Cytotoxic and Anti-inflammatory Activities of Medicinal Plants and Women's Health Remedy Found in "Mahachotarat Scripture" of Thai Traditional Medicine

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Background: *"Mahachotarat" is a lesson for woman's care from the Thai traditional medicine book, composed of both medicinal plants and herbal remedies that can treat both pain and cervical cancer. Medicinal plants and herbal remedies, which have often been used in the treatment of pain and cancer, were selected to investigate for biological activity related to woman's health. They were Boesenbergia rotunda Linn, Piper nigrum Linn, Zingiber cassumunar Roxb, Zingiber officinale Roscoe, Zingiber zerumbet (L) Smith, Dioscorea birmanica Prain & Burkill including its ingredient; Prosapogenin A of dioscin and Leard-ngam remedy.*

Objective: *The objective was to investigate cytotoxic and anti-inflammatory activities of all sample plants.*

Material and Method: *Medicinal plants and Leard-ngam remedy were extracted similarly to that practiced by Thai traditional practitioners (ethanol and water extraction). Bioassay guide fractionation was used for isolating pure compound. The structure elucidation of pure compound was proven by spectrophotometry technique. These extracts were tested for their cytotoxic activity against Hela cells, cervical cancer cells, by sulforhodamine B assay, inhibition of nitric oxide and prostaglandin E_2 production in lipopolysaccharide-stimulated mouse macrophage RAW 264.7 cells.*

Result: *This study showed that P. nigrum, Z. officinale, B. rotunda and Z. cassumunar showed potent inhibitory activity on nitric oxide and PGE2 production. The 95% ethanolic extract of Z. zerumbet had the highest cytotoxic activity on Hela cells with IC_{50} value of 4.42±0.20 μg/ml. Diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside or Prosapogenin A of dioscin, which was purified from the 95% ethanolic extract of D. birmanica, showed strong cytotoxic activity on Hela cells with IC_{50} value of 6.07±0.02 μg/ml.*

Conclusion: *Thai medicinal plants and LG remedies which have often been used in the treatment of pain and cancer by Thai traditional practitioners, showed high anti-inflammatory properties on both pathways which represent chronic and acute inflammation. Interestingly, some medicinal plants were used daily in Thai food. In addition, Z. zerumbet and D. birmanica, which has often been used in the treatment of cancer, also showed high cytotoxic activity against cervical cancer cells.*

Keywords: *Anti-inflammatory, Cytotoxic, Medicinal plants, Leard-ngam remedy*

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Dysmenorrhea, one of the most frequently encountered gynecological disorders, refers to painful menstruation. It typically occurs in the first few years after menarche[1] and affects as many as 50% of post pubertal females[1]. In Thailand dysmenorrhea is the most prevalent complaint in women (42.2%)[2]. Dysmenorrhea is classified as primary or secondary dysmenorrheal[3,4]. Primary dysmenorrhea is defined as menstrual pain that is not associated with macroscopic pelvic pathology (i.e, occurs in the absence of pelvic disease). Secondary dysmenorrhea is defined as menstrual pain resulting from anatomic or macroscopic pelvic pathology, as is seen in women with endometriosis or chronic pelvic inflammatory disease[3,4]. Natural products have been used in the treatment of cancer and inflammation for thousands of years. Thai traditional medicine is a cultural heritage and indigenous wisdom which has been used for cancer and inflammation for over a thousand years. The preparation for cancer and inflammation composed of many plants to balance the four body elements and kill the cancer cells. The principle of the Thai

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herbs preparation must have the main ingredients to exert the main action or to destroy the cancer cell. Thai traditional practitioners use taste for indicating action; a spicy taste shows that could be an anticancer plant. The second group of plants is to support the main ingredients; their actions are as a tonic to reduce side effects or other symptoms e.g. pain, fever, nausea, abscesses and laxative plants, thought to be necessary. *Boesenbergia rotunda* Linn, *Piper nigrum* Linn, *Zingiber cassumunar* Roxb., *Zingiber officinale* Roscoe., *Zingiber zerumbet* (L) Smith, Prosapogenin A of dioscin, *Dioscorea birmanica* Prain & Burkill (Hua-Khao-Yen) and Leard-Ngam remedy (LG) have all been used as treatments in women's health as shown in Mahachotalat Scripture[5]. The use of Thai herbs preparation can describe as follows: LG remedy is a dysmenorrhea preparation in the Thai National List of Herbal Medicinal Products AD 2011[5]. In addition, Thai traditional practitioners also use other herbs such as, *B. rotunda*, *P. nigrum*, *Z. cassumunar*, *Z. officinale*, *Z. zerumbet* to treat primary dysmenorrhea including pain. For secondary dysmenorrhea, *D. birmanica* has been used as a cure for women cancer for over a hundred years and it had also high cytotoxic activity against A 549 and COR-L23 lung cancer cells (IC_{50} = 7.45±0.31, 8.71±0.29 µg/ml, respectively) but is less cytotoxic against normal lung cells; MRC-5 (IC_{50} = 94.76±1.25 µg/ml)[6]. There is no report in cervical cancer cells of *D. birmanica*. In addition, previous research showed the ethanolic extracts of *P. nigrum* had an inhibitory effect on nitric oxide production in RAW 264.7 cells[7]. Diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside or prosapogenin A of dioscin (DBS1) was isolated from the ethanolic extract of *D. birmanica* and had high cytotoxic activity against A 549 and COR-L23 lung cancer cells (IC_{50} = 1.81±0.03, 1.84±0.05 µg/ml, respectively)[6] but it was less cytotoxic against MRC-5 normal lung cells (IC_{50} = 37.09±0.67 µg/ml)[6]. Thus, the objectives of this study were to investigate the cytotoxic properties against cervical cancer cells (HeLa cells) and anti-inflammatory activities of *B. rotunda*, *P. nigrum*, *Z. cassumunar*, *Z. officinale*, *Z. zerumbet*, *D. birmanica* including its compound prosapogenin A of dioscin and LG remedy. The results should support the use of Thai traditional medicine to reduce a range of symptoms of health disorders in women.

