รายงานฉบับสมบูรณ์

โครงการวิจัยเรื่อง

ผลของกวาวเครือขาวต่ออัตราการเจริญของเซลล์ปกติและเซลล์มะเร็งของมดลูก

Possible effect of *Pueraria mirifica* on growth of primary culture of porcine endometrial cells and human endometrial cancer cells

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CONTENT

ผลของกวาวเครือขาวต่ออัตราการเจริญของเซลล์ปกติและเซลล์มะเร็งของมดลูก

บทคัดย่อ

กวาวเครือขาวเป็นพืชสมุนไพรพื้นบ้านที่ใช้เป็นยาอายุวัฒนะ มีฤทธิ์เหมือนฮอร์โมนเอสโตรเจน สามารถกระตุ้นการเจริญของเต้านมและอวัยวะสืบพันธุ์ บำรุงผิวพรรณให้เต่งตึง กวาวเครือขาวประกอบด้วยสารหลายชนิดที่มีคุณสมบัติของ phytoestrogen การศึกษาครั้งนี้มีวัตถุประสงค์ในการศึกษาฤทธิ์เหมือนฮอร์โมนเอสโตรเจน (estrogenic activity) ของสารสกัดอย่างหยาบของกวาวเครือขาวต่อการเพิ่มจำนวนเซลล์เยื่อบุมดลูกของหมู (primary endometrial epithelial cell, PE cell) และเซลล์มะเร็งของเยื่อบุมดลูกคน (Ishikawa cell) ด้วยวิธี hemocytometer และ MTT colorimetric cell proliferation assay โดยทำการศึกษาเปรียบเทียบกับผลของฮอร์โมนเอสโตรเจน และสารแทม็อกซิเฟน (tamoxifen) ซึ่งเป็นสารที่ยับยั้งการทำงานของตัวรับฮอร์โมนเอสโตรเจน (estrogen receptor antagonist) เซลล์เยื่อบุมดลูกจะถูกเลี้ยงตามปกติในน้ำเลี้ยงเซลล์ DMEM ที่ประกอบไปด้วย 5 % FBS เมื่อต้องการทดสอบผลของสารต่าง ๆ ต่อการเพิ่มจำนวนเซลล์ เซลล์จะถูกนำไปเลี้ยงในน้ำลี้ยงเซลล์ชนิด phenol red free-DMEM และ 2 % charcoal-stripped FBS เป็นเวลา 2 วัน หลังจากนั้นสารทดสอบจะถูกใส่ลงไปในน้ำเลี้ยงเซลล์เพื่อทดสอบผลเป็นเวลา 24-48 ชั่วโมง ผลการทดลองพบว่า 17β-estradiol (E_2) ความเข้มข้น 0.01-100 นาโนโมลาร์ สามารถเพิ่มจำนวนเซลล์ทั้งสองชนิดตามระดับความเข้มข้นที่เพิ่มขึ้น โดยเห็นผลชัดเจนหลังจากทดสอบเป็นเวลา 48 ชั่วโมง การตอบสนองของเซลล์ทั้งสองชนิดต่อฮอร์โมนเอสโตรเจนสอดคล้องกับการแสดงออกของโปรตีนตัวรับเอสโตรเจนที่วิเคราะห์ด้วยวิธี Western blot analysis การทดสอบด้วยน้ำสกัดจากผงกวาวเครือความเข้มข้น 75, 150 และ 300 กรัมต่อ 1 มิลลิลิตรไม่มีผลเปลี่ยนแปลงจำนวนของ PE cells และ ishikawa cells อย่างมีนัยสำคัญทางสถิติเมื่อทดสอบเป็นเวลา 24 และ 48 ชั่วโมง ถึงแม้นว่ากวาวเครือมีแนวโน้มเพิ่มจำนวนเซลล์ของ Ishikawa cells ในการทดสอบเป็นเวลา 24 ชั่วโมง การให้สาร tamoxifen ความเข้มข้น 1 ไมโครโมลาร์มีผลเพิ่มจำนวน PE cells และ Ishikawa cells คิดเป็น 160 % และ 188 % ตามลำดับ ในขณะที่ความเข้มข้น 10 ไมโครโมลาร์มีผลลดจำนวนของ PE cells เหลือ 51 % แต่ไม่มีผลเปลี่ยนแปลงจำนวน Ishikawa cells เมื่อเปรียบเทียบกับค่าควบคุม 100 % การให้ E_2 ร่วมกับ tamoxifen ไม่มีผลต่อเปลี่ยนแปลงจำนวน PE cells และ Ishikawa cells เมื่อเปรียบเทียบกับการให้ tamoxifen เพียงอย่างเดียว จากผลการศึกษาครั้งนี้สรุปได้ว่า น้ำสกัดจากผงกวาวเครือขาวไม่มีผลเปลี่ยนแปลงจำนวนเซลล์เยื่อบุมดลูกปกติของหมูและเซลล์มะเร็งของเยื่อบุมดลูกของคน ในทางตรงข้ามฮอร์โมนเอสโตรเจนมีผลเพิ่มจำนวนเซลล์ของทั้งสองชนิด โดยเห็นผลชัดเจนหลังจากทดสอบเป็นเวลา 48 ชั่วโมง อย่างไรก็ตามจากผลการตอบสนองของ PE cells ที่มีต่อฮอร์โมนเอสโตรเจนร่วมกับการแสดงออกของโปรตีนตัวรับเอสโตรเจนที่พบใน PE cells นี้ แสดงให้เห็นถึงตัวอย่างเซลล์ที่มีคุณสมบัติเหมาะสมในการนำไปประยุกต์ใช้ในการทดสอบสารที่มีฤทธิ์คล้ายเอสโตรเจน และการศึกษากลไกการทำงานของเซลล์ผ่านตัวรับเอสโตรเจนได้เป็นอย่างดี

Possible effect of *Pueraria mirifica* on growth of primary culture of porcine endometrial cells and human endometrial cancer cells

ABSTRACT

Peuraria mirifica (Kwao Keur) is an herbal plant that has been used in Thai folk as a rejuvenating drug. It has been known to have estrogenic effect on the growth of mammary gland and reproductive organs in animals. We hypothesized that *P. mirifica* may have a potential role to regulate endometrial cell function. The present study was conducted to investigate the effect of *P. mirifica* on the proliferation of primary porcine endometrial epithelial cells (PE cells) and human endometrial cancer cell, Ishikawa cell by hemocytometer and MTT colorimetric cell proliferation asssay. The proliferative effects of *P. mirifica* was compared with those of 17β-estradiol (E_2) under conditions where PE cells and Ishikawa cells ($2x10^4$ /well) were maintained in phenol red-free DMEM supplemented with 2% heated-inactivated and charcoal-stripped fetal bovine serum. The compounds were added in day 2 after seeding and treated for 24-48 h. PE cells and Ishikawa cells treated for 24 and 48 hours with E_2 (10^{-11}-10^{-7} M) revealed increases in proliferation in a concentration-dependent manner, especially following 48 h treatment. These findings were consistent with the expression of estrogen receptor proteins (ERα) in both PE cells and Ishikawa cells as determined by Western blot analysis. Treatment with the water extract of *P. mirifica* 75, 150 and 300 mg/ml for 24-48 h did not produce significant changes in proliferation of PE cells and Ishikawa cells, although a slightly increase in cell numbers was observed with Ishikawa cells following 24 h. Tamoxifen 1 μM significantly increased proliferation of PE cells and Ishikawa cells from 100 % in control groups to 160 % and 188 %, respectively whereas the higher concentration (10 μM) of tamoxifen significantly decreased proliferation of PE cells to 51 % with no effect on that of Ishikawa cells. The addition of tamoxifen plus E_2 (10 nM) or *P. mirifica* (300 μM) did not change cell proliferation of PE cells and Ishikawa cells when compared with those of tamoxifen alone. From the results, we concluded that the water extract of *P. mirifica* powder has no effect on growth of normal porcine endometrial epithelial cells and human endometrial cancer cell. In contrast to *P. mirifica*, estrogen was shown to increase proliferation of both PE cells and Ishikawa cells, especially following 24 h treatment. The estrogen responsiveness and the presence of estrogen receptor provided the PE cells a useful cell model to screen the estrogenic and antiestrogenic effects of unknown compounds.

INTRODUCTION

Peuraria mirifica or "Kwoa Keur" is an herbal plant that people pay much more attention on its biological activity. It has been traditionally used in Thai folk as rejuvenating drugs and for its effects on breast enlargement, good skin complexion and life long. The scientific name of white Kwoa Keur is *Peuraria mirifica Airy-Shaw et Suvatabandhu*. It is composed of several groups of compounds such as coumarins, flavonoids, chromene, steroids and others. Most of these compounds are phytoestrogen. *P. mirifica* was first shown to have biological actions similar to estrogen. In 1940, one of biologically active compounds, miroestrol was isolated and found to have structure similar to estrogen (Schoeller *et al*, 1940). Miroestrol when given subcutaneously in rat exerted estrogenic effect by stimulating growth of ductule part of mammary gland, which was accounted for 70 % of 17β-estradiol activity. This miroestrol effect was 2.2 times more potent than estrone in mice (Cain, 1960; Jones and Pope, 1961). An unusual estrogenic phenol mirosterol was also isolated as an active principle from Kwao Keur (Cain, 1960). Recently, deoxymiroestrol, a new potent phytoestrogen, was isolated (Chansakaow *et al.*, 2000).

