# Chapter II

# Literature review

*P. mirifica, B superba, M. collettii and P. lobata* were classified into the family of Leguminosae (Ridley, 1967; Pengklai, 1977; Savatti, 1978; Starr, Martz and Loope, 1999) The local name for these plants was varied in various part of Thailand as shown in **Table 1**

**Table 1** Common name of *P.mirifica, B. superba, M collettii* and *P. lobata* in various part of Thailand (Qicheng, 1980 ;Panriansaen, 2000; Smitinand, 2001)

| Species | Common name | Location |
|---|---|---|
| *P. mirifica* | - Tong Kwao, Thong Krua, Hua Kwao, Tan Krua, White Kwao Krua, Chan Krua | - Northeast Thailand |
| | - Potagu, Thao Thong | - Kanchanaburi |
| *B. superba* | - Thong Khrua, | - Central Thailand |
| *M. collettii* | - Tao Hom, Haen Hao Hon | - Loei |
| | - Beng-ke | - Kanchanaburi (Karen) |
| | - Maba Maeng | - Chiengmai |
| | - Yang Dam | - Nakhornratchasima |
| | - Saba Ling | - Kanchanaburi |
| | - Saba Ling Dam | - Central Thailand |
| | - Mak Ba Luem Dam | - Sukhothai |
| *P. lobata* | - Kudzu | - Japan |
| | - Ge-gen | - China |
| | - Pak Peep Phee | - Chiengmai province |

## I Botanical background and chemical constituents

### *1.1 P. mirifica*

The plant was a long live twining wood. The leaves were pinnately 3 foliate; terminal leaflet. The tuberous roots were varied in sizes and shapes. The flower was bluish-purple typically legume shaped. Flowering occurred during late January to early April. The flower contained five petals and the petals were one stand with two keels. The pod was slender typically short or elongate, hairy or smooth, including 1-10 single seeds when fully matured and dried which turn into various color. (Smitasiri and Wungjai, 1986 ; Cherdshewasart unpublished data)

*P. mirifica* contained at least 20 chemicals which were categorized into a group of phytoestrogens. The first isolated chemical named miroestrol (Bound and Pope, 1990) was believed to be the most active compound, found in approximately 15 mg/kg of the dried tuber. Miroestrol was found to act as estrogen evens the chemical structure was not steroid. (Benson, Cowie and Howsking, 1961). Miroestrol was recently claimed to be an artifact of deoxymiroestrol, an unstable compound. (Chansakaow *et. al.*, 2001). The other compounds, mainly found in *P. mirifica* were coumarins, isoflavones, chromenes, sterols and macromolecule such as protein, lipid and starch. The list of found chemicals in *P. mirifica* tuberous root was shown in **Table2**

*P. mirifica* was interesting not only in term of research but also in term of product development. The tubers were long-term consumed in Thailand as traditional remedies (Suntara, 1931). At least 5 submitted patents were already applied for *P. mirifica* in varied aspects from cosmetics to a drug for anti-menopausal treatment. (Hirose, Katayama and Hirata; 2000; Sanrittowanicha; 2000; Hoshino, Sakata and Higuchi, 2001; Ishikawa and Sekine, 2001; Moriyama, Hoshino and Higuchi, 2001)

**Table 2.** Summary of the chemical constituents of *P. mirifica*
(After Panriansaen, 2000)

| Category | Chemical | Reference |
|---|---|---|
| Coumarin | Coumestrol | Ingham, Tahara and Dziedzic, 1986, 1988 |
| | Mirificoumestan | Ingham, Tahara,and Dziedzic, 1988 |
| | Mirificoumestan glycol | Ingham, Tahara,and Dziedzic, 1988 |
| | Mirificoumestan hydrate | Ingham, Tahara,and Dziedzic, 1988 |
| Isoflavone | Daidzein | Ingham et. al., 1986 |
| | Daidzin (daidzein-7-o-glucoside) | Ingham, Tahara,and Dziedzic, 1986 |
| | Genistein | Ingham, Tahara,and Dziedzic, 1986 |
| | Genistin (genistein-7-o-glucoside | Ingham, Tahara,and Dziedzic, 1986, 1989 |
| | Kwakhurin | Ingham, Tahara,and Dziedzic, 1986 |
| | Kwakhurin hydrate | Ingham, Tahara,and Dziedzic, 1989 |
| | Mirificin (puerarin 6"-o-β-apiofuranoside) | Ingham, Tahara,and Dziedzic, 1986<br>Ingham et. al., 1986 |
| | Puerarin (daidzein-8-glucoside) | Nilandihi et. al., 1957,<br>Ingham, Tahara, and Dziedzic, 1986, 1989<br>Ingham et. al., 1986 |
| | Puerarin 6"-monoacetate | Ingham et. al., 1989 |
| Chromene | Miroestrol | Schoeller, Dohrn and Hohweg, 1904<br>Bound and Pope, 1960<br>Jones and Pope, 1960 |
| | Deoxymiroestrol | Chansakaew et. al.,2000 |
| Sterol | β- sitosterol | Hoyodom, 1971 |
| | Stigmasterol | Hoyadom, 1971 |