Material and Method

Animal cell lines and reagents

Mouse leukaemic macrophage RAW264.7 cells were established and kindly provided by Assoc. Prof. Dr. Supinya Tewtrakul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. RPMI Medium 1640 (RPMI1640) Powder with L glutamine, Fetal Bovine Serum (FBS), Penicillin-Streptomycin (P/S), Tripsin-EDTA and trypan blue were purchased from Gibco, USA. Phosphate Buffer Saline (PBS) was from Amresco (USA), Sodium bicarbonate was from BDH (England), lipopolysaccharide (LPS from *Escherichia coli*), 3-(4,5-dimethy-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) were from Sigma (USA).

Preparation of the plant extracts

Preparation of single herbs

Five medicinal plants *B. rotunda*, *P. nigrum*, *Z. cassumunar*, *Z. officinale*, *Z. zerumbet* were cleaned, sliced and dried at 50°C in an oven and ground to be powder. Dried powder of each plant was macerated with 95% ethanol and boiled in distilled water for 15 minutes, 3 times. All extracts were concentrated to dryness under reduced pressure. The crude extracts were then pooled and kept in a freezer (-20°C) until required. All extracts were studied for cytotoxic and anti-inflammatory activities.

Preparation of LG remedy

Leard-ngam remedy (LG) consists of twenty herbs as follows, *Allium sativum* Linn, *Amomum xanthioides* Wall, *Artemisia vulgaris* Linn, *B. rotunda*, *Citrus aurantifolia* (Christm *et* Panz) Swingle, *Citrus hystrix* DC, *Cymbopogon citratus* (DC Stapf), *Glycyrrhiza glabra* Linn *Metha cordifolia* Opiz, *Myristica fragrans* Houtt, *Ocimum sanctum* Linn, *Oroxylum indicum* (L) Kurz, *P. nigrum*, *Piper retrofractum* Vahl, *Piper sarmentosum* Roxb, *Plumbago indica* Linn, *Syzygium aromaticum* (Linn), *Z. cassumunar*, *Z. officinale* and *Z. zerumbet* in the ratios shown in Table 1. Components of LG preparation were extracted in a similar way to that practiced by Thai traditional doctors. They were divided into two equal parts; the first part was macerated in 95% and 50% ethanol for 3 days, 3 times and percentage of yield determined, while the second part was boiled in distilled water for 15 minutes, 3 times. The extracts were concentrated to dryness under reduced pressure. All extracts were then pooled and kept in a freezer (-20°C) until required. And then all extracts were studied for cytotoxic and anti-inflammatory activities.

Table 1. The plant ingredient, part used and ratio in the formula of LG remedy

Scientific name	Common name	Family name	Part used	Ratio in remedy (percentages)
Allium sativum Linn.	Garlic	LILIACEAE (ALLIACEAE)	Bulb	5
Amomum xanthioides Wall.	Bustard cardamom	ZINGIBERACEAE	Seed	5
Artemisia vulgaris Linn.	Mugwort/ Common wormwood	ASTERACEAE (COMPOSITAE)	All parts	5
Boesenbergia rotunda (Linn) Mansf	Fingerroot	ZINGIBERACEAE	Rhizome	5
Citrus aurantifolia Christm. et Panz Swingle	Common lime	RUTACEAE	Leaf	5
Citrus hystrix DC	Leech lime	RUTACEAE	Peel	5
Cymbopogon citratus (DC) Stapf	Lemongrass	POACEAE (GRAMINEAE)	All parts	5
Glycyrrhiza glabra Linn	Licorice	FABACEAE (LEGUMINOSAE)	Root	5
Mentha cordifolia Opiz	Kitchen mint	LAMIACEAE (LABIATAE)	All parts	5
Myristica fragrans Houtt	Nutmeg tree	MYRISTICACEAE	Seed	5
Ocimum sanctum Linn	Holy basil	LAMIACEAE (LABIATAE)	Leaf	5
Oroxylum indicum (Linn) Kurz	Broken bones tree	BIGNONIACEAE	Bark	5
Piper nigrum Linn	Pepper	PIPERACEAE	Seed	5
Piper retrofractum Vahl	Long pepper	PIPERACEAE	Flower	5
Piper sarmentosum Roxb	Wildbetalleafbush	PIPERACEAE	All parts	5
Plumbago indica Linn	Rose-colored leadwort	PLUMBAGINACEAE	Root	5
Syzygium aromaticum Linn	Clove	MYRTACEAE	Flower	5
Zingiber cassumunar Roxb	Cassumunar ginger	ZINGIBERACEAE	Rhizome	5
Zingiber officinale Roscoe	Ginger	ZINGIBERACEAE	Rhizome	5
Zingiber zerumbet (Linn) Smith	Shampoo ginger	ZINGIBERACEAE	Rhizome	5

