BIOLOGICAL ACTIVITIES AND STABILITY TESTING OF A THAI TRADITIONAL REMEDY FOR FEMALE REPRODUCTIVE CANCER TREATMENT

BY

DUANGPACHARAPORN KWANCHIAN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
IN APPLIED THAI TRADITIONAL MEDICINE
FACULTY OF MEDICINE
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ENTITLED

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was approved as partial fulfillment of the requirements for
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Thesis Title	BIOLOGICAL ACTIVITIES AND STABILITY TESTING OF A THAI TRADITIONAL REMEDY FOR FEMALE REPRODUCTIVE CANCER TREATMENT
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ABSTRACT

Ya Kae Ma Reng Nai Mod Look (MRM) is Thai traditional medicine used to treat female reproductive cancer described in the Thai traditional medicine book by Krom Luang Chumphon Khet Udomsak. It consists of *Smilax corbularia* Kunth., *Dioscorea membranacea* Pierre ex Prain & Burkill., *Stemona collinsiae* Craib., *Rauwenhoffia siamense* Scheff., *Canna indica* L., and sodium chloride. It has not ever been researched into the biological activity of this remedy. Therefore, this study aimed to investigate the cytotoxic, antioxidant, anti-inflammatory, and antifungal activities of MRM extracts and its plant ingredients. Furthermore, this study determined the marker compound and also tested the stability of MRM extract.

The ethanolic extracts of MRM and its plant ingredients were extracted by maceration with 95% ethanol, and decoction which gave the ethanolic and aqueous extracts of MRM and its plant ingredient extracts. The water extract of *C. indica* (CIW) had the highest yield of 49.37% w/w. The yields of ethanolic extracts of MRM (MRME) and water extract of MRM (MRMW) were 11.09 and 47.70% w/w, respectively.

All extracts were investigated cytotoxic activity against three cancer cell lines including endometrioid adenocarcinoma cell line (HEC-1-A), cervical cancer cell line (HeLa), ovarian cancer cell line (SKOV-3), and two normal cell lines, including normal lung fibroblast line (MRC-5) and human keratinocyte cell line (HaCaT). The result found that MRME and MRMW had no cytotoxic effect against HEC-1-A and SKOV-3. Nevertheless, MRME was highly toxic to HeLa cells with an IC_{50} value of 0.1 µg/mL, while MRMW had no toxicity to this cell line. Meanwhile, the plant ingredients of MRME, including the ethanolic extract of *Dioscorea membranacea* (DME), the ethanolic extract of *Stemona collinsiae* (SCLE), and the ethanolic extract of *Rauwenhoffia siamense* (RSE) showed cytotoxicity against cancer cell lines. The results found that RSE, DME, and SCLE exhibited significantly inhibitory effect on the growth of HEC-1-A with IC_{50} values of 30.06±0.79, 48.26±4.88, and 69.29±4.47 µg/mL, respectively. In addition, the RSE exhibited the highest cytotoxic activity against HeLa with IC_{50} of 0.01µg/mL, followed by SCLE, DME, and the water extracts of *Rauwenhoffia siamense* (RSW) showed cytotoxic effect with IC_{50} of 12.59±0.88, 35.80±1.45, and 84.48±2.24 µg/mL, respectively. Moreover, the RSE also showed significant cytotoxicity against SKOV-3 with IC_{50} of 54.46±0.67 µg/mL. Besides, RSE showed the highest toxicity significantly to MRC-5 with IC_{50} of 0.004±0.00 µg/mL, followed by MRME, SCLE, DME, and RSW with IC_{50} of 0.10±0.02, 36.84±4.07, 71.06±0.13, and 79.37±1.75 µg/mL, respectively. Meanwhile, the results of cytotoxic activity against HaCaT showed that only RSE and DME was moderate cytotoxic activity against HaCaT with IC_{50} of 24.12±0.64 and 90.57±0.13 µg/mL.

For antioxidant activities of MRM and its plant ingredients extracts found that MRME was higher ferric-reducing power than MRMW, with FRAP and TEAC values of 384.15±9.18 mgFe2+/g extract and 144.59±3.54 mgTrolox/g, respectively. The RSW showed the highest FRAP and TEAC value with 881.40±26.03 mgFe^{2+}/g extracts and 336.19±10.03 mgTrolox/g, followed by SCE, RSE, CIE, DME, and SCLE, respectively. Moreover, astilbin, the chemical marker of MRM showed the highest FRAP value and TEAC value of 1,227.82±17.51 mgFe2+/g extract and 572.98±9.25 mgTrolox/g, respectively. Furthermore, MRME had good superoxide anion scavenging activity with

IC_{50} of 45.06±7.80 µg/mL, while MRMW had no effect. Interestingly, the RSE had the highest significant superoxide radical scavenging activity with IC_{50} of 21.30±1.34 µg/mL, followed by RSW, SCE, CIE, SCW, and CIW, respectively.