### *1.2 P. lobata*

*P. lobata* was a climbing, soft, perenial vine. The deciduous leaves were alternate and compound with three broad leaflets up to 4 inches across. Hairy leaflets may be entired or deeply 2-3 lobed. Individual purple flower was about ½ inch long. It was highly fragrant and borne in long hanging clusters. Flowering occured in winter (December in Thailand, Cherdshewasart unpublished data) The pod was brown or black, mild hairy, rod-shaped seedpod. Each of the seedpod contained three to ten hard seeds. Flowers were in blue or purple similar to that of *P. mirifica*, standard with a yellow central patch, 15-25mm long keel darker in color. Pods were 4-13 cm long. Seeds were reddish brown with black mosaic, ovoid to ellipsoid, slightly laterally flattened, 4-5 mm long (after Starr, Martz and Loope, 1999; Cherdshewasart unpublished data)

The tuberous root of *P. lobata* was reported to contain high isoflavone including puerarin (Gurerry *et. al.*, 2000), daidzein (950 mg/kg dry weight) genistein (<400mg/kg dry weight) (Kaufman *et. al.*, 1997) and daidzin (Li and Lin, 1998). The recent studies of *P. lobata* were emphasized on the effects on cardiovascular system and alcoholism treatment. Isoflavonoid glycoside showed excellent clinical results in the treatment of hypertension (Qicheng, 1980) There were evidences that puerarin which was also abundant in the plant could reduced the alcohol absorption from the gastrointestinal system (Carai *et. al.*, 2000) In addition, puerarin acted as a beta-adenoreceptor antagonist in isolated arteries and vein (Wang, Zhao and Chai, 1994), reduced blood flow velocity in cerebrum (Chen *et al.*, 1995) and play an important role in regulating the imbalance of endothelin, renin activity and angiotensin II in acute myocardial infraction patients (Li, Liu and Chen, 1997)

The other two isoflavone, daidzin and daidzien, could suppress voluntary alcohol consumption in alcohol-preferring rat ( Lin *et. al.*, 1996; Lin and Li, 1998) and ethanol preferring golden Syrian hamsters (Keung and Vallee, 1998).

Not only developed for medical applications, a patent was also submitted in the application of the plant product for ample breast that was similar to the claim derived from *P. mirifica.*

### *1.3 B. superba*

*B. superba* was a large size climber, with thick stem at a size of a man's leg. The leaflet was acuminate chartaceous. The flowers were large with gorgeous orange color. The peticels were three time longer than the calyx. The pods were 3-4 inches long, oblong shaped with silvery silky short hair. (Kruz, 1877; Brandis, 1990) and only one seed was present (Cherdshewasart, unpublished data)

*B. superba* tuberous root was found to contain 5 groups of chemical constituents namely: carboxylic acid, steroid, steroid glycoside, flavonoid and flavonoid glycoside (**Table 3).** The flavonoid glycoside could inhibit cAMP Phosphodiesterase activity (Roengsamran et. al., 2000). β-sitosterol, Campesterol and stigmasterol were reported to be the anti-inflamatory agent, enhancing activity of T-Helper-1 (TH-1) cell, reduce blood sugar by increase insulin secretion (Altern Med., 2001). β-sitosterol, Campesterol and stigmasterol might offer protection and treatment for the common cancers (Awad and Fink, 2000) such as colon (Awad, Chen, Fink and Hennessey, 1996; Normen *et. al*,, 2001), prostate (von Holtz, Fink and Awad, 1998; Awad, Fink, Wiliam and Kim, 2001) and breast cancer (Awad, Downie, and Fink, 2000; Awad, Downie, Fink and Kim, 2000; Awad, Williams and Fink, 2001).