Preparation of A diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside from Dioscorea birmanica Prain & Burkill

The rhizomes of *D. birmanica* were collected from Chantaburi province, Thailand. Authentication of plant material was carried out at the herbarium of the Department of Forestry, Bangkok, Thailand where the herbarium voucher (SKPA062001002) is kept. Specimens are also kept in the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla, Thailand. Dried powdered rhizome of *D. birmanica* (1 kg) was macerated with 95% ethanol, the extract concentrated to dryness under reduced pressure, to obtain 11.13% of crude extract. Ten gram aliquot of the ethanolic extract of *D. birmanica* (DBE) was separated by vacuum liquid chromatography (VLC) using C_6H_{14} (1,000 ml), C_6H_{14}: $CHCl_3$ (1:1) (1,000 ml), $CHCl_3$ (1,000 ml), $CHCl_3$: MeOH (8:2) (1,000 ml), $CHCl_3$: MeOH (1:1) (1,000 ml) and MeOH (1,000 ml), respectively and drying by rotary evaporation. DBCM, fraction of chloroform: methanol (1:1), was select to discover a cytotoxic compound because of the previous research that showed it had high against lung cancer cells. And then an aliquot (2 g) of DBCM fraction was separated by column chromatography (silica gel with a gradient of solvents as follow $CHCl_3$ (400 ml), $CHCl_3$: MeOH (9.5:0.5) (400 ml), $CHCl_3$: MeOH (9:1) (400 ml), $CHCl_3$: MeOH (8:2) (400 ml), $CHCl_3$: MeOH (7:3) (400 ml), $CHCl_3$: MeOH (6:4) (400 ml), $CHCl_3$: MeOH (5:5) (400 ml), $CHCl_3$: MeOH (4:6) (400 ml), $CHCl_3$: MeOH (3:7) (400 ml), $CHCl_3$: MeOH (2:8) (400 ml), $CHCl_3$: MeOH (1:9) (400 ml) and finally MeOH (400 ml). Each 20 ml fractions was collected

for each eluting solvent and fractions combined, following TLC examination (silica gel/$CHCl_3$: MeOH (7:3) detection with acidic anisaldehyde spray.

Structure elucidation

The pure compound of the isolates was determined by their NMR data [^{1}H and[13] C on a Varian Unity Inova 500 spectrometer (500 MHz for ^{1}H; 125 MHz for ^{13}C)], UV spectra [a Hewlett Packard 8452A Diode array spectrometer], IR spectra [Jasco IR-810 spectrometer]. The pure compound was identified as diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside (prosapogenin A of dioscin) by comparison of its spectral features with literature values and it was identical with published data for prosapogenin A of dioscin[8,9]. This compound was also identical in chromatographic behavior when compared with authentic samples previously isolated[10]. DBS1 (Diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside or Prosapogenin A of dioscin): white amorphous solid (9 mg, 0.225% w/w of crude extract): mp 240-243°C (dec), IR (KBr disc) 3,420 (broad), 2,950, 2,900, 2,875, 1,650, 1,450, 1,380, 1,240 (acetate carbonyl), 1,140, 1,050, 980, 960, 920, 900 (intensity of 900 >920, 25 (R)-spiroketal) cm-[11] H-NMR ($CDCl_3$ and CD_3OD, 400 MHz) ppm 0.79 (3H, d, J = 5.4 Hz, H-27), 0.85 (3H, s, H-18), 1.04 (3H, s, H-19), 1.25 (3H, d, J = 6.3Hz, H-21), 1.75 (3H, d, J = 7.3 Hz, H-6'' of rhamnose), 3.2-3.7 (peak of sugar proton, H-3, H-16, H-26) 3.86 (1H, dd, J = 2.9, 9.9 Hz, H-6') 4.1 (1H, dd, J = 1.8, 3.3 Hz, H-2''), 4.44 (1H, m, H-3''), 4.5 (1H, d, J = 7.7 Hz, H-1') 5.19 (1H, d, J = 1.1 Hz, H-1") 5.36 (1H, br s, H-6) and ^{13}C NMR (see Table 2 and Fig. 1). The extract was concentrated to dryness under reduced pressure. The extract was then pooled and kept in freezer (-20°C) until required. The extract was studied for cytotoxic and anti-inflammatory activities.