MRME had a better inhibition effect on nitric oxide production than MRMW, with IC_{50} of 45.93±2.70 and more than 100 µg/mL, respectively. Moreover, the DME had the highest significant effect on inhibiting nitric oxide production with IC_{50} of 15.75±2.45 µg/mL, followed by RSE, SCLE, and CIE. In addition, this study investigated for inhibition of IL-6 production in RAW 264.7 cells using sandwich ELISA and found that MRME had the highest inhibition effect of IL-6 cytokine with IC_{50} of 35.47±3.29 µg/mL. Still, MRMW showed no inhibition effect on IL-6. On the other hand, DME and RSE inhibited IL-6 production with IC_{50} values of 39.57±2.34 and 99.39±0.09 µg/mL, respectively. Furthermore, MRME, MRMW, and astilbin had no inhibition effect on TNF-α releasing with IC_{50} more than 100 µg/mL.

The antifungal activity results found that MRME inhibited *C. albicans* with a MIC value of 2.5 mg/mL and MFC value of more than 5 mg/mL. Moreover, RSE had good antifungal activity against *C. albicans* with a MIC value of 0.625 mg/mL and DME inhibited *C. albicans* with a MIC value of 2.5 mg/mL and MFC value of 5 mg/mL.

The results of the biological activity of MRME showed high antioxidant activity. The main compound in the MRME was astilbin, a flavonoid by astilbin contents in MRME extracts was 32.54 ± 0.90 mg/g of extract. MRME was investigated through stability testing under the accelerated condition at 40±2°C with 75±5% RH for six months. After that, all samples were determined for cytotoxic activity against cervical cancer cells (HeLa) by SRB assay, antioxidant activity by FRAP assay, and anti-inflammatory activity by inhibiting nitric oxide production assay. The results of MRME showed a significantly decreased cytotoxic activity against Hela cells after 60 days. However, the cytotoxic activity of MRME under accelerated had not significantly difference after 180 days. The antioxidant activity of MRME under accelerated conditions showed that FRAP values and TEAC values of MRME were less changed after 180 days. Moreover, the inhibition of nitric oxide production of MRME was investigated. The results showed that the inhibition effect of MRME on nitric oxide production

decreased significantly after 30 days. It showed no inhibition effect on nitric oxide production with an IC_{50} value was more than 100 µg/mL.

These results can be concluded that MRME showed the greatest inhibition specific to cervical cancer cells. Its plant ingredients, including SCLE and DME, were active cytotoxicity against cervical cancer cell lines. Furthermore, MRM remedy extract and its plant ingredients showed good antioxidant, anti-inflammation, and antifungal activities, which would be related to the effect of anti-cancer. The primary compound of MRME was astilbin which is used to determine the marker of quality control for antioxidant activity. Thus, these results can support preliminary medicinal properties of this remedy for cancer treatment, but relevant MRM biological activities and safety in animal model should be further studied.

Keywords: YA-KAE-MA-RENG-NAI-MOD-LOOK, Thai traditional medicine, Cancer

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Duangpacharaporn Kwanchian

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
%	Percent
°C	Degree(s) Celsius
>	More than
<	Less than
=	Equal
/	Per
β	Beta
α	Alpha
µM	Micromolar
µg	Microgram
µL	Microliter
mM	Milli Molar
mg	Milligrams
mL	Milliliter
g	Gram(s)
kg	Kilogram
w/w	Weight by weight
CO_2	Carbon dioxide
NO	Nitrous oxide
HEC-1-A	Endometrioid adenocarcinoma cell line
HeLa	Cervical cancer cell line
SKOV-3	Ovarian cancer cell line
MRC-5	Normal human lung tissue cell line
HaCaT	Immortalized human keratinocyte cell line
ATCC	American type culture collection
DMEM	Dulbecco's Modified Eagle Medium

