta, and Vaughan, 1996) and / or phytoestrogen containing plant (Lamartiniere et al., 1995b, Murrill et al., 1996).

It had been previously demonstrated for the *in vitro* anti-proliferative effect of the high dose of *P. mirifica* to MCF-7 cells. The result was more effective in the supplement estrogen environment (Cherdshewasart et, al. 2004[a]). The result is similar to what had done *in vivo* in this experiment in the sense that *P. mirifica* treatment to breast cancer cells is most effective only at the highest dose of treatment. This phenomenon confirms that phytoestrogen from *P. mirifica* initiated both *in vitro* and *in vivo* effective competitive binding to ERα with estrogen only in the condition of high amount. Besides, the strong competitive binding to ERα is a main cause of protective *in vivo* did by the plant. There was a demonstration in *P. mirifica* treated rats that consumption of *P. mirifica* at the same high dose as did in this experiment (1,000 mg/kgBW) could result in disruption of estrogen activity by reducing the estrogen amount present in serum (Malaivijitnond et.al., 2004,). This may be one of the reason that the development of breast tumor mass and number was inhibited partially not totally in the treatment with high dose of *P. mirifica*.

The consumption of *P. mirifica* in ordinary women is thus an alternative to soy for the purpose of prevention of breast cancer. There had been a popular consumption of *P. mirifica* in Thai menopausal women in the past not in the present. It is a reason why there is no possibility to create a research on breast cancer protective roles of this plant as there had been done in soy consumption in Japanese population (Markies et al., 1998 and Adlercreutz, 1999). It should realize that *P. mirifica* treatment at a high dose, 1,000 mg/kgBW in monkeys which have a similar estrous cycle with human resulted in prolongation of the menstruation period (Trisomboon et., al. 2004[a]) and could block human ovulation (Trisomboon et., al. 2004[b]). Thus the consumption of *P. mirifica* for breast cancer protective purpose should be done with a long-term period and with the low to medium dose, not high dose, to avoid such effect.

B. superba treatment resulted in an one week delay of tumor development at the dose of 10 and 1,000 but not at the dose of 100 mg/kgBW. Only the dose of 10 mg/kg resulted in reducing the size of developed tumors but no effect on the

number of developed tumor. This dose also exhibited no papillary pattern of tumor tissue as did in the control group. The dose of 1,000 mg/kgBW showed a reduction of 25.40% in multiplicity of the induced tumor. The dose of 100 mg/kgBW initiated promotion effect to the tumor size. The effectiveness of preventive effect to mammary tumor initiated by *B. superba* is therefore not in a dose dependent manner and is not much effective as did by *P. mirifica*. The main active ingredients in the plant were found to be flavonoid and flavonoid glycoside with strong inhibitory effect to cAMP phosphodiesterase (Roengsamran et., al. 2000). That activity leads to a promotion of vasodilation. The preventive effect to breast cancer of the plant is thus not related with phytoestrogen. In the rat uterotrophic assay, the treatment with 40 mg/kg BW of *B. superba* extract caused the significant increase of the vagina weight but no response for uterus weight (Kim et. al., 2003). It implies that if the plant contains phytoestrogen, it may contains few amount or not include the one with a strong estrogenic effect. In the contrary, the promotion effect to breast tissues at the dose of 100 mg/kgBW may caused by the optimum blood supply to cancer tissues cause by vasodilation.

5.2 Anti-tumor activities of *P. mirifica* and *B. superba* in DMBA-induced rat carcinoma

For tumor induction using a single intragastric dose of 80 mg/kg BW DMBA, there was no death evidence of toxicity after administration and all tumors were found during the fourth to the tenth weeks of the experiment. Following the further treatment of 10, 100, and 1000 mg/kgBW *P. mirifica*, no death was found. These results are in agreement with the published plant toxicity tests (Chivapat et al., 2000 and Cherdshewasart, 2003). The ovarian weight was found normal. The uterine weight was significantly higher only in the PM-100 group. This should not have resulted from the *P. mirifica* treatment, as the withdrawal period was approximately 10 weeks before necropsy. In carcinogen-fed rats, the onset of the first tumor nodule was detectable by palpation as early as 4 weeks after DMBA administration. The induction period of this study was shorter than that seen with dosages of 12-20 mg DMBA, which was 3-4 months (Melby and Alman, 1974). DMBA-induced mammary cancer was predominately adenocarcinoma that was similar to its human counterpart. Upon necropsy, an effect of *P. mirifica* on tumor location was found in all cases. The thoracic and cervical regions were found to have more tumors than the abdominal region, and the distributions on the right and left sides of the abdomen

were found to be different. This result is consistent with the protective effects of soy protein isolate and bovine whey (Baggott et al., 1990).