Table 2. ^{13}C chemical shift (MHz, in ppm) of DBS1 compared with ^{13}C chemical shift of prosapogenin A of dioscin (A) from literature[10]

^{13} C	A[a]	DBS1[b]
1	37.4	38.9
2	30.2	31.2
3	78.4	77.9
4	39.2	39.9
5	140.9	142.2
6	121.7	123.2
7	32.2	33.7
8	31.6	31.9
9	50.2	51.9
10	37.0	38.5
11	21.1	22.5
12	39.9	41.4
13	40.4	41.9
14	56.6	58.2
15	32.2	33.2
16	81.1	82.7
17	62.8	63.2
18	16.3	17.6
19	19.4	20.6
20	41.9	43.3
21	15.0	15.7
22	109.4	111.2
23	32.2	32.9
24	29.2	30.3
25	30.5	31.9
26	66.9	68.4
27	17.3	18.3
Glu-1'	100.6	101.1
C-2'	79.6	79.9
C-3'	78.0	79.5
C-4'	72.1	72.8
C-5'	78.0	79.6
C-6'	62.9	63.9
Rha-1"	101.9	102.6
C-2"	72.5	72.2
C-3"	72.9	72.8
C-4"	74.2	74.3
C-5"	69.4	70.2
C-6"	18.6	18.6

[a] in pyridine-d_5, [b] in $CDCl_3$ and CD_3OD

Bioassay

Assay for inhibitory effect on nitric oxide production[11]

Inhibitory effect on nitric oxide (NO) production in mouse leukaemic macrophage cell lines (RAW264.7) was studied. Briefly, the RAW264.7 cell line was cultured in RPMI1640 medium supplemented with 10% heated fetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. Cells were harvested using trypsin-EDTA and diluted to a suspension in fresh medium, and seeded into 96-well plates to obtain $1x10^5$ cells/well and allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with fresh medium containing the test sample at various concentrations and then incubated for 48 hours. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. The supernatant

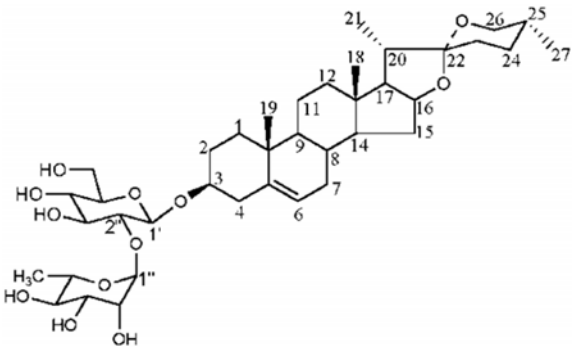


Fig. 1 DBS1 ($C_{39}H_{62}O_{12}$); Diosgenin 3-O-α-L-rhamno-pyranosyl (1→2)-β-D glucopyranoside.

(100 µl) was removed and added to 96-well plates. Griess reagent (100 µl) was added to 96-well plates and absorbance was read with a microplate reader at 570 nm. The stock solution of each test sample was dissolved in DMSO, and the solution was added in to the medium RPMI (final DMSO is 2%). The inhibition (%) was calculated by using the following equation. IC_{50} values were determined by Prism program (n = 3)

Inhibition (%) = ((A-C)-B/A-C) x 100

[A: 2 % DMSO + LPS, C: 2 %DMSO + Media, B: Sample-blank]

IC_{50} value (effective concentration of sample required to inhibit NO release by 50%) was obtained by linear regression analysis of dose-response curve-plotting between % inhibition and sample concentrations and calculated by Prism program. All extracts were tested for anti-inflammatory effects, starting with concentrations of 1, 10, and 50 to 100 µg/ml. The extracts that showing more than 50% inhibition of NO production were evaluated for their anti-inflammatory activity and toxicity compared to the positive controls (Indomethacin and MTT assay).

Cytotoxic activity by MTT assay [12]

Mouse leukaemic macrophage RAW264.7 cells were used. Briefly, the RAW264.7 cells were cultured in RPMI1640 medium supplemented with 10% heated fetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. Cells were harvested using trypsin-EDTA and diluted to a suspension in fresh medium. Cells were then seeded in 96-well plates to obtain $1x10^5$ cells/well and allowed to adhere for 1 hour at 37°C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with fresh RPMI medium containing test sample at various concentrations and then incubated for 48 hours. The supernatant was added to 96-well plates. Cytotoxicity was determined using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric method. Briefly, after 48 hours incubation with test samples, MTT solution (10 µl, 5 mg/ml in PBS) was added to each well. After 2 hours incubation, the medium was removed, and isopropanol containing 0.04 M HCl was added to dissolve the formazan production in the cells. Optical density of formazan solution was measured with a microplate reader at 570 nm. The test compound was considered cytotoxic when the optical density of the formazan solution was less than 80% of that of the control (vehicle-treated) group. Indomethacin was used as positive control. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final DMSO is 2%). Percent inhibition was calculated using the following equation, and IC_{50} values were determined by Prism program. The experiment was done in triplicate. The result is expressed as the percentage of growth inhibition (% Cytotoxicity) and IC_{50} by mean and standard error of mean (SEM) of three determinations.

% Cytotoxicity = $[(OD_{control}-OD_{sample})/OD_{control}]$ x 100

[OD = Optical density]

Assay for inhibitory effect on PGE_2 production [13,14]

This assay is based on the competition between PGE_2 and PGE_2-acetylcholineesterase (AChE) conjugate (PGE_2 Tracer) for a limited amount of PGE Monoclonal antibody. Because the concentration of the PGE_2 Tracer is held constant while the concentration of PGE_2 varies, the amount of PGE_2 Tracer that is able to bind to the PGE_2 Monoclonal antibody will be inversely proportional to the concentration of PGE_2 in the well. This antibody-PGE_2 complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to AChE) is added to the well. Inhibitory effects on the release of PGE_2 in Raw 264.7 cells were evaluated using Prostaglandin E_2 EIA kit-monoclonal. Firstly, the cells were seeded in 96-well plate to obtain $1x10^5$ cells/well and allowed to adhere for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with fresh medium containing the test sample at various concentrations and then incubated for 24 h. The supernatant 50 µl was transferred into