**Table 3.** Summary of the chemical constituents of *B. superba* (Raksilpa, 1995; Yavada and Reddy, 1998; Roengsamran *et. al.*, 2000)

| Category | Chemical |
|---|---|
| Carboxylic acid | Straight chain carboxylic acid ($C_{22}$-$C_{26}$) |
| Steroid | Campesterol, stigmasterol, β-sitosterol |
| Steroid glycoside | β-sitosteryl 1-3-o-β-D-glucopyranoside, Stigmasteryl 1-3-o-β-D-glucopyranoside |
| Flavonoid | 3, 7, 5'-trihydro-4'-methoxyflavone |
| Flavonoid glycoside | 3-5'-dihydroxy-4'-methoxyflavone-7-o-β-D-glucopyranoside |

***1.4 M. collettii***

*M. collettii* was a large woody climber, 30-40 m height scattered by stems in evergreen forest. The leaves were trifoliate; leaflets 4-8 by 2-4 inches sparsely hairy, entire margin; petiole 5-10 cm long, base stout. The flowers were hanging on the stem up to 12 inches long with 5 sepals covered with brown rough hair and unite into a bell-shaped tube. The petals were blackish-purple pea-like shaped. The stamens were two bundles. The pods were linear-oblong shaped up to 16 inches long. The seeds were hard and flattened. The flowers were blooming during January-March. (Pengklai, 1977)

The whole stem of *M. collettii* was found to contain 3 interested chemical constituents namely: Kaempferol, Quecertin and Hopeaphenol. The median inhibitory concentration ($IC_{50}$) for cAMP Phosphodiesterase inhibiting effect were found to be 281.83, 80.91 and 22.75 μg/ml, respectively (Roengsamran *et. al.*, 2000). The effect of phytoestrogens, kaempferol and qucertin, on mammary cancer cell culture was analized conducted. Hopeaphenol was reported to be highly cytotoxic on KB cell line (an epidermal carcinoma of the mouth) with $IC_{50}$ value of 1.2 μg/ml (Ohyama *et al.*, 1999). The *in vivo* study indicated that *M.collettii* affected the reproductive system by causing the abnormality of spermatozoa (Wutteeraphon *et. al.*, 2001)

## II Phytoestrogens

Phytoestrogens were plant-derived compounds with estrogen-like bioactivity. The compounds could regulate gene expression mediated by an estrogen responsive element (ERE), in a manner either agonistic or apparently antagonistic to 17β-estradiol, as a result of binding to estrogen receptor (ER). (Clarke *et. al.* 1996; Murkies, Wilcox and Devis, 1998; Stahl, Chun and Gray, 1998). The use of some plants in traditional medicine and remedy could show evidence on their estrogenic effect. As described above, *P. mirifica* was used as a rejuvenant and aphrodisiac purpose (Murkies, Wilcox and Devis, 1998) as well as a crude drug for memopause symptom treatment (Muangman and Cherdshewasart, 2000). The consumption of diets containing large amount of phytoestrogens, such as soybean and its products, were associated with a lower risk of cancer and cardiovascular disease (Messina *et. al.*, 1994).

Phytoestrogens were classified into 3 main classes; namely isoflavones (e,g. genistein, daidzein) coumestans (e.g. coumestrol) and lignans (e.g. enterolactone) A single plant often contained more than one class of phyrtoestrogens for example soybean was found to be rich in isoflavone (genistein and daidzein) while its sprout was a potent source of coumestan (coumestrol) (Murkies, Wilcox and Devis, 1998 and USDA, 1999)

### 2.1 Sources of phytoestrogens

Phytoestrogens could be found in various plants including beans, peas, clover sprouts, alfalfa seeds, flaxseeds and tea (USDA, 1999) or even in cabbage. (Ju *et. al.*, 2000). The most famous source of phytoestrogens was soybean with high content of genistein and daidzein. *P. mirifica* was also reported to contain high amount of isoflavones . (Cherdshewasart, unpublished data)

