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Anti-Breast Cancer Activity of Thai Herbal Medicine Formulation

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ABSTRACT

Thai traditional medicine formulations have been used as folk medicine to treat breast cancer patients, balance element in the patient's body or increase their immunities. It is composed of five Thai medicinal plants; *Albizia procera* bark, *Amphineurion marginatum* root and stem, *Diospyros mollis* heartwood, *Smilax corbularia* and *Smilax glabra* rhizome. Nonetheless, biological testing for anti-cancer activity against breast cancer and biological effect of this formulation on cell line models have not yet been fully investigated. This research aimed to identify species of plant materials and investigate cytotoxic effects of the formulation and its constituent plants on normal mouse fibroblast (L929) and human breast cancer (MCF-7) cells. Phylogenetic identification of plant materials was evaluated using genetic markers on chloroplast DNA. Two non-coding regions; *trnL* intron and *trnL-F* IGS, were amplified by PCR technique using universal primers. These PCR products were identified by DNA sequencing and aligned with ClustalW using BioEdit program. The nucleotide sequences were submitted to GenBank database and accession numbers were assigned. BLAST results of *A. procera* and *A. marginatum* showed the highest percent identities at 100% in both non-coding regions when compared with reference sequences on GenBank database. Then, ethanol maceration was performed which was similar to those practiced by Thai folk preparation. As the results, ethanolic extract of formulation had potent anti-cancer activity on MCF-7 cells and low cytotoxicity on L929 cells with IC₅₀ values of 52.51 and 81.48 µg/mL, respectively. These results will support the biological evidences of Thai folk medicines to treat breast cancer cells.

Keywords: Anti-cancer activity, Cytotoxicity, Formulation, MCF-7 cells, Thai traditional medicine

INTRODUCTION

Cancer is a major health problem worldwide. Breast cancer is the most commonly occurring cancer and the second leading cause of cancer death in women. Unfortunately, the trends of breast cancer also occurred in Thailand. Nowadays, there are many kinds of treatment methods for breast cancer, especially chemotherapy, it is effectively used for lowering the total cancer cells and reduce cancer spreading. However, it also causes many side effects (Partridge *et al.*, 2001). Thus, to come across these disadvantages, it has an alternative treatment for breast cancer, such as traditional herbal medicine.

Traditional medicine is a system of methods and practices, such as herbal medicine or bodywork that is native to the region. Many folks and traditional herbal medicines are found on complex mixtures of plant phytochemicals. The herbal medicine formulation is the complex mixtures of plant phytochemicals. It consists of three major ingredients, including active ingredients, adjuvants and correctives. It has been reported to relieve chemotherapy-induced vomiting and peripheral neuropathy. In addition, the anti-cancer effects of some traditional herbal components have been reported to involve the improvement of immune functions *in vitro* and *in vivo* models (Kim *et al.*, 2015; Ruangnoo S. and Itharat A., 2010; Prayong *et al.*, 2008). In Thailand, many cancer patients used traditional medicine, which is commonly made by boiling the plant materials in water or soaking in alcohol as an alternative for cancer treatment and there are many kinds of herbal medicine formulation available (Itharat *et al.*, 2004). Therefore, it comes across for this research attention on another formulation, which consists of the barks of *Albizia procera*, the roots and stems of *Amphineurion marginatum*, the heartwoods of *Diospyros mollis*, the rhizomes of *Smilax corbularia* and *Smilax glabra*. This traditional formulation has been used as folk medicine to treat breast cancer patients, balance element in the patient's body or increase their immunities. Currently, these five medicinal plants had been studied individually for phytochemical compositions, chemical characterization, biological properties and anti-cancer activities *in vitro* models. The wide varieties of these medicinal herbs might be used to inhibit the proliferation of tumor cells and may increase immunity in the patient's body.

Before starting the experiments, phylogenetic analysis of plant species is also very important process to identify the species of plant materials using genetic markers. A genetic marker is a gene or DNA sequence that can be used to identify species and described as a variation. The non-coding regions of chloroplast DNA, which displayed the highest frequency of mutation, have been used as tools for evolutionary studies, phylogenetic classification and discrimination different plant genus. Therefore, two loci of non-coding regions, including *trnL* intron and *trnL-trnF* intergenic spacer (*trnL-F* IGS), on chloroplast DNA of medicinal plants will be investigated

Although some pharmacological studies of these Thai medicinal plants have been reported, biological testing for the cytotoxic activity against breast cancer and biological effect of the anti-breast cancer formulation on cell line models have not been fully investigated for scientific approved. In present study, the five Thai medicinal extracts, including *A. procera*, *A. marginatum*, *D. mollis*, *S. corbularia* and *S. glabra* which are plant ingredients of herbal medicine formulation will be evaluated for phylogenetic identification of plant species and cytotoxic activity against human breast cancer (MCF-7) and normal mouse fibroblast (L929) cell lines.

