P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia Science & Technology Asia
Vol. 25 No.3 July - September 2020 Page: [38-50]
Original research article

Cytotoxicity against Cervical and Breast Cancer Cells of Leard-Ngam Remedy and Its Plant Compositions

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Received 9 October 2019; Received in revised form 3 April 2020
Accepted 10 June 2020; Available online 21 September 2020

ABSTRACT

The Leard-Ngam (LG) remedy has been used in Thai traditional medicine to balance women's health. Previous studies reported that some herbs in the LG remedy, such as *Boesenbergia rotunda*, *Myristica fragrans, Piper nigrum, Zingiber officinale* and *Zingiber zerumbet*, were active against cancer cells but there was no report for the LG remedy. The objectives of this study were to investigate the cytotoxicity of the LG remedy and its plant compositions against two breast cancer cells (MCF-7, T47D) and cervical cancer cells (HeLa) in comparison with keratinocyte cells (HaCaT). The stability of the remedy was also studied at accelerated conditions for 6 months and its markers were evaluated by HPLC method. These extracts were prepared according to those practiced by Thai traditional doctors i.e. by maceration in 95% ethanol and decoction with water. These extracts were evaluated for cytotoxic activity by sulphorhodamine B (SRB) assay. The results showed that the LG remedy extract showed good activities against breast cancer cells (T47D and MCF-7) with a selective index of 3.33 and 2.99, respectively. For single herbs, the 95% ethanolic extract of *Oroxylum indicum* showed the best activities. It exhibited specific cytotoxicity against breast (MCF-7, T47D) and cervical (HeLa) cancer cells with a selective index value of 4.31, 3.43, and 4.18, respectively. The phytochemical compounds of the LG remedy were Eugenol and Piperine. They were not stable under accelerated conditions. In summary, the LG remedy and some single herbs were effective in inhibiting breast cancer cells. It should be further studied in other types of cells and the identification of active ingredients should be conducted for future drug development.

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Keywords: Leard-Ngam remedy (LG); Sulforhodamine B (SRB) assay; Cytotoxicity; Cervical cancer cells; Breast cancer cells

1. Introduction

The leading cause of death among Thai women is cervical and breast cancers [1]. In Thailand, breast and cervical are general cancers in women, especially breast cancer which is the first cause of death in women [1]. The most common type is of surrounding tissue and which becomes invasive breast cancer [2]. Breast cancer can also begin in the cells of the lobules and other tissues of the breast [3]. Cervical cancer occurs in the cells of the cervix. The greatest cause of cervical cancer is human papillomavirus (HPV) infection [4]. Chronic inflammation can cause DNA damage and lead to cancer. The unregulated nitric oxide production can cause cell damage through oxidative stress [5]. At present, there are many types of cancer treatments such as surgery, chemotherapy, radiation, and immunotherapy [3]. These treatments cause side effects that include anemia, bleeding and bruising, etc.

Thai traditional medicine is one of the alternative treatments for natural healing and maintaining human health. The principle of the Thai traditional medicine is that the human body consists of four elements (fire, wind, water, and earth). If the four elements are not balanced, it will cause illness. For this reason, Thai remedies always have many herbs to balance the four elements [6].

The Leard-Ngam(LG) remedy consists of twenty herbs. It is an herbal remedy in the Thai National List of Essential Medicines [7] and has been used for treating menstrual pain or primary dysmenorrhea by restoring elemental balance and also to reduce other symptoms that occur during menstruation, e.g. fever, nausea, dyspepsia including also in the formulation of cancer drugs. Thai traditional practitioners believe that menstrual disorders could develop into cancer [8]. It is normal if there are some symptoms during menstruation, such as dark color, fever, diarrhea, and muscle pain. However, if these symptoms persist they may develop into ulcers or abscesses in the uterus or secondary dysmenorrhea and eventually become cancer. LG is used for treating menstrual pain and reducing the risk of cancer [9].

Previous studies have reported that ethanolic extracts of *Piper nigrum, Zingiber officinale*, and *Citrus hystrix DC.* and the 95% ethanolic extract of the LG remedy showed potent anti-inflammatory activity against NO production, with IC_{50} values of 1.31 ± 0.42, 2.87 ± 0.31, 3.03 ± 3.27 µg/mL, respectively [10]. The LG remedy and its herbal components also showed inhibitory activity against PGE_2 production, which was the main cause of uterine pain [11]. In addition, several studies have reported that many herbs in the LG remedy were cytotoxic against cancer cells but there was no report for this remedy against breast and cervical cancer cells. Thus the objective of this study was to investigate cytotoxicity against breast cancer cells (T47D, MCF-7) and cervical cancer cells (HeLa), compared with keratinocyte cells (HaCaT). The stability of the remedy was also studied at accelerated conditions for 6 months and evaluated by HPLC method (Eugenol and Piperine which were previously established as appropriate markers) and anti-inflammatory by nitric oxide (NO) inhibition.

2. Materials and Methods

2.1 Preparation of plant extracts

The LG remedy consists of twenty herbs; each plant was collected at different

Table 1. Plant components of LG remedy.