### 2.2 Effects of phytoestrogens on cancer

Phytoestrogens, especially isoflavonoids, were considered as anti-tumor agent. They might reduce the risk of development of many cancer types such as breast cancer. (Pagliacci *et. al.*, 1994: Barnes *et al*, 1996: Peterson *et. al.*, 1996 : Zawa and Duwe, 1997 : Constantinou *et. al.*, 1998), colon cancer (Agullo *et. al.*, 1996; Deschner *et. al.*, 1991; Kou, 1996; Kou, Morehouse, and Lin,, 1998; Pool-Zobel *et. al.*, 2000), lukemia (Constantinou *et. al.*, 1990), thyroid cancer (Horn-Ross, Hoggatt and Lee, 2002), prostate cancer (Mitchell, Duthie and Collins, 2000; Steiner, Raghow and Neubauer, 2001; Strom *et. al.*, 1999), cervical cancer (Wang *et. al.*, 2001) and uterine cancer (Newbold *et. al.*, 2001). Due to the fact that estrogen was related to breast tumor growth (Shafie, 1980) and the consumption of soya phytoestrogens decreases ovarian hormones (17β-estradiol and progesterone) (Lu *et. al.*, 2000). The case-control study in breast cancer patients showed that the increased excretion of some phytoestrogens was associated with a substantial reduction in breast cancer risk (Ingram *et. al.*, 1997).

The effects of phytoestrogens on breast cancer were mostly emphasized. Many phytoestrogens could not only inhibit the proliferation of the estrogen receptor positive ($ER^+$) mammary cancer cell line, MCF-7 and T47D but also showed the proliferation effect. (as summarized in **Table 4**).

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines.

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Genistein** | MCF-7* | not described | - Relative binding affinity to SBG = 27% compared with $E_2$<br>- Markedly enhance tumor cell proliferation.<br>- Competed with $E_2$, resulted in rapid ER decrement. | Martin *et. al.*, 1978 |
| | MCF-7* | 0, 1, 5, 50, 500 μM/L | - Inhibited cell growth in dose dependent manner<br>- $ID_{50}$ = 4μM/L after 72 hr. of incubation.<br>- The cell exhibited DNA content decrement and nuclear fragmentation characteristic of apoptosis. | Pagliacci *et. al.*, 1994 |
| | MCF-7* | $10^{-9}$ –$10^{-5}$ M | - Stimulated growth at lower concentrations ($10^{-8}$-$10^{-6}$M) but inhibited growth at higher concentrations (>$10^{-5}$)<br>- Compete with $E_2$ for binding to ER. | Wang *et. al.*, 1996 |
| | | Genistein + Estradiol | - The expression of *pS2* was lower than each compound treatment. | |

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines. (Continued)

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Genistein** (continued) | MDA-MB-231** | $10^{-9}$-$10^{-5}$ M | - Inhibited growth at high concentration ($>10^{-6}$)<br>- The expression of *pS2* was lower than each compound treatment. | Wang *et. al.*, 1996 (continued) |
| | MCF-7* | 0, 0.01, 0.1, 1, 10, 50, 100 μM | - Showed biphasic effect<br>- Induced DNA synthesis at 0.01-10 μM but inhibited DNA synthesis at >10μM<br>- $IC_{50}$ = 41 μM<br>- Showed continuous DNA synthesis stimulation at low concentration (10 days treatment) | Wang and Kurzer, 1997 |
| | MDA-MB-231** | 0, 0.01, 0.1, 1, 10, 50, 100 μM | - Inhibited DNA synthesis at high concentration with no stimulation shown at any concentration<br>- $IC_{50}$ = 26.7 μM | |

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines. (Continued)

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Genistein** (continued) | MCF-7* | 1, 10, 100 nM 1, 10, 100 μM | - Stimulated growth at 1 nM – 10 μM but inhibited growth at >10 μM<br>- The maximal growth stimulation (0.1 –1 μM) was equal to that of estradiol at 1 nM.<br>- *pS2* level in the growth medium was rose steadily (in dose dependent manner) and peaking at 20 μM | Zava and Duwe, 1997 |
| | MDA-MB 468** and HMEG** | 1, 10, 100 nM 1, 10, 100 μM | - genistein had little effect or was slightly growth inhibition at10 nM – 1μM | |
| | T47D* | 1, 10, 100 nM 1, 10, 100 μM<br><br>100 nM-20μM<br><br>1, 10, 100 nM 1, 10, 100 μM + 0.3 nM $E_2$ | - Increased growth from 10nM to 10 μM but then inhibited at >20 μM<br><br>- Markedly inhibited cell growth at 20 μM<br><br>- Genistein had little effect on the growth-promoting effects of 0.3 nM $E_2$ over the concentration range from 0.3-10 nM ( but was slightly inhibitory to $E_2$ action from 80-300 nM | |