MATERIALS AND METHODS

Chemicals and reagents

NucleoSpin[®] Plant II kit was purchased from Macherey- Nagel (Germany). 2X Phusion flash PCR master mix was purchased from ThermoFisher (USA). E.Z.N.A.[®] Cycle Pure Kit was purchased from Omega Bio-tek (USA). Ethanol absolute was purchased from Merck (USA). Penicillin-streptomycin was purchased from Caisson Laboratories (USA). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (United Kingdom). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), agarose A, dimethyl sulfoxide (DMSO) and phosphate buffer solution (PBS) were purchased from Bio Basic (Canada). All chemicals in this research were ACS reagent and analytical grades.

Plant materials

The dried plant materials and powders of *Albizia procera* (barks), *Amphineurion marginatum* (roots and stems), *Diospyros mollis* (heartwoods), *Smilax corbularia* (rhizomes) and *Smilax glabra* (rhizomes) were purchased from Parinam Osot, Samut Sakhon province, Thailand. The parts of plant materials were used as shown in Table 1.

Table 1. Information of several Thai medicinal herbs formulation which were used in this study

Plant name	Plant species	Family	Parts used
Thing-Thon	<i>Albizia procera</i>	Fabaceae	Barks
Ma-Duea-Din	<i>Amphineurion marginatum</i>	Apocynaceae	Roots and stems
Ma-Kluea	<i>Diospyros mollis</i>	Ebenaceae	Heartwoods
Khao-Yen-Neua	<i>Smilax corbularia</i>	Smilacaceae	Rhizomes
Khao-Yen-Tai	<i>Smilax glabra</i>	Smilacaceae	Rhizomes

Phylogenetic identification

DNA extraction. The dried plant materials were powdered in liquid nitrogen by pestle and mortar. The genomic DNAs were extracted using NucleoSpin[®] Plant II kit and analyzed by 0.8% agarose gel electrophoresis. The quality and quantity of genomic DNAs were determined by Nanodrop spectrophotometer (BioDrop, UK).

PCR amplification. The universal primers, designed by Taberlet *et al.*, 1991, were used to amplify non-coding regions; *trnL* intron and *trnL*-F IGS, from the genomic DNAs of medicinal plants using polymerase chain reaction (PCR) technique as shown in Table 2 and Figure 1. The optimization of annealing temperature and PCR conditions for *trnL* and *trnL*-F IGS were performed, as follows: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 53 to 57°C for 30 sec and extension at 72°C for 2 min, with final extension step of 7 min at 72°C. PCR amplification was performed in the total reaction volume of 50 µL containing 2.5 µL of genomic DNA, 1.25 µL of 10 µM of each primer, 20 µL of sterile deionized water and 25 µL of 2X Phusion flash PCR master mix. The PCR reactions were applied in automate thermal cycling (Biometra, Germany) and PCR products were separated by 1.2% agarose gel electrophoresis

PCR products purification. The non-coding regions PCR products were purified using E.Z.N.A.[®] Cycle Pure Kit.

DNA sequencing. The purified PCR products were submitted to First BASE Laboratories Sdn Bhd (Malaysia) to perform nucleotide sequencing by BigDye[®] Terminator v3.1 cycle sequencing kit chemistry using universal primers as shown in Table 2 and Figure 1.

DNA sequence data analysis. The nucleotide sequences of *trnL* intron and *trnL*-F IGS were aligned with ClustalW using BioEdit sequence alignment editor v7.0.5. Then, the nucleotide sequences were compared with reference

sequences on GenBank nucleotide sequence database by BLAST program and submitted to GenBank database for accession numbers.

Table 2. Nucleotide sequences of universal primers used for PCR amplification and direct DNA sequencing

Locus	Primer	Type	Sequence (5' → 3')	Reference
<i>trnL</i> intron	C	Forward	CGAAATCGGTAGACGCTACG	Taberlet <i>et al.</i> , 1991
	D	Reverse	GGGGATAGAGGGACTTGAAC	
<i>trnL</i> -F IGS	E	Forward	GGTTCAAGTCCCTCTATCCC	
	F	Reverse	ATTGAACTGGTGACACGAG	

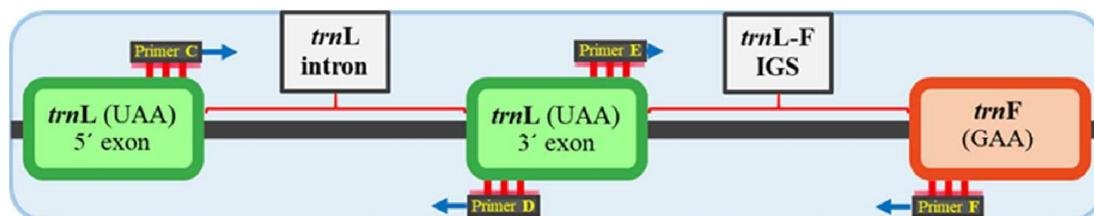


Figure 1. Positions and directions of universal primers were used to amplify and sequence two non-coding regions of chloroplast DNA