Scientific name	Family	Common name	Part used	Specimen No.
Allium sativum L.	LILIACEAE	Garlic	Bulb	SKP 006 01 19 01
Amomum xanthioides Wall.	ZINGIBERACEAE	Bustard cardamom	Seed	SKP 206 01 24 01
Artemisia vulgaris L.	COMPOSITAE	Wormwood	All parts	SKP 051 01 01 01
Boesenbergia rotunda (L.) Mansf	ZINGIBERACEAE	Finger root	Root	SKP 095 02 18 01
Citrus aurantifolia (Christm.) Swingle	RUTACEAE	Common lime	Leaf	SKP 166 03 01 01
Citrus hystrix DC.	RUTACEAE	Leech lime	Peel	SKP 166 03 08 01
Cymbopogon citratus (DC.)	GRAMINEAE	Lemon grass	All parts	SKP 081 03 03 01
Glycyrrhiza glabra L.	LEGUMINOSAE	Licorice	Root	SKP 072 07 07 01
Mentha cordifolia Opiz.	LAMIACEAE	Kitchen Mint	All parts	SKP 095 13 03 01
Myristica fragrans Houtt.	MYRISTICACEAE	Nutmeg	Seed	SKP 121 13 06 01
Ocimum sanctum Linn.	LABIATAE	Holy Basil	Leaf	SKP 095 15 19 01
Oroxylum indicum Linn	BIGNONIACEAE	Broken Bones Tree	Bark	SKP 025 15 09 01
Piper nigrum Linn.	PIPERACEAE	White pepper	Seed	SKP 146 16 14 01
Piper retrofractum Vahl	PIPERACEAE	Long Pepper	Flower	SKP 146 16 19 01
Piper sarmentosum Roxb.ex Hunter	PIPERACEAE	Wildbetel Leafbush	All parts	SKP 148 16 09 01
Plumbago indica	PLUMBAGINACEAE	Rosecolored leadwort	Root	SKP 123 19 01 01
Syzygium aromaticum (L.)	MYRTACEAE	Clove	Flower	SKP 206 26 03 01
Zingiber cassumunar Roxb.	ZINGIBERACEAE	Cassumunar ginger	Rhizome	SKP 206 26 15 01
Zingiber officinale Roscoe	ZINGIBERACEAE	Ginger	Root	SKP 206 26 26 01
Zingiber zerumbet (L.) Sm.	ZINGIBERACEAE	Shampoo Ginger	Rhizome	SKP 146 16 19 01

locations and their voucher specimens were deposited at the Faculty of Pharmacy, Prince of Songkla University (Table 1)

The twenty medicinal plants are *Allium sativum, Amomum xanthioides, Artemisia vulgaris, Boesenbergia rotunda, Citrus aurantifolia, Citrus hystrix, Cymbopogon citratus, Glycyrrhiza glabra, Mentha cordifolia, Myristica fragrans, Ocimum sanctum, Oroxylum indicum, Piper nigrum, Piper retrofractum, Piper sarmentosum, Plumbago indica, Syzygium aromaticum, Zingiber cassumunar, Zingiber officinale, and Zingiber zerumbet.* Equal weights of each herb were cleaned, sliced and dried at 50 °C in an oven and powdered. The dried powder of single plants was macerated with 95% ethanol and boiled 3 times in distilled water for 15 minutes. All extracts were concentrated to dryness; the ethanolic extracts were evaporated under reduced pressure and aqueous extracts were dried with lyophilization. The crude extracts were kept at -20°C until required.

2.2 Cell lines and reagents

Cancer cells used were human breast adenocarcinoma (MCF-7) (ATCC® HTB-22™), mammary gland, ductal carcinoma (T47D) (ATCC® HTB-133™), human cervical adenocarcinoma (HeLa) (ATCC® CCL-2™) and human keratinoc-yte cells (HaCaT). RPMI Medium 1640 (RPMI1640) powder with L- glutamine, Minimum Essential Medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin (P/S), trypsin- EDTA and trypan blue were purchased from Gibco, USA. Sodium bicarbonate was purchased from BDH, England. Hydrochloric acid (HCl) was purchased from Univar, Australia. Fetal bovine serum (FBS) was purchased from Biochem, Germany. Dimethyl sulfoxide [$(CH_3)_2SO$)] (DMSO) was purchased from RCl Labscan, Thailand. Phosphate buffered saline (PBS) was purchased from Amresco, USA. Trichloroacetic acid (Cl_3CCOOH) (TCA) was purchased from Merck, Germany. Sulforhodamine B sodium salt ($C_{27}H_{29}N_2NaO_7S_2$) and Tris (hydroxymethyl) aminomethane ($(HOCH_2)_3CNH_2$) were purchased from Sigma-Aldrich, USA.

2.3 Stability study

Stability tests of ethanolic extract of the LG remedy were performed according to the guideline of the Thai FDA. The samples were kept at 40°C and 75% relative humidity (RH) for 0, 15, 30, 60, 90, 120,

150, and 180 days. The purpose of this investigation was to obtain a suitable form of this extract, which implies that the extract is stable when kept in a closed container protected from light and stored at room temperature for at least two years. After testing, the samples were analyzed for the quantity of chemical compounds by using the HPLC method and the biological activity of anti- inflammatory activity by inhibitory effects of nitric oxide production.