P. mirifica was shown to have both estrogenic and non-estrogenic effects in animal model. *P. mirifica* exerted many estrogen-related biological activities; for example, stimulating mammary gland growth and uterine weight, contraceptive activity, antifertility activity and inhibiting lactation in lactating animals (ยุทธนาและคณะ 2532, ยุพดีและยุทธนา 2528; Sornsrivichai *et al.*, 1987). The estrogenic effects of *P. mirifica* have been extensively studied in mammary glands and reproductive organs, which are under control of steroid hormones. Previous studies have shown that oral administration of *P. mirifica* caused increases in the growth of mammary gland in both male and female rats and mice (Cain, 1960) and the size and weight of uterine tissues in dogs and ovariectomized mice (พูลศิลป์และคณะ 2530; Sornsrivichai *et al.*, 1987; Benson *et al.*, 1961). Studies in Japanese quail demonstrated that *P. mirifica* increased the size and number of seminiferous tubule (Sornsrivichai *et al.*, 1987). The antifertility effect of *P. mirifica* was supported by several studies. Oral administration of *P. mirifica* powder to male rats at the doses range 100-200 mg/kg/day reduced number of sperm and sperm motility (ยุพดีและยุทธนา 2528). *P. mirifica* 1 g/rat completely acted as contraceptive and prevented pregnancy in rats receiving *P. mirifica* for 10 days after implantation (ยุทธนาและเสรี 2530). *P. mirifica* was also found to inhibit the growth of mammary glands and milk production in rats nursing baby,

resulting in low body weight and death of young rats (ยุทธนาและคณะ 2532). Besides the estrogenic effects, *P. mirifica* was shown to inhibit the growth of chicken (สมบูรณ์และยุทธนา 2530), decrease the number of red blood cells and lymphocytes (ปกรณ์และคณะ 2536) and increase the level of cholesterol in the blood of japanese quails (สมบูรณ์และสุวิทย์ 2532).

The pharmacological actions of *P. mirifica* at the cellular level have not been extensively studied. Many studies were carried out using an active constituent of *P. mirifica* with estrogenic activity such as miroestrol, deoxymiroestrol, genistein etc. Miroestrol is phytoestrogen that has been thought to possess the highest potency of estrogenic effect. Recent studies demonstrated that miroestrol and deoxymiroestrol promoted the growth of MCF7 human breast cancer cells (Welshons *et al.*, 1987; Chansakaow *et al.*, 2000). In contrast, enterolactone, eneterodiol and their synthetic derivatives inhibited the growth of ZR-75-1 mammary cell line (Hirano *et al.*, 1990). Studies using estrogen-dependent and independent mammary cell line demonstrated that genistein and equol acted as estrogen agonists and only genistein inhibited the growth of cell line (Zawa and Duwe, 1997). However, little was known about the direct effect of *P. mirifica* the growth of mammary cell. Particularly, the effect of *P. mirifica* on the growth of endometrial cell has not been investigated.

Endometrium consists of different cell populations such as epithelial cells and stromal cells that are mainly regulated by sex steroid hormones. Estrogen is known to regulate several endometrial functions including proliferation, differentiation and secretory functions. However, the proliferative effect of estrogen was only studied in endometrial cancer cell model that were highly responsive to estrogen. Since the crude extract of *P. mirifica* has been shown to contain estrogenic activity, we hypothesized that *P. mirifica* may exert estrogen-like effects to regulate cell function of primary endometrial cells or. Therefore, the aim of the present study was to investigate the possible effect of *P. mirifica* on the growth of endometrial cells. In addition, the effect of *P. mirifica* will be compared with estrogen and estrogen antagonist. The study was performed using both primary culture of porcine endometrial epithelial cells and human endometrial cancer cell, Ishikawa cell. The primary endometrial epithelial cells were isolated from immature, noncycling pig uterus using a modified technique to get the purity of the cell population (Deachapunya and O'Grady, 1998). These cells can be grown as a complete monolayer and formed tight junction with high resistance suitable for studying ion transport. Although the presence of estrogen receptor has not been identified in porcine endometrial epithelial cells, previous study using epithelial cells

grown on the permeable support demonstrated that the cell monolayer responded to17β-estradiol by increasing electrolyte and water secretion (Deachapunya and O'Grady, 1998). This observation suggested that the normal endometrial cells were estrogen responsiveness. The Ishikawa cells represent one of the few cell lines derived from uterine tissue that retains estrogen responsiveness in long term culture. Several studies have demonstrated the effect of estrogen on proliferation of Ishikawa cells (Anzai *et al.*, 1989; Kayisli *et al.*, 2002). Therefore both primary endometrial cells and endometrial cancer cells are deserved to be the effective model for studying the effect and regulation of estrogen and estrogen-like compounds on cell proliferation. The effect of *P. mirifica* on the growth of primary endometrial cells, representing normal cells, has not been studied and is very interesting regarding to the application of this herbal plant. Therefore, the results obtained from the present study will provide valuable basic knowledge for consideration and application of using *P. mirifica* as traditional medicine in the future.

RATIONALE

P. mirifica contains diverse groups of compounds such as isoflavonoids and chromene that are phytoestrogen and known to have estrogenic effects on various tissues. The estrogenic activity of *P. mirifica* has been widely studied in the growth of mammary glands and reproductive organs that are under the influence of estrogen hormone. Among reproductive organs, few studies have been conducted regarding the effects of *P. mirifica* on endometrial cell function and uterine weight. Previous studies demonstrated that oral administration of powder form and ethanol extract of *P. mirifica* resulted in increased uterine weight of mice (Sornsrivichai *et al*, 1987). Administration of food mixed with dried and blended *P. mirifica* to female puppy 1-2 g/day for consecutive 26 days resulted in increases in size and weight of uterus (Benson *et al.*, 1961). In addition, oral feeding of *P. mirifica* to growing and ovariectomized rats for 7 days or feeding of *P.mirifica* combined with prolactin injection was shown to increase the weight of uterine and pituitary gland (วัชรีวรรณ 2529). All of these results may suggest the estrogenic effect of *P. mirifica* on the endometrial cell function and proliferation of endometrium, resulting in increased uterine weight. At present, no evidences have been reported about the direct effect of *P. mirifica* on proliferation of endometrial cells. Therefore, in the present study, the direct effects of *P. mirifica* was carried out to study its estrogenic effect on the growth of endometrial cells, both in normal and cancer cell line.

Although the estrogenic effect of *P. mirifica* on at the cellular level of estrogen-responsive tissues has not been reported, several cytotoxicity studies on several cell lines have been conducted using miroestrol, deoxymiroestrol and genistein, the active ingredients of *P. mirifica*. Mirostrol and deoxymiroestrol has been demonstrated to increase growth of human mammary cell line MCF-7 which was not inhibited by estrogen antagonist toremifene. The growth-promoting effect of these compounds was more potent than 17β-estradiol (10 nM) (Chansakaow *et al.*, 2000). Studies using both estrogen-dependent and estrogen-independent mammary cell line demonstrated that genistein and equol acted as estrogen agonist and only genistein that exhibited inhibitory effect on cell growth, with different mechanism of action from estrogen antagonist tamoxifen (Zawa and Duwe, 1997). Experiments using rat have shown that administration of genistein before treatment with dimethylbenz [a] anthracene (DMBA) can prevent cancer (Lamartiniere *et al.*, 1995). However, coumestrol, genistein and enterolactone when given at low doses were found to stimulate DNA synthesis in estrogen-dependent MCF-7 but no effect on estrogen-independent mammary cell line MDA-MB-231 while the higher doses inhibited DNA synthesis in both mammary cell lines (Wang and Kurzer, 1997). Studies using cultured human endometrial stromal and glandular (Ishikawa cells) have demonstrated that isoflaviods (genistein, genistin, daidzein, daidzin) possesed the estrogenic activity as assessed by cell proliferation and alkaline phosphatase activity (Kayisli *et al.*, 2002). All of these studies illustrated the effects of several phytoestrogen on cellular model, although some effects could not be concluded. Since these compounds are active ingredient of *P. mirifica*, it is more likely for *P. mirifica* to exert some estrogenic effect on cell functions. In the present study, we evaluated the possible estrogenic effect of *P. mirifica* on the proliferation of endometrial cells. We have developed the techniques for isolation the primary endometrial cells that allows us to compare the effects of *P. mirifica* on the proliferation of normal endometrial cell with the human endometrial cell line.

OBJECTIVES

The objectives of the present study are:

1. To investigate the effect of water extract of *P. mirifica* powder on growth of porcine endometrial epithelial cells and human endometrial cancer cell, Ishikawa cells
2. To compare the effect of *P. mirifica* with estrogen or estrogen receptor antagonist tamoxifen and combination of *P. mirifica* and tamoxifen on growth of porcine endometrial epithelial cells and Ishikawa cells

MATERIALS AND METHODS

Materials :

17β-estradiol, insulin, non-essential amino acid and high purity grade salts were purchased from Sigma Chemical Co., (St Louis, MO, USA). Tamoxifen ([Z]-1-[p-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene; citrate salt) was purchased from Wako Co., (Japan). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffer saline (DPBS), phenol red-free DMEM, fetal bovine serum (FBS), collagenase (type 1), 0.05 % trypsin-0.53 mM ethylenediaminetetraacetic acid (EDTA), kanamycin, penicillin-streptomycin and fungizone were purchased from GIBCO BRL (Grand Island, NY). Charcoal-stripped FBS was purchased from Biowest Co., (Australia).