96-well ELISA plate and reagents added respectively (reference from PGE_2 EIA kit-monoclonal) and then incubated for 24 h. When the incubation time had elapsed plates were washed by microplate washer, Ellman's reagent added and then incubated for 1 h. Percent inhibition was calculated using the following equation, and IC_{50} values were determined by Prism program.

$$\% \text{ Inhibition} = [(OD_{control}-OD_{sample})/OD_{control}] \times 100$$

***Cytotoxic activity by SRB assay*[15,16]**

The sulforhodamine B (SRB) assay was used to estimate cell numbers indirectly by staining total cellular protein with the SRB. The protocol was based on that originally described by Skehan[15]. In brief; cells at the exponential growth phase were detached with 0.25% trypsin-EDTA to make single cell suspensions. The viable cells were counted by trypanblue exclusion using a hemocytometer and diluted with medium to give a final concentration of $1x10^4$ cells/ml for Hela cells (ATCC® CCL-2™) as cervical cancer cells. 100 µl/well of these cell suspensions would be seeded in 96-well microtiter plates and incubated to allow cell attachment. After 24 hours the cells were treated with various concentrations of the extracts. The extracts were diluted in medium to produce the required concentrations and 100 µl/well of each concentration was added to the plates to obtain final concentrations of 1, 10, 50, 100 µg/ml for the extract and 0.1, 1, 10, 50 µg/ml for pure compound. The final mixture was used for treating cells containing not more than 1% of the solvent, the same as in solvent control wells. The plates were incubated for selected exposure time of 72 hours. At the end of each exposure time, the medium was removed. The wells were washed with medium. And 200 µl of fresh medium were added to each well. The plates were incubated for a recovery period for 72 hours. On the seventh day of culture period, cells were fixed by 100 µl of ice-cold 40% trichloroacetic acid (TCA) per well, incubated at 4°C for 1 hour in the refrigerator and washed 5 times with tap water to wash non-viable cells, so viable cells were fixed as monolayer in each well. 50 µl of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and left in contact with the cells for 30 min; then the plates were washed 4 times with1% acetic acid until only dye adhering to the cells was left. The dry plates and 100 ml of 10 mMTris base [trishydroxy methyl) aminomethane, pH 10.5] were added to each well to dissolve the dye. The plates were shaken gently for 20 minutes on a gyratory shaker. The absorbance (OD) of each well (4 replicate) was read on Micro plate reader at 492 nm as an indication of cell number. 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.

Results

Primary dysmenorrhea is defined as menstrual pain that is associated with inflammation. Thai medicinal plants have been used in the treatment of cancer and inflammation for thousands of years as showed in the result of anti-inflammatory and cytotoxic activities of all extracts in Table 3 and Fig. 2, 3. In the present study, the ethanolic extract of *D. birmanica* and LG remedy showed moderate inhibition activity of nitric oxide production, where most water extracts were apparently inactive; whereas, five ethanolic extracts namely of *P. nigrum*, *Z. officinale*, *B. rotunda*, *Z. cassumunar* and *Z. zerumbet* showed potent inhibitory activity with IC_{50} values of 1.31 ± 0.42, 2.87 ± 0.31, 4.92 ± 1.43, 4.93 ± 0.42 and 22.18 ± 2.89 µg/ml, respectively. Interestingly, these plant extracts exhibited nitric oxide production with an inhibitory effect higher than the positive control (IC_{50} value of indomethacin was 25.04 ± 3.79 µg/ml); to the contrary, the 95% ethanolic extract of *D. birmanica* (DBE) and the fractions that were isolated using vacuum liquid chromatography (VLC), with ordered polarity of solvents and coded as DBCM, were apparently inactive (IC_{50} > positive control) except for DBS1, which showed potent inhibitory activity with IC_{50} value of 4.29 ± 0.52 µg/ml. For LG remedy, the nitric oxide inhibitory effect was less than the positive control with IC_{50} value of 28.18 ± 4.63 µg/ml. All extracts were non-toxic on RAW 264.7 cells (IC_{50} >100 µg/ml by MTT assay).

Prostaglandins are formed from arachidonic acids by the action of COX and involved in various pathophysiological processes including inflammation and carcinogenesis. COX2 is inducible in response to inflammatory stimuli and control of cell growth. Thus, medicinal plants that exhibited PGE_2 production inhibitory effect might be reduce the incidence of cancer and inflammation. In the present study, The ethanolic extract of spicy herbs and LG remedy exhibited PGE_2 production inhibitory activity, where most extracts were less than positive control and described as follow, four ethanolic extracts namely of *P. nigrum*, *Z. officinale*, *Z. cassumunar* and *B. rotunda* showed potent inhibitory activity with IC_{50} value of 1.20 ± 0.05, 4.78 ± 1.60, 7.45 ± 0.01 and 7.89 ± 0.01 µg/ml, respectively. Interestingly, these plant extracts exhibited PGE_2

Table 3. Biological activities showing cytotoxicity in cervical cells (Hela cells), anti-inflammatory activity by inhibition of NO and PGE_2 production in mouse leukaemic macrophage RAW 264.7 cells