2.3.1 Quantitative analysis of markers by HPLC method

Eugenol and piperine were analyzed by using HPLC (Agilent, LC1200) with Photodiode array detector (DAD) and auto sample on ZORBAX Eclipse XDB-C18 (4.6 mm × 250mm; 5µm) column by Gradient elution using mobile phase of acetonitrile and 0.1% Phosphoric acid at a flow rate of 1 ml/min. with 10 µl injection volume. Eugenol and piperine were detected at 210 and 254 nm, respectively. (Table 2)

Table 2. The percent of gradient elution and time setting for eugenol and piperine content analysis.

Time (min)	Acetonitrile (%)	0.1% Phosphoric acid (%)
0.00	5.00	95.00
5.00	5.00	95.00
45.00	50.00	50.00
60.00	95.00	5.00
65.00	100.00	0.00
65.10	5.00	95.00
70.00	5.00	95.00

2.3.2 Assay for anti-inflammatory by NO inhibitory effect [12]

Leukemic macrophage cell lines (RAW 264.7) were seeded in 96-well plates with 1×10^5 cells/well and allowed adhesion for 24 hours at 37 °C in a humidified atmosphere containing 5% CO_2. After that, the medium was removed and filled with fresh medium containing 100 µg/ml of LPS together with ethanol solutions dissolved in DMSO and water solutions dissolved in DI water at various concentrations (1,10,30,50,100 µg/ml) and then incubated for another 24 hours. The Supernatant (100 µl) was transferred to another 96 well plates and 100 µl of Griess reagent was added to each well to measure the accumulation of NO production; the absorbance was measured with a microplate reader at 570 nm. The first plate had 10 µl of MTT added and was incubated at 37 °C under CO_2 for 2 hours. Then the supernatant was discarded and 100 µl of 0.04M HCl in isopropanol was added. The OD was measured at 570 nm. The percentages of inhibition (Griess reagent) and percentage of survival (MTT) were calculated from OD measurements and the IC_{50} value was calculated by the Prism program.

2.4 Cytotoxicity study on human cells

2.4.1 Preparation of samples for cytotoxicity test

The ethanolic extracts were dissolved in sterile dimethylsulfoxide (DMSO) and aqueous extracts were dissolved in sterile deionized water (10 mg/ml) and then filtered through a 0.22-micron filter. The concentration was further diluted to 50 mg/ml for the screening test against cancer cells; if the result showed more than 50% inhibition, the extract was further diluted to give concentrations of 100, 50, 10, 1 µg/ml. The final mixture in 96 well plates contained not more than 2% of the DMSO, the same as in the solvent control well. Also, there is media control as well.

2.4.2 Sulforhodamine B (SRB) assay [13], [14]

The sulforhodamine B (SRB) assay was used to measure cell viability numbers by the SRB to stained total cellular protein. In brief, cells were secluded with 0.25% trypsin- EDTA to make cell suspensions. The trypan blue was used to count viable cells by hemocytometer. A 100 µl/well of cell suspensions was seeded in 96- well

plates and incubated to allow cell adhesion for 24 hours. After that, the cells were treated with the extracts and incubated for 72 hours. At the end of each exposure time, the medium was discarded. The wells were then washed with PBS, and 200 µl of fresh medium was added to each well and incubated for 72 hours. On the eighth day of the culture cell period, 100 µl of ice-cold 40% trichloroacetic acid (TCA) was used to fixed cells in each well, incubated at 4°C for 1 hour in the refrigerator, and the supernatant removed to wash away non-viable cells. The staining available cells had 50 µl of SRB solution (0.4% w/v in 1% acetic acid) added to each well for 30 min; then removed and washed with 1% acetic acid until only dye adhering to the cells was left. A 100 µl of 10 mM Tris base (trishydroxy methyl aminomethane, pH 10.5) was added to each well to dissolve the dye. The plates were shaken for 10 minutes on a gyratory shaker. The absorbance (OD) was read on a microplate reader at 492 nm. Cell survival was measured as a percentage of absorbance compared with the control (non-treated cells). The IC_{50} values were calculated from the Prism program. The National Cancer Institute guidelines for the toxicity of extracts were followed, with IC_{50} value < 20 µg/mL being toxic. [15] SI = selective index calculated by IC_{50} of normal cells / IC_{50} of cancer cell.

2.5 Statistical analysis

All data of cytotoxic activities are the mean of three replications. The values of different parameters were expressed as the mean ± standard error of the mean.

Student's t-test and one-way ANOVA were used for statistical analysis.

3. Results and Discussion

3.1 The Stability of LG Remedy

For determination of the stability of the LG remedy under accelerated conditions, high-performance liquid chromatography (HPLC) was used to identify and quantify the phytochemical compounds, and the inhibitory effect of NO production was used to identify the inflammatory activity under accelerating conditions.

3.1.1 The phytochemical compounds

The chromatogram of active compounds is shown in Figs. 1 and 2; eugenol and piperine tend to decrease at 60 days from Day 0 and continue to reduce until 180 days. There were significant differences at each period when compared to Day 0 (p-value < 0.05) (Fig. 3a). Eugenol is volatile and can easily evaporate off when stored at high temperate. Therefore, eugenol content at day 180th became lower than the starting point. Piperine is an alkaloid which could have altered under high temperature and high humidity. This explained the reduction of piperine content. Eugenol is the active compound in *S. aromaticum* and *O.sanctum* and Piperine is the major alkaloid in *P. nigrum*, *P. retrofractum* and *P. sarmentosum.* The previous study found that Eugenol and Piperine could reduce the production of prostaglandins E_2 [16], [17] which is related to menstrual pain. Therefore, eugenol and Piperine were chosen as markers of the LG remedy.