Preparation of the extracts

The ethanolic extracts of five plants were obtained by macerating the powdered plant materials in 300 mL of absolute ethanol at room temperature for three days. Samples were filtered (Whatman no. 1), and the total solvent extracts were concentrated to dryness under reduced pressure (40°C) using a rotary evaporator. The ethanolic extracts were dried to constant weight by vacuum evaporation and stored in air-tight glass container at room temperature until used. The ethanolic extracts of five plants were mixed in the same ratio to provide the formulation. The percentage yield of ethanolic extracts were shown in Table 3.

Table 3. Yields (%w/v) of five medicinal plants and formulation from the ethanolic extracts.

Plants	Parts used	Type of extract	Yield (%)
<i>A. procera</i>	Bark	Ethanolic extract	14.19

<i>A. marginatum</i>	Root and stem	Ethanollic extract	2.00
<i>D. mollis</i>	Heartwood	Ethanollic extract	2.38
<i>S. corbularia</i>	Root	Ethanollic extract	5.94
<i>S. glabra</i>	Root	Ethanollic extract	4.61

Cytotoxicity and anti-breast cancer activity of the plant extracts

The cytotoxicity and anti-breast cancer activity of the ethanolic extracts and formulation were evaluated on normal mouse fibroblast L929 (ATCC[®] CCL-1[™]) and human breast cancer MCF-7 cell lines (ATCC[®] HTB-22[™]) by MTT assay. The L929 and MCF-7 cells were seeded at a concentration of 1x10⁴ cells/mL and cultured in 96-well plates for 24 h. These cells were cultured in a RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin in an incubator at 37°C, in a 95% humidified atmosphere containing 5% CO₂. The ethanolic extracts and formulation at 0, 10, 25, 50, 75, 100, 150, 200, 250, 300, 400 and 500 µg/mL concentrations and doxorubicin (0-10 µg/mL) as positive control for MCF-7 cells, were added to the plates and further incubated for 48 h. The cells were washed with PBS (pH 7.4) and then 80 µL serum-free of RPMI 1640 medium supplemented with 20 µL of 5 mg/mL MTT solution was added and incubated at 37°C for 4 h. Then, the medium was replaced with 100 µL of DMSO to dissolve the MTT formazan crystals. Finally, the absorbance was measured at 540 nm and 620 nm by microplate reader (SpectraMax[®] i3x, Molecular Devices, USA). The percentage of cell viability was calculated by the following equation:

$$\text{Cell viability (\%)} = \frac{(\text{absorbance of formazan product} - \text{mean absorbance of blank})}{\text{mean absorbance of positive control}} \times 100$$

RESULTS

Phylogenetic identification of five plant materials

The genomic DNA concentrations of five plant materials obtained the ranges between 2.06 to 82.45 ng/ μ L using Nanodrop spectrophotometer. Then, the nucleotide sequences of *trnL* intron and *trnL*-F IGS of chloroplast DNA from five Thai medicinal plants were successfully amplified by PCR using two sets of universal primers and purified using E.Z.N.A.[®] Cycle Pure Kit. As seen in Figure 2, the band sizes of *trnL* intron and *trnL*-F IGS purified PCR products of five medicinal plants had the ranges started from 500 to 600 and 300 to 500 base pairs, respectively.

