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Experimental Therapeutics in Breast Cancer Cells

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1. Introduction

Many cancer patients have medicated themselves with traditional herbal remedies, which are mostly composed of a single or combined medicinal plants. The effectiveness of the remedies can be explained by the presence of the phytochemicals and the bioactivity. The investigators select the traditional-used and evidence-based herbs for the research and development. The investigation of the following Thai herbs and their reasonable utilization are presented.

2. The selected medicinal plants

2.1 Single plant

2.1.1 *Trichosanthes cucumerina* L.

It is a cucurbitaceous vine, grooved stem with scattered hairs and tendrils. The leaf is simple, alternate, five-lobed margin and 8-12 cm blade width. It is dioecious, the male flower is white and the female yellow. The fruit is round with pointed ends and green striped lengthwise, usually 3-4 cm wide and 5-6 cm long (Fig. 1).

It is distributed wildly in the tropical Asia, climbs over the big trees along the riverside and mixed forest. The fruit is very bitter and inedible. It is used for shampooing to relieve itching, dandruff and lice. The fruit and the aerial plant parts are the ingredients in traditional Thai herbal medicine for dizziness. The dried fruit is ground, mixed with tobacco and smoked to alleviate the asthma. The seed (20-30 seeds) is an emetic, antidysentery, febrifuge, emenagogue and anthelmintic. The root decoction is laxative, febrifuge and used to relieve headach and bronchitis. The vine is decocted and used as febrifuge, laxative and tonic.

The bitterness of *T. cucumerina* fruit arises from the presence of cucurbitacins, the highly oxygenated C30-triterpenes, which are frequently found in the cucurbitaceous plants. The fruit juice was extracted with diethyl ether. The extract was concentrated and the crystalline solid was produced. The marc was continuously extracted with petroleum ether, chloroform and methanol in a Soxhlet apparatus. We tested the biological activities of the fruit juice, crystalline solid (TC compound) and the methanol extract. The fruit juice which was prepared as lyophilized (spray dried) solid, did not inhibit the standard strains of the microorganism (*E. coli*, *S. aureus*, *B. subtilis*, *M. smegmatis* and *C. albicans*), whereas the

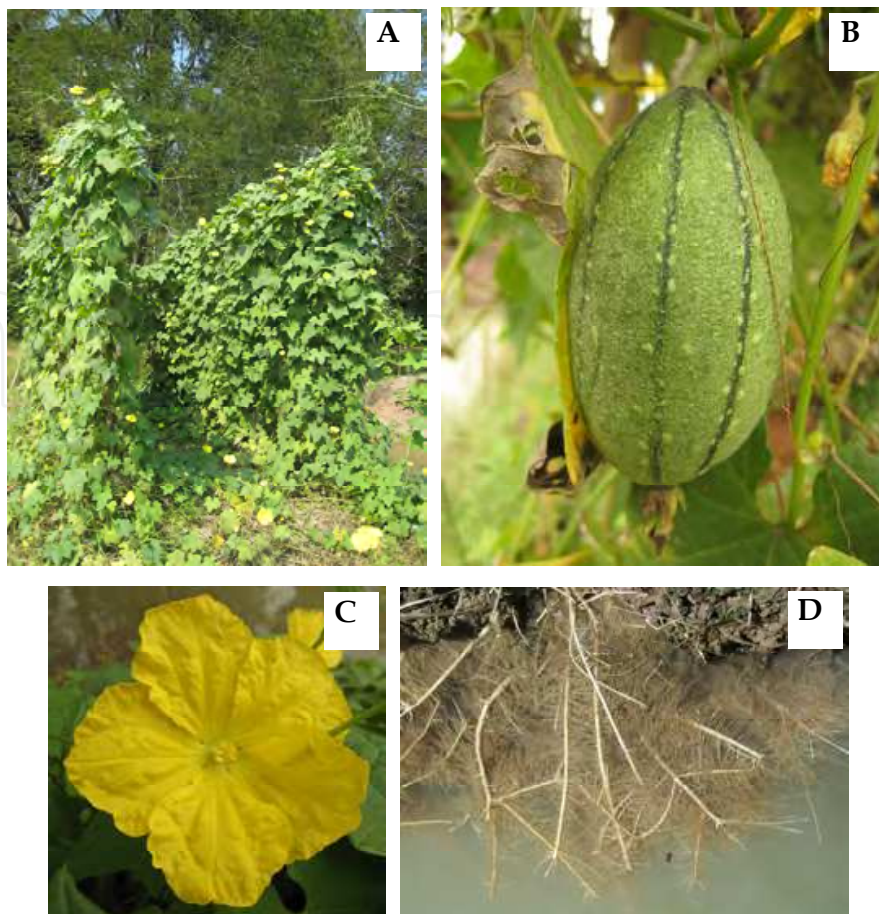


Fig. 1. *Trichosanthes cucumerina* L. : A whole plant; B fruit; C flower; D root.

methanol extract inhibited *S. aureus*, *M. smegmatis* and had a bactericidal action against *C. diphtheriae* (Tiangda et al, 1986). TC compound comprised cucurbitacin B (Fig. 2) and dihydrocucurbitacin B, which had a strongly cytotoxic action, in vitro, against KB cell (human nasopharynx carcinoma cell) (Silapaarcha et al, 1981; Jiratchariyakul et al, 1992).

The MTT colorimetric assay for cytotoxicity against breast cancer cell line (SKBR3) of the TC compound, cucurbitacin B and dihydrocucurbitacin B resulted the ED₅₀ of 0.48, 0.05 and 0.40 µg/mL, respectively (Jiratchariyakul et al, 1999). The lyophilized fruit juice of 5 mg/kg body weight was injected peritoneally to Swiss albino mice exhibited no sign of toxicity as in the control group. The LD₅₀ of the juice was 13 mg/kg body weight, which was considered as moderate toxicity according to Casarett and Doull (Tiangda et al, 1986; Casarett & Doull, 1975).

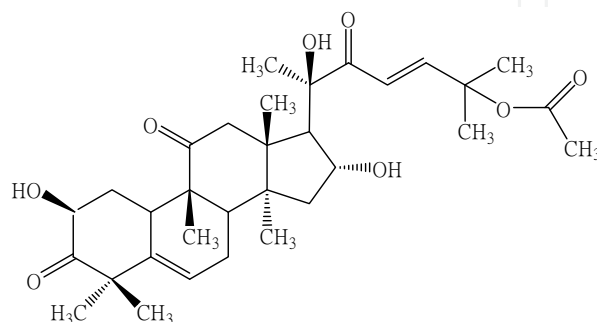


Fig. 2. Structure of cucurbitacin B.

The antiproliferative effect of the fruit juice and the isolated cucurbitacin against four breast cancer cell lines was further carried out. The cell lines included SKBR3, MCF7, T47D and MDA-MB435. SKBR3 is a human breast cancer cell line with overexpression of the HER2/neu receptor and absence of the ER receptor. In contrast, T47D and MCF7 are breast cancer cell lines that are ER receptor positive and HER2/neu negative, whereas the MDA-MB435 breast cancer cell line, both the ER receptor and HER2/neu expressions are absent (Table 1).