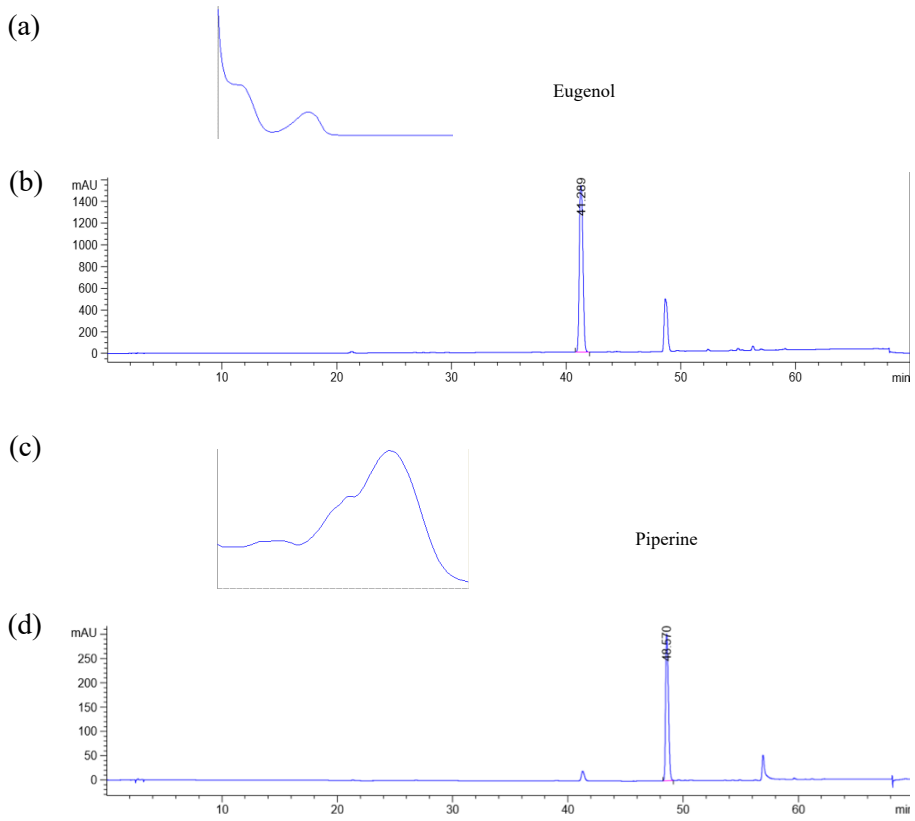
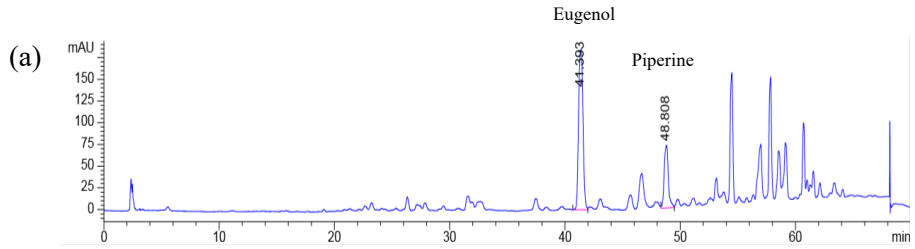
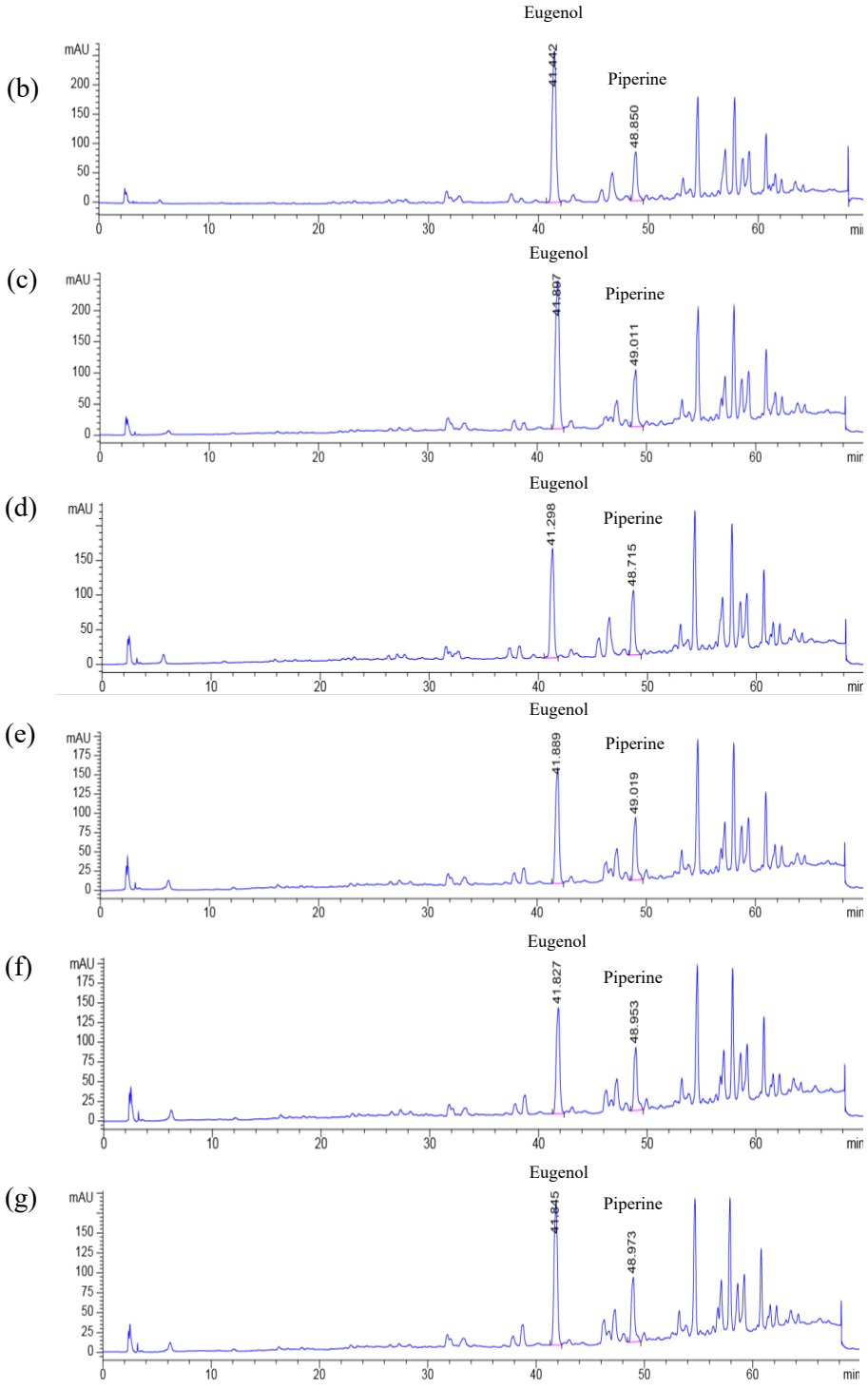