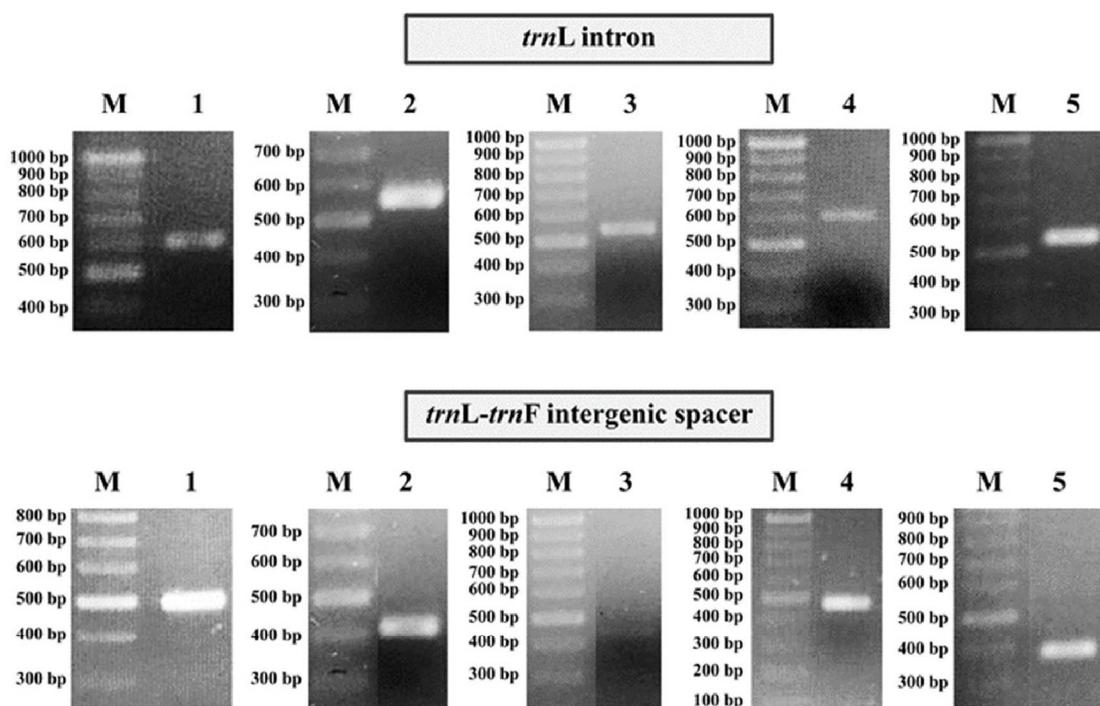


Figure 2. 1.2% agarose gel electrophoresis of two non-coding regions of purified PCR products. Lane M: VC 100 bp Plus DNA Ladder. Lane 1-5: purified PCR products of *A. procera*, *A. marginatum*, *D. mollis*, *S. corbularia* and *S. glabra*, respectively.

Then, all purified PCR products were successfully sequenced using these universal primers. All nucleotide sequences of *trnL* intron and *trnL*-F IGS were aligned with ClustalW using BioEdit sequence alignment editor

v7.0.5. The sizes of purified PCR products were widely distributed ranging from 510 to 599 bp for *trnL* intron and from 360 to 449 bp for *trnL*-F IGS among the medicinal plants used in this study as shown in Figure 3 and 4.



Figure 3. Nucleotide sequences of *trnL* intron from medicinal plants; *A. procera*, *A. marginatum*, *D. mollis*, *S. corbularia* and *S. glabra*, were aligned with ClustalW using BioEdit sequence alignment editor.

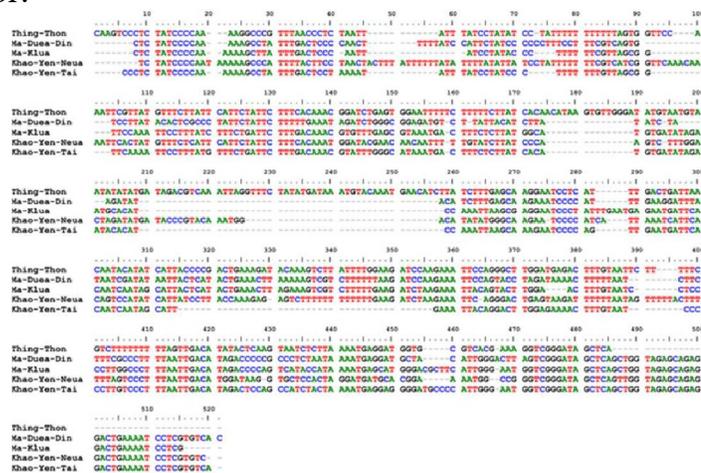


Figure 4. Nucleotide sequences of *trnL*-F IGS from medicinal plants; *A. procera*, *A. marginatum*, *D. mollis*, *S. corbularia* and *S. glabra*, were aligned with ClustalW using BioEdit sequence alignment editor.

All nucleotide sequences were subjected to nucleotide BLAST analysis on GenBank database for searching against the GenBank nucleotide database to identify the most closely related sequences. Interestingly, BLAST analysis results showed that *A. procera* and *A. marginatum* sequences matched the same species of reference sequences on GenBank database with the highest percent identities at 100% in both *trnL* intron and *trnL-F* IGS. In contrast, for the other nucleotide sequences remaining, *D. mollis*, *S. corbularia* and *S. glabra*, could not be obtained the close match with percent identities at 98 to 100% as shown in Table 4. Moreover, the 10 nucleotide sequences data reported in this research had been submitted in the GenBank nucleotide database with the accession numbers were shown in Table 5.