Breast cancer cell lines	ER receptor	HER2/neu	IC ₅₀ (µg/mL)		Doxorubicin (positive control)
			Lyophilized fruit juice	Cucurbitacin B	
MDA-MB435	Negative	Negative	155.77 ± 0.14	25.84 ± 0.83	0.48 ± 0.05
SKBR3	Negative	Overexpressed	130.52 ± 0.17	73.29 ± 0.88	0.65 ± 0.13
MCF7	Positive	Negative	374.77 ± 0.19	34.52 ± 0.11	0.62 ± 0.04
T47D	Positive	Negative	249.32 ± 0.40	60.26 ± 0.61	0.63 ± 0.04

Table 1. Cytotoxicity of the fruit juice of *T. cucumerina* (Kongtun et al., 2009).

The mechanism that cucurbitacin B inhibits the breast cancer cell can be explained through the increased telomerase expression, which is associated with the neoplastic growth. Over 90% of breast cancer has highly telomerase expression. We investigated the effect of cucurbitacin B on telomerase activity. Cucurbitacin B inhibited telomerase activity especially in the ER-negative breast cancer cell SKBR3 with IC₅₀ of 3.29 µg/mL, whereas TC compound 4.6 µg/mL and the lyophilized fruit juice less than 10 µg/mL. The activity of human telomerase reverse transcriptase (hTERT) and the level of c-Myc protein were also decreased by cucurbitacin B. Thus cucurbitacin B possibly inhibited the breast cancer cell by reducing the telomerase activity via down regulating both hTERT and c-Myc expressions (Duangmano et al, 2010). Cucurbitacin B inhibited also lung and especially colon cancer cell lines (Kummalue et al, 2009). The recent research indicated the strong cancerostatic action of cucurbitacins B which worked through Jak/STAT-signal ways (Jak = Janus-kinase; STAT = signal transducer and activator of transcription). Cucurbitacin B specifically inhibited STAT 3 which involved in the formation of interleukin 6 (IL-6), an important mediator in immune system (Haensel & Sticher, 2007). Cucurbitacin B was possible to act as immunosuppressive agent according to its inhibition of PHA-activated PBMC (U-pratya et al, 2010).

2.1.2 *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy

M. loriformis is commelinaceous, and monocotyledonous, perennial herb, about 10 cm high. The leaves are simple, glabrous, and alternate. The leaf blade is linear about 1.5-2.0 cm wide, 15-20 cm long. The flowers are inflorescence, terminal and densely panicle. The pedicels slightly curved, translucent bracts about 4 mm, sepal ovate-elliptic, about 3 mm; petals blue or bluish violet (Fig.3). The plant is also used in traditional Chinese medicine as a remedy for detoxification and respiratory tract complaints (Book of traditional Chinese medicine in Sichuan, 1992).

For over 30 years pressed herb juice from *M. loriformis* has been used to support self-medication in Thailand by patients who suffer from different types of cancer. It is supposed to prolong the patient's life and reduce the side effects resulting from modern therapy (radiation and chemotherapy) (Report from the first seminar on herbs and cancer, 1988).



Fig. 3. *Murdannia loriformis* Rolla Rao et Kammathy.

The phytochemical work on this plant indicated the presence of cytotoxic glycosphingolipid namely 1 β -O-D-glucopyranosyl-2-(2'-hydroxy-6'-ene-cosamide)-sphingosine (Fig. 4) which had moderate cytotoxicity with ED₅₀ of 10.7, 15.6, 18.9 and 32.5 μmolL^{-1} against human breast, lung, colon and liver cancer cell lines, respectively, whereas the positive control (quercetin) had the ED₅₀ of 23.2, 13.2, 24.8 and 59.6 μmolL^{-1} , respectively (Jiratchariyakul et al., 1997, 1998, 2006).

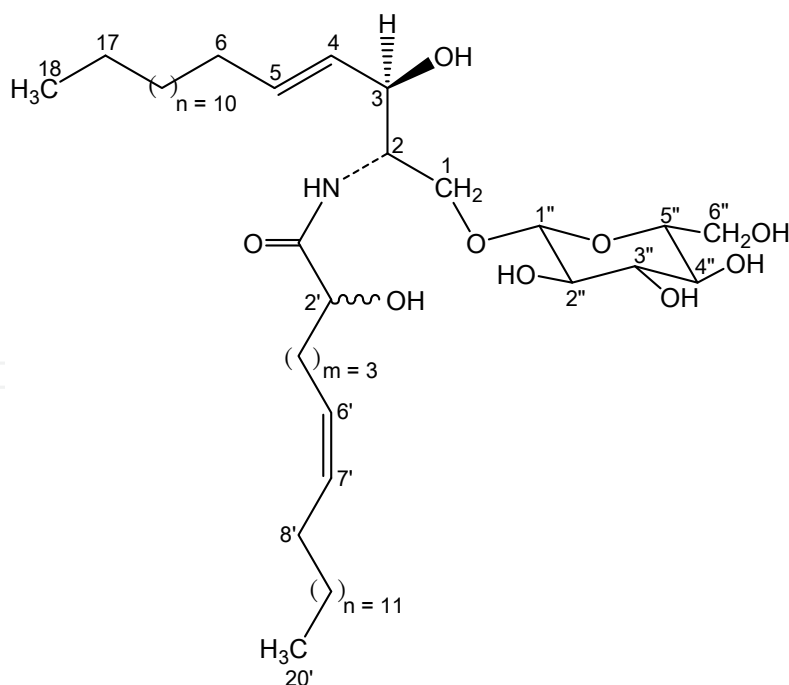


Fig. 4. Structure of 1 β -O-D-glucopyranosyl-2-(2'-hydroxy-6'-ene-cosamide)-sphingosine, a glycosphingolipid from *M. loriformis*.

The investigation of the indirect (immune-mediated) cytotoxicity was also carried out. The pressed herb juice and the isolated cytotoxic glycosphingolipid were not toxic to the

peripheral blood mononuclear cells (PBMCs) in vitro. Both increased PBMCs proliferation in the presence of the mitogen PHA (phytohaemagglutinin); the glycosphingolipid had the stronger effect. The pressed juice slightly increased the CD3, 4 : CD3, 8 ratio in the 3 day culture and the glycosphingolipid increased this ratio at both the 3 and 7 day cultures. They were the indicative of an in vitro immunomodulatory effect of the pressed juice and the glycosphingolipid (Jiratchariyakul et al., 2006).

The pressed juice had LD₅₀, orally in rats, more than 120 g/kg body weight, and did not damage the growth, the blood chemistry and the pathology of organs. It was safe according to World Health Organization (Tappayuthpijarn et al., 1991; Intayot et al., 2002). The sub-chronic toxicity study of *M. loriformis* in rats for 3 months did not damage the growth, the blood chemistry and the pathology of organs (Tappayuthpijarn et al., 1991). The ethanol extract of this plant induced in vitro DT-diaphorase, a detoxifying enzyme (Vinitketkumnun, 1996), and decreased in vivo the formation of aberrant crypt foci (ACF), which was related to the colon cancer (Intayot et al., 2002).

2.1.3 *Ficus hispida* L. f.

F. hispida is a traditional Thai plant from family Moraceae. It is a moderate sized tree and grows well in damp and shady place (Fig. 5). Almost all parts of this plant can be used in traditional medicine for the treatment of various ailments, for example, an anti-diarrhea, emetic, astringent, hepatoprotective, antitussive, antipyretic, antiinflammatory, depurative, vulnerary, hemostatic, antiulcer as well as in the treatment of anemia (Wuttidhamaved, 1997; Mandal and Ashok Kumar, 2002; Peraza-Sanchez et al., 2002).