P. mirifica was kindly provided by Dr Yuthana Samitasiri from the Northern part of Thailand as a blended, white powder. The dried *P. mirifica* was freshly prepared in distilled water at the concentration of 15 mg/ml. After leaving for sedimentation, the supernatant was then collected and sterile-filtered before testing with the cells.

Cell Isolation and culture

Primary endometrial epithelial cell

The endometrial epithelial cells were isolated from porcine uterus obtained from Metropolitan slaughterhouse, Klongtoey, Bangkok. Uterine tissues from adult, precycling animals were used to minimize variability in proliferative properties at different stages of the estrus cycle. Uterine tissues were immediately collected from the killed animals and placed in ice cold porcine Ringer solution containing (mM): 130 NaCl, 6 KCl, 3 $CaCl_2$, 0.7 $MgCl_2$, 20 $NaHCO_3$, 0.3 NaH_2PO_4, 1.3 Na_2HPO_4, gassed with 95% O_2/5 % CO_2, pH

7.4. After remove serosal muscle layer, the endometrial tissues were cut into small pieces (~1 mm^3) and washed twice with Ca^{2+} and Mg^{2+} -free DPBS. The tissue fragments were then digested overnight with 0.2% collagenase and the epithelial glands were isolated as described previously (Deachapunya and O'Grady, 1998). Briefly, after overnight collagenase digestion, the tissues were dissociated with a Pasteur pipette and sieved with a mesh screen (100 μm) to get rid of undigested tissues. The suspension was spinned down with low-speed centrifugation to recover endometrial glands which were further collected using gravitational sedimentation method. The epithelial glands were suspended in DMEM supplemented with 3.7 g/L sodium bicarbonate, 10% heat-inactivated fetal bovine serum, 850 nM (5 μg/ml) insulin, 1% non-essential amino acid, 5 μg/ml fungizone, 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml kanamycin. They were then plated onto cell culture dishes and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Culture medium was changed after 24 hours and then every 2-3 days. After 80 % confluence (within 2-3 days), the remaining stromal cells were easily removed by trypsinization for 5 min. The purity of epithelial cells was greater than 90% as assessed by staining isolated cells with cytokeratin (Deachapunya and O'Grady, 1998). Cell viability assessed with the trypan blue exclusion method was more than 90%. The cells used in the proliferation experiment were within 2 weeks after isolation.

Ishikawa cells

The Ishikawa cells were human endometrial cell line that contained estrogen-dependent responsiveness. The cells were generously provided by Dr Guy Hagerman, University of Gent, Belgium. The cells were routinely maintained in Dulbecco's modified Eagle's medium containing 10 mM L-glutamine, 1% non essential amino acid, 100 U/ml penicillin, 100 μg/ml streptomycin and 5% heat-inactivated fetal bovine serum. The cells were trypsinized one every 1-2 weeks before reaching confluence and subcultured at 1:6-1:10 split ratio.

Proliferation study

To study the proliferation, both porcine endometrial epithelial cells (PE cells) and Ishikawa cells were preconditioned for 3 days before starting experiments, by changing to phenol red-free DMEM containing 2% charcoal-stripped FBS, 10 mM L-glutamine and 1% non essential amino acid. This conditioned medium was used to eliminate the estrogenic effect of phenol red and the serum was treated with charcoal to remove steroid

hormones (low concentration of steroid hormone) in order to minimize the estrogenic effect of the medium.

On day 0 of the experiment, the cells were plated in the preconditioning medium at the density of $2x10^4$ cells/well onto 24-well Costar culture plates and allowed to adhere for 18-24 h. On day 2, the seeding medium was aspirated, the cells were washed once with DPBS and fed with the same medium. On day 3, the medium was aspirated and filled with the experimental medium. The experimental medium consisted of phenol red-free medium fresh supplemented with 2% charcoal-stripped FBS, 10 mM L-glutamine, 1% non essential amino acid and dilutions of 17-β estradiol (E_2) or water extract of *P. mirifica* with or without tamoxifen. Each chemical was tested in triplicate for one time. Estrogen was dissolved in ethanol and tamoxifen in methanol, which were prepared at final concentrations of 0.01%. Control groups containing 0.01% ethanol, 0.01% methanol or in combination of both were also included. The cells were incubated in the experimental medium for 24-48 h and the number of cells was estimated using MTT cell proliferation assay or hemocytometer.

MTT cell proliferation assay

Cell proliferation was assessed by a colorimetric assay using MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The MTT assay is based on the mitochondrial metabolic reduction of soluble MTT to insoluble formazan crystals by mitochondria dehydrogenase of viable cells (Alley *et al.*, 1988; Scudiero *et al.*, 1988). After incubation period in the experimental medium, the cells were washed once with DPBS and incubated for four h in 0.25 ml medium containing 125 μg MTT at 37 C. The MTT solution was then aspirated and the formazan crystals were dissolved in 200 μl DMSO at 37 C for 2 h. The DMSO-solubilized solution was transferred to a 96 well Elisa plate and the absorbance of formazan was read at 550 nm using an automatic plate reader (Biorad, USA). Relative cell numbers were proportional to the absorbance of formazan products (data not shown). Cell numbers in the vehicle control group were considered 100 % and those in the treatment groups were calculated as percent of controls.

Hemocytometer

In some experiment, the cells were trypsinized and counted in a hemocytometer under an inverted light microscope. The cell viability was assessed using trypan blue dye exclusion method.

Western blot hybridization

PE cells or ishikawa cells seeded on the 100-mm cell culture dish were allowed to grow to 80% confluence. After 48 hours growing in serum free medium, 2% fetal bovine serum medium or treatment with estradiol, cells were trypsinized and centrifuged and the resulting pellets washed with iced-cold phosphate-buffered saline. The cells were lysed in 1000 µl of lysis buffer (50 mM tris HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 20 µg aprotinin and 1 mM NaF, pH 7.4). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., USA). To detect estrogen receptor alpha (ERα), 30 µg of total protein per sample were run on 8% SDS-Polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Proteins were electrically transferred to a polyvinylidene difluoride (PVDF) membrane (Pall Life Sciences, USA). After blocking with TBST-3 % non-fat dry milk (25 mM tris, 140 mM NaCl, 0.1% tween and 3% non-fat dry milk) for 30 minutes, the membrane was reacted with antihuman ERα-rabbit polyclonal antibody which detects toward the carboxyl terminal domain of human ERα isoforms (HC-20, Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1: 1000 dilution at 4 C overnight. The secondary antibody, horse raddish peroxidase (HRP)-conjugated anti rabbit antibody (Zymed Laboratories Inc., San Francisco, CA) were incubated on the next day at a 1:5000 dilution for 2 hours at room temperature. The immunocomplex was visualized using the enhanced chemiluminesence (ECL) detection system (Santa Cruz Biotechnology Inc., Santa Cruz, CA) according to manufacturer instuction.

Data analyses

Each experiment included three culture wells and the experiments were usually performed at least four times. Values from all experiments were expressed as mean ± standard error of mean (SEM). The differences between control and experimental means were analyzed using Student T-test or Analysis of Variance (ANOVA) when appropriate. Difference among means following a significant ANOVA was identified by Dunnett's multiple comparison test (PrismTM 2.0, GraphPad Software, Inc., San Diego, CA). A value of $P<0.05$ was considered significant.

RESULTS

Cell growth curve

To study the growth rate of endometrial epithelial cells (PE cells) and Ishikawa cells under basal conditions without adding any compounds, the cells were cultured in DMEM supplemented with 2 % FBS for 7 days and the fresh media were changed every two days. The cell numbers were assessed every 1-2 day using hemocytometer for PE cells and MTT colorimetric assay for Ishikawa cells. The growth curve of PE cells demonstrated that the cells started increasing in cell numbers on day 3 after seeding on day 0 and continuously increased up to day 7 (Fig. 1). After day 7 the PE cells reduced in growth rate and reached the lag phase. The growth curve of Ishikawa cells was found in the same manner as that of PE cells (Fig. 2). The Ishikawa cells increased cell numbers up to day 4 and declined on day 5, possibly due to cell confluence. From the growth curves obtained in the present study, we therefore performed the experiments by treatment of both PE cells and Ishikawa cells with various compounds on day 2 after seeding and the effects of these compounds on cell proliferation was examined for 24-48 h.

Effects of estrogen on endometrial cell proliferation

To investigate the effect of estrogen on proliferation of PE cells, we treated the cells with various concentration of E_2 ranging from 0.01 to 100 nM for 24-48 h. As shown in Fig.3, estrogen treatment induced concentration-dependent increase in growth of PE cells. The stimulatory effect of estrogen was less observed with 24 h incubation period and more pronounced with 48 h treatment. Treatment with E_2 at concentration of 0.01, 0.1, 1, 10 and 100 nM tended to increase cell numbers from 100 % in control group to 117.32 ± 10.65, 117.04 ± 7.9, 124.62 ± 7.9, 142.50 ± 20.53 and 128.27 ± 21.28 % for 24 h treatment and 110.32 ± 3.65, 110.31 ± 1.13, 132.95 ± 13.36, 159.04 ± 17.70 and 206.95 ± 36.48 % for 48 h treatment, respectively (Fig. 3A).