Table 4. BLAST analysis of plant materials according to *trnL* intron and *trnL-trnF* intergenic spacer

Sample	Best match species on GenBank database	Accession number	Maximum percent identity (%)	
			<i>trnL</i> intron	<i>trnL-F</i> IGS
<i>A. procera</i>	<i>Albizia procera</i>	EU440009.1	100.0	100.0
<i>A. marginatum</i>	<i>Amphineurion marginatum</i>	MG963253.1	100.0	100.0
<i>D. mollis</i>	<i>Thunbergia erecta</i>	AF061821.1	98.0	-
	<i>Thunbergia grandiflora</i>	KT075034.1	-	100.0
<i>S. corbularia</i>	<i>Clausena lansium</i>	EU369568.1	99.0	
	<i>Smilax aspera</i>	GU945061.1	-	99.27
<i>S. glabra</i>	<i>Premna nana</i>	MG836410.1	99.79	-
	<i>Premna odorata</i>	HQ412925.1	-	99.39

Table 5. GenBank accession numbers of plant materials according to *trnL* intron and *trnL-trnF* intergenic spacer

Plant name	Common name	GenBank accession number	
		<i>trnL</i> intron	<i>trnL-F</i> IGS
<i>Albizia procera</i>	Thing-Thon	MK547598	MK547603
<i>Amphineurion marginatum</i>	Ma-Duea-Din	MK547599	MK547604
<i>Diospyros mollis</i>	Ma-Kluea	MK547600	MK547605
<i>Smilax corbularia</i>	Khao-Yen-Neua	MK547601	MK547606
<i>Smilax glabra</i>	Khao-Yen-Tai	MK547602	MK547607

Determination of cytotoxicity on L929 cells and anti-breast cancer activity on MCF-7 cells

According to study of several Thai medicinal plant, which included plant genus, species, family and plant parts, were used in this study as shown in Table 1. Percentage of yields of ethanolic extracts and formulation were shown in Table 2. The cytotoxicity and anti-breast cancer activities were evaluated of each plant extract and formulation as IC_{50} values ($\mu\text{g/mL}$) which were summarized in Table 6. The result revealed that the ethanolic extracts of *D. mollis*, *S. glabra* and formulation had the highest anti-cancer effects against MCF-7 cells with IC_{50} values of 11.75, 36.18 and 52.51 $\mu\text{g/mL}$, respectively. The ethanolic extract of formulation showed the potent activity against cancer cells MCF-7 ($IC_{50} = 52.51 \mu\text{g/mL}$) which was low cytotoxicity on normal cells L929 ($IC_{50} = 81.48 \mu\text{g/mL}$). These results indicated that the formulation possessed high anti-cancer activity against breast cancer cells and less cytotoxicity on normal fibroblast cells. In addition, the comparison of anti-cancer activity of ethanolic extracts from individual plants against breast cancer cells could be concluded that *D. mollis* extract showed the highest activity ($IC_{50} = 11.75 \mu\text{g/mL}$), but also had cytotoxic effect on normal fibroblast cells ($IC_{50} = 11.52 \mu\text{g/mL}$). Thus, ethanolic extract of *D. mollis* significantly suppressed proliferation of breast cancer cells *in vitro* assay.

Table 6. The cytotoxicities and anti-breast cancer activities of ethanolic extracts from each medicinal plant and formulation on L929 and MCF-7 cells treated for 48 h. (n = 2)

Crude extract and pure compound	Type	Cell lines, IC ₅₀ value (µg/mL)	
		L929	MCF-7
<i>Albizia procera</i>	EtOH	164.52 ± 11.90	138.57 ± 46.06
<i>Amphineurion marginatum</i>	EtOH	194.37 ± 57.26	238.75 ± 72.15
<i>Diospyros mollis</i>	EtOH	11.52 ± 1.32	11.75 ± 0.72
<i>Smilax corbularia</i>	EtOH	290.33 ± 49.08	290.94 ± 97.56
<i>Smilax glabra</i>	EtOH	43.39 ± 15.69	36.18 ± 8.54
Formulation	EtOH	81.48 ± 17.01	52.51 ± 2.94
Doxorubicin (+)	Pure compound	ND	3.07 ± 1.06

Note: (+) = positive control, EtOH = ethanolic extract, ND = no detection

DISCUSSION

In the genomic DNA separation on agarose gel electrophoresis, it was found that the yield of genomic DNA was low and RNAs were contaminated. Moreover, during DNA extraction, DNA was degraded and contained high amounts of polysaccharide, polyphenols and secondary metabolites which were commonly found in plants. However, *trnL* intron and *trnL*-F IGS in chloroplast DNA are short fragments which are around 300 to 700 base pairs. So, these DNAs can be used as DNA template for PCR reaction. The band sizes of *trnL* intron and *trnL*-F IGS PCR products of five medicinal plants had the ranges started from 500 to 600 and 300 to 500 base pairs, respectively. In addition, all bands revealed obviously clear without non-specific band. These results indicated that primers can specifically complementary to *trnL* intron and *trnL*-F IGS, and these purified PCR products can be used for direct DNA sequencing.