Plant name	Code	Cytotoxicity (Hela) (IC_{50} ± SEM)	PGE_2 inhibitor (RAW 264.7) (IC_{50} ± SEM)	Nitric oxide (RAW 264.7) (IC_{50} ± SEM)
		µg/ml	µg/ml	µg/ml
Boesenbergia rotunda	BR1	24.45±4.73	7.89±2.84	4.92±1.43
(Linn) Mansf	BR2	>100	>100	43.85±1.29
Piper nigrum Linn	PN1	34.39±1.22	1.20±0.05	1.31±0.42
	PN2	>100	>100	>100
Zingiber cassumunar Roxb	ZC1	56.12±0.21	7.45±0.01	4.93±0.42
	ZC2	>100	>100	>100
Zingiber officinale Roscoe	ZO1	42.07±2.01	4.78±1.60	2.87±0.31
	ZO2	>100	93.50±3.50	73.78±2.31
Zingiber zerumbet (Linn) Smith	ZZ1	4.42±0.20	11.34±0.28	22.18±2.89
	ZZ2	>100	>100	41.13±2.46
Leard-ngam remedy	LG1	75.31±4.37	8.59±0.89	28.18±4.63
	LG2	>100	>100	>100
	LG3	>100	>100	>100
Dioscorea birmanica	DBCM	33.03±1.36	17.23±1.1	31.16±1.18
Dioscorea birmanica (EtOH)	DBE	40.79±0.41	62.39±3.01	84.99±1.01
Diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside	DBS1	6.07±0.02	29.11±1.15	4.29±0.52
Indomethacin	IDM	ND	1±1.02	25.04±3.79

* Code number 1 is 95% ethanolic extract, 2 is water extract and 3 is 50% ethanolic extract

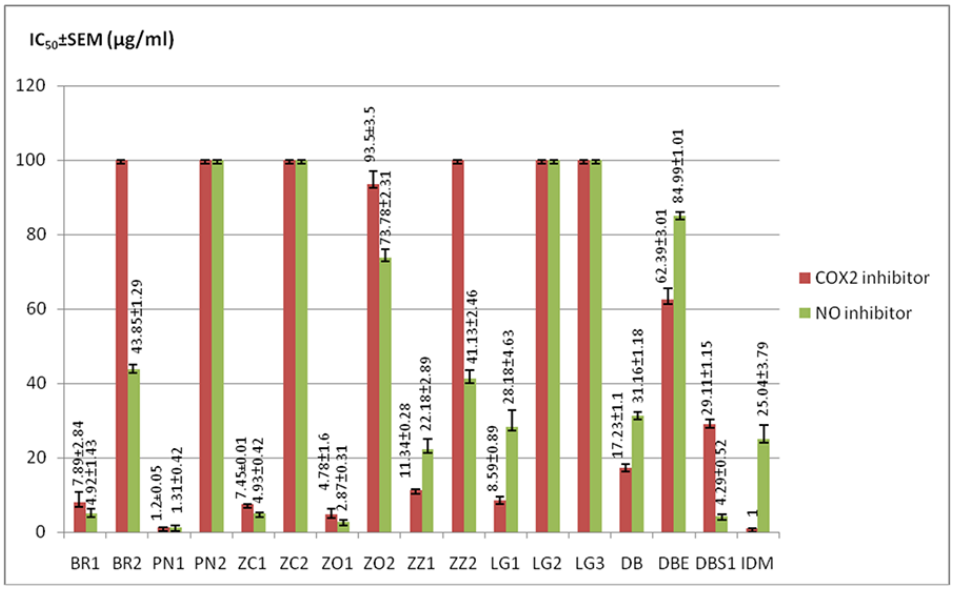
production inhibitory effect very close to the positive control (IC_{50} value of indomethacin was 1±1.02 µg/ml). The DBE and all fractions from DB were apparently inactive (IC_{50} >positive control). The 95% ethanolic extract of LG remedy exhibited PGE_2 production inhibitory effect, which was less than the positive control with IC_{50} value of 8.59±0.89 µg/ml. All extracts were non-toxic on RAW 264.7 cells (IC_{50} >100 µg/ml by MTT assay).

Secondary dysmenorrhea is defined as menstrual pain resulting from anatomic or macroscopic pelvic pathology, as is seen in women with endometriosis or chronic pelvic inflammatory disease. Untreated pelvic inflammatory disease can result in long-term complications including chronic pelvic pain and cancer. This result showed that some herb samples exhibited the inhibitory activity on cervical cancer cells. The ethanolic extract of spicy herbs, *D. birmanica* and LG remedy were tested for cytotoxic activity against Hela cells (Table 3 and Fig. 3), the data showed that the 95% ethanolic extract *Z. zerumbet* had the highest cytotoxic activity on Hela cells with IC_{50} value of 4.42±0.20 µg/ml. DBS1, which was purified from *D. birmanica* ethanolic extract, showed strong cytotoxic activity on Hela cells with IC_{50} value of 6.07±0.02 µg/ml. Meanwhile, DBS1 is non-toxic on normal cell lines (MRC-5)[6].

According to National Cancer Institute guidelines extracts with IC_{50} value <20 µg/ml and <4 µg/ml for pure compounds are plants with cytotoxic activity.