Ishikawa cells are highly estrogen-responsive and represent a good cell model for studying the estrogenic actions on endometrial cell function and proliferation. In many studies, estrogen was shown to increase proliferation of Ishikawa cell in short term and long term culture (Holinka *et al.*, 1986; Anzai *et al.*, 1989). In this study, we also compared the effect of estrogen on proliferation in PE cells with those of Ishikawa cells.

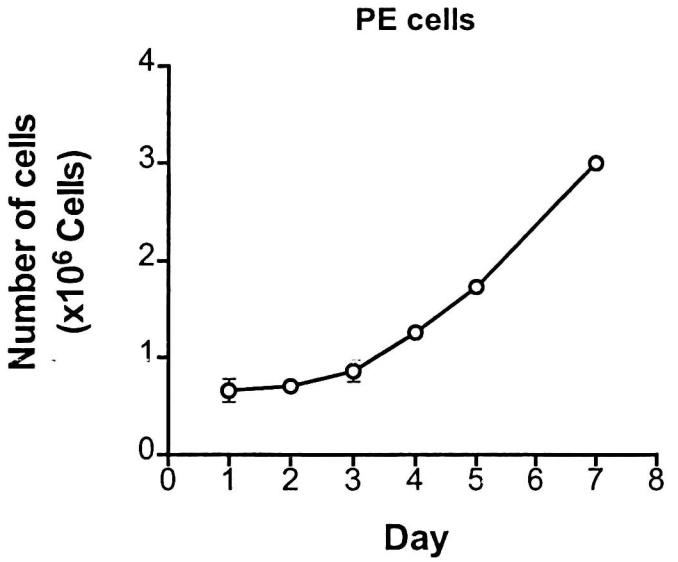

Figure 1 Growth rate of endometrial epithelial cells (PE cells) for 7 days. Cells ($1x10^6$ cells/well) were seeded on day 0 in 12-well plates containing DMEM with 2 % FBS. The fresh media was changed every two days. Cell numbers were counted with hemocytometer. Values are expressed as mean ± SEM of 2-3 experiments, each experiment was done triplicate.

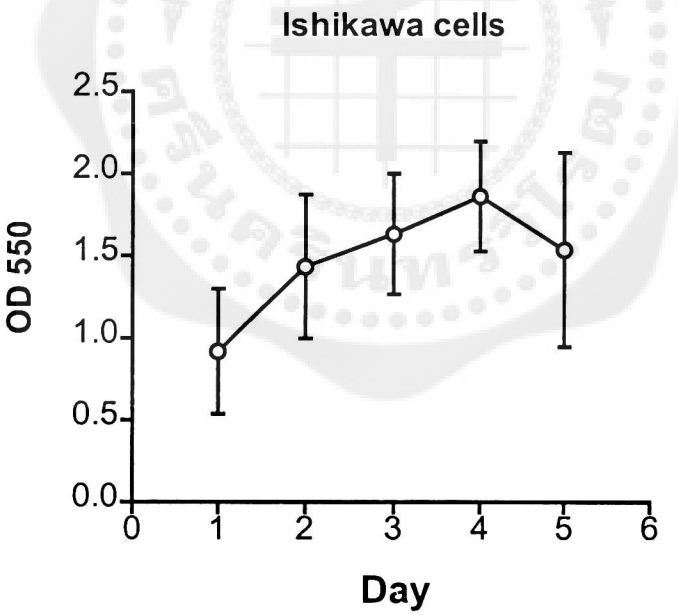

Figure 2 Growth rate of Ishikawa cells for 5 days. Cells ($12.5x10^4$ cells/well) were seeded on day 0 in 24-well plates containing DMEM with 2 % FBS. The fresh media was changed every two days. Cell proliferation was assessed by MTT colorimetric assay. Values are expressed as mean ± SEM of 2-3 experiments, each experiment was done triplicate.

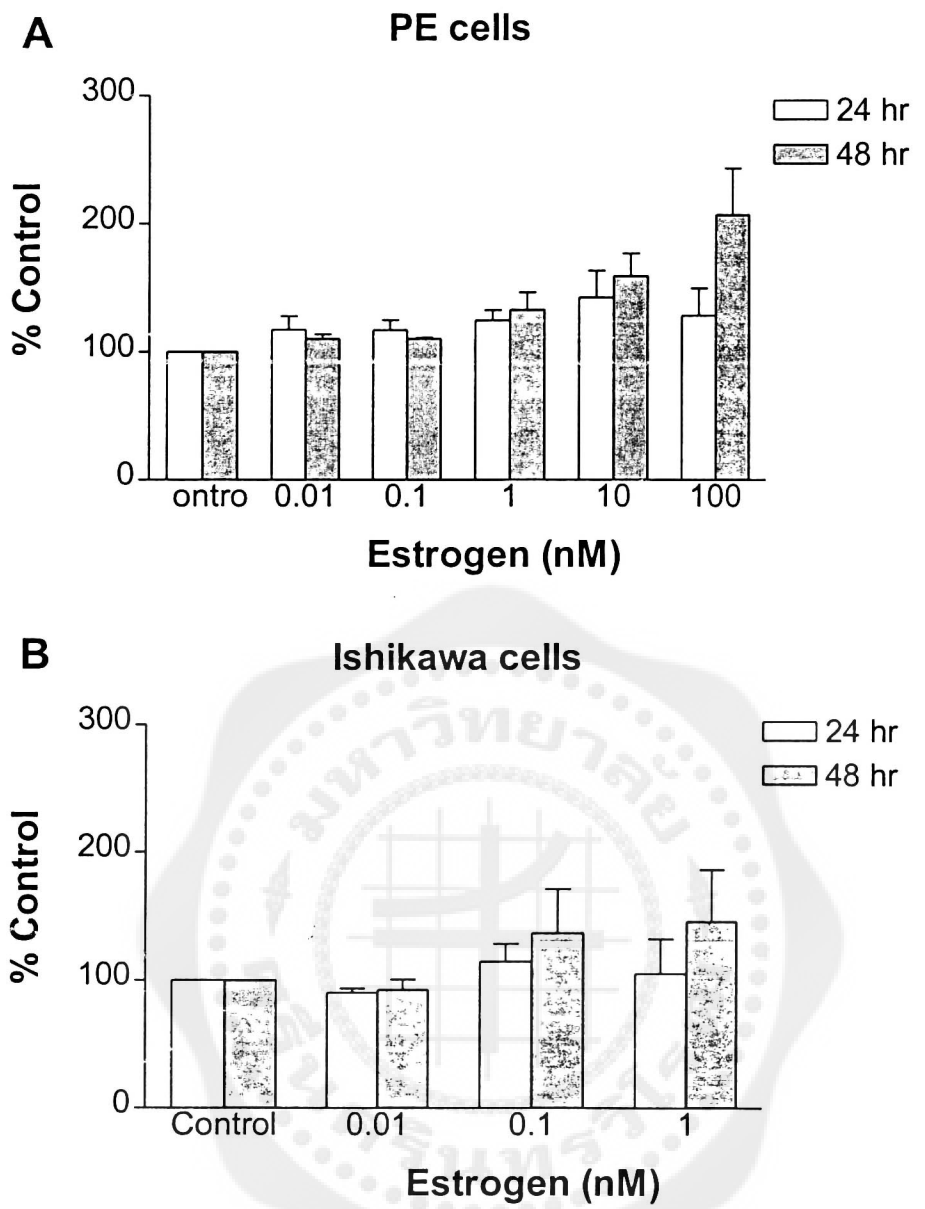

Figure 3 Effects of 17β-estradiol (E_2) on proliferation of normal endometrial cells (PE cells) (A) and Ishikawa cells (B). Cells (2 x 10^4 cells/well) were seeded in 24-well plates containing phenol red-free media with 2 % charcoal-stripped FBS. Two days after seeding, cells were treated with 17β-estradiol (E_2) at the concentrations of 0.01-100 nM (for PE cells) or 0.01-1 nM (for Ishikawa cells) or ethanol in control group for 24-48 h. Cell proliferation was analyzed by hemocytometer and MTT colorimetric assay. Values are expressed as mean ± SEM of 4-7 experiments. Each experiment was done triplicate.

As well as PE cells, Ishikawa cells stimulated cell proliferation in concentration-dependent manner in both 24 and 48 h treatment period. In all concentrations tested, the proliferative effect of estrogen is more responsive at 48 h than 24 h (Fig. 3B). Treatment of E_2 for 24 h increased cell numbers from 100 % in control groups to 90.41 ± 3.25, 114.69 ± 13.80 and 105.11 ± 27.36 % for 0.01, 0.1 and 1 nM E_2, respectively. Following 48 h treatment, E_2 at concentration of 0.01, 0.1 and 1 nM increased cell proliferation to 92.68 ± 7.96, 137.03 ± 34.44 and 146.23 ± 40.26 % compared with 100 % in control groups, respectively.

ERα expression in endometrial cell culture

To investigate the possible involvement of estrogen receptor in the development and response to the estrogenic or antiestrogenic drugs in this study, we assessed the expression of ERα in the PE cells and Ishikawa cells using Western blot analysis. Both PE cells and Ishikawa cells grown in serum-free DMEM or DMEM + 2 % FBS with or without 10 nM E_2 expressed 66 kDa and 32 kDa of ERα. However, the Ishikawa cells also expressed 44 kDa isoform of ERα (Fig. 4).