According to BLAST analysis results, *A. procera* and *A. marginatum* nucleotide sequences matched the same species of reference sequences on GenBank database with the highest percent similarities at 100% in both *trnL* intron and *trnL*-F IGS. On the other hand, for other sequences remaining, including *D. mollis*, *S. corbularia* and *S. glabra* could be obtained the close match with percent identities at 98 to 100% with *Thunbergia* spp., *Clausena lansium*, *Smilax aspera* and *Premna* spp. Despite of the reference sequences of *D. mollis*, *S. corbularia* and *S. glabra*, which studied on *trnL* intron and *trnL*-F IGS, were not available on GenBank database to date. These indicated that

trnL intron and *trnL*-F IGS of *D. mollis*, *S. corbularia* and *S. glabra* have not been studied before. For *trnL* intron and *trnL*-F IGS of *D. mollis*, no close match could be obtained, because only 20 genetic markers of *D. mollis* which were investigated on *matK*, *rbcL*, *ndhF*, ITS1, ITS2, 5.8S rRNA, *atpB*, *ncpGS*, *trnS*-G IGS and PHYA genes were available in GenBank database (Turner *et al.*, 2013; Wang *et al.*, 2013; Duangjai *et al.*, 2009; Yonemori *et al.*, 2008; Duangjai *et al.*, 2006). For *trnL* intron and *trnL*-F IGS of *S. corbularia*, no close match could be obtained, because only 27 genetic markers of *S. corbularia* which were investigated on 5.8S, 18S, 28S of rRNA, *matK*, *rbcL*, ITS1, ITS2, *atpB*-*rbcL* IGS, *trnS*-G IGS and *rpl16* genes were available in GenBank database. And the last one, *trnL* intron and *trnL*-F IGS of *S. glabra*, no close match could be obtained, because only 55 genetic markers of *S. glabra* which were investigated on *trnH*, *trnH*-*psbA* IGS, *psbA*, *psbA*-*trnH*, *rbcL*, *ndhA*, *ndhF*, 5.8S, 18S, 28S of rRNA, *rpl16*, *matK*, ITS1, ITS2, *atpB*-*rbcL* IGS, *trnS*-G IGS and EF1-a genes were available in GenBank database (Chen *et al.*, 2017; Jin *et al.*, 2016; Liu *et al.*, 2015; Sun *et al.*, 2015; Chen *et al.*, 2014; Qi *et al.*, 2013; Kritpetcharat *et al.*, 2011). Thus, these results suggested that the other regions on chloroplast DNA could be used as the genetic markers, which are available on the GenBank nucleotide database, such as *matK*, ITS, *rbcL* or *trnH*-*psbA* gene, for herbal plant identification (Mishra *et al.*, 2016; Ganie *et al.*, 2015; Li *et al.*, 2015; Techen *et al.*, 2014).

According to the cytotoxicity by MTT assay, all ethanolic extracts and formulation were treated at various concentrations on L929 and MCF-7 cells. This investigation showed that the ethanolic extracts from single plant and anti-breast cancer formulation had no cytotoxic effect on normal cells ($IC_{50} > 50$ $\mu\text{g/mL}$), except ethanolic extracts of *D. mollis* and *S. glabra* ($IC_{50} = 11.52$ and 43.39 $\mu\text{g/mL}$). For comparison of anti-cancer activity between ethanolic extract from single plant and the formulation against breast cancer cells, it was found that *D. mollis* and *S. glabra* extracts had the higher anticancer activity potential ($IC_{50} = 11.75$ and 36.18 $\mu\text{g/mL}$, respectively) rather than the formulation ($IC_{50} = 52.51$ $\mu\text{g/mL}$). It was possible that the active compounds in *D. mollis* and *S. glabra* which were ingredients of the formulation, affected to MCF-7 cells. In previous study, diospyrin and derivatives which isolated from *Diospyros* spp. had the anti-cancer activity. Acetylamine derivatives exhibited cytotoxic effect on colon cancer cells HT-29 and involved in mitochondrial pathway of cell death. Diospyrin, diethylether, bisnaphthoquinonoid derivatives revealed the oxidative stress-dependent apoptosis in several human cancer cells and tumor models. In addition, diospyrin diethylether could triggered the increment in cytosolic calcium which led to the apoptotic cell death in MCF-7 cells.

Moreover, diospyrin and derivatives exerted their anti-cancer actions, one of these mechanisms implicated reactive oxygen species (ROS) as a key factor in causing the anti-cancer activity (Rauf *et al.*, 2017). Meanwhile, *S. glabra* extract was consistent with a previous finding that glycoproteins isolated from the rhizomes of *S. glabra* had anti-proliferative effects in MCF-7 cells which mediated the apoptosis of MCF-7 cells through the sub-G1 phase of the cell cycle *in vitro* (Ooi *et al.*, 2008). Furthermore, crude extract of *S. glabra* rhizome inhibited the growth of MCF-7, HT-29 and BGC-823 cells (IC_{50} = 42.95, 40.85, and 40.45 mg/mL, respectively) (Gao *et al.*, 2011). In addition, based on this present study, doxorubicin which was the most effective anti-cancer drug significantly inhibited the cell proliferation of breast cancer cells with IC_{50} value of 3.07 μ g/mL. Doxorubicin binds to nucleic acids, presumably through specific intercalation of the planar anthracycline moiety and produces DNA strand breaks (Filyak *et al.*, 2007).

