

In Vitro Cytotoxic Activity of Sa-Tri-Lhung-Klod Remedy and Its Herbal Ingredients on Ovarian and Cervical Carcinoma Cell Lines

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Background: Sa-Tri-Lhung-Klod is a Thai traditional medicine remedy for postpartum in the lists of The National Drug List of Herbal Medicine Products AD. It consists of seventeen herbs and were obtained by maceration and used in the form of liquor for women's health care such as treatment of amenorrhea, menopause and blood tonic. In addition, it also used for postpartum care for being anti-inflammation in postpartum and prevention of cancer in women.

Objective: To investigate cytotoxic activity of Sa-Tri-Lhung-Klod remedy extracts and its herbal ingredients against human ovarian carcinoma cell line (SKOV-3) and cervical adenocarcinoma (HeLa) cell line.

Material and Method: Sa-Tri-Lhung-Klod remedy and its plant ingredients were extracted by maceration in 95% ethanol and dried using evaporator. All extracts were tested for cytotoxic activity by sulforhodamine B (SRB) assay.

Results: Ethanolic extract of Sa-Tri-Lhung-Klod remedy displayed cytotoxic activity against SKOV-3 and HeLa with IC_{50} values of 72.84 ± 1.07 and 47.24 ± 2.83 $\mu\text{g/ml}$, respectively. It was classified as "less-active" according to the NCI guideline. However, *Caesalpinia sappan*, *Mammea siamensis* and *Curcuma comosa* showed high cytotoxic activity against SKOV-3 with IC_{50} values of 9.55 ± 1.38 , 13.45 ± 0.82 and 14.21 ± 1.30 $\mu\text{g/ml}$, respectively. The ethanolic extracts of *Caesalpinia sappan* and *Mammea siamensis* also exhibited cytotoxic activity against HeLa with IC_{50} values of 6.30 ± 0.06 and 7.72 ± 0.11 $\mu\text{g/ml}$, respectively.

Conclusion: These results support the use of Sa-Tri-Lhung-Klod remedy in Thai traditional medicine for preventing of ovarian cancer and cervical cancer. *Caesalpinia sappan*, *Curcuma comosa* and *Mammea siamensis* were strikingly active against ovarian and cervical cancer cells. Their extracts have the potential for developing as new anti-cancer drugs for women health.

Keywords: Sa-Tri-Lhung-Klod remedy, Cytotoxicity, Ovarian cancer, Cervical cancer

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Women's cancers, including ovarian and cervical cancer, lead to premature deaths among women worldwide⁽¹⁾. In Thailand, the National Cancer Institute in Bangkok reported that, the most common cancer in women is breast cancer (37.5%), cervical cancer (14.4%) and ovarian cancer (4.5%)⁽²⁾. Many factors related to ovarian cancer and cervical cancer. There are both external and internal factors; i.e. having sex at a young

age, age, chemicals such as oral contraceptives, smoking, foods and, most important are the chronic inflammation, infections and genetic disorders⁽³⁾. Nowadays, the treatments of ovarian and cervical cancer are several methods such as surgery (19.7%), radiation therapy (21.5%), chemotherapy (3.8%), etc⁽¹⁾. Surgery is the main treatment for most ovarian and cervical cancers, but there are many several side effects such as pain, fatigue, infection and bleeding or organ dysfunction⁽⁴⁾. However, alternative medicine is now commonly used for cancer patients⁽⁵⁾ because generally, it is believed that herbal medicine is safe and has less side-effects than surgery, radiation therapy and chemotherapy⁽⁶⁾.

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Thai traditional medicine in Thai National List of Essential Medicine called Sa-Tri-Lhung-Klod remedy was used to treat symptoms of women. It was used for treatment such as treating irregular menstruation, amenorrhea, stimulating blood circulation, menopause, postmenopausal, blood tonic, anti-inflammation in postpartum and astringent. These symptoms were predicted as cancer of women such as ovarian cancer and cervical cancer. This remedy consists of seventeen plants. There is no report of cytotoxic activity of this preparation, but the previous studies reported that some single herbs showed cytotoxic activity against cervix cancer cell line (HeLa) such as *Caesalpinia sappan*⁽⁷⁾, *Curcuma comosa*⁽⁸⁾, *Mammea siamensis*⁽⁹⁾, *Mesua ferrea*⁽¹⁰⁾, *Piper nigrum*⁽¹¹⁾, *Piper ribesoides*, *Piper samentosum* and *Plumbago indica*⁽¹²⁾. Therefore, the objectives of this study are to investigate the cytotoxic activity of Sa-Tri-Lhung-Klod remedy extracts and each of its herb ingredients against two types of human cancer cells from women such as ovarian carcinoma cell line (SKOV-3) and cervical adenocarcinoma cell line (HeLa). These results will support the use of this remedy in Thai traditional medicine for ovarian and cervical cancer.