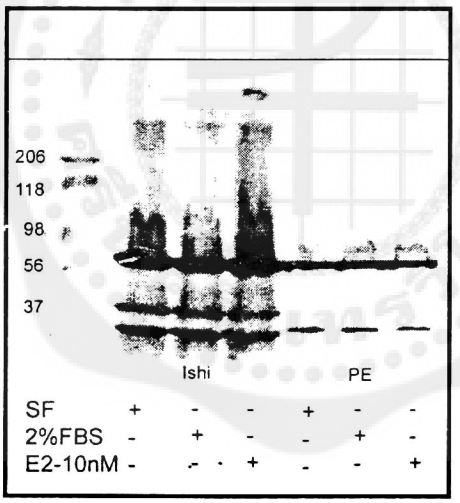

Figure 4 Western blot analysis of estrogen receptor α (ERα) in Ishikawa cells (Ishi, lanes 1-4) and porcine endometrial epithelial cells (PE, lanes 4-6). A 66 kDa band corresponding to the known size of ERα was detected in the Ishikawa and PE cells cultured in the serum free medium (SF), 2% fetal bovine serum (2% FBS) or 10 nM estradiol (E2-10nM) for 4 days. Proteins from whole-cell lysates were reacted with rabbit polyclonal antibody against carboxy terminal domain of human ER α as indicated in materials and methods. This analysis was repeated twice with similar results.

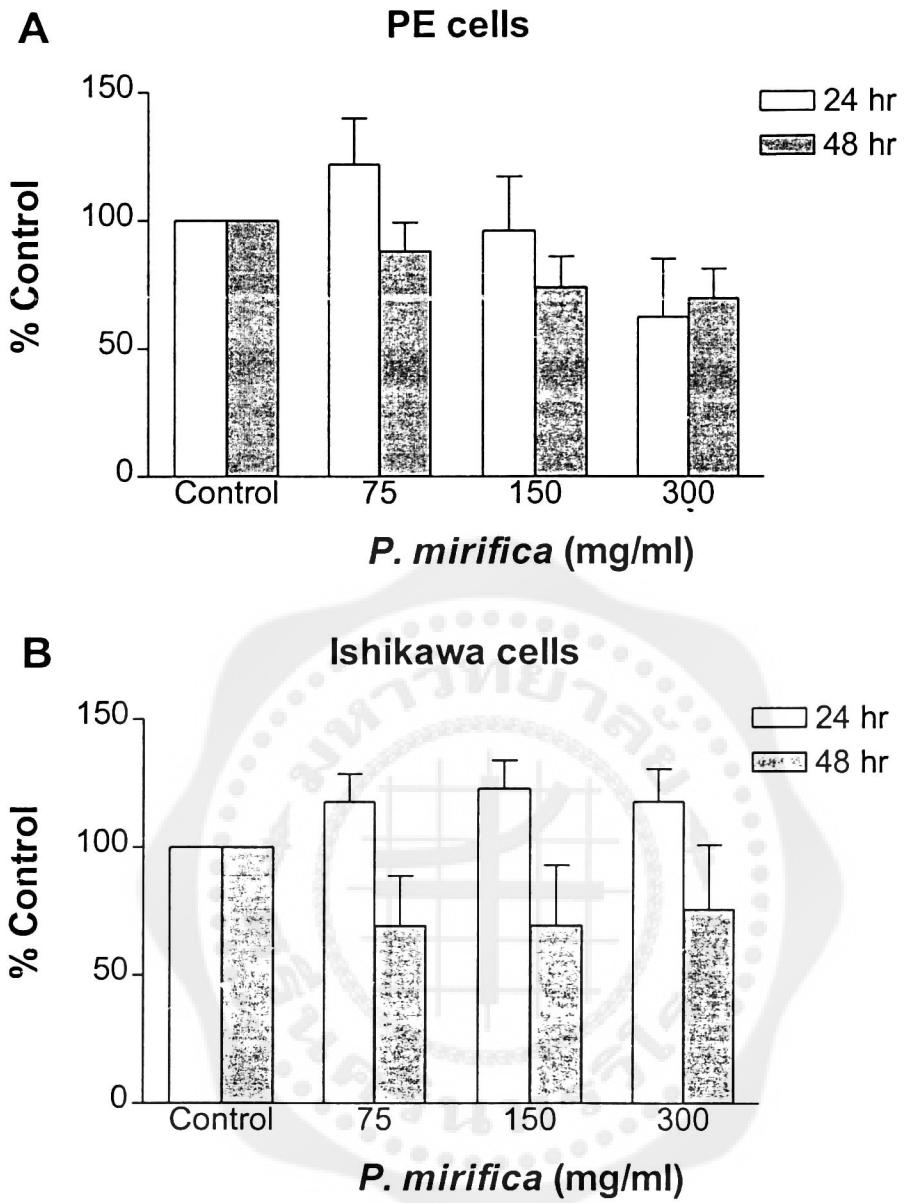

Figure 5 The effect of water extract of *P.mirifica* on the growth of normal endometrial epithelial cells (PE cells) (A) and Ishikawa cells (B). Cells at the number of 2 x 10^4 cells were seeded in 24-well plates containing phenol red-free media with 2 % charcoal-stripped FBS at day 0. After incubation for 48 h, cells were treated with water extract of *P. mirifica* 75, 150 and 300 mg/ml or distilled water as control for 24-48 h. Cell proliferation was analyzed by hemocytometer and MTT colorimetric assay. Values are expresses as mean ± SEM of 4-7 experiments. Each experiment was done triplicate.

Effects of water extract of P. mirifica on endometrial cell proliferation

To investigate the possible effect of *P. mirifica* on the growth of endometrial cancer cell and normal endometrial epithelial cells, we treated the cells with water extract of *P mirifica* at the concentration of 75, 150 and 300 mg/ml on day 2 after seeding cells. The proliferative effects were assessed after treatment for 24 and 48 h. As shown in Fig 5A, treatment of PE cells with all concentrations of *P. mirifica* for 24 and 48 h had no significant effect on cell numbers, although a slightly increase in cell number was observed at low concentration of *P. mirifica* following 24 h treatment. The numbers of PE cells were changed from 100 % in vehicle control groups to 121.92 ± 18.01 % (n=5), 96.05 ± 21.16% (n=5) and 62.44 ± 22.60 % (n=4) for 24 h treatment and to 88.08 ± 11.25 % (n=7), 73.96 ± 12.03 % (n=6) and 69.64 ± 11.57 % (n=6) for 48 h treatment, corresponding to *P. mirifica* 75, 150 and 300 mg/ml respectively. When the same protocol was employed with the Ishikawa cells, it was found that as shown in Fig. 4B, treatment of *P. mirifica* 75, 150 and 300 mg/ml for 24 h tended to increase the growth of Ishikawa cells to 117.72 ± 10.86 % (n=5), 122.83 ± 11.01 % (n=4) and 117.73 ± 12.77 % (n=4), respectively. In contrast, the treatment of *P. mirifica* for 48 h tended to decrease the growth of Ishikawa cells to 69.27 ± 19.57 % (n=6), 69.44 ± 23.52 % (n=6) and 75.67 ± 25.18 % (n=6), respectively.

Effect of estradiol and tamoxifen on endometrial cell proliferation

To investigate whether the effects of estrogen on cell proliferation are mediated through estrogen receptor, we treated the cells with E_2 or *P. mirifica* in combination with tamoxifen for 48 h. Tamoxifen is estrogen receptor antagonist that has been commonly used to treat breast cancer, although it was evident to increase proliferation of endometrial cells. Tamoxifen was used in this study to evaluate its effect on the proliferative response to estradiol in normal endometrial cells and endometrial cancer cells. In PE cells, treatment of E_2 100·nM resulted in a 158.56 ± 20.96 % (n=6, $P<0.01$) increase in cell proliferation as shown in Fig.6A. Tamoxifen at the concentration of 1 μM significantly increased the cell number to 160.92 ± 29.22 % (n=3, $P<0.01$) wheras at 10 μM tamoxifen decreased the number of cells to 51.27 ± 8.55 % (n=3, $P<0.05$) compared with 100 % in vehicle control. The cell numbers of groups receiving the combination of E_2 100 nM and tamoxifen 1 or 10 μM were not different from those receiving tamoxifen alone (150.86 ± 30.03 % (n=3) vs 160.92 ± 29.22 % (n=3) and 48.56 ± 19.74 % (n=3) vs 51.27 ± 8.55 % (n=3) in the presence of tamoxifen 1 μM and 10 μM, respectively).