Fig. 5. *Ficus hispida* L. f.

F. hispida, was investigated for antiproliferative activity on human breast cancer cell lines: SKBR3, MDA-MB435, T47D, and MCF7. Based on the results, the methanolic extract exhibited the most powerful activity on T47D breast cancer cell line with ED₅₀ of 110.3±9.63 µg/mL as compared to DMSO, the negative control. Confirmation of the result using colony-forming assay (clonogenic assay) yielded the effect in a dose - dependent manner. The cell cycle analysis of this plant extract revealed non-specificity with prominent in apoptosis. Therefore, *F. hispida* might provide some benefits in the treatment of breast cancer. (Pratumvinit et al., 2009)

2.1.4 *Erycibe elliptilimba* Merr. & Chun.

Erycibe elliptilimba, belonging to the Convolvulaceae family, is a Thai medicinal plant that has long been used for many decades to relieve symptoms from fever caused by infection, inflammation and prescribed in the mixture of traditional medicine for treating of various malignancies (Wuttidhamaved, 1997; Sintusarn, 2002).



Fig. 6. *Erycibe elliptilimba* Merr. & Chun.

The ED₅₀ values of the methanol fraction against SKBR3 and MDA-MB435 were 56.07 and 30.61 µg/mL, respectively. Doxorubicin as a positive control. This inhibitory growth activity of the treated cells acted in a dose-dependent manner which was also confirmed by the cell viability with the trypan blue exclusion assay. (Kummalue et al., 2007).

2.2 Herbal mixture

Two Thai remedies (TR1 and TR2) were tested for antiproliferative effect. TR1 was composed of following ingredients : *Albizia procera* (stem), *Diospyros mollis* (stem), *Ficus hispida* (stem), *Smilax glabra* (stem), *Smilax china* (stem) and cobra bone. TR2 was composed of *Gelonium multiflorum* (stem), *Erycibe elliptilimba* (stem), *Balanophora abbreviate* (stem), *Smilax glabra* (stem), *Smilax china* (stem) and *Millingtonia hortensis* (stem). TR1 and TR2 had no antiproliferative effect on breast, lung and colon cancer cell lines. However the combination of the remedies (either TR1 or TR2, 30 µg/mL) with doxorubicin (0.5 µg/mL) could significantly inhibit growth of lung cancer cell line (A549) at G2/M phase stronger than doxorubicin alone (Srisapoomi et al., 2008).

In addition two Thai medicinal plants, they were *P. indica* and *S. rarax*, were tested for antiproliferative effect. *P. indica* inhibited the leukemic cancer cells (NB4, HT93A) (U-pratya et al., 2008) and *S. rarax* inhibited the lung cancer cell (A549) (Kummalue et al., 2011). Both did not inhibit the breast cancer cell.

3. The herbal preparation

The usage of herbs nowadays has been developed according to the laboratory evidence. Herbs are prepared as drug materials, which are conformed to the following pattern (Dingermann, 2000).

- The nature (crude drug, tincture, pressed juice, fluidextract, dry extract, etc.) of the drug material (drug composition) must be specified.
- The quantity of the drug material in each dose of the solid dosage form (tablets) or in each package of the liquid dosage form must be specified.
For example: 200 mg dry extract / tablet or 100 mL fluidextract
- The drug extract ratio (DER), the amount (g) of crude drug to produce one g extract or drug material, and the extraction method must be specified.
- The solvent or solvent mixture used for the extraction must be specified.
- The indication must be conformed to the monograph established by the Ministry of the Public Health, or supported by the clinical evidence.
- The daily dose must be specified as the weight of the crude drug.

For example :

labeled DER	=	4-6.7 : 1
recommended daily dose	=	500 mg extract
thus, the daily dose	=	2-3.35 g crude drug.

3.1 The preparation of *Trichosanthes cucumerina*

- Declaration
The drug material comprises pressed juice from *T. cucumerina* fruit, which is standardized with cucurbitacin B, a specific marker compound.
- DER
10.5:1
One g pressed juice is prepared from 10.5 g fresh fruit.
- According to the preparation which is made from the pressed juice it is non-drug and can be considered as traditional remedy (Haensel & Hoelzl, 1996). The indication is supported by the laboratory evidence and the traditional use. It is supposed to be used as natural cytostatic agent.
- The daily dose
10.5 g fresh fruit

3.2 The preparation of *Murdannia loriformis*

- Declaration
The drug material comprises pressed juice from *M. loriformis* herb, which is standardized with glycosphingolipid namely 1 β -O-D-glucopyranosyl-2-(2'-hydroxy-6'-ene-cosamide)-sphingosine, a specific marker compound.
- DER
1.7:1
One g pressed juice is prepared from 1.7 g fresh herb.

- According to the preparation made from the pressed juice it is non-drug and can be considered as traditional remedy (Haensel & Hoelzl, 1996). The indication can be supported by the traditional use over 30 years and the case reported by the physicians. To use as an adjunct to anticancer therapy in order to reduce the side effects and prevent the cancer metastasis.
- The daily dose
100 g fresh herb (aerial plant parts)

4. Breast cancer cells and experimental procedure in antiproliferative activity background

Breast cancer is one of the leading causes of cancer death around the world. In Thailand, the incidence of breast cancer disease accounts for 20.5 per 100,000 female population (Chaiwerawatana, 2007). Though breast cancer treatment nowadays has dramatically improved due to new drug emerging such as monoclonal antibody, and tyrosine kinase inhibitor, the resistance to the drug itself, unfortunately, has significantly increased. Therefore, searching for the new drug to treat breast cancer is very essential. Based on this important issue, four specific breast cancer cell lines, i.e., SKBR3, MCF7, T47D, and MDA-MB435 have been used to evaluate the potential and promising chemotherapeutic agents from medicinal plants sources.

These four breast cancer cell lines, SKBR3, MCF7, T47D, and MDA-MB435, have different origins and properties which are considered as the very useful tools for investigating the medicinal plants effects. Actually, medicinal plants have long been prescribed for over centuries to treat various diseases including infections and malignancies such as breast cancer in many countries, for example, China, and Thailand. This traditional medicine nowadays plays an important role in healthcare system with approximately 80% of people around the world using it (Itharat and Oraikul, 2007). Determination of the plants activities especially growth inhibition and their mechanisms for cancer therapy is, indeed, undergoing in much effort.

In this section, four breast cancer cell lines and their experiments on medicinal plants are demonstrated. These include antiproliferative activity and cytotoxicity, cell cycle study, apoptotic study, mechanisms of action in plants, and future direction for testing the drugs from plant sources. Most of the experiments are performed in the biosafety level 1 which is suitable enough for manipulating these breast cancer cell lines *in vitro*.

4.1 Breast cancer cell lines: Their origins and properties

Breast cancer cell lines can be categorized into three groups according to their phenotypes and invasiveness (Lacroix and Leclercq, 2004).

The first group is luminal epithelial-like cells. This group expresses high amount of typical luminal epithelial phenotype of breast cells such as estrogen receptor (ER), E-cadherin (gene CDH1), zonula occludens-1(TJP1), desmoplakin I/II (DSP), and desmosomal junctions. This group of breast cancer cells will grow as interconnected colonies of polygonal cells on plastic and as fused colonies in Matrigel, which is semisolid medium. This kind of cells are weakly invasive. BT-483, MCF7, T47D, and ZR-75 are all in this group.