Material and Method

Chemicals

Dimethyl sulfoxide [(CH₃)₂SO](DMSO) was purchased from RCI Labscan, Thailand. Fetal bovine serum (FBS) was purchased from Biochem, Germany. Hydrochloric acid (HCl) and Sodium hydroxide (NaOH) were purchased from Univar, Australia. Minimum Essential Medium (MEM), Penicillin-Streptomycin (P/S), RPMI medium 1640, Trypan blue stain 0.4% and Trypsin-EDTA were purchased from Gibco, USA. Phosphate buffered saline (PBS) was purchased from Amresco, USA. Sodium bicarbonate (NaHCO₃) was purchased from BHD, England. Sulforhodamine B sodium salt (C₂₇H₂₉N₂NaO₇S₂) and Tris (hydroxymethyl) aminomethane ((HOCH₂)₃CNH₂) were purchased from Sigma-Aldrich, USA. Trichloroacetic acid (Cl₃CCOOH) (TCA) was purchased from Merck, Germany.

Plant materials

The parts of plants of Sa-Tri-Lhung-Klod remedy were collected from several parts of Thailand in 2012, voucher specimens shown in Table 1. The voucher specimens were carried out at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

Extraction

The parts of these plants were washed with water, sliced to small pieces, dried in an oven at temperature 50°C and ground to powder. Sa-Tri-Lhung-Klod remedy (270 grams) and its herbal ingredients (50 grams of each) were macerated with 95% ethanol for 3 days, filtered using Whatman No. 1 filter paper and concentrated to dryness by evaporator (Rotavapor R-205, Germany). The marc was macerated twice more times. Filtrates were pooled and dried using an evaporator.

In vitro assay for Cytotoxic activity by SRB assay

Preparation of sample solution

Each sample was initially dissolved in sterile dimethylsulfoxide (DMSO) for ethanolic extracts and prepared sample at concentration 10 mg/ml. The first screening, final concentration of sample solution was prepared at 100 µg/ml. If the percent inhibition of cancer cell by the extract is more than 50%, the extract will be further tested for IC₅₀ value. IC₅₀ values were determined by preparing as four serial dilutions (200, 100, 20 and 2 µg/ml, respectively) to give the final concentration of 100, 50, 10, 1 µg/ml, respectively.

Human cell lines

Human ovarian carcinoma (SKOV-3) and cervical adenocarcinoma (HeLa) cell lines were purchased from the American Type Culture Collection (ATCC, USA). SKOV-3 was cultured in RPMI-1640 medium and HeLa was cultured in Minimum Essential Media (MEM) with Earle's salt (without glutamine). There supplemented with 10% heated fetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin. All cell lines were maintained at 37°C in an incubator with 5% CO₂ and 95% humidity.

Cytotoxicity assay

Cells growing as monolayer in 75 cm² flask were washed with Phosphate-buffered saline (PBS) and cells detached with 0.025% trypsin-EDTA to make a single cell suspension. 3 ml medium was then added to flask to inactivate trypsin-EDTA. The viable cells were counted in haemocytometer using Trypan Blue Exclusion Test of cell viability⁽¹³⁾. Single cell suspension was diluted with medium to give a final concentration of 3x10³ cells/well for each of SKOV-3 or HeLa, respectively. One hundred microlitres per well of these cells suspensions were added in 96-well microtiter plates and incubated at 37°C in 5% CO₂ atmosphere with 95% humidity for 24 hours. Cells were then treated with