Fig. 1. the standard spectrum and chromatogram of eugenol and piperine using by HPLC (Agilent, LC1200) with Photodiode array detector (DAD) and auto sample on ZORBAX Eclipse XDB-C18 (4.6 mm x 250mm; 5µm) column by Gradient elution. (a) The spectrum of eugenol's standard (b) the chromatogram of eugenol's stand was detected at 210 nm. (c) The spectrum of piperine's standard (d) the chromatogram of piperine's standard was detected at 254 nm.




(b)
Eugenol
Piperine
41.442
48.850
(c)
Eugenol
Piperine
41.897
49.011
(d)
Eugenol
Piperine
41.298
48.715
(e)
Eugenol
Piperine
41.889
49.019
(f)
Eugenol
Piperine
41.827
48.953
(g)
Eugenol
Piperine
41.845
48.973
mAU
min

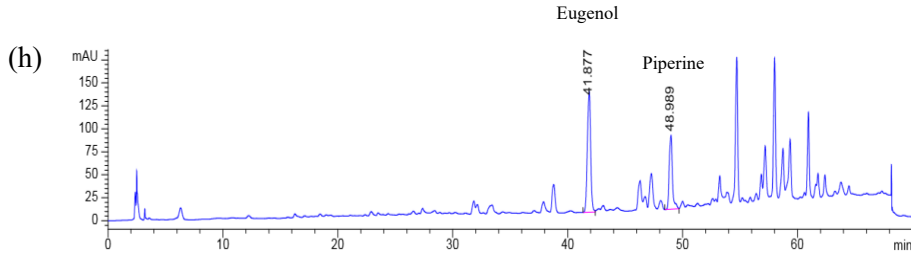
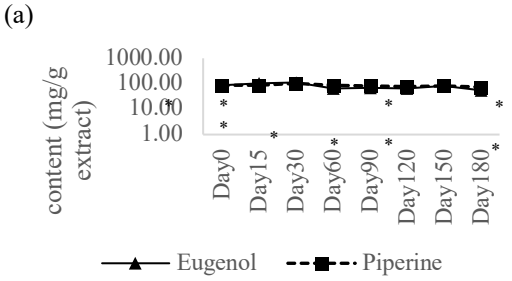


Fig. 2. Chromatogram of eugenol and piperine in LG remedy using by HPLC (Agilent, LC1200) with Photodiode array detector (DAD) and auto sample on ZORBAX Eclipse XDB-C18 (4.6 mm x 250mm; 5µm) column by Gradient elution. Piperine was detected at 254 nm. (a) Chromatogram of eugenol and piperine was not kept under the accelerated condition (Day0), (b) Chromatogram of eugenol and piperine was kept under the accelerated condition for 15 days, (c) Chromatogram of eugenol and piperine was kept under the accelerated condition for 30 days, (d) Chromatogram of eugenol and piperine was kept under the accelerated condition for 60 days, (e) Chromatogram of eugenol and piperine was kept under the accelerated condition for 90 days, (f) Chromatogram of eugenol and piperine was kept under the accelerated condition for 120 days, (g) Chromatogram of eugenol and piperine was kept under the accelerated condition for 150 days, (h) Chromatogram of eugenol and piperine was kept under the accelerated condition for 180 days

3.1.2 The result of NO inhibitory effect under accelerating condition

The results of inhibitory activity against LPS induced NO production of the LG remedy under the accelerated condition (40 ºC 75% relative humidity, for 6 months or 180 days) IC_{50} values of NO inhibitory effect were increasing in value each time (Fig. 3b) but with no significant difference at each period when compared to Day 0 (p-value > 0.05). This result can indicate that the LG remedy had unaltered NO inhibitory effect when kept at the accelerated conditions and could be kept for 2 years.