Symbols/Abbreviations	Terms
MEM	Minimum essential medium
IMDM	Iscove's Modified Dulbecco's Medium
RAW 264.7	Murine macrophage cell line
DMSO	Dimethylsulfoxide
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
P/S	Penicillin Streptomycin
$NaHCO_3$	Sodium bicarbonate
IC_{50}	The half maximal inhibitory concentration
SI	Selective index
OD	Optical density
ELISA	Enzyme-linked immunosorbent assay
$CHCl_3$	Chloroform
EtOH	Ethanol
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
pH	Power of hydrogen ion
UV	Ultraviolet
MIC	Minimum inhibitory concentration
MFC	Minimum fungicidal concentration
R^2	Correlation coefficient
As	Arsenic
Cd	Cadmium

Symbols/Abbreviations	Terms
Pb	Lead
ppm	Parts per million
RH	Relative humidity
rpm	Round per minute
min	Minute
RT	Retention time
SEM	Standard error of mean
SRB	Sulforhodamine B
WHO	World Health Organization
TCA	Trichloroacetic acid
EDTA	Ethylenediamine tetra acetic acid
MHB	Mueller Hinton Broth
SDA	State Drug Administration
TMB	Tetramethylbenzidine
H_2SO_4	Sulfuric acid
cm^2	Square meter
NBT	Nitroblue tetrazolium
Cont	Continued

CHAPTER 1
INTRODUCTION

1.1 General introduction

Uterine cancer refers to cancer originating in the uterus. There are two major types of uterine cancer, uterine carcinoma and uterine sarcoma (Faizan et al., 2020). Uterine carcinoma or endometrial cancer is the most common genital cancer in women that can occur from the endometrium and the myometrium (Roett, 2015). In contrast, uterine sarcoma is found in 3% of uterine cancer that arises from the middle of muscular tissue (Angelo & Prat, 2010). In 2020, the World Health Organization reported that endometrial cancer was the sixth most commonly diagnosed cancer in women between 50 and 59 years, as shown in **Figure 1-1** (WHO, 2020). In addition, approximately 5.1% of the female cancer burden in Thailand is uterine cancer (National Cancer Institute, 2020). Although the incidence rate of uterine cancer is moderate, patients with endometrial cancer were reported to metastasize to the ovary and cervical (Takeshima et al., 1998; Fanning et al., 1991).

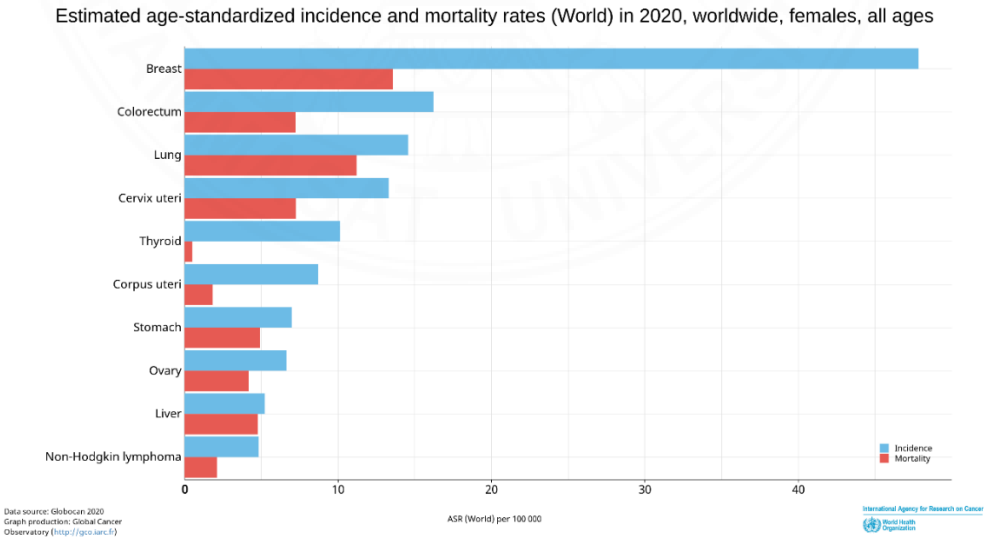

Figure 1-1 Estimated age-standardized incidence and mortality rates in women globally in 2020 (WHO, 2020)

Postmenopausal (90% of patients) is the most common presenting symptom of uterine cancer. Other common symptoms are vague abdominal pain, pelvic discomfort, nausea, and dysuria (Faizan, 2020). Moreover, the risk factors associated with uterine cancer include unopposed estrogen, early menarche, atypical endometrial hyperplasia, infertility, late menopause, and family history of endometrial. In addition, age older than 50 years, hypertension, diabetes mellitus, obesity, thyroid disease, and Lynch syndrome were also associated with uterine cancer (Braun et al., 2016). In addition, *Candida* infection can produce carcinogen compounds such as nitrosamines that activate cancer initiation (Krogh, 1990).