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines. (Continued)

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Genistein** (continued) | T47D* | 1, 10, 100 nM 1, 10, 100 μM + 1 mM TAM | - The dose-response curve was shift 1 log to the right (from genistein only curve) | Zava and Duwe, 1997 (continue) |
| | | 1, 10, 100 nM 1, 10, 100 μM + 100 nM HTAM | - The dose-response curve was shift 2 log to the right (from genistein only curve) | |
| | MCF-7* | 0-100 μ mol/L | - Stimulated growth at low concentration (5 μmol/L) but inhibited growth at higher concentrations in dose dependent manner.<br>- $IC_{50}$ = 31 μ mole/ L<br>- Caseine, lipid and the membrane protein ICAM1 were optimally expressed after the treatment.<br>- The cells became differentiated in response to the treatment. | Constantinous *et. al.*, 1998 |
| | MCF-7* nude mice xenograph | 30 μ mol/L | - Deminished the cells tumorigenic potential. | |

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines. (Continued)

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Genistein** (continued) | MDA-MB-468** | 0-100 μ mol/L | - Genistein showed more efficient in inhibiting MDA-MB-468 cell growth than MCF-7 cell with no stimulatory effect.<br>- $IC_{50}$ = 21 m mol/L<br>- The cells become differentiated in response of the treatment. | Constantinous *et. al.*, 1998 (continued) |
| | MDA-MB-468** nude mice xenograph | 30 μ mol/L | - Deminished the cells tumorigenic potential. | |
| | MCF-7* | 0.5-20 μM | - Exerted a biphasic effect.<br>- Stimulated growth at concentrations less than 5 μM and caused dose dependent inhibition at high concentrations.<br>- $IC_{50}$ = 10 μM | Miodini *et. al.*, 1999 |

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines. (Continued)

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Genistein** (continued) | MCF-7*, T47D*, 549* | 0–40 μg/ml+$10^{-9}$M $E_2$ | - Stimulated growth at lower concentrations (1-5 μg/ml) but inhibited growth at higher concentrations (20-40 μg/ml).<br>- Resulting in down regulation of ER mRNA level in dose dependent manner.<br>- Anti-proliferative effect are estrogen dependent.<br>- Inhibited PTK activity in Phenol Red media.<br>- Inhibited $E_2$ upregulation of *pS2* and TGF-α mRNA at high concentration<br>- In the absence of $E_2$, increased ERE-CAT activity at lower concentration (<20μg/ml).<br>- In the presence of E2, at both low and high concentrations ahown decrement of ERE-CAT activity. | Shao *et. al.*, 2000 |

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines. (Continued)

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Genistein** (continued) | MDA-MB-231** and MDA-MB-468** | 0–40 μg/ml + $10^{-9}$ M $E_2$ | - Inhibited growth at high concentration (>10 μg/ml) with no stimulatory effect at any concentration.<br>- Anti-proliferative effects are estrogen independent.<br>- Showed no effect on PTK activity<br>Showed no effect on ERE-CAT activity. | Shao *et. al.*, 2000 (continued) |
| | MCF-7* nude mice xenograph | 15, 150, 300 ppm suplemented in diet. | - Cell proliferation was greatest in tumors of animals given 150, 300 ppm.<br>- The dosage 150, 300 ppm resulted in the increment of pS2 expression | Allred *et. al.*, 2001 |

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines. (Continued)

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Daidzein** | MCF-7* | $10^{-6}$ M | - Cellular proliferation was 79% of that seen with $10^{-10}$ M $E_2$ | Sathyamoorthy *et. al.*, 1994 |
| | MCF-7* | 0.01, 0.1, 1, 10, 50, 100 μM | - Showed biphasic effect.<br>- Induced DNA synthesis at 0.01-10 μM but inhibited DNA synthesis at >10 μM.<br>- $IC_{50}$ = not describe | Wang and Kuzer, 1997 |
| | MDA-MB231** | 0.01, 0.1, 1, 10, 50, 100 μM | - Inhibited DNA synthesis at high concentration with no stimulation shown at any concentration.<br>- $IC_{50}$ = 81.2 μM | |
| | MCF-7*and MDA-MB231** | 0-100 μmol/L | - No growth stimulatory effect at low concentration but show growth inhibitory effect at higher concentration.<br>- Did not induced cell differentiation. | Constantinou *et. al.*, 1998 |