With regard to anti-tumor activity, it was found that *P. mirifica* in a dose of 1000 mg/kg BW had an inhibitory effect on the multiplicity of mammary tumors which was clearly seen at weeks 17^{th} –20^{th} week after DMBA administration. The significant antiproliferative action on breast cancer cells in this study led to a 34.60 % decrease in tumor numbers. The anti-breast cancer effect occurred only at the highest treatment dose of *P. mirifica* and was based mainly on the disruption of ERα estrogen activation of the induced mammary tissue, and partly on the metabolism of DMBA, as previously reported in the case of phytoestrogen (Wang et al., 1996) and confirmed in the MCF-7 test (Cherdshewasart et al, 2004). The proliferative effect on MCF-7 cells of a low dose of the plant extract confirmed that phytoestrogen in this plant could bind effectively to ERα and promote an estrogenic response at the same level as did by estrogen. At high dose, the plant extract in combination with a physiological dose of estrogen exhibited a strong competitive binding to ERα and resulted in a stronger anti-proliferative effect on MCF-7 cells. The results of that study, in combination with our results, demonstrate that a high dose of *P. mirifica* has the strongest anti-proliferative effect on induced breast cancer cells, through strong disruption of estrogen binding to ERα in the developing breast cancer cells. *P. mirifica* may also cause other hormonal disruptions, as hormones act as key regulators of mammary proliferation (Hakkak et al., 2000 and Baggott et al., 1990). An effect of *P. mirifica* on the secretion of gonadotropins in rats has been reported (Malaivijitnond et al., 2004). Phytoestrogens show a preference for binding ERβ (Gutendorf and Westendorf, 2001). In breast cancer, high levels of ERβ are associated with poor differentiation. A paradoxical effect was also found (Tanos et al., 2002), however. Genistein (1-10 μg/ml) inhibits the growth of dysplastic and malignant epithelial breast cancer cells *in vitro*. The ER does not modulate these effects.

The anticancer activity of *P. mirifica* has now been evaluated against two systems, the *in vitro* MCF-7 cell culture proliferation assay (Cherdshewasart et al., 2004) and, in this study, the *in vivo* rat mammary induction model. The plant was administered in the early 4-week period when the tumors were still small in size. Even though the plant treatment was discontinued throughout the later stages of the experiment, the tumors were always at remote sites. The results suggest that the

inhibition of an existing tumor by the plant produces a local anti-tumor effect in that tumor bed that persists after PM treatment is ceased. During the early treatment period, it was demonstrated clearly for the first time that consumption of phytoestrogen could inhibit the proliferation of pre-existing breast cancer, as it was found that the size of the tumors was decreased. Tumor development was interrupted and resulted in a reduction of tumor size during the 5^{th} –7^{th} week in the PM-1000 rats. Later, this group was not different from the others. This effect might derived from a strong competitive binding to ERα as the period during which a difference was detected overlapped with the period of daily feeding with *P. mirifica*.

In the present study, the same batch of plant material was used as in the previous studies in MCF-7 cell cultures (Lee et al., 2002) The plant contains significant amounts of isoflavonoid as analyzed by HPLC and could exhibit estrogenic effects (Chansakaew et al., 2000). Isoflavonoids in the plant might play an anti-tumor role, as found in the treatment with genistein (Tanos et al., 2002). There are indications that genistein has anti-promoter or therapeutic potential at the beginning of, and/or during carcinogenesis (Tanos et al., 1994 and Barnes, 1995).

It has been suggested that the activity of the soy extract constituent in tumor inhibition was greater than that of genistein provided in the diet alone. The mixtures of isoflavones or other components of the soy extract, such as saponins, in combination with genistein may contribute to a greater cancer inhibitory action of the soy extract (Hewitt and Singletary, 2003). A hundred grams of dry *P. mirifica* powder contains various phytoestrogens, including 169.1 mg of total isoflavone (Muangman and Cherdshewasart, 2000).