The second group is called weakly luminal epithelial-like cells. This group of cells shows the expression closely to the first group with a reduced extent or at least some of those markers.

Cells are weakly invasive *in vitro*. In Matrigel, most of these cells grow as non-fused spheres. Moreover, on plastic, they will accumulate in clusters of loosely attached cells and rarely reach full confluency. Breast cancer cell lines in this group are BT-474, CAMA-1, MDA-MB134, MDA-MB361, MDA-MB453, MDA-MB468, and SKBR3.

The third group is mesenchymal-like or stromal-like cells. It does not express the markers found in the first and second group. In contrast, it exhibits a high level of vimentin (gene VIM) which is the marker of mesenchymal cells. These cells have fibroblastoid phenotype on plastic and grow as colonies with large stellate projections in Matrigel. They are highly invasive *in vitro*. MDA-MB435S, MDA-MB231, Hs578T, and BT-549 are classified in this group.

Herein, details of breast cancer cell lines which have been employed in our medicinal plant research are demonstrated.

4.1.1 SKBR3 breast cancer cell line

SKBR3 is a human breast carcinoma cell line established in late 1970s and derived from pleural effusion of Caucasian patient diagnosed as invasive ductal carcinoma (CLS, Germany). It is an adherent cell which is the usual character found in most breast cancer cell lines. Ultra structures of cells show microvilli and desmosomes, large lysosomes, and bundles of cytoplasmic fibrils. In nude mice, these cells can form poorly differentiated adenocarcinoma (<http://www.cell-lines-service>). In addition in nude mice, c-Jun, the ending molecule of signal transduction pathways, is demonstrated to have the critical role in the tumorigenesis and metastasis in SKBR3 breast cancer cells (Zhang et al, 2007).

Estrogen receptor (ER) is found to be absent or expressed at the very low level in SKBR3 cells and progesterone receptor is also absent in this type of cell (Lacroix and Leclercq, 2004). In contrast, Her2/neu receptor including leptin receptor are overexpressed in this cell line whereas insulin-like growth factor receptor- I is expressed at the lower level when compared with those in MCF7 cells (Ozbay and Nahta, 2008). Interestingly, recent report revealed that SKBR3 expressed NMDAR1 and NMDAR2 receptors, both of which are important calcium channels. These receptors are reported to be essential for the growth of human breast cancer xenografts in mice (North et al, 2010).

This SKBR3 cell line with high expression of HER2 protein has long been used in various research. Recently, it has been shown to be the suitable candidate for reference materials in quality control of HER2 testing together with MCF7 cell line. Based on this report, SKBR3 and MCF7 breast cancer cells provide valuable controls for quantitative measurement of HER2 amplification and production (Xiao et al, 2009). The photograph of SKBR3 cultured in the flask is shown in Figure 7.

4.1.2 MCF7 breast cancer cell line

MCF7 is a human breast carcinoma cell line derived from pleural effusion of Caucasian patient diagnosed with breast adenocarcinoma (CLS, Germany). It is a relatively resistant to cisplatin treatment (Westmose Yde and Issinger, 2006). Morphology of this cell line exhibits epithelial-like cell including the ability to process estradiol via cytoplasmic estrogen receptors and domes formation. This cell line has oncogene, *wnt7h* and can induce tumor in nude mice (<http://www.cell-lines-service>).

This MCF7 cell line expresses both estrogen and progesterone receptors whereas the expression of Her2/neu is absent (Lacroix and Leclercq, 2004). The cells can be suppressed by catechin hydrate, product from plant sources such as green tea, through TP53/caspase-

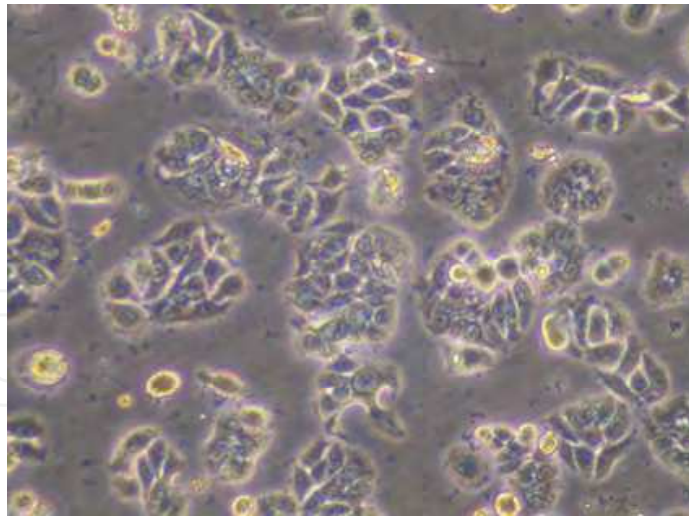


Fig. 7. SKBR3 cells grow in DMEM supplemented with 10% fetal bovine serum and 1% penicillin+ streptomycin. Cells show monolayer when grow in plastic with polygonal shaped. This cell line is categorized as weakly luminal epithelial-like cell.

mediated apoptosis (Alshatwi, 2010). Some vasoactive peptides such as endothelin 1 is found at low level in MCF7 while in SKBR3 is expressed at the higher level. This expression might correlate with high invasiveness phenotype in breast cancer (Hagemann et al, 2005). MCF7 has been extensively used as the model for breast cancer and breast cancer therapy. However, different sources of MCF7 show the differences in response to 17beta-estradiol resulting from activation or inhibition of insulin-like growth factor I (IGF-1) (Hamelers et al, 2003). Therefore, the different responses of MCF7 should be realized due to the expression of IGF-1. As mentioned above, MCF7 is suitable to be candidate of reference materials in quality control for HER2 testing (Xiao et al, 2009).

4.1.3 T47D breast cancer cell line

T47D is a human breast carcinoma cell line derived from pleural effusion of an infiltrating ductal carcinoma of the breast (CLS, Germany). It contains cytoplasmic junctions, receptors to 17 beta estradiol including steroids and calcitonin (Moseley et al, 1983). Receptors for estrogen and progesterone are also present (Hevir et al, 2011). This cell line has tumorigenicity in nude mice and has three oncogenes, i.e., wnt3, wnt7h, and wnt7b (<http://www.cell-lines-service>).

By activation Janus kinase2/signal transducer and transcription 5 pathway, this cell line expresses ample growth hormone receptor and prolactin receptor (Xu et al, 2011). Both receptors are structurally similar cytokine receptor superfamily members. Direct protein identification by MALDI post-source decay (PSD) or MALDI collision-induced dissociation (CID) in T47D cells showed only histone H2B (Pevsner et al, 2007). Chemokine CXCL12 and its promoter activity are found to be increase in human T47D breast cancer cells (Chen et al, 2010). Indeed, T47D cell line, as well as MCF7, represents the good model of estrogen-dependent breast cancer with capacity of 17beta-estradiol local production (Hevir et al, 2011).