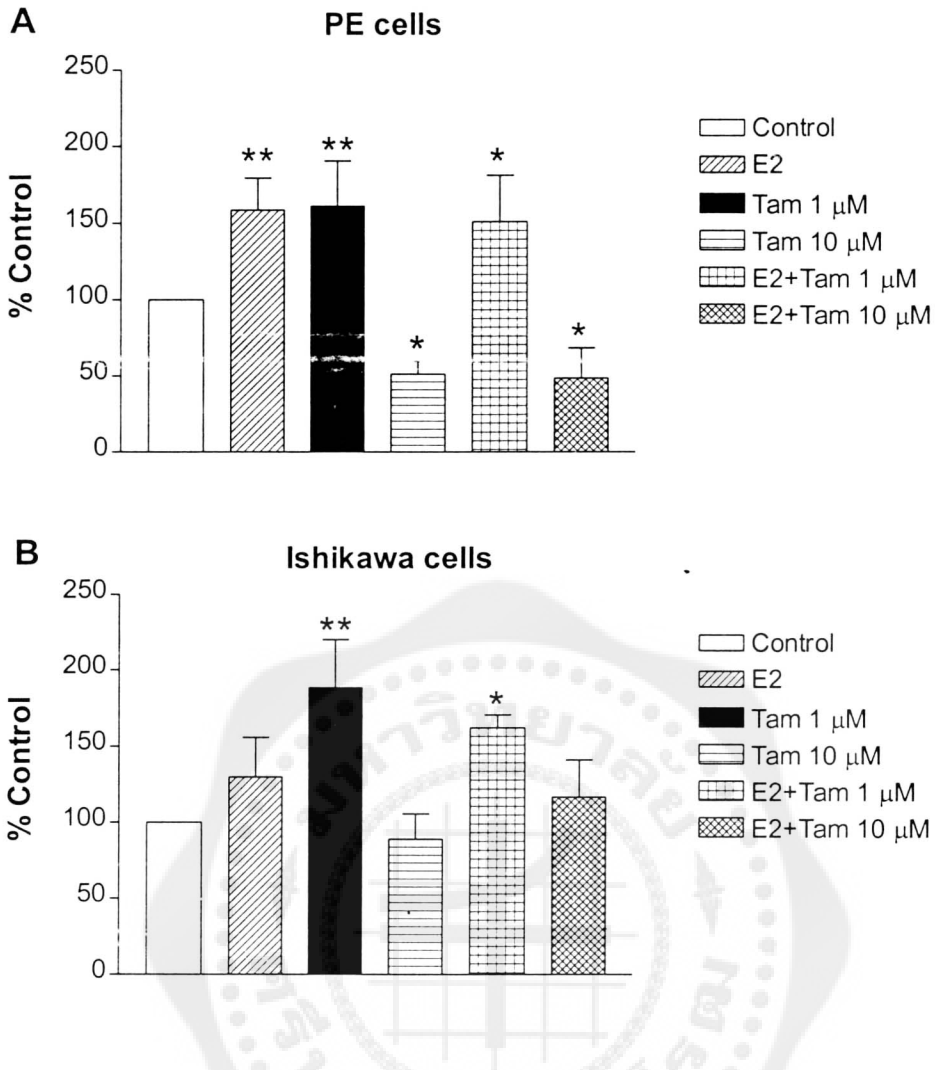

Figure 6 The effect of E_2 and tamoxifen on the growth of PE cells (A) and Ishikawa cells (B). Cells at the number of 2 x 10^4 cells were seeded in 24-well plates containing phenol red-free media with 2 % charcoal-stripped FBS. After incubation for 48 h, the cells were treated with the fresh media in the presence of 10 nM E_2 with or without 1 and 10 μM tamoxifen. After 48 h incubation period, the number of cells was estimated by MTT colorimetric assay. Values are expressed as mean ± SEM of 3-6 experiments. Each experiment was done triplicate. ** *P< 0.001* and * *P< 0.05* (Dunnett's test) were considered significantly difference from vehicle.

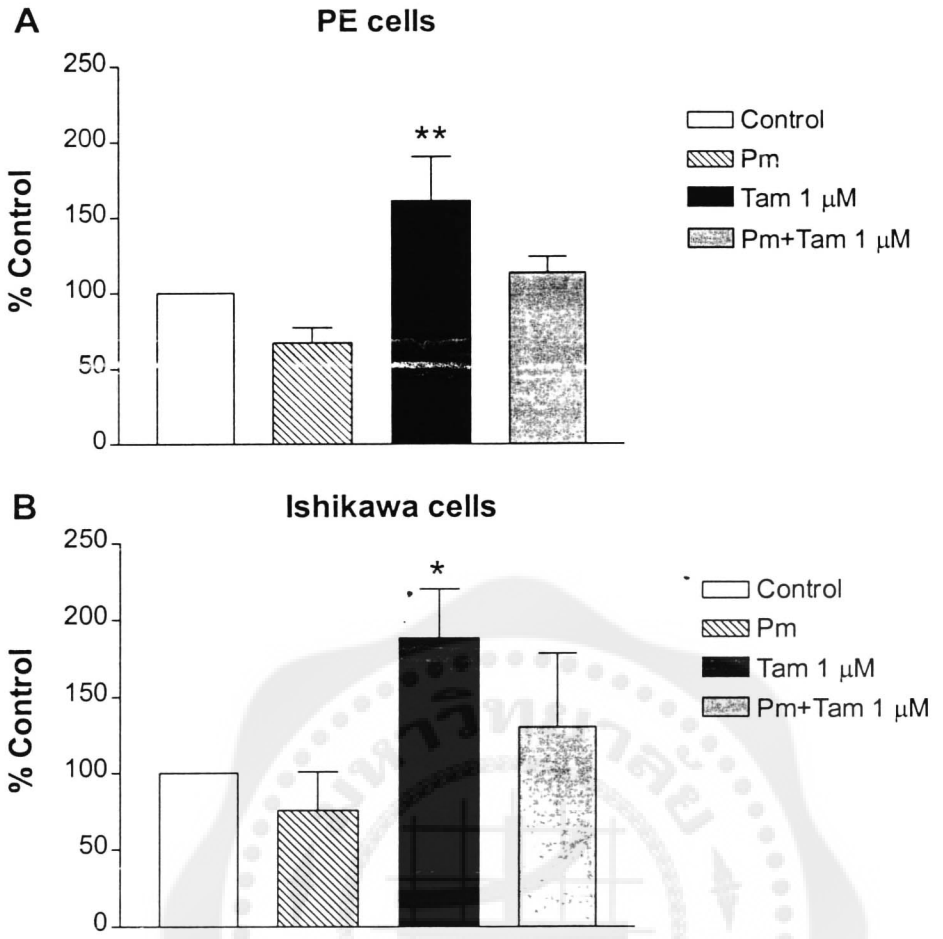

Figure 7 The effect of E_2, tamoxifen and water extract of *P.mirifica* on the growth of PE cells (A) and Ishikawa cells (B). Cells at the number of 2 x 10^4 cells were seeded in 24-well plates containing phenol red-free media with 2 % charcoal-stripped FBS. After incubation for 48 hr, the cells were treated with the fresh media in the presence of 300 mg/ml *P. mirifica*, with or without 1 μM tamoxifen. After 48 hr incubation period, the number of cells was estimated by MTT colorimetric assay. Values are expressed as mean ± SEM of 3-6 experiments. Each experiment was done triplicate. ** $P< 0.001$ and * $P< 0.05$ (Dunnett's test) were considered significantly difference from vehicle.

In Ishikawa cells (Fig. 6B), treatment of E_2 100 nM increased the cell numbers to 129.78 ± 25.74 % (n = 5) but not statistically difference from control group. Tamoxifen 1 μM produced a marked increase in numbers of Ishikawa cells to 188.33 ± 31.61 % (n=3, P<0.001) whereas cell numbers were not significant different from the control group when exposed to 10 μM tamoxifen (88.95 ± 16.38 %, n=3). In the presence of E_2, the stimulatory effect of tamoxifen 1 μM was still observed. The cell numbers obtained with the combination of E_2 10 nM and tamoxifen 1 or 10 μM were not different from those exposed to tamoxifen alone (162.00 ± 8.39 % (n=3) vs 188.33 ± 31.61 % (n=3) and 116.36 ± 24.63 % (n=3) vs 88.95 ± 16.38 % (n=3) in the presence of tamoxifen 1 μM and 10 μM, respectively).

Effect of P. mirifica and tamoxifen on endometrial cell proliferation

To investigate estrogenic or antiestrogenic effects on cell proliferation assay and its mechanism mediated through estrogen receptor, we also treated the normal porcine endometrial cells or Ishikawa cells with *P. mirifica* in combination with tamoxifen for 48 h. In contrast to estrogen treatment, treatment of *P. mirifica* 300 mg/ml did not change the number of PE cells compared to the vehicle control (75.67 ± 25.18 %, n=6) (Fig. 7A). However, there was a tendency to increase the cell number in groups receiving *P. mirifica* 300 mg/ml and tamoxifen 1 μM, although the increase was not significantly diferent from the control group (113.44 ± 10.61 % vs 100.00 ± 0%) or group receiving *P. mirifica* alone.

The effect of *P. mirifica* on the growth of Ishikawa cells was shown in Fig. 7B. Consistent with the results obtained from PE cells, there were no significant differences from the vehicle control when Ishikawa cells were treated with *P. mirifica* 300 μg/ml with (75.67 ± 25.18 %, n=3) or without tamoxifen 1 μM (129.89 ± 48.53 %, n=3).