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines. (Continued)

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Coumestrol** | MCF-7* | Not described. | - Relative binding affinity to SBG = 14% compared with $E_2$<br>- Markedly enhance tumor cell proliferation.<br>- Competed with $E_2$, resulted in ER decrement. | Martin *et. al.*, 1994 |
| | MCF-7* | 0.01, 0.1, 1, 10, 50, 100 μM | - Show biphasic effect.<br>- Induced DNA synthesis at 0.01-10 μM but inhibited DNA synthesis at >10 μM.<br>- $IC_{50}$ = 42.5 μM | Wang and Kuzer, 1997 |
| | MDA-MB231** | 0.01, 0.1, 1, 10, 50, 100 μM | - Inhibited DNA synthesis at high concentration with no stimulation shown at any concentration.<br>- $IC_{50}$ = 24.4 μM | |

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines. (Continued)

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Quercertin** | MCF-7* | $10^{-6}$ M | - Resulted in no growth stimulation. | Sathyamoorthy *et. al.*, 1994 |
| | MCF-7* | 5.2 μg/ml ($IC_{50}$) + 100 nM $E_2$ | - The addition of $E_2$ was unaffected to the cells treated with quercertin. | So *et. al.*, 1997 |
| | MCF-7* | 0.01, 0.1, 1, 10, 50, 100 μM | - $IC_{50}$ = 88.0 μM | Wang and Kuzer, 1997 |
| | MDA-MB231** | 0.01, 0.1, 1, 10, 50, 100 μM | - $IC_{50}$ = 37.0 μM | |
| | T47D* | 100 nM-20μM | - Markedly inhibited cell growth at 20 μM | Zava and Duwe, 1997 |
| | MCF-7* | 0.5-20 νM | - Did not influence cell growth up to 2.5 μM and dramatically inhibited growth at higher concentrations.<br>- $IC_{50}$ = 4.9 μM | Miodinet *et. al.*, 1999 |

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines. (Continued)

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Kaempherol** | MCF-7* | $10^{-6}$ M | - Cellular proliferation was 66% of that seen with $10^{-10}$ M $E_2$ | Sathyamoorthy *et. al.*, 1994 |
| | MCF-7* | 0.01, 0.1, 1, 10, 50, 100 μM | - Showed biphasic effect.<br>- Stimulated DNA synthesis to 225% of control at 10 μM and inhibited DNA synthesis at high concentrations<br>- $IC_{50}$ = 69.0 μM | Wang and Kuzer, 1997 |
| | MDA-MB231** | 0.01, 0.1, 1, 10, 50, 100 μM | - Inhibited DNA synthesis at high concentration with no stimulation shown at any concentration.<br>- $IC_{50}$ = 42.2 μM | |
| | T47D* | 100 nM-20μM | - Markedly inhibited cell growth at 20 μM | Zava and Duwe, 1997 |

**Table 4.** Summary of the study of the effects of phytoestrogens on breast cancer cell lines. (Continued)

(* = Estrogen receptor positive breast cancer cell line, **= Estrogen receptor breast cancer cell line)

| Chemical | Cell | Dosage | Results | Reference |
|---|---|---|---|---|
| **Others** | MCF-7* | Equol $10^{-6}$ M | - Cellular proliferation was 79% of that seen with $10^{-10}$ $E_2$ | Sathyamoorthy *et. al.*, 1994 |
| | MCF-7* | Galangin 4.2 μg/ml<br>Baicalein 5.3 μg/ml<br>Hesperetin 12.0 μg /ml<br>Naringenin 18.0 μg /ml | - The addition of $E_2$ was unaffected to the cell treated with these flavonoids at their $IC_{50}$ concentration. | So *et. al.*, 1997 |
| | MCF-7* nude mice xenograph. | Genistin 750, 1200 ppm. Supplemented in diet. | - Increased tumor growth, *pS2* expression and cellular proliferation<br>- Removal .of genistin from the diet caused tumor regression. | Allred *et. al.*, 2001 |

## III The interaction of phytoestrogens with estrogen receptor

### 3.1 Estrogen receptor

Estrogen receptor (ER) was first found in the rat uterine (Jansen and Jacob, 1992). ER was categorized into a group of nuclear hormone receptor, the group of hormone-activated transcription factor that could initiate or enhance the transcription of genes containing specific hormone response element. (Green *et. al.*, 1986: Greene *et. al.* 1986) The first type of ER was named ERα, consisted of 595 amino acids that separated into six different functional regions. (Kuma *et. al.*, 1987)

The second type of ER was cloned from a rat prostate complementary DNA (cDNA) library and was named ERβ. (Kuiper *et. al.*, 1996) The ERβ protein consisted of 485 amino acids and separated into six functional regions.