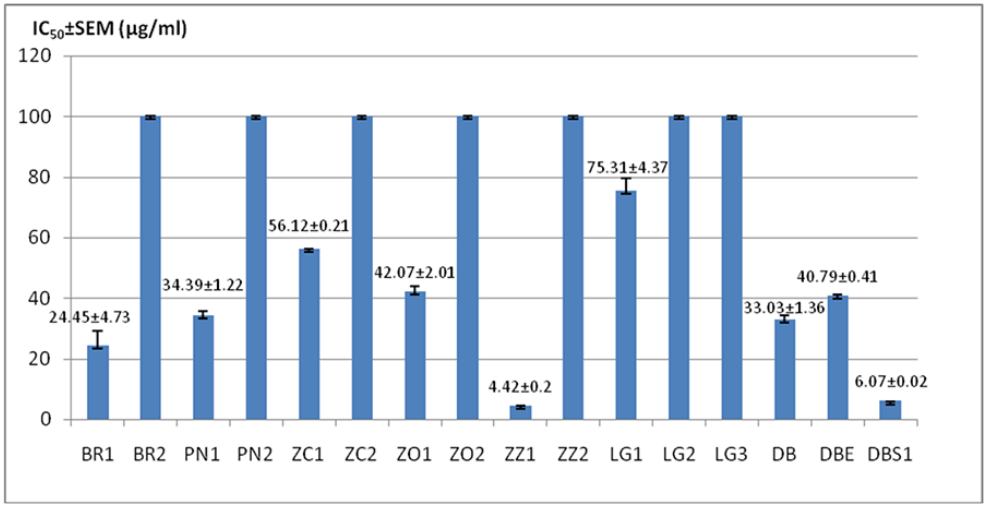
Discussion and conclusion

This investigation of medicinal plants, *B. rotunda*, *P. nigrum*, *Z. cassumunar*, *Z. officinale*, *Z. zerumbet*, *D. birmanica* and LG remedy, were based on their use by Thai traditional doctors to treat inflammation which is the leading cause of dysmenorrheal and cervical cancer. Interestingly, some plant extracts which have often been used in Thai medicine and food. In Mahachotalat Scripture, spicy herbs are used by Thai traditional practitioners to treat cancer patients. The data show that the 95% ethanolic extract *Z. zerumbet* had the highest cytotoxic activity on Hela cells with IC_{50} value of 4.42±0.20 µg/ml, DBS1 showed strong cytotoxic activity on Hela



* *B. rotunda* (BR), *P. nigrum* (PN), *Z. cassumunar* (ZC), *Z. officinale* (ZO), *Z. zerumbet* (ZZ), Leard-ngam remedy (LG), *D. birmanica* (DB), *D. birmanica* (95% ethanolic extract; DBE), Diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside (DBS1), Indomethacin (IDM)
** 1 is 95% ethanolic extract, 2 is water extract and 3 is 50% ethanolic extract

Fig. 2 Anti-inflammatory activity on inhibition of NO and PGE_2 production in mouse leukaemic macrophage cells (RAW 264.7).



* *B. rotunda* (BR), *P. nigrum* (PN), *Z. cassumunar* (ZC), *Z. officinale* (ZO), *Z. zerumbet* (ZZ) Leard-ngam remedy (LG), *D. birmanica* (DB), *D. birmanica* (95% ethanolic extract; DBE), Diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside (DBS1), Indomethacin (IDM)
** 1 is 95% ethanolic extract, 2 is water extract and 3 is 50% ethanolic extract

Fig. 3 Cytotoxic activity, IC_{50} value, of all extracts exhibited against cervical cancer cells (Hela).

cells with IC_{50} value of 6.07±0.02 µg/ml. These results are consistent with previous research of DBS1 which had high cytotoxic activity against A 549 and COR-L23 lung cancer cells (IC_{50} = 1.81±0.03, 1.84±0.05 µg/ml,

respectively)[6] but was less cytotoxic against normal lung cells MRC-5 (IC_{50} = 37.09±0.67 µg/ml)[6]. In addition, herbs which have often been used by practitioners as *P. nigrum*, *Z. officinale*, *Z. cassumunar* and *B. rotunda* also showed potent inhibitory activity on nitric oxide and PGE_2 production. This research is consistent with the research of Tewtrakul et al, 2009 that found Panduratin A and hydroxypanduratin A, which were purified from *B. rotunda* methanolic extract, showed strong inhibitory activity against nitric oxide, with IC_{50} values of 5.3 µM and 13.3 µM, respectively[17]. For LG remedy, dysmenorrhea preparation is used by Thai traditional practitioners in hospitals and medical clinics, had potent inhibitory activity on nitric oxide and PGE_2 production with IC_{50} value of 28.18±4.63 and 8.59±0.89 µg/ml, respectively.

It can be concluded that these results support the use of Thai traditional medicine for treating acute and chronic inflammation which is the leading cause of dysmenorrhea and cancer according to records of the medical health care system.

What is already known on this topic?

"Mahachotarat" is a lesson for women's care from Thai traditional medicine book. Some spicy herb, medicinal plant and a remedy have often been used in this scripture. They were *B. rotunda* Linn., *P. nigrum* L, *Z. cassumunar* Roxb, *Z. officinale* Roscoe, *Z. zerumbet* (L) Smith, *D. birmanica* Prain & Burkill including its ingredient; Prosapogenin A of dioscin, and Leard-ngam remedy (LG). This plants and remedy were used to treatment dysmenorrhea, women cancer, and also used in pospartum. Thus they were selected to investigate for biological activity related woman's health such as anti-inflammatory activity represents acute and chronic inflammation including cytotoxic against cervical cancer.