DISCUSSION

Peuraria mirifica contained several active constituents that possessed the estrogenic activity. In many studies using animal model, *P. mirifica* when orally given was shown to have estrogenic effects by stimulating the growth of mammary gland and uterine weight (Caine, 1960; Sornsrivichai *et al.*, 1987). However, the effect of *P. mirifica* on the cellular level of estrogen-responsive organs has not been illucidated. It has been believed that lifetime exposure to estrogens or antiestrogen agents may be

important risk factors for both mammary and endometrial cancer (Parazzini *et al.*, 1994). The organ, tissue and cellular approaches will provide the useful information for consideration of using estrogenic and anti-estrogenic agents. In the present study, we employed endometrial epithelial cell model, porcine endometrial epithelial cells (PE cells) and human endometrial cancer cells (Ishikawa cells), to study the estrogenic of *P. mirifica* on the cell proliferation. Primary endometrial epithelial cells (PE cells) represented a normal cell model for studying cell growth. Although estrogen receptors have not been characterized in these cells, previous study using PE cells grown on permeable support demonstrated that long-term culture with 17β-estradiol (E_2) increased anion secretion across cell monolayer (Deachapunya and O'Grady, 1997). The Ishikawa cells represented few of endometrial cancer cells that have been shown to possess estrogen receptors and highly functional responses to estrogen (Holinka *et al.*, 1986; Sakomoto *et al.*, 2002).

It is known that estrogen regulates several endometrial functions including proliferation, differentiation and secretion. Most of the proliferative and estrogenic effects are produced via estrogen receptor in endometrial cells. In the present study, we demonstrated that 17β-estradiol (E_2) produced concentration-dependent increase in cell numbers of both PE cells and Ishikawa cells, although the effects was evident at 48 h treatment period (Fig. 3). The proliferative effects of E_2 were consistent with the expression of estrogen receptor alpha (ERα) isoform protein in the PE cells and Ishikawa cells as determined by western blot analysis using rabbit monoclonal antibody against human ERα (Fig. 4). This antibody can detect human ERα 66 which are the full length estrogen receptors (Green *et al.*, 1986). These findings suggested that the effect of E_2 on cell proliferation was mediated through estrogen receptors. The presence of ERα and the E_2-increased proliferation of Ishikawa cells in this study were in agreement with previous studies (Holinka *et al.*, 1986; Sakomoto *et al.*, 2002). While the present findings that PE cells possessed ER receptor and estrogen responsiveness, up to 2-3 weeks in culture, was relatively new evidence and indicated PE cells a good model for study the estrogenic and anti-estrogenic activities of estrogenic compounds including *P. mirifica*.

Most studies with estrogenic and anti-estrogenic drug screening have been widely performed in cancer cells that express high estrogen receptors. The existence of ERα and estrogen responsiveness of PE cells was very interesting. This was not only because it represented non-cancer endometrial cells for studying cell growth and estrogen-related cell functions under non-pathogenic conditions, but it also served as a comparative model

for human since the porcine tissues were the most homology to the human tissues. Even though the pattern of female sex hormone in pig during the estrous cycle is considered to be different from that in human, the presence of estrogen receptor alpha (ERα) and estrogenic functions was found to be similar as shown in the present study.

It has been known that the serum-containing media affected various degrees on cell proliferation study due to the presence of growth factors, steroid hormones as well as pH indicator phenol red. In order to minimize the proliferative effect of the medium, the cells were preconditioned with phenol red-free medium supplemented with charcoal-treated fetal bovine serum. Under this condition, the cells were stripped of steroid hormone (or low concentration of steroid hormone). In most cells, a preconditioning treatment, which involved growing the cells in charcoal-dextran-treated serum containing medium for a certain period of time, can improve the estrogenic-induced proliferation of Ishikawa cells or other estrogen-dependent cells, MCF7 (Welshons and Jordan, 1987). This was attributed to up-regulation of estrogen receptors in Ishikawa cells (Holinka *et al.*, 1989). In our study, PE cells and Ishikawa cells produced a comparable increase in cell numbers within nanomolar range. However, the Ishikawa cells show only a small increase in cell proliferation after estrogen treatment of 48 hours when compared to the other study that revealed a marked increase in cell number after 4-5 days of estrogen treatment (Anzai *et al.*, 1989). In addition, the expression of ERα under serum-containing media or serum-free media in the absence or presence of estrogen seemed not to be different.

In the present results, we found that treatment of water extract of *P. mirifca* had no significant effect on proliferation of both PE cells and Ishikawa cells using MTT cell proliferation assay (Fig. 4), although the slightly increase in cell numbers of Ishikawa cells was observed following 24 h treatment. This suggested that the water extract of *P. mirifica* had no estrogenic effect. Previous *in vivo* studies demonstrated that the oral administration of crude extract of *P. mirifica* increased the growth of mammary ducts in both female and male rats (Caine, 1960) and the uterine weight in dogs (พูลศิลป์และคณะ, 2530) and mice (Sornsrivichai et al., 1987). If the increase in organ weight was presumably due to the increase in cell numbers, it indicated that the water extract of *P. mirifica* was depleted of estrogenic compounds that produced estrogenic effect on cell growth. In addition, the route of administration should be considered in such a way that some orally given ingredients may be metabolized in the body to the more active compound with estrogenic activity. However, the further study will be required to isolate and characterize

the active ingredient of *P. mirifica* extract and the interactions between these substances and their targets in the normal and carcinoma endometrial cells.

Several active ingredient of *P. mirifica* was shown to have estrogenic effects on proliferation of many cell lines. Miroestrol and deoxymiroestrol was a major component extracted from *P. mirifica* (Chansakaow *et al*, 2000). Deoxymiroestrol was found to be more potent than miroestrol and estrogen to produce the growth of MCF-7 mammary cancer cell (Chansakaow *et al*, 2000). *P. mirifica* also contained isoflavones that are a major subclass of the phytoestrogen family. Proliferative study using human stromal and glandular epithelial cells demonstrated that isoflavones acted as estrogen receptor agonist and estrogen receptor antagonist in the presence of E_2 (Kayisli *et al.*, 2002). Although the present results as illustrated in Fig. 4 demonstrated that the water extract of *P. mirifica* had no significant effect on cell numbers of PE cells, but it showed a tendency to inhibit PE cell proliferation after 48 h treatment. However, when we closely compared the proliferative effect of *P. mirifica* 300 mg/ml with the control group alone, this *P. mirifica* group turned out to be significant different from the control groups (Fig. 7A). All of these results with PE cells suggested possibilities that 1) the aqueous extract of *P. mirifica* was not composed of plant hormones or phytoestrogens that was previously shown to exert estrogenic effect on proliferation of estrogen-sensitive cell and tissues. 2) the aqueous extract of *P. mirifica* may contain other substances that can inhibit the growth of endothelial cells, by acting as estrogen receptor antagonist or acting through other different pathway, not mediated through estrogen receptors.

In this study, we also investigated and compared the proliferative effects of *P. mirifica* with an estrogen receptor (ER) antagonist tamoxifen. Tamoxifen has been widely used for the treatment of breast cancer but it can increase the risk of endometrial cancer. Basically, tamoxifen was classified as antiestrogens type I, which have mixed estrogenic or antiestrogenic actions (MacGregor and Jordan, 1998). Several *in vitro* studies have reported that tamoxifen stimulates the growth of endometrial cancer cells, but not breast cancer cells, in cell culture (Anzai et al., 1989; Jamil et al., 1991). In the present study, we found the biphasic response of tamoxifen on proliferation of both PE cells and Ishikawa cells. Tamoxifen when given at 1 μM increased the cell numbers whereas at 10 μM had no effect on cell numbers of Ishikawa cells compared to the vehicle control group (Fig. 5B). The stimulatory effect of tamoxifen (1 μM) on proliferation of Ishikawa cells was in agreement with the previous of Anzai et al (1989) and Jordan (1984). Studies in breast cancer cells revealed that the very low concentrations of

triphenylethylene-type antiestrogen (antiestrogens type I) caused a single round replication, which was completely inhibited at the high concentrations. In addition, the antiestrogens type I (i.e., tamoxifen and its metabolite 4-hydroxytamoxifen) have been known for the drugs that can form complex to cytosolic estrogen receptors causing either the inhibition of estrogen to bind to its receptor or the initiation of estrogen-responsive gene transcription depending on the shape of drug-receptor complex (Metzger et al., 1988; McDonnell *et al.,* 1995). These explanations were attributed to the estrogenic and antiestrogenic of tamoxifen. From these mechanisms together with the biphasic actions of tamoxifen in the present study indicated that tamoxifen at low concentrations exerted estrogenic effects whereas high concentrations exerted antiestrogenic effects.

The combination treatment of low concentration of tamoxifen and estrogen did not show synergistic effects on the growth of PE or ishikawa cells (Fig. 6), suggesting that the estrogenic effects of tamoxifen were probably mediated by different binding site of estrogen receptors or different pathways, not mediated by estrogen receptor system. Tamoxifen at low concentration may bind to the different site on estrogen receptor and result in activation of gene transcription. This binding complex may prevent estrogen to bind to its receptor sites. Recent study with Ishikawa cells has been demonstrated the stimulatory effect of 4-hydroxytamoxifen on proliferation of Ishikawa cells (Sakamoto et al., 2002). In this study, they suggested that the stimulatory effect may involve the activation of transcriptional activity of ERα via the AF1 domain, which is activated by constitutively activated mitogen-activated protein (MAP) kinase pathway. In addition, high concentration of tamoxifen combined with E_2 did not reverse the antiproliferative activity of tamoxifen in PE cells. This finding suggested that high concentrations of antiestrogens type I may bind to the different sites on estrogen receptor and completely block the estrogen to bind to its receptor. However, the exact mechanism is unknown.