4.1.4 MDA-MB435 breast cancer cell line

MDA-MB435 is derived from the pleural effusion of metastatic ductal adenocarcinoma of the breast. Recent data have pointed out that MDA-MB435 cells are from melanoma

(Lacroix, 2009; Rae et al, 2004). This cell line is found to express certain genes commonly transcribed in melanocyte which are not seen in breast cancer cell lines such as DCP gene. Moreover, expression of melanocyte proteins tyrosinase and melan-A has also been demonstrated in MDA-MB435. However, some evidences have been presented that MDA-MB435 might be breast cancer cell line expressing a poorly differentiated aggressive breast tumor together with epithelial and melanocyte markers (Chambers, 2009; <http://www.cell-lines-service>).

Investigation on protein identification in MDA-MB435 cells was performed and found 30 important proteins expressing in this cell line (Chandramouli et al, 2009). For example, caspase 14 precursor, heat shock protein 60, cell adhesion proteins, and some immune response proteins such as complement C3 were also demonstrated.

4.2 Breast cancer cells: Experimental procedures in antiproliferative activity

Antiproliferative and cytotoxic activity are the basic and very common investigations used for determination in the efficacy of plant extracts for searching the novel drugs. The measurement of herb treated mammalian cells either surviving or proliferating cells could be achieved by several techniques. Counting cells that include or exclude a dye such as trypan blue dye with the use of a hemocytometer under a microscope or in an automated cell counter is one assay. Quantitation of suspension cells could be performed by counting them directly whereas adherent cells which grow in monolayer culture require proteolytic agents such as trypsin prior to quantitation. This method is, therefore, not only time consuming, labor intensive, but also dependent upon individual skills.

A number of various indirect methods to quantitate living or dead cell number has been developed. Based on the fact that active or living cells will increase or decrease some specific proteins or even nucleic acids that can indicate cellular proliferation or cytotoxicity. Measuring released ⁵¹Cr-labeled protein after cell lysis, and measuring incorporation of ³H-thymidine during cell proliferation have been employed (Mosmann, 1983). These traceable radioactive moieties are very efficient and specific. However, the radioactive methods could handle very limited numbers of samples at each time and also require long sample preparation procedure with inherent dangers and high cost (Haslam et al, 2000). Therefore, several non-radioactive methods have been developed instead of an old traditional method, the cell counting.

In this section, breast cancer cells and experimental procedures in antiproliferative activity, three non-radioactive assays, i.e., MTT assay, Lactate dehydrogenase (LDH) cytotoxicity assay, and clonogenic assay, commonly used have been demonstrated in details.

4.2.1 MTT assay

Principle & mechanism of action

This colorimetric assay was first developed and reported by Mosmann (Mosmann, 1983). Tetrazolium salt MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) is tested and used in this assay. These tetrazolium salts can measure the activity of several dehydrogenase enzymes especially mitochondrial enzyme succinate-dehydrogenase (Slater et al., 1963). The reaction is known to be occurred in the living cells by cleaving the tetrazolium ring in the metabolically active mitochondria, not in dead cells or erythrocytes. From the MTT pale yellow substrate, the dark blue (purple) formazan products are generated in the direct proportion to the living cell numbers. Notably, this MTT assay

cannot distinguish between the cytotoxic and cytostatic effect of the tested drug or herb (Plumb, 2004).

Interestingly, MTT solution at 1 mg/mL increases the formazan product whereas MTT solution at 1-2 mg/mL will slow down the production (Sylvester, 2011). This is the reason why MTT solution is usually used at the concentration of 1 mg/mL. From the kinetics of formazan production, incubation time over 4 hours will get the plateau production curve. Therefore, standard incubation time at 3-4 hours is usually enough for the production time (Denizot and Lang, 1986).

As reported, the blue formazan products are partially dissolved in the medium. To induce complete solubilization of these MTT formazan products, several organic solvents have been tested and used such as ethanol, dimethylsulfoxide (DMSO), and isopropanol which is reported to be the most suitable solvent. After getting the homogeneous solution, optical density (OD) will be measured by reading on a scanning multiwell spectrophotometer (ELISA reader). The absorption spectrum of MTT formazan crystal is pH dependent. At pH 10.5, the maximum absorption is 570 nm. whereas, at pH 7.0, the absorption peak will show at 500 and 570 nm. (Plumb, 2004). The use of flat-bottomed well will help in increasing the sensitivity due to the doubled light-path length in the final optical density reading (Denizot and Lang, 1986).

In general, the accurate and reliable results from the MTT assay depend upon various important parameters which need to be optimized first. The proper cell number for seeding should be characterized by using standard curve. Changing into fresh medium prior to adding MTT solution is also critical in getting the good yield because of reduction of MTT activity in nutrient-depleted medium. Time and dose of MTT solution in each experiment may be different for each cell or cell lines used (Sylvester, 2011). Importantly, chemotherapeutic agents and some drugs such as valproic acid can yield the lower results from the MTT assay (Ari et al, 2010; Ulukaya et al, 2008). The experimental procedure is as followed (Kummalu et al, 2005).

Experimental procedure:

- Breast cancer cells will be seeded at 1×10^4 cells per well in 96 flat-bottomed well plate containing 100 μ l of the culture medium DMEM supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic.
- After 24 hours, the cells will be treated with herbal extract at final concentrations from 1, 10, 100, and 500 μ g/mL for 48 hours. These serial concentrations are used for determining the activity in crude extracts only.
- After 48 hours incubation, medium will be changed into fresh medium, and MTT 50 μ l (stock concentration at 1mg/mL in PBS) will be added to each well.
- After 4 hours incubation, the MTT solution will then be removed by rapid flick-off of the medium and 100 μ l of 100% DMSO will be added to solubilize the formazan products. The dark purple solution from formazan products can be seen as shown in Figure 8.
- Plate will be rotated on the platform rotator for 10 minutes before further measurement the O.D. by a microplate Elisa reader at 595 nm.
- Calculation of ED₅₀ (50% of inhibition of cancer cell growth) will be performed by using the formula:

$$\text{percentage of viability} = \text{OD sample} / \text{OD control} \times 100 \quad (1)$$

$$\text{percentage of inhibition} = 100 - \% \text{ viability}$$

Special comments:

- For pure compounds, serial concentrations can be diluted to the final concentrations as low as 0.1 $\mu\text{g}/\text{mL}$. This is because pure compounds are active compounds which are purified from crude extracts.
- After adding MTT solution, remember to wrap plate with aluminium foil because MTT substrate is light sensitive.
- We usually perform the O.D. measurement at 595 nm. because of the limitation of our instrument.
- Rotate the cell culture plate at the rotator platform for 10 minutes is important to induce the homogeneous solution that will be easy for the O.D. measurement and to achieve the correct data.
- The rapid flick-off of the medium after the MTT incubation will be done only in adherent cell lines such as breast cancer cell lines. For suspension cells such as leukemic cell lines, K562, the 100 μl of the stop solution (10% SDS dissolved in 0.01 N HCl) will be added into each well and incubated overnight.
- If dilute the herbal extract with DMSO, it is better to keep the percentage of DMSO in the final concentration not more than 0.5% to avoid cell damage and false positive result. In our experience, 1% DMSO still can cause some effect on cell viability.
- Please remember to set the control wells (both positive and negative control). We usually use doxorubicin or vinca alkaloid for positive control. Only cells in the medium will be used as negative control.
- The number of the seeding cells used in our experiment is from the standard curve at the O.D 0.7-1.2.
- We always perform the MTT assay with three independent experiments.