Table 1. Plants and part of plants component in Sa-Tri-Lhung-Klod remedy

Botanical name	Family	Plant collected from	Voucher specimen number	Part of used	Thai traditional medicine used
<i>Angelica sinensis</i>	UMBELLIFERAE	China	SKP199010901	Root	Blood tonic, menopause and premenstrual syndrome
<i>Artocarpus heterophyllus</i>	MORACEAE	Nakhon Ratchasima	SKP117010801	Stem	Haemostatic, anti-inflammation, blood tonic and astringent
<i>Caesalpinia sappan</i>	LEGUMINOSAE	Bangkok	SKP098031901	Stem	Blood tonic, menorrhagia, leucorrhoea and diabetes
<i>Carthamus tinctorius</i>	COMPOSITAE	Chiang Mai	SKP051032001	Flower	Blood tonic, cardiac tonic and reduce cholesterol
<i>Curcuma comosa</i>	ZINGIBERACEAE	Phetchabun	SKP201030301	Rhizome	Menorrhagia, leucorrhoea, postpartum uterine bleeding and perimenopausal bleeding
<i>Jasminum sambac</i>	OLEACEAE	Nakhonpathom	SKP129101901	Flower	Asthma, cardiac tonic and fatigue
<i>Maclura cochinchinensis</i>	MORACEAE	Prachuap Khiri Khan	SKP117130301	Stem	Fever, antispasmodic and astringent
<i>Mammea siamensis</i>	GUTTIFERAE	Ratchaburi	SKP083131901	Flower	Cardiac tonic, fever-lowering and enhancement of appetite
<i>Mesua ferrea</i>	GUTTIFERAE	Ratchaburi	SKP083130601	Flower	Astringent, carminative, blood tonic and cardiac tonic
<i>Mimusops elengi</i>	SAPOTACEAE	Ratchaburi	SKP171130501	Flower	Cardiac tonic, expectorate and sore throat
<i>Nelumbo nucifera</i>	NELUMBONACEAE	Ratchaburi	SKP125141401	Pollen	Cardiac tonic, vertigo, faintness and fever
<i>Piper longum</i>	PIPERACEAE	Chanthaburi	SKP146160301	Fruit	Carminative, element tonic and antidiarrheal
<i>Piper nigrum</i>	PIPERACEAE	Chanthaburi	SKP146161401	Fruit	Carminative, element tonic, expectorate and diuretic
<i>Piper ribesoides</i>	PIPERACEAE	Sakhonnakhon	SKP146161801	Stem	Carminative, antitulant and element tonic
<i>Piper samentosum</i>	PIPERACEAE	Ratchaburi	SKP146161901	Root	Expectorant, carminative and flatulence
<i>Plumbago indica</i>	PLUMBAGINACEAE	Bangkok	SKP148160901	Root	Carminative, stomachache, antidiarrheic and hemorrhoids
<i>Salacia chinensis</i>	CELASTRACEAE	Ratchaburi	SKP044190301	Stem	Blood tonic, cardiac tonic, carminative and muscle pain
Sa-Tri-Lhung-Klod	-	-	-	-	Amenorrhea, blood tonic, menopause and astringent

extracts. 100 µl sample solution was added and mixed where 1% DMSO solution was used as control solvent. Cells in 96-well plate were incubated in CO₂ incubator for 72 hours. Medium was removed and washed with PBS. 200 µl fresh medium was added, incubated for recovery period of 72 hours and the survival percentage was measured calorimetrically using SRB assay and IC₅₀ values were calculated using prism program.

Sulphorhodamine B (SRB) assay

The anti-proliferative assay, SRB (Sulphorhodamine B) assay was performed essentially according to the method of Skehan et al 1990⁽¹⁴⁾. This assay is used for cell density determination, which was performed to assess growth inhibition by a colorimetric assay by staining total cellular protein with the dye SRB. Cell cultures were fixed with 100 µl of ice-cold 40% Trichloroacetic acid (TCA) per well, incubated at 4°C for 1 hour in refrigerator and washed with distilled water to wash non-viable cells. 50 µl of SRB solution (0.4% w/v in 1% acetic acid) was added per well and allowed contact with cells for 30 minutes and washed with 1% acetic acid. The plate was dried and 100 µl of 10 mM Tris Base pH 10.5 (Tris (hydroxyl methyl) aminomethane) was added. The absorbance (OD) of each well was read at 492 nm. The IC₅₀ values were

calculated using prism program by plotting the percentage of survival versus the concentrations, interpolated by cubic spine. According to National Cancer Institute guidelines⁽¹⁵⁾ extracts with IC₅₀ values <20 µg/ml were considered active.

Statistical analysis

All data are the mean of three replications. Values of different parameters were expressed as the mean ± standard error of mean. Statistical analysis was performed using SPSS (SPSS 13 for windows) statistical software.

Results

The percentage of yields (w/w) of the 95% ethanolic extracts of Sa-Tri-Lhung-Klod remedy and its herbal ingredients were shown in Table 1. The effect of the ethanolic extracts of Thai medicinal preparation and each of its herbal components were studied on two different cancer cell lines: human ovarian carcinoma cell line (SKOV-3) and human cervical adenocarcinoma cell line (HeLa). IC₅₀ values are summarized in Table 2.