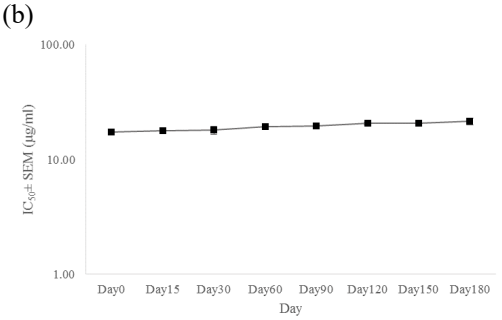


Fig. 3. The stability test of LG remedy after being kept under the accelerated condition for 6 months. The difference in each parameter was represented as the mean ± SEM. One-way ANOVA was used to compare the value change from baseline and day15, and comparison tests conducted until day 180 for (a) the chemical marker's content of LG remedy (eugenol and piperine) (b) the inhibitory effect of nitric oxide production of LG remedy
*Significant difference at p-value < 0.05

3.2 Cytotoxicity study on the human cell lines

The percent inhibition of the LG remedy and its plant composition against breast cancer cells (T47D and MCF-7) and cervical cells (HeLa) is shown in Fig. 4. The results of cytotoxic activity show that the aqueous extracts of the LG remedy and herbs that are components of the LG remedy did not show cytotoxicity against T47D, MCF-7 and HeLa cancer cells, while the 95% ethanolic extract of the LG remedy exhibited cytotoxicity against T47D and MCF-7 cells with IC_{50} values of 24.98 ± 1.22 µg/mL [selective index = 3.13 and 26.13 ± 1.19 µg/mL] selective index = 2.99, respectively, without cytotoxic activity against keratinocyte cells (HaCaT). The ethanolic extract of twenty herbs in the LG remedy showed more cytotoxicity against breast cancer cells than against cervical cancer cells. These extracts were as follows: 95% ethanol of *Z. zerumbet* was specific to breast cancer cells (MCF-7) with selective index 7.96 and IC_{50} value was 7.17 ± 1.04 µg/mL. Five ethanolic extracts of *B. Rotunda, Z. officinale, P. nigrum, P. Indica*, and *P. sarmentosum* were specific to breast cancer cells (T47D) with IC_{50} values of 11.89 ± 1.86, 12.33 ± 1.42, 12.70 ± 0.05, 19.44 ± 1.86 and 29.58 ± 3.29 µg/mL (selective index = 2.88, 2.89, 2.84, 2.08 and 2.80), respectively. Three ethanolic extracts that are components of the LG remedy were specific to two types of breast cancer cells (T47D and MCF-7). These are described as follows: *Z. cassumunar, M. Fragrans,* and *P. retrofractum* were specific to T47D with an IC_{50} value of 7.17 ± 1.04, 20.83 ± 1.68, 27.72 ± 2.38 µg/mL (selective index = 7.37, 3.74 and 2.64), respectively. Also, they were specific to MCF-7 with IC_{50} value of 11.13 ± 1.48, 28.71 ± 2.09, 27.22 ± 0.39 µg/mL (selective index = 4.75, 2.68 and 2.71), respectively. In addition, *O. Indicum* exhibited cytotoxicity and selective against two types of the breast (MCF-7, T47D) and cervical (HeLa) cancer cells with an IC_{50} value of 23.21 ± 1.97, 29.13 ± 2.70 and 23.95 ± 1.08 µg/ml (selective index = 4.31, 3.43 and 4.18), respectively. For aqueous extracts which are extracted similarly to that practiced by Thai traditional practitioners, most aqueous extracts were inactive. (IC_{50}> 50 µg/ml) as shown in Table 3.

From these results, the LG remedy and ten herbs that are its components showed more cytotoxicity against breast cancer cells than against cervical cancer cells. In addition, herbs frequently used in cancer treatment and medicinal foods that Thai people eat daily, such as *M. fragrans, O. indicum, P. retrofractum, P. sarmentosum, P. indica, B. rotunda, P. nigrum* and *Z. officinale* also showed cytotoxic activity on breast cancer cells but were not toxic on keratinocyte cells. These results show consistency with previous studies that reported the LG remedy and its components *P.nigrum, Z.officinale, B.rotunda,* and *Z.cassumunar* had the potential to inhibit nitric oxide and PGE_2 production that is associated with preventing cancer cells. [18]