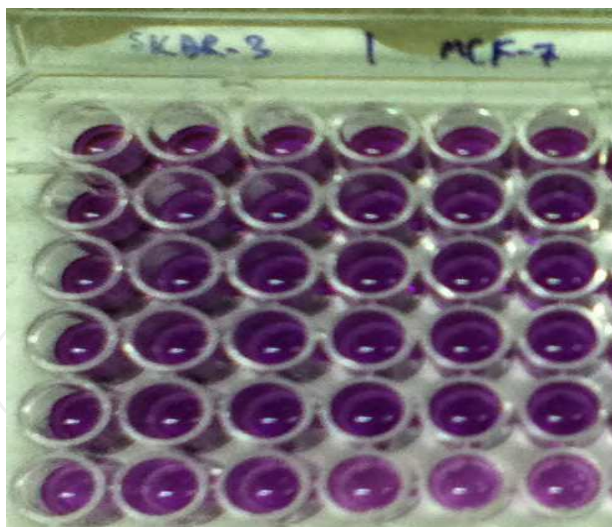


Fig. 8. The MTT assay showed the dark purple formazan products in the wells after dissolving the crystal with 100 μl of 100% DMSO.

4.2.2 Lactate dehydrogenase (LDH) cytotoxicity assay**Principle & mechanism of action**

For MTT tetrazolium salt assay, the method measures the surviving cells after treatment with chemical substances. However, for the very low toxic reagents, calculation from the

data of control cells and treated cells from MTT assay results in low sensitivity. In this case, direct measurement of dead cells will give more accurate information (Sasaki et al, 1992). Therefore, development of assay using cytolytic enzymes releasing from dead cells has been initiated which can represent proportionally to the dead cell number (Sasaki et al, 1992). Detection of lactate dehydrogenase enzyme, one of the stable cytolytic enzyme found in animal cells, has been established for this purpose (Decker and Lohmann-Matthes, 1988). Lactate dehydrogenase (LDH) enzyme has the function to catalyze lactate to pyruvate under anaerobic condition. It is a soluble enzyme in the cytoplasm. This enzyme will be released to the culture medium when the cell is ruptured or dead. Usually the assay will contain two steps. First, LDH releasing from the cells will oxidize lactate to generate NADH and H⁺. The second step, diaphorase in the reaction will use NADH and H⁺ to catalyze the tetrazolium salt such as WST-8, or INT to generate colored formazan which will absorb strongly at wavelength 490-520 nm. The amount of the formazan production will correlate in the proportion to the damaged cells in the reaction (Haslam et al, 2000; Rodney et al, 1966). The experimental procedure is shown below (Kummalue et al, 2009; LDH-cytotoxicity assay kit with WST-8, MBL international corporation).

Experimental procedure:

- Breast cancer cells will be seeded at a density of 1×10^4 cells per well in 96 well plates flat-bottom in 100 μ l of culture medium DMEM supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic.
- The next morning, cells will be treated with the herbal extract at various final concentrations from 1 to 500 μ g/mL for 48 hours in the incubator. These concentrations are used to evaluate the ED50 for crude extracts only.
- Centrifuge the cells at 1500 rpm or 250 g for 10 minutes.
- Transfer 10 μ l of culture medium into the new 96 well plate with corresponding well.
- Add 100 μ l of LDH reaction mix (containing LDH and WST-8) to 10 μ l of tested culture medium and incubated at 37 °C with a 5% CO₂ incubator for an additional 30 minutes.
- The absorbance at 450 nm of the dissolved solution will be measured by using an Elisa plate reader.

Special comments:

All MTT, WST-8, XTT, and INT are commonly used tetrazolium salts. They have different chemical structures and properties such as solubility. These properties depend on the substituents on the tetrazole rings in the compounds (Zhivich et al, 1990).

4.2.3 Clonogenic assay

Principle & mechanism of action

This method for cell sensitivity assay is considered as the gold standard technique and has been used for a long time (Plumb, 2004). It is a well known method for testing the effects of drugs on the proliferation of treated cell lines. This assay can measure the ability of cells to proliferate and form colonies after treating cells with chemical substances or herbal extracts (cytostatic effect of drug) whereas cells that are killed by the drugs cannot grow and form colonies (cytotoxic effect of drug). In addition, clonogenic assay is reported to be able to detect cell survival as low as 1% (Plumb, 2004).

Staining technique is a very important step because the accuracy of counting colonies depends on the good visualization. Colony staining has been performed with several dye

solutions such as methylene blue, ethidium bromide, and crystal violet (Guda et al, 2007). Counting colony can be done by using manual counting with microscope or using the specific software (Niyazi et al, 2007). The experimental procedure is demonstrated below (Pratumvinit et al, 2009).

Experimental procedure:

- Breast cancer cell lines will be plated in 12 well plate at a density of 7×10^2 cells per well.
- The next morning, cells will be treated with the herbal extract at the concentrations of 0, 50, 100, and 150 $\mu\text{g}/\text{mL}$.
- After 48 hours incubation, culture medium will be changed into fresh medium.
- Cells will be further cultured for 7-14 days until colonies are observed.
- Plates will be stained with 1 gm of crystal violet in 50% methanol for 30 minutes.
- Pour off the stain, carefully rinse with running water.
- Colonies will be counted by using ChemiDocXRS with specific software. Figure 9 showed the colonies detected in our experiment.

Special comments:

- The number of cells seeded in each well will be vary depending on the area of the well.
- The concentrations used in the experiment depend upon the ED_{50} value of each medicinal plant.
- Changing into fresh medium (after treated cells) before continue keeping cells in culture is very important for cell growth and colony formation.
- The time for culturing after exposure to the herbal extract depend upon the cell growth curve. For breast cancer cell lines, small colony can be detected after culturing cells for at least 7 days.
- Counting the colonies can be achieved by using only light microscope, no software use (but may be too difficult to count in some cases).
- We always perform the clonogenic assay with three independent experiments.

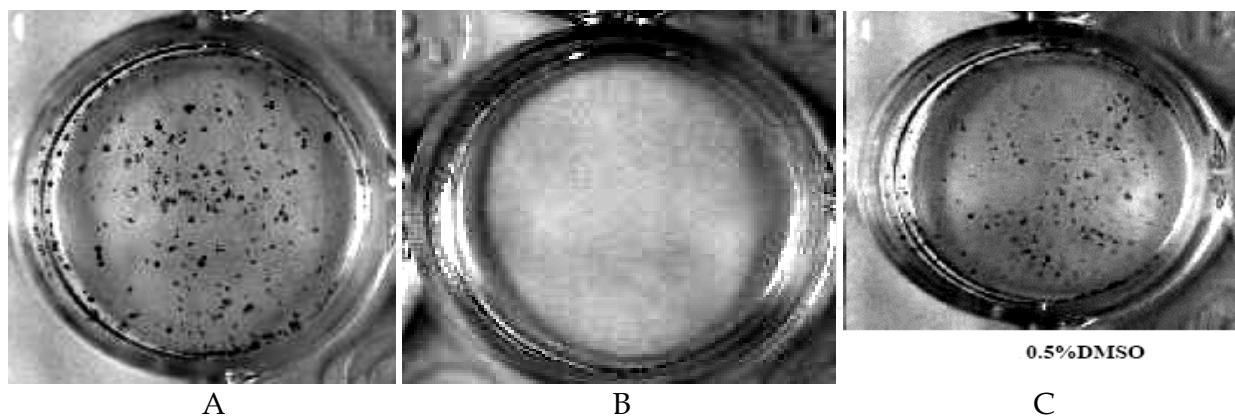


Fig. 9. Demonstration of colonies detected in the well. A. T47D breast cancer cells as a negative control. B. T47D treated with plant extract at 0.5 times ED_{50} . No colony was detected which was due to the cytotoxicity of the extract. C. T47D treated with 0.5% DMSO as a control because the extract was diluted with 0.5% DMSO. Colonies could be seen as dots at the bottom of the wells after staining with crystal violet in this experiment and counted by using the software.