Both ER subtypes played many important roles in the reproductive system (Gorodeski and Pal, 2000), cardiovascular system (Makela *et. al.*, 1999); development (Cassanova *et. al.*, 1999), reproduction-related behaviors (Ogawa *et. al.*, 1998) as well as the regulation of the $Na^+/H^+$ exchanger (Ediger *et. al.*, 1999)

#### 3.1.1 The distribution of ER

Both ER subtypes could be found through out the body including cardiovascular system, central nervous system, reproductive system, gastrointestinal system, breast and bone. The amount of each ER subtype was depended upon the tissue type. For example, ERα was found predominately in the reproductive system (Gustafsson, 1999) whereas the gastrointestinal tract was bared only ERβ. (Foley *et. al.*, 2000)

The ratio of ERα and ERβ might be important in determining the susceptibility of a tissue to estrogenic-induced carcinigenesis. (Gustafsson, 1999).

The changes in the ratio of ER subtypes could help to predict the pathological state of the tissue such as colon. (Foley *et. al.*, 2000), myometrium (Benassayag *et. al.*, 1999) and breast (Gustafsson, 1999).

### 3.1.2 Functional region of ER

The six functional regions of both type of ER had been defined based on the putative functions that were contained in each area (A-F region). (Macgregor and Jordan, 1998)

| A/B | C | D | E | F |
|---|---|---|---|---|
| AF-1 | DBD | Hinge | HBD/AF-2 | C-terminal |

**Figure 1** Schematic structural of ER (A-F region)

**1) A/B region**

The A/B region contained one of the two transcriptional AFs presented in ER. Activation Function –1 (AF-1) activated transcription in a cell and motor context specific manner. (AF-1 was not discovered during the early study due to ER deletion mutation.) (Kumar *et. al.*, 1987)

**2) C region**

The C region contained the DNA binding domain (DBD) and dimerization domain. The DBD was the most conserved region in the nuclear hormone receptor superfamily. The DBD consisted of two zinc fingers, which were the essential components of the ER. When the ER was lack of the DBD, it could not bind to DNA both *in vivo* and *in vitro* (Kumar *et. al.*, 1987; Kumar and Chambon, 1998). Another basic requirement for DBD activity was the heat shock protein 90 (molecular chaperone). This protein bound to ER molecule and resulted in the ER conformation change into the inactive form. DBD could not bind directly to DNA (Chambraud *et. al.*, 1990).

**3) D region**

The D region was the hinge region. When the ER conformation change, this region would swing in and out to bear DBD to bind to DNA.

**4) E region**

The E region was the hormone binding domain (HBD), which contained AF-2 ligand-dependent and promoter-specific, heat shock protein binding function, hormone binding site (ligand-dependent) and the dimerization domain. The HBD was responsible for ligand recognition because it controlled transcription in a specific and selective manner. The HBD worked together with DBD and the heat shock protein. (Macgregor and Jordan, 1998)

**5) F region**

The F region was the C-terminus. (Macgregor and Jordan, 1998)

The comparison of the amino acid sequence on HBD and DBD of both ER subtypes indicated that DBD was the most conserved region (97.5% conserved). It should implied that both ER subtypes might regulate the same gene. In contrast, HBD was the less conserved than DBD (59.1% conserved). ER subtypes might be different in its ability to bind to different chemical compounds. AF-1 site was the least conserved region (15.5% conserved) Therefore, these two receptors might interact with different set of protein. (Kuiper *et. al.*, 1998; Gustafsson, 1999) **(Figure 2)**

| A/B | C | D | E | F | Region |
|---|---|---|---|---|---|
| AF-1 | DBD | Hinge | HBD/AF2 | C-terminal | ERα |
| 15.5 | 97.0 | 30.0 | 59.1 | 17.9 | ERβ |

**Figure 2** Comparison of the primary structures of human estrogen receptors (hERα and hERβ respectively). The numbers within the ERβ represent the degree of homology (%) between respective domains in the two receptor subtypes (Macgregor and Jordan, 1998).