Discussion

Plant components in Thai traditional medicine, Sa-Tri-Lhung-Klod remedy can be divided into several

Table 2. Cytotoxic activity of 95% ethanolic extracts from Sa-Tri-Lhung-Klod remedy and its herbal ingredients against of human ovarian carcinoma (SKOV-3) and cervical adenocarcinoma cell line (HeLa) by SRB assay (n = 3)

Botanical name	Wt. in remedy (g)	% yield	IC ₅₀ (µg/ml ± SEM)	
			SKOV-3	HeLa
<i>Angelica sinensis</i>	20	16.33	67.03±2.44	75.93±9.77
<i>Artocarpus heterophyllus</i>	20	9.34	32.58±0.76	33.37±0.39
<i>Caesalpinia sappan</i>	20	8.84	9.55±1.38	6.30±0.06
<i>Carthamus tinctorius</i>	10	15.64	>100	>100
<i>Curcuma comosa</i>	20	6.20	14.21±1.30	35.58±0.10
<i>Jasminum sambac</i>	10	13.10	>100	>100
<i>Maclura cochinchinensis</i>	20	12.20	>100	84.99±0.82
<i>Mammea siamensis</i>	10	32.60	13.45±0.82	7.72±0.11
<i>Mesua ferrea</i>	10	23.98	49.51±3.59	40.05±1.96
<i>Mimusops elengi</i>	10	6.35	>100	>100
<i>Nelumbo nucifera</i>	10	10.72	>100	>100
<i>Piper longum</i>	20	11.96	72.14±1.12	76.39±1.13
<i>Piper nigrum</i>	20	6.01	35.16±1.07	37.20±0.49
<i>Piper ribesoides</i>	20	6.49	40.68±0.32	43.14±6.42
<i>Piper samentosum</i>	10	4.28	77.21±2.04	71.03±4.34
<i>Plumbago indica</i>	20	9.37	73.50±2.16	37.01±0.07
<i>Salacia chinensis</i>	20	6.30	>100	>100
Sa-Tri-Lhung-Klod	270	8.51	72.84±1.07	47.24±2.83

groups according to its use for treatment as follows: *Angelica sinensis*, *Caesalpinia sappan*, *Carthamus tinctorius* and *Curcuma comosa* were used for treatment of menopause, premenstrual syndrome^(16,17) and as blood tonic⁽¹⁸⁾. *Artocarpus heterophyllus* and *Maclura cochinchinensis* were used for treatment as antimycobacterial⁽¹⁹⁾, anti-inflammation and astringent⁽²⁰⁾. *Piper longum*, *Piper nigrum*, *Piper ribesoides*, *Piper samentosum* and *Plumbago indica* were carminative^(21,22) and element tonic⁽²³⁾. *Jasminum sambac*, *Mammea siamensis*, *Mesua ferrea*, *Mimusops elengi* and *Nelumbo nucifera* were used as cardiac tonic⁽²⁴⁾, and for vertigo⁽²⁵⁾ and fever. The last group, *Salacia chinensis* was used for treatment of muscle pain. Thus, it is obvious that Sa-Tri-Lhung-Klod remedy is of various advantages quite good for woman health.

The results show that the ethanolic extract of Sa-Tri-Lhung-Klod remedy displayed cytotoxic activity against human ovarian carcinoma cell line (SKOV-3) and human cervical adenocarcinoma cell line (HeLa) with IC₅₀ values of 72.84±1.07 and 47.24±2.83 µg/ml, respectively. It was classified as “less-active” according to the NCI guideline. However, *Caesalpinia sappan*, *Mammea siamensis* and *Curcuma comosa* showed the high cytotoxic activity against human ovarian carcinoma cell line (SKOV-3) with IC₅₀ values of 9.55±1.38, 13.45±0.82 and 14.21±1.30 µg/ml, respectively. The ethanolic extracts of *Caesalpinia sappan* and *Mammea siamensis* also exhibited the cytotoxic activity against human cervical adenocarcinoma cell line (HeLa) with IC₅₀ values of 6.30±0.06 and 7.72±0.11 µg/ml, respectively. The previous reports showed that methanolic extract of *Caesalpinia sappan* and theraphin C (compound of *Mammea siamensis*) had IC₅₀ values of 15.5 µg/ml and 4.7 µM, respectively^(7,9). In addition, other plants also showed cytotoxic activity against SKOV-3 cell line and HeLa cell line. These are ten plants, i.e. *Angelica sinensis*, *Artocarpus heterophyllus*, *Macluraco chinchinensis*, *Mesua ferrea*, *Piper longum*, *Piper nigrum*, *Piper ribesoides*, *Piper samentosum*, *Plumbago indica* and *Salacia chinensis*. Although their extracts were less-active, while pellitorine (compound of *Piper nigrum*), ethanolic extracts of *Piper ribesoides* and *Plumbago indica* were reported as showed high cytotoxic activity against human cervical adenocarcinoma cell line (HeLa) with IC₅₀ values of 13.0, 4.6 and 1.5 µg/ml, respectively^(11,12).