Even though the daily oral administration of *P. mirifica* at dosages of 10, 100, and 1000 mg/kg BW for 28 days in adult Sprague-Dawley rats did not cause any observed toxicity, a decrease in survival in treated rats was recorded judging from the diameter of tumor burden reaching the termination criteria.

Although the present knowledge does not allow for the elucidation of the precise mechanism by which *P. mirifica* powder exerts its therapeutic action *in vivo*, it might depend on its action on the hormone regulatory system.

Our result demonstrates clearly that consumption of phytoestrogens from *P. mirifica* can strongly decrease the evidence of developing breast cancer after induction with a specific carcinogen in the rat. The result is similar to the tumor reduction reported in adult Sprague-Dawley rats fed a 10 g/kg diet of fermented soymilk (Ohta et al., 2000) or a 100 g/kg diet of miso (Hakkak et al., 2000). It thus serves as a first experiment to determine the efficacy of *P. mirifica* in breast cancer treatment. Treatment with phytoestrogens from *P. mirifica* also created a difference in the subcellular organization of the tumor mass. Interestingly, both malignant and benign tumor was observed in rats that received high doses of *P. mirifica*, while the others displayed only malignancy. The plant may interfere with tumor development and result in the production of less aggressive tumor types. In rats, it is probable that a fast-growing tumor will show at least one criterion of malignancy. This might be the reason why the growth of the tumor mass is lower at high dose of *P. mirifica*.

Although the precise mechanism by which *P. mirifica* inhibits mammary cancer occurrence requires further clarification, the present results may have important implications for public health. Note that such results have been obtained from *P. mirifica* treatment but never after treatment with soy. It can thus be concluded that *P. mirifica* contains high amounts of phytoestrogens with stronger estrogenic effects than soy phytoestrogens. The consumption of *P. mirifica* may be more beneficial than consumption of soy for the purpose of phytoestrogen replacement therapy, especially during menopause. The ordinary daily dose consumed by the native Thai people is 2-4 mg/kg BW (Muangman and Cherdshewasart, 2001 and Lermlertkittikul and Chandeying, 2004). The dosages of 10, 100, and 1000 mg/kg BW *P. mirifica* in rats are 2.5-5, 25-50, and 250-500 times the amount that is consumed by a person, respectively. In a therapeutic regimen, the plant may be consumed for a short period of time and adverse effects may be mild. In addition, treatment with the plant extract at a specific area may be a possible alternative route and more effective, with fewer systemic adverse effects.

The rats' body weights were significantly different during some weeks. We noticed that the growth rate increased rapidly in the early period. This may be related to the early growing phase of the rats (Melby and Alman, 1974). On the day of necropsy, the body weight gains of the PM-100 and PM-1000 groups were greater than the weight gain of the control. This result is not consistent with a sub-chronic study in which 100 and 1000 mg/kg BW *P. mirifica* was administered to rats. A slower growth rate and less appetite were found, compared with the control (Chivapat et al.,

2000). During the period of *P. mirifica* administration (28 days), there was no substantial effect on survival. However, the survival rate of the PM-1000 group was also significantly lower than the control survival rate.

It was found that the result of ER-analysis by immunohistochemistry revealed that the plant had effect on the ERα amount. The ERα was 66.35% reduced and the ERβ was 23.63% reduced as compared with the control group at the treatment of 1,000 mg/kgBW. It was found that the ERα/ERβ ratio was shift from 0.83 (37.18/44.98) to 0.36 (12.51/34.35). It was noticed that the changing of ERα, ERβ amount and ERα/ERβ ratio is less than the pretreatment experiment. It implies that pretreatment at weanling stage could affect more to the expression of ERα and ERβ genes and their products. The application of *P. mirifica* for anti-breast cancer purpose is thus recommended to be pretreatment at weanling stage rather than treatment after the body developed the first breast tumor at a mature stage.

To our knowledge, this is the first demonstration of the anti-tumor efficacy of *P. mirifica* in specific carcinogen-induced mammary tumors. The carcinogenic effect of DMBA can be significantly suppressed but not totally eliminated. The results of this study should be applied to promote the daily consumption of *P. mirifica* for the purpose of inhibiting breast cancer.

In term of therapeutic application, *B. superba* showed no any effect. In the contrary *B. superba* at the dose of 10 mg/kgBW increased the tumor size for 4-5 weeks during the tumor development and also resulted in the increasing tumor mass at the end of the experiment.