4.3 Breast cancer cells and experimental procedures in mechanism of actions

This is usually the next step of investigation in searching for the new drug from plant sources. Plant extracts that exhibit the promising ED₅₀ values, i.e., the plant extracts show some antiproliferative or cytotoxic activities on specific breast cancer cell lines, the extracts will be further studied to find the mechanism of actions in view of inhibition of cancer cells growth or even killing cells. Cell cycle analysis and apoptotic study are the most two common methods used in the field for searching the mechanism of actions in plants.

4.3.1 Breast cancer cells and experimental procedure in cell cycle study

Based on the knowledge of normal cell cycle, breast cancer cells actively synthesize DNA in the same process (Ross et al, 2003). Normally, the cell cycle phase contains G₀, G₁, S, G₂, and M phase in order. Cells in the G₀ phase or resting state contain diploid DNA which will enter the second step G₁ (Gap 1). After this step, cells increase their DNA content to two folds with twice the diploid DNA content at the end. This accumulation state of DNA is S phase. Cells then enter the G₂ phase (Gap 2) and finally go into the M phase, while M means the mitotic state. Finally, each mitotic cell will end up with 2 daughter cells.

In breast cancer cells, DNA ploidy has been reported to correlate with the prognosis in patients. The first retrospective report using flow cytometry to study DNA content as a predictor in breast cancer was done by Auer et al with pure diploid showing an excellent prognosis (Auer et al, 1980). However, the use of DNA ploidy as a prognostic indicator in clinical practice still remains in controversy (Ross et al, 2003). Estrogen has also been reported to induce breast cancer which might due to the inappropriate activation of cyclin dependent kinase and consequently abnormal transition through G₁ phase (Foster et al, 2001).

The most popular technique used for demonstration DNA content in the cells is by flow cytometry (Nunez, 2001). Four distinct phases could be categorized, i.e., G₁, G₂, S, and M phases with G₂ and M phase will represent at the same DNA content (so called G₂/M phase). The staining techniques to distinguish these cell cycle phases are the measurement of the incorporation of bromodeoxyuridine (BrdU) and propidium iodide (Kummalue et al, 2002; Pratumvinit et al, 2009). Interference from RNA may result in misinterpretation as false positive. Therefore, the use of RNase should be added in the protocol. The experiment in breast cancer demonstrated here will share the experience in using the cell cycle kit which is much easier than the traditional technique. (The details of cell cycle study using BrdU incorporation and propidium iodide can be found in the references as mentioned in the text.) Belowed is the experimental procedure (Kummalue et al, 2007).

Experimental procedure:

- Breast cancer cells will be seeded at 1x10⁶ cells in 100 mm tissue culture dish and incubated overnight.
- The next morning, cells will be treated with the plant extract at the dosage ranging from 0.5 times ED₅₀ to 2 times ED₅₀.
- After 48 hours incubation, cells will be harvested and incubated with reagents as described in the protocol of the CycleTEST™PLUS DNA reagent kit (Becton Dickinson, USA).
- Measurement of DNA content of cells will be performed within 3 hours by flow cytometry and analysed by CellQuest Software.

Special comments:

- The incubation time for cell cycle study depends on the time that is used for cell sensitivity assay.
- Dosage used in the experiment to investigate the effect of plant on cell cycle depends upon the ED₅₀ of the plant extract.

4.3.2 Breast cancer cells and experimental procedure in apoptotic study

Apoptosis or programmed cell death is a very interesting investigation in drug discovery and development especially for cancer therapies. Searching for the anticancer agents that can induce apoptosis is necessary and needed for the effective treatment. For example, tamoxifen, which is prescribed as an adjuvant therapy in breast cancer, has been shown to induce apoptosis by down regulation of bcl-2 (Zhang et al, 1999). Therefore, most of medicinal plant researchers keep looking on finding these properties of plants. By the way, high cost and time consumption are unavoidable to face.

Apoptosis is a process that organisms, including human beings, use to tightly regulate the cell numbers and tissue size (Hengartner, 2000). It is caused by a group of cysteine proteases known as caspases. There are 2 major apoptotic pathways. One is via death receptor pathway which is triggered by death receptor superfamily such as CD95 and also tumor necrosis factor receptor I. This results in activation of caspase 8. The other pathway is mitochondrial pathway. This activates pro-apoptotic member of the Bcl-2 family and then pro caspase 9. Both pathways converge at the caspase 3 activation (Hengartner, 2000). To evaluate the apoptotic activity of plant extracts, caspase activity is usually investigated either by detection at the level of the enzyme caspase itself or by the level of its cleavage product, poly (ADP-ribose) polymerase-1 (PARP-1) (Los et al, 2002; Kummalue et al, 2011).

In summary, characterization of apoptosis is chromatin condensation, fragmentation of DNA (seen as ladder pattern in gel electrophoresis), and reduction of cell volume. Externalization of phosphatidylserine at the cell surface also plays an important role in macrophages recognition and consequently eradication of these apoptotic cells (Koopman et al, 1994). With these specific expression of phosphatidylserine, the new method was developed for detection of apoptotic cells by flow cytometry and has been frequently used nowadays. (Koopman et al, 1994). The experimental procedure is shown below.

Experimental procedure:

- Cells will be seeded on 100 cm tissue culture dish at the density of 1×10^6 cells.
- After 24 hours, cells will be treated with plant extracts at the various final concentrations ranging from 0.5 times ED₅₀, to 2 times ED₅₀.
- After incubating cells for various time points, cells will be harvested. The time points used to treat cells depend on the efficacy of the plant extract itself.
- Cells will be labeled with Annexin V-PI following the protocol of Annexin V-FITC Apoptosis Detection Kit from the manufacture and analysis by flow cytometry.

Special comments:

Detection of early and late apoptosis using annexin V depends upon the doses and time that treating the cells.

4.3.3 Breast cancer cells and specific mechanism of action of the plant extracts

Elucidation of the mechanism of action will greatly help in understanding the activity from plant sources on these breast cancer cell lines. Following the cell cycle and apoptotic studies as the basis, the mechanisms of these activities such as telomerase activity, antioxidant activity, and details of special signaling pathways are always considered for studying.

Telomerase is a eukaryotic ribonucleoprotein complex that helps in stabilizing telomere length in human stem cells, reproductive cells, and cancer cells (Shay and Bacchetti, 1997; Shay et al, 2001). The activity of telomerase increases in almost all human cancers except for the normal cells (Shay et al, 2001; Kim et al, 1994). Based on several studies, there are correlations between telomere shortening and growth failure of human cells (Harley et al, 1990; Shay et al, 2001). Therefore, human cancer cells show to have short telomeres with high telomerase, which is in contrast to normal human cells. Notably, cancer cells need to maintain telomeres for their immortalization. Based on this correlation, investigations of human cancer on telomerase activity have been done for over decades due to the potential development in drug targeting cancers.