In this study, *A. procera* ethanolic extract showed no cytotoxic effect on L929 cells and MCF-7 cells. The IC_{50} value of MCF-7 cells were reliable with a previous report that echinocystic acid 3,16-O-bisglycosides, isolated compound from *A. procera* bark, were no inhibitory effect for their cytotoxicities against HepG2, A549, HT-29 and, MCF-7 cells when evaluated by MTT assay (Miyase *et al.*, 2010). In contrast, the previous finding reported that triterpenoid saponins isolated from the bark exhibited cytotoxic effect against human liver cancer cell line HepG2 with IC_{50} values of 9.13 μ g/mL and 10 μ g/mL, respectively, using SRB cytotoxic assay (Melek *et al.*, 2007). However, the properties of substances (crude extracts and isolated pure compounds) which used to treat cells and type of cells which used for testing were different, resulting in the trends of these IC_{50} values were inconsistent. In addition, Sivakrishnan and Kottaimuthu, (2014) suggested that the ethanolic extract of aerial parts of *A. procera* had high phytochemical contents, including triterpenoids, carbohydrates, glycosides, phytosterols, phenolic compounds, saponins, tannins and flavonoids. These active constituents showed different activities against different diseases, including cancer, liver disorders, diabetes, atherosclerosis and inflammatory diseases. It also possessed antioxidant properties. Therefore, this study indicated that it could be a high medicinal value and could be involved in a medicinal plant category.

Recently, *A. marginatum* has been widely used as traditional medicine in many countries. For example, the decoction of roots is used as a treatment for anemia, loss of appetite and urinary diseases, a tonic against fevers and an aid to menstruation (Nooteboom, 2018). In this research, the cytotoxicity of ethanolic extract from *A. marginatum* showed no cytotoxic effect on normal

mouse fibroblast (L929) cells and human breast cancer (MCF-7) cells. The previous reports suggested that the ethanolic extract of *A. marginatum* had low anti-liver cancer activity on malignant human hepatoma (HepG2) cells ($IC_{50} = 866 \pm 125 \mu\text{g/mL}$) and had inactive on normal african green monkey kidney (Vero) cells ($IC_{50} > 1000 \mu\text{g/mL}$) (Prayong *et al.*, 2008). Thus, the cytotoxicity information from this previous study correlated with this data suggesting that *A. marginatum* ethanolic extracts provided non-toxic effects on MCF-7 and L929 cells. Furthermore, Khay *et al.* (2012) also reported the results of cytotoxicity screening of several Cambodian traditional medicinal plants, including *A. marginatum*, against colon adenocarcinoma (HT-29) cells and hepatoma (HepG2) cells using MTT assay. Recently, dehydropyrrolizidine alkaloids (dehydroPAs) ; 9- *O*- angeloylretronecine, amphineurine and marginatine, were found in the roots, leaves and stems of *A. marginatum*. However, the use of *A. marginatum* as medicine need to be addressed for the presence of dehydroPAs and may be considered seriously in the conditions of human health (Colegate *et al.*, 2018). Interestingly, this medicinal plant has been widely used as traditional medicine in many countries, but the few scientific reports are available with aspect to formula, bioactive secondary metabolites or proven therapeutic functions.

According to the cytotoxicity assay in this research, the ethanolic extract of *D. mollis* demonstrated the highest anti-breast cancer activity on MCF-7 cells. These data were consistent with a previous finding that the presence of naphthalene derivatives and triterpenes in *D. mollis* which might be involved the inhibition on proliferation and metastasis of cancer cells. In addition, it had been reported that the isolates from this plant were tested for hyaluronidase inhibitory activity, and only lupeol caffeate, which was triterpenoid, showed moderate inhibitory activity. These results suggested that some traditional uses of this plant were supported by inhibiting hyaluronidase and they also suggested that the plant had the potential for biological activity (Suwama *et al.*, 2017). However, *D. mollis* which are used in folk and traditional medicines still available in the few scientific evidences and reports on cancer cells and normal cells testing.