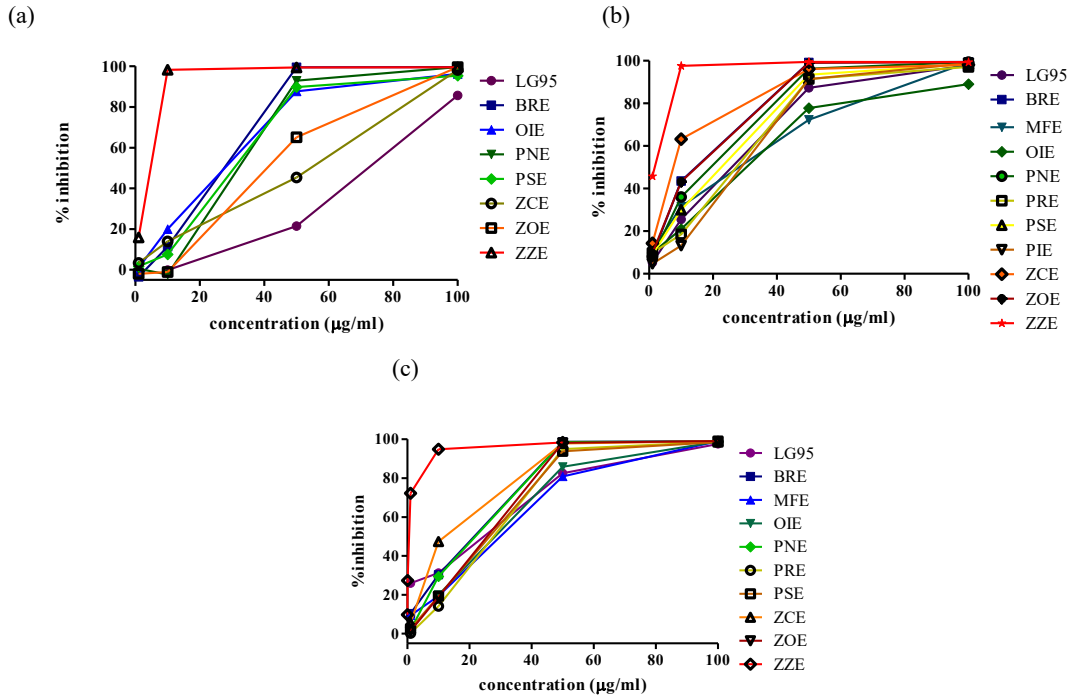


Fig. 4. The percent inhibition of LG remedy and its plant compositions against three types of female cancer cells. (a) The percent inhibition of LG remedy and its plant compositions against cervical cells (HeLa), (b) The percent inhibition of LG remedy and its plant compositions against breast cancer cells (T47D), (c) The percent inhibition of LG remedy and its plant compositions against breast cancer cells (MCF-7).

Table 3. Cytotoxicity of the LG remedy and its ingredient plants against cervical cancer cell line (HeLa), breast cancer cell lines (T47D, MCF-7), and normal cell line (HaCat) using SRB assay (n=3). *According to the National Cancer Institute guidelines, extracts with IC_{50} value < 20 µg/ml. are toxic.*

Botanical name	Extract	Code	IC_{50} (µg/ml ±SEM) [selective index] [E_{max}]			
			HeLa	T47D	MCF7	HaCat
Allium sativum L.	95%Ethanol	ASE	>50	>50	>50	NT
	Water	ASW	>50	>50	>50	NT
Amomum xanthioides Wall.	95%Ethanol	AXE	>50	>50	>50	NT
	Water	AXW	>50	>50	>50	NT
Boesenbergia rotunda (L.) Mansf	95%Ethanol	BRE	24.45 ± 4.73 [SI = 1.40] [E_{max} = 99.58%]	11.89 ± 1.86* [SI = 2.88] [E_{max} = 99.43%]	19.20 ± 0.32* [SI = 1.78] [E_{max} = 98.99%]	34.24 ± 0.61
	Water	BRW	>50	>50	>50	NT
Citrus aurantifolia (Christm.) Swingle	95%Ethanol	CAE	>50	>50	>50	NT
	Water	CAW	>50	>50	>50	NT
Citrus hystrix DC.	95%Ethanol	CHE	>50	>50	>50	NT
	Water	CHW	>50	>50	>50	NT
Cymbopogon citratus (DC.)	95%Ethanol	CCE	>50	>50	>50	NT
	Water	CCW	>50	>50	>50	NT
Mentha cordifolia Opiz.	95%Ethanol	MCE	>50	>50	>50	NT
	Water	MCW	>50	>50	>50	NT