What this study adds?

Plants and remedies were extracted by two methods as ethanol and water extraction. These extracts were tested for their cytotoxic activity against cervical cancer cell by SRB assay, inhibition of nitric oxide (NO) production and COX-2 inhibitory assay by measuring prostaglandin E_2 production in lipopolysaccharide-stimulated mouse macrophage RAW 264.7 cells. The results were discussed and it was concluded that medicinal plant and LG remedy which were used to be treatment of women's care showed high anti-inflammatory on both pathways which represent chronic and acute inflammation. In addition they also showed high cytotoxic activity against cervical cancer cells.

Acknowledgements

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Potential conflicts of interest

None.

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ฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งและฤทธิ์ต้านการอักเสบของสมุนไพรและตำรับยาโรคสตรีที่พบในคัมภีร์มหาโชตรัตจากตำราการแพทย์แผนไทย

นวลจันทร์ ใจอารีย์, อรุณพร อิฐรัตน์, ศรีโสภา เรืองหนู

ภูมิหลัง: คัมภีร์มหาโชตรัตเป็นตำราทางการแพทย์แผนไทยสำหรับโรคทางสตรี ซึ่งมีทั้งสมุนไพรเดี่ยวและสมุนไพรตำรับที่สามารถรักษาโรคได้ทั้งอาการปวดประจำเดือนและมะเร็งปากมดลูก ในการศึกษาวิจัยครั้งนี้ได้คัดเลือกสมุนไพรเดี่ยวและสมุนไพรตำรับที่มีการใช้บ่อยในการรักษาอาการปวดประจำเดือนและมะเร็งปากมดลูกได้แก่กระชายพริกไทยไพลขิงกะทือหัวข้าวเย็นสารบริสุทธิ์ที่แยกได้จากหัวข้าวเย็นและตำรับยาเลือดงาม

วัตถุประสงค์: เพื่อศึกษาความเป็นพิษในเซลล์มะเร็งปากมดลูกและฤทธิ์ต้านการอักเสบของสารสกัดจากสมุนไพรเดี่ยว สารบริสุทธิ์ที่แยกได้จากสมุนไพรหัวข้าวเย็น และสารสกัดจากตำรับยาเลือดงาม

วัสดุและวิธีการ: สารสกัดจากสมุนไพรเดี่ยวและสารสกัดจากตำรับยาเลือดงามจะสกัดโดยวิธีตามแบบดั้งเดิมที่มีการใช้ในทางการแพทย์แผนไทยโดยจะหมักด้วยแอลกอฮอล์และต้มด้วยน้ำ สำหรับสารบริสุทธิ์จากสมุนไพรหัวข้าวเย็นใช้วิธีการ Bioassay guide fractionation และพิสูจน์โครงสร้างโดยใช้เทคนิคสเปกโตรสโคปี สารสกัดทั้งหมดจะถูกทดสอบฤทธิ์ความเป็นพิษในเซลล์มะเร็งปากมดลูกด้วยวิธีใช้สีย้อมซัลโฟโรดามีนบี การทดสอบฤทธิ์ต้านการอักเสบโดยวิธีการยับยั้งการหลั่งไนตริกออกไซด์และวิธีการยับยั้งการหลั่งโพรสตาแกลนดินอีทูในเซลล์แมคโครฟาจ RAW 264.7

ผลการศึกษา: พบว่าสารสกัดชั้น 95% เอทานอลของพริกไทยขิงกระชายและไพลมีฤทธิ์ยับยั้งไนตริกออกไซด์และโพรสตาแกลนดินอีทู ส่วนสารสกัดชั้น 95% เอทานอลของกะทือมีฤทธิ์ยับยั้งเซลล์มะเร็งปากมดลูกดีที่สุดโดยมีค่า IC_{50} เท่ากับ 4.42±0.20 ไมโครกรัมต่อมิลลิลิตร รองลงมาคือสารบริสุทธิ์ที่แยกได้จากหัวข้าวเย็นชื่อ Diosgenin-3-O-α-L-rhamnosyl (1$\rightarrow$2)-β-D-glucopyranosideor Prosapogenin A of dioscin มีฤทธิ์ยับยั้งเซลล์มะเร็งปากมดลูกโดยมีค่า IC_{50} เท่ากับ 6.07±0.02 ไมโครกรัมต่อมิลลิลิตร

สรุป: สมุนไพรไทยและตำรับยาไทยที่มีการใช้บ่อยเพื่อรักษาโรคทางสตรีนั้น มีฤทธิ์ต้านการอักเสบทั้งชนิดเฉียบพลันและเรื้อรัง ซึ่งที่น่าสนใจคือสมุนไพรบางตัวนั้นเป็นอาหารในชีวิตประจำวันของคนไทย นอกเหนือจากนี้ยังสรุปได้อีกว่าสมุนไพรกะทือและหัวข้าวเย็นที่มีการใช้บ่อยในการรักษามะเร็งปากมดลูกมีฤทธิ์ต้านมะเร็งปากมดลูกได้