Human telomerase consists of a catalytic protein component (hTERT) and integral RNA which are essential for the function of telomerase (Shay et al, 2001). hTERT is reported to be the critical for the production of telomerase (Nakamura et al, 1997). Investigation on telomerase activity can usually perform by detection the hTERT expression using RT-PCR. RNA extraction from treated breast cancer cells will be done before further processing to quantitate the level of transcripts. The method can be undertaken by real time RT-PCR and specific software for interpretation (Duangmano et al, 2010).

5. Breast cancer cells in medicinal plants research: Future direction in drug testing

Generating of induced pluripotent stem cells (iPS) for the first time in the world by Professor Dr. Shinya Yamanaka in Year 2006 has very high impact on several fields such as stem cell therapies, regenerative medicine, replacement therapy, and also drug screening and toxicity (Inoue and Yamanaka, 2011). Researchers worldwide are very interested in this iPS technology because of the high possibility in its usefulness and application to human diseases.

Induced pluripotent stem cells or iPS has first been generated from mouse tip tail fibroblasts which were somatic cells (Takahashi and Yamanaka, 2006). The process involved in transduction of fibroblasts with retrovirus carrying 4 transcription factors, i.e., Sox2, Klf4, c-Myc, and Oct3/4. These four factors are now known as Yamanaka's four factors. iPS cells exhibit several features characteristics of embryonic stem cells such as positive for alkaline phosphatase, forming teratoma in mice, and expression of specific embryonic antigen (Park et al, 2008). Nowadays, iPSs have been generated from various kinds of animals cells such as monkey, mouse, etc., including normal human cells, human cancer cell lines, and patients' cells to generate the patient-specific iPSs (Chun et al, 2010; Raya et al, 2009).

In drug screening and toxicity testing, human iPSs offer the high values in this field. For example, breast cancer patients' fibroblasts can be induced with transcription factors either 2 or 4 factors to create patient-specific iPS. Based on the fact that the iPS cells are generated from patients' own somatic cells, therefore, these specific iPS cells can be used to identify and test with the novel drug developing for cancer patients including the toxic effects of the drugs themselves (Chun et al, 2010). Interestingly, the advantage of iPS is the generation of

the library for human cancer that will represent the genetic and epigenetic variation of the population (Chun et al, 2010). In addition, since iPSs grow in culture like cell lines, therefore, this technology can provide the unlimited sources of cells for desired situation.

6. Discussion and conclusion

The investigation of the traditional used and evidence-based medicinal plants provides the chance to discover natural anticancer agents. The breast cancer cell lines with specific properties and the progress in antiproliferative and cytotoxic experiments are the important tools for the investigation of the medicinal plants. Clear differences in specific properties including receptors of these 4 breast cancer cell lines have been demonstrated so far. Comparing specific receptors, i.e., estrogen receptor, progesterone receptor, and Her2/neu in these 4 breast cancer cell lines: SKBR3, MCF7, T47D, and MDA-MB435 are demonstrated in Table 2. These 4 breast cancer cell lines are classified in different groups and express different receptors. Therefore, treatment of plant extracts on these 4 breast cancer cell lines can help, at least, in the decision whether or not the extracts will exert activities in breast cancer with different subtypes. However, the established cell lines from patients do not always represent the genotypes of parental tumor tissues (Tsuji et al, 2010). Therefore, testing in cell lines may not yield the same results when performing in vivo though most of these cells deregulate in signaling along the Egfr-MAPK pathway (Heiser et al, 2009).

Characters	SKBR3	MCF7	T47D	MDA-MB435
Category	Second group	First group	First group	Third group
Estrogen receptor	-	+	+	-
Progesterone receptor	-	+	+	-
Her-2/neu	overexpression	-	-	-

(-) = not expressed; (+) = expressed

Table 2. Characters of receptors on four breast cancer cell lines: SKBR3, MCF7, T47D, and MDA-MB435 (<http://www.cell-lines-service>; Lacroix and Leclercq, 2004; Hevir et al, 2011).

Searching for the novel chemotherapeutic agents from plant sources usually involves with the initial process of antiproliferative activity. These three non-radioactive assays are commonly used nowadays in the determination of antiproliferative and cytotoxic activities. Several aspects from them are summarized in Table 3, i.e., sensitivity, cost and time consuming for each assay. Notably, each assay has its own advantages and disadvantages which should be carefully considered.

Prediction of toxicology and therapeutic responses induced by the novel drugs can be approached by iPS technology as mentioned above. Lots of current disease specific based patients iPS have been reported since then (Chun et al, 2010). This application as a personalized approach leads to the specialized model in vitro. However, the safety and risk in applying to the clinical trials should be aggressively considered because of the techniques in generation of iPS with viral gene transfer.

The elucidation of the action mechanism provides the concrete evidence of the study plant. The utilization of herbs as natural anticancer agents can solve the problem of the

	MTT assay	LDH assay with WST-8	Clonogenic assay
Sensitivity	++ (high sensitivity for antiproliferative activity)	+++ (high sensitivity for cytotoxic activity)	+ (low sensitivity and high false negative result due to small colony formation)
Cost consuming	++ (high cost reagent)	++ (high cost reagent)	+ (low cost staining)
Time consuming	++ (moderate term culture)	+ (short term culture)	+++ (long term culture and time spending in counting)

+++ represent the highest scores

+ represent the lowest scores

Table 3. Comparison of three non-radioactive methods for assaying the antiproliferative activities (Kosaka et al, 1996; Kawada et al, 2002; Fotakis and Timbrell, 2006; Miyamoto et al, 2002).

unaccessible anticancer drugs. We investigated thoroughly two Thai herbs and discovered the possible benefit to the breast cancer patients. *T. cucumerina* fruit juice exerted strongly antiproliferative effect. The juice itself can be considered as a natural cytostatic agent because of its potent effect. According to the presence of cucurbitacins, the juice irritates the mucous membrane of the gastrointestinal tract. The juice produced toxicity to the central nervous system and the respiratory tract, the usage of the herbal juice should be under the supervision of the physician.

For the part of *M. loriformis* herb juice, it can be used as an adjunct to the modern therapy according to the moderate cytotoxicity, immunomodulatory effect and safety. The patient cases, which were reported by the physicians, have supported the use of herbal juice to reduce the side effects from the modern therapy. Several active compounds in the pressed juice worked together to exert the activities, which were expected to have broad spectrum and lowered toxicity. The appropriate daily doses of cytotoxic effect from *T. cucumerina* and immunomodulatory effect from *M. loriformis* require further clinical investigation.

F. hispida and *E. elliptilimba* are interesting herbs for further investigation. It was also noticeable that herbal remedy could potentiate the effect of anticancer drug.

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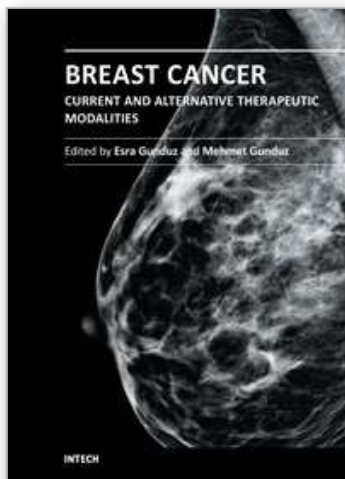
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various therapeutic modalities from signaling pathways through various anti-tumor compounds as well as herbal medicine for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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