In western medicine, treatment of uterine cancer consists of surgery, radiation therapy, chemotherapy, hormonal therapy, or combining these treatments. Unfortunately, there are several side effects of treatment such as nausea, vomiting, loss of appetite, loss of hair, hand, and foot rashes, mouth sores, increased risk for infections, easy bruising, bleeding, and fatigue (Thongsong et al., 2011; American Cancer Society, 2021).

Currently, alternative medicine and natural products are the choice for cancer treatment (Dutta et al., 2019). The Paet-Thai-derm-book (Paet-Sart-Song-Kroh No.1) is a traditional Thai medicine textbook that describes the four elements involved with the human body. Four elements consist of Pathavi (earth element, i.e., muscle, skin, tendon), Semha (water element, i.e., blood, lymph, urine), Vata (wind element, i.e., circulatory system or movements in the body), and Pitta (fire element, i.e., temperature, metabolism in the body or inflammation). They can be increased, decreased, deformed, or imbalanced, which may cause abnormal body function and illness (Foundation of Thai Traditional Medicine and Ayurved Thamrong School, 2007). In addition, cancer is caused by the imbalance of four elements in traditional medicine theory. Therefore, Thai traditional theory for the development of cancer may be explained that consisting of two main steps (Foundation of Thai Traditional Medicine and Ayurved Thamrong School, 2007): the beginning step and during the cancer step. In the beginning step, the cause of cancer such as obesity, hypertension, infection, smoking, taking oral contraceptives for a long time, or other increase Pitta to raise

Semha (blood and lymph) and Vata (circulatory system). Conversely, long-term Pita lasting decreases Semha and Vata and subsequently by activating the abnormal effect of Pathavi. Finally, the tumor appearance is triggered by the abnormality of four elements, as shown in **Figure 1-2**.

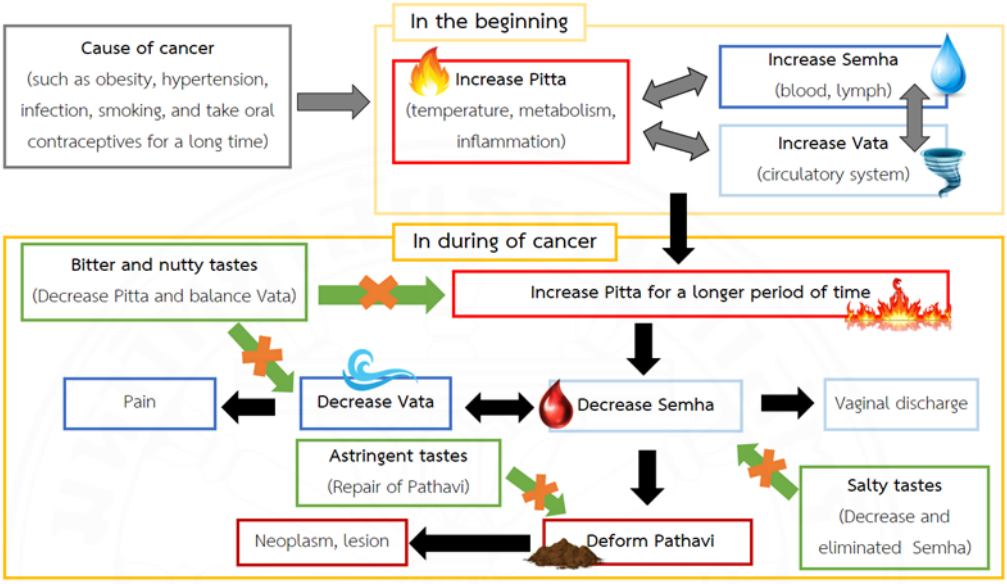

Figure 1-2 The mechanism of female reproductive cancer as stated by Thai traditional medicine theory