In addition, the results from this research showed that ethanolic extract of *S. corbularia* had no inhibitory effect on MCF-7 cells and L929 cells. In the previous reports, the antioxidant and cytotoxic activities of the water and ethanolic extracts of *S. corbularia* rhizomes were investigated. In SRB cytotoxicity assay, water and ethanolic extracts of *S. corbularia* showed no cytotoxic activities against two types of human lung cancer cell lines; A549 and COR-L23, and human normal lung (MRC5) cell lines ($IC_{50} > 50 \mu\text{g/ml}$),

but the both extracts of *S. corbularia* exhibited high antioxidant activity using DPPH assay. Thus, *S. corbularia* could be used in cancer preparation due to its high antioxidant activity (Ruangnoo and Itharat, 2010). From the IC₅₀ values of above cells (A549, COR-L23 and MRC5), the cytotoxic effects of *S. corbularia* ethanolic extract on human breast cancer (MCF-7) cells and normal mouse fibroblast (L929) cells, which used in this research, tended to be inactive in the same direction as previous reports. Moreover, as for the anti-inflammatory effects of *S. corbularia*, the previous studies suggested that oral administration of ethanolic extract from *S. corbularia* rhizomes (1,600 mg/kg) significantly suppressed carrageenin-induced edema in rats compared with aspirin as reference drug. So, these studies suggested that *S. corbularia* ethanolic extract possessed anti-inflammatory activity. It was possible that active constituents included in *S. corbularia* ethanolic extract might be involved in the inhibition of inflammatory mediators (Reanmongkol *et al.*, 2007).

Ethanolic extracts of *S. glabra* in this research showed more cytotoxicity against MCF-7 cells rather than L929 cells. These data were consistent with a previous finding that glycoproteins isolated from the rhizomes of *S. glabra* also had anti-proliferative effects in human breast cancer cell lines MCF-7 which mediated the apoptosis of MCF-7 cells through the sub-G1 phase of the cell cycle *in vitro*. Its anti-proliferative potential for cancer cells that was dependent on different levels of glycosylation of three core proteins made this plant more interesting and important in biomedical research (Ooi *et al.*, 2008). Furthermore, crude extract of *S. glabra* rhizome inhibited the growth of MCF-7, HT-29 and BGC-823 cells in a dose-dependent, with IC₅₀ values of 42.95, 40.85, and 40.45 mg/mL, respectively. In addition, mitochondrial membrane permeabilization (MMP), production of reactive oxygen species (ROS), elevation of intracellular [Ca²⁺], relocation of cytochrome C and activation of caspase-3 were associated with the initiation of apoptosis by rhizomes of *S. glabra* treatment. In microarray analysis, it was found that the changes in expression profiles of genes related to apoptosis, proliferation and cell cycle control in the cells which treated with the rhizomes from *S. glabra* (Gao *et al.*, 2011). These previous studies suggested that *S. glabra* rhizome might be the potential anti-cancer agent and used as promising anti-cancer drug preparation for cancer treatments.

However, MTT measured metabolic activity within cells rather than cytotoxicity. Therefore, it should be noted that the herbal formulations impaired metabolism rather than killing the cells. Thus, for further, the investigation of cytotoxic effects and mechanism of these ethanolic extracts

and formulation on breast cancer and normal fibroblast cell lines need to be addressed. The cells in different phases of cell cycle arrest, the forms of programmed cell death, and DNA damage and repair will be investigated for further experiments.

These results in this study is the first report that investigate the cytotoxicity of anti-breast cancer formulation on human breast cancer cells and normal fibroblast cells. Therefore, this report can conclude that the formulation had the inhibitory effect on cancer cells and had a low cytotoxicity in normal cells. These suggested that this formulation which consisted of wide varieties of these medicinal herbs, might be used to inhibit proliferation of tumor cells formulation could be the alternative of anti-cancer drug for treating breast cancer patients and may increase immunity in the patient's body.

CONCLUSION

The nucleotide sequences of *trnL* intron and *trnL*-F IGS of chloroplast DNA from five Thai medicinal plants were successfully amplified by PCR, identified by direct DNA sequencing, aligned with ClustalW using BioEdit program and submitted to GenBank nucleotide sequence database. GenBank accession numbers of these nucleotide sequences were assigned. The sizes of purified PCR products were widely distributed ranging from 510 to 599 bp for *trnL* intron and from 360 to 449 bp for *trnL*-F IGS. In addition, BLAST results of *A. procera* and *A. marginatum* revealed the highest percent identities at 100% in both *trnL* intron and *trnL*-F IGS non-coding regions when compared with reference sequences in GenBank database. The results obtained in this study demonstrate that sequence analysis of *trnL* intron and *trnL*-F IGS can be used to distinguish various medicinal plants. For their cytotoxicities on normal cells and breast cancer cells, the ethanolic extract of formulation had the moderate anti-breast cancer activity with IC_{50} value of 52.51 $\mu\text{g}/\text{mL}$ and low cytotoxicity on L929 cells with IC_{50} value of 81.48 $\mu\text{g}/\text{mL}$. These results will support the biological evidences of Thai folk medicines to treat breast cancer cells.

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