Conclusion

Purposes of the present study were to investigate the cytotoxicity effects of ethanolic extract

of Sa-Tri-Lhung-Klod remedy and each of its plant ingredients. The results indicated that the ethanolic extract of Sa-Tri-Lhung-Klod remedy showed mild cytotoxic activity against ovarian cancer and cervix cancer cell lines. However, the ethanolic extract of some plants as a remedy possesses high potency of cytotoxic activity. These results support the use of this Thai medicinal remedy for preventing cancer in women in the future. The biological activity of this Sa-Tri-Lhung-Klod remedy has never been studied. Thus, the present study will be very useful to provide this information for further research in the development of herbal medicine for use in treating symptoms in women such as preventing cancer in women and for women's overall health care.

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Potential conflicts of interest

It is known that Sa-Tri-Lhung-Klod remedy and its plant ingredients had been used for treating symptoms in women such as menstrual disorders, anti-inflammation in postpartum and as blood tonic for preventing cancer in women. However, the mechanism of action of these components is not yet known. Thus, it should be further tested in animals and humans to support the experimental results.

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การทดสอบฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งของตำรับยาสตรีหลังคลอดและสมุนไพรเดี่ยวที่เป็นส่วนประกอบในตำรับต่อเซลล์มะเร็งรังไข่และมะเร็งปากมดลูก

จันทร์จิรา อินทรประสิทธิ์, ศรีโสภา เรืองหนู, อรุณพร อิจูรัตน์

ภูมิหลัง: ตำรับยาสตรีหลังคลอด เป็นตำรับยาไทยอยู่ในบัญชียาหลักแห่งชาติ ประกอบด้วยพืชสมุนไพร 17 ชนิด ใช้โดยการหมักเป็นยาดองเหล้าเพื่อดูแลสุขภาพของผู้หญิง เช่น รักษาภาวะขาดประจำเดือน อาการวัยหมดประจำเดือน บำรุงโลหิต นอกจากนี้ยังใช้สำหรับเป็นยาบำรุงสุขภาพหลังคลอด เพื่อป้องกันการอักเสบหลังคลอด ซึ่งอาการดังกล่าวนี้อาจก่อให้เกิดโรคมะเร็งในผู้หญิงได้

วัตถุประสงค์: เพื่อศึกษาฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งรังไข่ชนิด SKOV-3 และเซลล์มะเร็งปากมดลูกชนิด HeLa ของสารสกัดตำรับยาสตรีหลังคลอด และสารจากพืชสมุนไพรเดี่ยว

วัสดุและวิธีการ: ทำการสกัดสารจากพืชแต่ละชนิดและคำนวณโดยวิธีการหมักด้วยเอทานอล 95% และทำให้แห้งด้วยเครื่องกลั่นระเหยแห้ง หลังจากนั้น นำสารสกัดที่ได้ทั้งหมดมาทดสอบฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งด้วยวิธี SRB assay

ผลการศึกษา: สารสกัดเอทานอลของตำรับยาสตรีหลังคลอด มีความสามารถน้อยในการยับยั้งเซลล์มะเร็งรังไข่และมะเร็งปากมดลูก โดยมีค่า IC_{50} เท่ากับ 72.84 ± 1.07 และ $47.24 \pm 2.83 \mu\text{g/ml}$ ตามลำดับอย่างไรก็ตาม แก่นฝางเสน ดอกสารภี และวุ้นชันชั่งมดลูก มีศักยภาพสูงในการยับยั้งเซลล์มะเร็งรังไข่ โดยมีค่า IC_{50} เท่ากับ 9.55 ± 1.38 , 13.45 ± 0.82 และ $14.21 \pm 1.30 \mu\text{g/ml}$ ตามลำดับ และฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปากมดลูกของสารสกัดแก่นฝางเสน และดอกสารภีมีค่า IC_{50} เท่ากับ 6.30 ± 0.06 และ $7.72 \pm 0.11 \mu\text{g/ml}$ ตามลำดับ

สรุป: ผลเหล่านี้ใช้เป็นข้อมูลสนับสนุนการใช้ตำรับยาสตรีหลังคลอดในการแพทย์แผนไทย เพื่อป้องกันการเกิดโรคมะเร็งรังไข่และมะเร็งปากมดลูก โดยเฉพาะอย่างยิ่งแก่นฝางเสน วุ้นชันชั่งมดลูก และดอกสารภี มีศักยภาพสูงในการยับยั้งเซลล์มะเร็งทั้งสองชนิด