Thai traditional practitioners use herbal preparation to treat and prevent illness. Ya-Kae-Ma-Reng-Nai-Mod-Look remedy (MRM) was described as an anti-cancer drug in the Mor-Ya-Sa-Mun-Prai book of Krom Luang Chumphon Khet Udomsak scripture. It has been used to treat uterine cancer, which consists of the rhizome of *Smilax corbularia* Kunth. (nutty tastes), rhizome of *Dioscorea membranacea* Pierre ex Prain & Burkill. (nutty tastes), the whole plant of *Stemona collinsiae* Craib (nutty tastes), the root of *Rauwenhoffia siamensis* Scheff (bitter and astringent tastes), and rhizome of *Canna indica* L. (bitter and astringent tastes), one part each, and Sodium Chloride or sea salt (salty tastes), one handful. All ingredients were put into the clay pot and boiled with water. Its recommended dosage was one of the teacups for uterine cancer patients. Moreover, the tastes of MRM remedy that correlate with the mechanism of Thai traditional medicine theory is described in Figure 1-2. The main tastes of MRM remedy consist of two tastes such as bitter and nutty tastes. Plant

ingredients that are bitter and nutty tastes can decrease Pitta element (anti-inflammation) and balance Vata element (induce blood circulation and relieve pain). Moreover, astringent plants are used to repair the Pathavi element that lead to wound healing. The salty taste is used to decrease and eliminate the Semha element or eliminate slime and discharge in this mechanism.

Plant ingredients of MRM have been reported on cytotoxic activity and biological activities related to anti-cancer, especially *D. membranacea* that showed inhibition effect on the growth of cancer cell lines such as lung cancer (COR-L23), breast cancer MCF-7, prostate cancer (PC3), liver cancer (HepG2), and cervical cancer (HeLa) cell lines and anti-inflammatory activity (Itharat et al., 2014; Tewtrakul & Itharat, 2007). However, there are no reports on the cytotoxicity activity of MRM remedy and some plant ingredients against uterine cancer cell line (HEC-1-A), cervical cancer cell line (HeLa), and ovarian cancer cell line (SKOV-3). Therefore, the present study performed the cytotoxic activity of MRM extract, its plant ingredients, and its marker compound against three cancer cell lines, consist of uterine cancer cell line (HEC-1-A), cervical cancer cell line (HeLa), and ovarian cancer cell line (SKOV-3), and including the biological activities related to anti-cancer.

1.2 Research Questions

1.2.1 Do the extracts of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy, its plant ingredient, and its chemical marker show cytotoxic activity on uterine, cervical, and ovary cancer cell lines?

1.2.2 Do the extracts of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy (MRM), its plant ingredient, and its chemical marker show biological activities related to anti-cancer?

1.2.3 What is the chemical marker compound of MRM extract?

1.2.4 How much chemical marker compound does MRM extract?

1.2.5 Is MRM extract stable?

1.3 Objectives

1.3.1 Overall objective

This research aims to investigate the physiochemical properties and biological activity related to the anti-cancer of MRM extract and its plant ingredients, including cytotoxic activity against cancer cell lines, antioxidant activity, anti-inflammatory activity, and antifungal activity. Moreover, the marker compound and stability testing of MRM extracts are performed in this study.

1.3.2 Specific objectives

1.3.2.1 To study the physicochemical properties of MRM extract and its plant ingredients to use as the standard of extracts in this study

1.3.2.2 To investigate the cytotoxic activity of 95% ethanolic and aqueous extracts of MRM, its plant ingredients, and its marker compound against three cancer cell lines (HEC-1-A, Hela, and SKOV) and normal cell line (MRC-5 and HaCaT).

1.3.2.3 To investigate the antioxidant activity of 95% ethanolic and aqueous extracts of MRM, its plant ingredients, and its marker compound by Ferric Reducing Antioxidant Power (FRAP) assay and Nitroblue tetrazolium (NBT) reduction assay.

1.3.2.4 To investigate the anti-inflammatory activity of 95% ethanolic and aqueous extracts of MRM, its plant ingredients, and its marker compound by inhibiting nitric oxide, TNF-**α**, and IL-6.

1.3.2.5 To investigate the antifungal activity of 95% ethanolic and aqueous extracts of MRM and its plant ingredients against *Candida albicans* by Microtiter Plate-based assay.

1.3.2.6 To determine the marker compound of MRM extract by HPLC technique quantitatively.

1.3.2.7 To study the stability testing of MRM extract by nitric oxide inhibition assay, FRAP assay, Cytotoxic activity against HeLa cell line, and HPLC technique.

1.4 Expected Benefits

This research provides the scientific knowledge of MRM remedy to use as essential knowledge for developing this remedy in the future.