In the present study, the estrogenic or antiestrogenic activities of tamoxifen on proliferation of PE cells and Ishikawa cells were dependent on the concentration. Treatment of *P. mirifica* 300 mg/ml decreased PE cells proliferation to the same degree as high concentration treatment of tamoxifen. The inhibitory effect of these two treatments was significant different from the control group. This evidence suggested that the water extract of *P. mirifica* may contain antiestrogenic activity on the proliferation of normal porcine endometrial cell. However, the antiestrogenic activity of *P. mirifica* mediated by estrogen receptor system or other different pathway cannot be concluded in the present study.

In conclusion, the water extract of *P. mirifica* had no effect on proliferation of normal endometrial epithelial cells and human endometrial cancer cell line except a slight inhibition of normal endometrial cell proliferation by high concentration of *P. mirifica*. The antiproferative effect of *P. mirifica* mediated via estrogen receptor requires further investigation. In contrast, 17β-estradiol increased the proliferation of both PE cells and Ishikawa cells in concentration-dependent manner. The presence of estrogen receptor and estrogen responsiveness of PE cells will serve as a non-pathogenic cell model used to screen chemicals for potential estrogenic properties. Finally the findings from our study will provide scientific data for condiseration of using *P. mirifica* for medical purposes in human in the future.

Acknowledgement

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References

ปกรณ์ ไทยานันท์ พิพิธ ตระกูลบุญ สมบูรณ์ อนันตลาโภชัย. 2536. อิทธิพลของกวาวเครือต่อนกกระทา: II. การสร้างเม็ดเลือดแดงและขาว. วารสารเทคนิคการแพทย์เชียงใหม่ 25(3): 107-114.

พูลศิป์ ไทยะโชติ กิตตินันท์ นิวาศะบุตร ยุทธนา มมิตะสิริ. 2530. การศึกษาเบื้องต้นเกี่ยวกับกวาวขาวในลูกสุนัข. การประชุมวิชาการวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ครั้งที่ 13 วันที่ 20-22 ตุลาคม 2530 ณ มหาวิทยาลัยสงขลานครินทร์: 498.

ยุพดี ลางคลิจันทร์ ยุทธนา สมิตะสิริ.2528. ผลของกวาวขาวต่อการสืบพันธุ์ในหนูขาวเพศผู้. การประชุมวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ครั้งที่ 11 วันที่ 24-26 ตุลาคม.

ยุทธนา สมิตะสิริ เสรี แปงจิตต์. 2529-2530. ฤทธิ์ในการคุมกำเนิดของกวาวขาวในหนูขาว. วารสารคณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ 13 -14(2/1): 75-80.

ยุทธนา สมิตะสิริ เสรี แปงจิตต์ สมบูรณ์ อนันตลาโภชัย. 2532. การยับยั้งการให้นมหนูที่กำลังให้นมด้วยกวาวขาวเปรียบเทียบกับเอสโตรเจน. วารสารคณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ 16: 7-11.

วัชรีวรรณ หวังดี 2529 ผลของกวาวขาว เอสโตรเจน และโพรแลคติน ต่อต่อมน้ำนม ต่อมใต้สมอง และมดลูกของลูกหนูที่ตัดรังไข่ รายงานปัญหาพิเศษ ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่.

สมบูรณ์ วิริยะ ยุทธนา สมิตะสิริ. 2530. ผลของกวาวขาวปริมาณต่ำต่อลูกไก่พันธุ์เนื้อ การประชุมวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ครั้งที่ 13 วันที่ 20-22 ตุลาคม ณ มหาวิทยาลัยสงขลานครินทร์: 373-374.

สมบูรณ์ อนันตลาโภชัย สุวิทย์ เจศรีชัย. 2532. ผลของสมุนไพรพื้นบ้านกวาวขาวปริมาณสูงต่อนกกระทาพันธุ์ญี่ปุ่น: ผลต่อการเปลี่ยนแปลงปริมาณแคลเซี่ยม โปรตีนและคอเลสเตอรอลในเลือด. วารสารคณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ 13(1): 29-37.

สมภพ ประธานธุรารักษ์. 2542. กวาวเครือและไฟโตเอสโตรเจน. ใน ณัฐนันท์ สินชัยพานิช และคณะ, บรรณาธิการ. การประชุมวิชาการเภสัชกรรมประจำปี 2542: เภสัชกรพัฒนาเพื่อการพึ่งพาตนเอง. 24-26 มีนาคม. กรุงเทพมหานคร: เภสัชกรรมสมาคมแห่งประเทศไทย. 25-41.

Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H. and Boyd, M.R. (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* 48: 589-601.

Anzai, Y., Holinka, C.F., Kuramoto, H., Gurpide, E. (1989). Stimulatory effects of 4-hydroxytamoxifen on proliferation of human endometrial adenocarcinoma cells (Ishikawa line). *Cancer Res.* 49: 2362-2365.

Benson, G.K., Cowie, A.T. and Hosking, Z.D. (1961) Mammogenic activity of miroestrol. *J. Endocrinol.* 21: 401-409.

Deachapunya C and O'Grady S.M. (1997) Estrogen regulates amiloride-sensitive Na absorption across glandular endometrium. *The Physiologist* 40: A5, 1997.

Deachapunya, C. and O'Grady, S. M. (1998) Regulation of chloride secretion across porcine endometrial epithelial cells by prostaglandin E_2. *J. Physiol.* 508(1): 31-47.

Caine, J.C. (1960) Miroestrol: an estrogen from the plant *Pueraria mirifica*. *Nature* 168: 774-777.

Chansakaow, S., Ishikawa, T., Seki, H., Sekine, K., Okada, M., and Chaichantipyuth, C. (2000) Identification of deoxymiroestrol as the actual rejuvenating principle of "Kwao keur", *Pueraria mirifica*. The known miroestrol may be an artifact. *J. Nat. Products* 63(2): 173-175.

Green, S., Walter, P., Kumar, V., Krust, A., Bornerte, J.M., Argos, P. and Chambon, P (1986) Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* 320(6058): 134-139.

Hirano, T., Fukuoka, K., Oka, K, *et al.* (1990) Antiproliferative activity of mammalian lignan derivatives against the human breast carcinoma cell line, ZR-75-1. *Cancer Invest* 8: 595-602.

Jamil, A., Croxtall, J.D. and White, J.O. (1991) The effect of antioestrogens on cell growth and progesterone receptor concentration in human endometrial cancer cells (Ishikawa). *J.Mol.Endocrinol.* 6: 215-221.

Jones, H.E.H. and Pope, G.S. (1961) A method for the isolation of miroestrol from *Pueraria mirifica. J. Endocrinol.* 22: 303-312.

Jordan, V.C. (1994) Long-term tamoxifen treatment for breast cancer. University of Wisconsin press, Madison.

Kayisli, U.A., Aksu, C.A.H., Berkkanoglu, M. and Arici, A. (2002) Estrogenicity of isoflavones on human endometrial stromal and glandular cells. *J.Clin.Endocrinol.Metab.* 87(12): 5539-5544.

Lamartiniere, C.A., Moore, J.B., Brown, N.M. *et al* (1995) Genistein suppresses mammary cancer in rats. *Carcinogenesis* 16(11): 2833-2840.

MacGreger, J.I. and Jordan, C. (1998) Basic guide to the mechanisms of antiestrogens. *Pharmaco. Rev.* 50: 151-196.

McDonnell, D.P., Clemm, D.L., Hermann, T., Goldman, M.E. and Pike, J.W. (1995) Analysis of estrogen receptor function reveals three distinct classes of antiestrogens. *Mol. Endocrinol.* 9: 659-669.

Metzger, D., White, J. and Chambon, P. (1988) The human estrogen receptor functions in yeast. *Nature* (Lond.) 334: 31-36.

Parazzini, F., Lavecchia, C., Negri, E. and Villa, A. (1994) Estrogen replacement therapy and ovarian cancer risk. *Int.J.Cancer* 57(1): 135-136.

Sakomoto, T., Eguchi, H., Omoto, Y., Ayabe, T., Moro, H. and Hayashi., S. (2002). Estrogen receptor-mediated effects of tamoxifen on human endometrial cancer cells. *Mol.Cell. Endocrinol.* 192: 93-104.

Somsrivichai, J., Liawruangrath, S., Kittakupt, P., Liawruangrath, B. and Smitasiri, Y. (1987) Pharmacological aspects of oestrogenic substances in tuberous root of *Pueraria mirifica.* Princess Congress I, 10-13 December, Bangkok: BC-4.

Schoeller, W., Dohrn, M. and Hohlweg, W. (1940) An estrogenic substance from the tubers of the Siamese vine, *Butea superba. Naturwissenschaften* 28: 532.

Scudiero, D.A., Shoemaker, R.H., Paull, K.D., Monks, A., Tierney, S., Nofziger, T.H., Currens, M.J., Seniff, D. and Boyd, M.R. (1988) Evaluation of a soluble

tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Research* 48: 4827-4833.

Wang, C. and Kurzer, M.S. (1997) Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. *Nutr. Cancer* 28(3): 236-247.

Welshons, W.V., Murphy, C.S., Koch, R., *et al.* (1987) Stimulation of breast cancer cells in vvitro by the environmental estrogen enterolactone and the phytoestrogen equol. *Breast Cancer Res. Treat*: 10(2): 169-175.

Zava, D.T. and Duwe, G. (1997) Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. *Nutr. Cancer* 27(1): 31-40.