Botanical name	Extract	Code	IC_{50} (µg/ml ±SEM) [selective index] [E_{max}]			
			HeLa	T47D	MCF7	HaCat
Myristica fragrans Houtt.	95%Ethanol	MFE	>50	20.83 ± 1.68* [SI = 3.74] [E_{max} = 99.27%]	28.71 ± 2.09* [SI = 2.71] [E_{max} = 99.08%]	77.98 ± 1.03
	Water	MFW	>50	>50	>50	NT
Ocimum sanctum Linn.	95%Ethanol	OSE	>50	>50	>50	NT
	Water	OSW	>50	>50	>50	NT
Oroxylum indicum Linn	95%Ethanol	OIE	23.95 ± 1.08* [SI = 4.18] [E_{max} = 96.24%]	29.13 ± 2.70* [SI = 3.43] [E_{max} = 89.04%]	23.21 ± 1.97* [SI = 4.31] [E_{max} = 98.78%]	>100
	Water	OIW	>50	>50	>50	NT
Piper nigrum Linn.	95%Ethanol	PNE	34.39 ± 1.22 [SI = 1.05] [E_{max} = 99.58%]	12.70 ± 0.05* [SI = 2.84] [E_{max} = 99.35%]	24.19 ± 1.34* [SI = 1.49] [E_{max} = 98.79%]	36.13 ± 1.41
	Water	PNW	>50	>50	>50	NT
Piper retrofractum Vahl	95%Ethanol	PRE	>50	27.72 ± 2.38* [SI = 2.64] [E_{max} = 97.15%]	27.22 ± 0.39* [SI = 2.68] [E_{max} = 98.76%]	73.07 ± 1.54
	Water	PRW	>50	>50	>50	NT
Piper sarmentosum Roxb.ex Hunter	95%Ethanol	PSE	31.74 ± 0.89* [SI = 1.27] [E_{max} = 95.38%]	19.44 ± 1.86* [SI = 2.08] [E_{max} = 98.41%]	24.84 ± 2.03* [SI = 1.63] [E_{max} = 98.52%]	40.39 ± 0.98
	Water	PSW	>50	>50	>50	NT
Plumbago indica Linn.	95%Ethanol	PIE	>50	29.58 ± 3.29* [SI = 2.80] [E_{max} = 98.88%]	>50	82.80 ± 0.78
	Water	PIW	>50	>50	>50	NT
Syzygium aromaticum (L.)	95%Ethanol	SAE	>50	>50	>50	NT
	Water	SAW	>50	>50	>50	NT
Zingiber cassumunar Roxb.	95%Ethanol	ZCE	52.49 ± 1.12 [SI = 1.00] [E_{max} = 98.20%]	7.17 ± 1.04* [SI = 7.37] [E_{max} = 99.34%]	11.13 ± 1.48* [SI = 4.75] [E_{max} = 98.96%]	52.86 ± 0.62
	Water	ZCW	>50	>50	>50	NT
Zingiber officinale Roscoe	95%Ethanol	ZOE	42.07 ± 2.01 [SI = 0.85] [E_{max} = 99.63%]	12.33 ± 1.42* [SI = 2.89] [E_{max} = 99.34%]	24.19 ± 1.34* [SI = 1.47] [E_{max} = 99.05%]	35.62 ± 0.12
	Water	ZOW	>50	>50	>50	NT
Zingiber zerumbet (L.) Sm.	95%Ethanol	ZZE	4.42 ± 0.2* [SI = 0.42] [E_{max} = 99.50%]	1.54 ± 0.08 [SI = 1.29] [E_{max} = 99.04%]	0.25 ± 0.02* [SI = 7.96] [E_{max} = 98.38%]	1.99 ± 0.32
	Water	ZZW	>50	>50	>50	NT
Leard-ngam remedy (LG)	95%Ethanol	LG95	75.31 ± 4.37 [SI = 1.04] [E_{max} = 85.69%]	24.98 ± 1.22* [SI = 3.13] [E_{max} = 97.93%]	26.13± 1.19* [SI = 2.99] [E_{max} = 97.56%]	78.15 ± 2.58
	50%Ethanol	LG50	>100	>100	>100	>100
	Water	LGW	>100	>100	>100	>100

Note: NT, not test; SI = selective index calculated, E_{max} is the value of %inhibition at the maximum herbal concentration test, mean difference of IC_{50} value of three types of cancer and keratinocyte cells were compare by student's t-test. * Significant difference at p-value < 0.05

4. Conclusion

The aqueous extracts of the LG remedy did not have activity against breast and cervical cancer cells. On the contrary, the 95% ethanolic extract of the LG remedy was specific to both breast cancer cells (T47D and MCF-7). The selective index values were 3.13 and 2.99, respectively, and had a mild activity with cervical cancer cells. Whereas, the 95% ethanolic extract of *M. fragrans, O. indicum, P. retrofractum,* and *P. indica* were safe with keratinocyte cells and also were toxic specific to cancer cells. Especially, *O. indicum* was toxic to breast and cervical cancer cells. *Z. zerumbet* showed the strongest activity against MCF-7, T47D, and HeLa cells, but also exhibited against keratinocyte cells. The results

followed the principle of Thai traditional medicine that *Z. zerumbet* should be used only in remedy. Interestingly, *B. rotunda*, *Z. officinale* and *P. nigrum,* which are common foods, showed no toxicity on keratinocyte cells but could inhibit the growth of both breast cancer cells (T47D and MCF-7) and cervical cancer cells (Hela).

The LG remedy exhibited moderate anti-inflammatory by nitric oxide inhibitory effect and mild activity against cervical cancer cells, more efficacy against breast cancer cells but no toxicity with keratinocyte cells. It should be studied with other cancer cells and other methods of anti-inflammation to support the use of Thai traditional medicine for treating cancer and related acute and chronic inflammation which is the leading cause of dysmenorrhea and cancer, and to develop this remedy for the treatment of breast cancer.

Acknowledgments

This project was supported by funding from the Faculty of Medicine, Thammasat University. The authors gratefully acknowledge the logistic support from the Department of Applied Thai Traditional Medicine and Herbal Medicine & Food Unit, Faculty of Medicine, Thammasat University, Thailand.

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