### 3.1.3 Estrogen action on ER.

Estrogen penetrated through the cell membrane and bound to ER (Headley, 1996) which resulted in the dissociation of the heat shock protein. The ER conformation was then altered into active form by phosphorylation. The hormone-receptor dimerization was subsequently occurred. The dimer bound to EREs represented in the promoter region of the estrogen-activated genes and resulted in activation of the transcription (Norris, 1997).

#### 3.1.3.1 Signal transduction of ER

Both ER subtuypes are estrogen-dependent transcriptional regulator which bind to estrogen responsive elements (EREs) on DNA or interact with the protein (Activator Protein-1 or AP-1) in the other pathway (Paech *et. al.*, 1997) as described.

1) **Hormone responsive element (HRE) pathway. (ER – DNA interaction)**

This classical pathway was also described in 3.1.3. When estrogen bind to ER and formed estrogen-receptor complex, this molecule would bind to another one estrogen-receptor complex and formed a dimer (Dimerization). The dimer bound to DNA at HRE and subsequently trigger transcription.

2) **Activator protein-1 (AP-1) pathway. (Protien - protien interaction)**

Estrogen-receptor complex bound to AP-1 protein, *Fos* and *Jun*. This molecule would bind to another estrogen-receptor complex and formed a dimer. This dimer would bind to DNA at AP-1 element and activate transcription.

### 3.2 Phytoestrogens action on estrogen receptor

Due to the fact that the ER was described as a nuclear transcription, this function was depend on the association with an appropriate ligan that bind to an ERE or AP-1. The ER was capable to bind several structurally diverse compounds with different affinities (Martin *et. al.*, 1978; Clarke *et. al.*, 1996). Some phytoestrogens such as coumestrol, genistein and kaempferol could compete stronger with $E_2$ for binding to ERβ than to ERα. ( Kuiper *et. al.*, 1998: Speirs and Kerin, 2000)

Both ER subtypes could mediate gene transcription in 2 pathways. When signaling was mediated via AP-1, ERα and ERβ signal in opposite way. The binding at AP-1 site would occur, in the presence of estradiol and phytoestrogens. ERα was activated to transcribe whereas ERβ transcription was inhibited. (Paech *et. al.*, 1997)

### 3.3 Selective estrogen receptor modulators

Selective estrogen receptor modulators (SERM) or estrogen analogs (previously called "anti-estrogen") were the agents that could maintain the benefits of estrogen but avoid the risk of estrogen. They were the additional choices for woman who was at a risk of diseases associated with chronic estrogen deficiency or those who was interested in using medication for overall health maintenance after menopause. Some of these agents could act as antagonists in human reproductive tissues, with partial agonists on the skeletal system and on serum lipoproteins. Each agent had its own unique activities, with quantitative and qualitative variability in its agonists and antagonist properties at different target tissues. The mechanisms involved with differential binding to different estrogen receptor subtypes, different conformations produced with each agent when bound to the estrogen receptor. (Cosman and Linsay, 1999) The examples for SERM were Tamoxifen and Raloxifen. It was found that phytoestrogens showed some hints to act like SERMs. (Diel *et. al.*, 2001)

A SERM could bind to either ERα and ERβ and the complexes could exhibit recruit co-activators or co-repressors. The complex might be homo- or hetero-dimerized and modulated genes by either ERE and AP-1. The different in AF-1 altered the SERM-ER complex, resulted in increased or decreased estrogenictity. It was now cleared that the ligan program the shape of the ER complex. Then the co-activators or co-repressors could bind to a SERM-ER complex. When the transcriptional complex was formed, the SERM-ERα and SERM-ERβ complexes must dimerized to be homo- or heterodimer, and resulted in initiation of the gene transcription. Thus SERMs could modulate gene transcription via two pathways, like estrogen. (Paech *et. al.* 1997),

Since dietary phytoestrogens from soybean were claimed to be the alternative choice for hormonal replacement therapy (HRT), cancer prevention and treatment. The other phytoestrogen-rich, *P. mirifica* might exhibit the higher potential to use in the same purpose. In addition, *P. mirifica* and *B. superba* herb products were available in the markets whereas their effects on cancer were still uncleared. The study of the effects upon some human cancer cell line would provide the evidence *in vitro* and thus resulted in a confidence of the consumers to make a choice on these products.