1.5 Conceptual framework

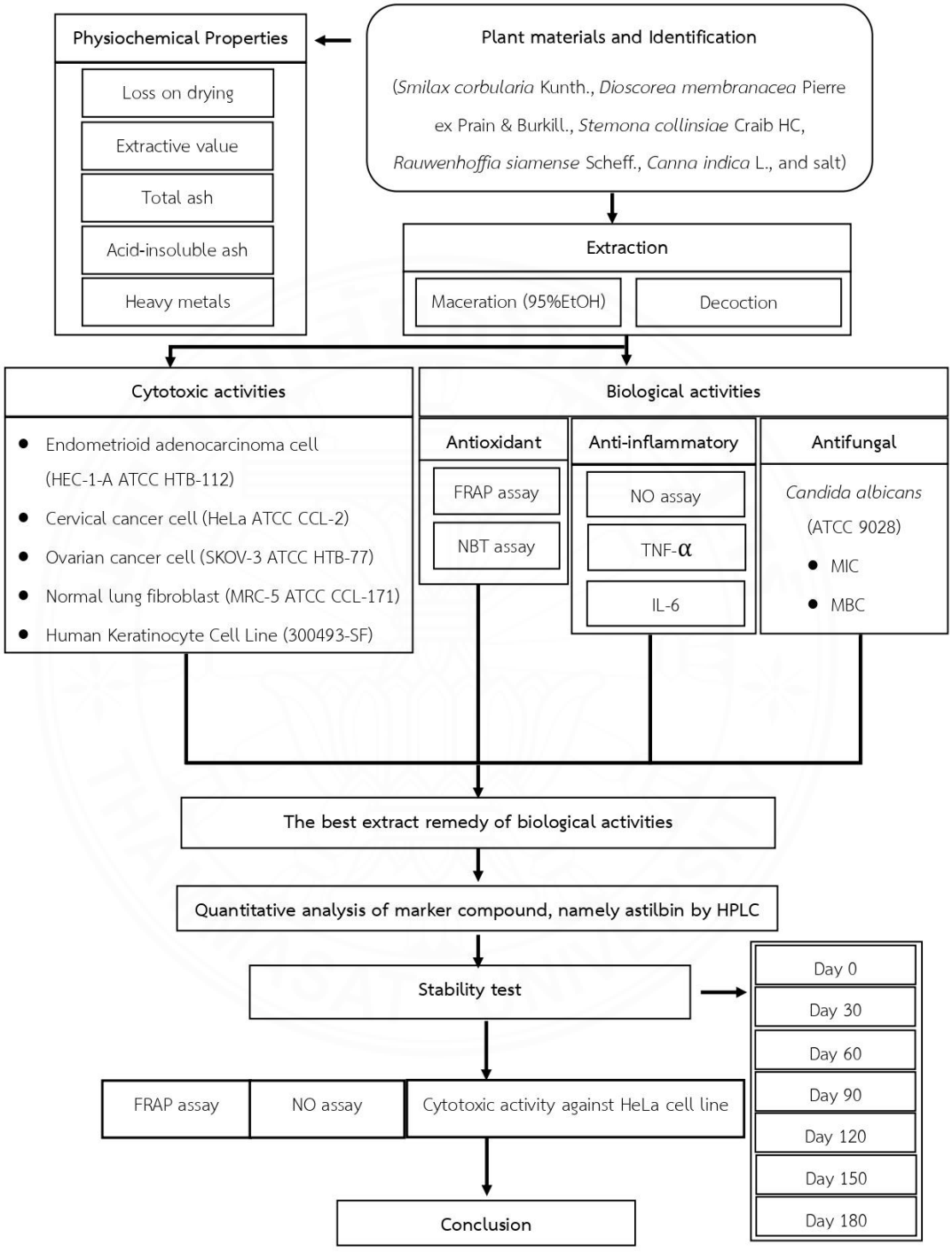

Figure 1-3 Conceptual framework in this study

CHAPTER 2
REVIEW OF LITERATURE

2.1 Cancer cells

Cancer has been long viewed as a disease consisting of transformed cells acquiring cell-autonomous hyperproliferative, invasive, and limitless survival capacities (Lorusso & Rüegg, 2008) and it is now clear that not only epithelial tumor cells play a role in carcinogenesis. The tumor microenvironment is composed of non-stromal cells, including endothelial cells, adipocytes, immune and nerve cells, and a stromal compartment composed of extracellular matrix, cancer-associated fibroblasts, and mesenchymal cells. Tumorigenesis is a dynamic process with constant interactions occurring between the tumor cells and their surroundings. The process of carcinogenesis is a complex multistep from mutation of a normal cell, which uncontrolled cells growth (Haykal et al., 2020).

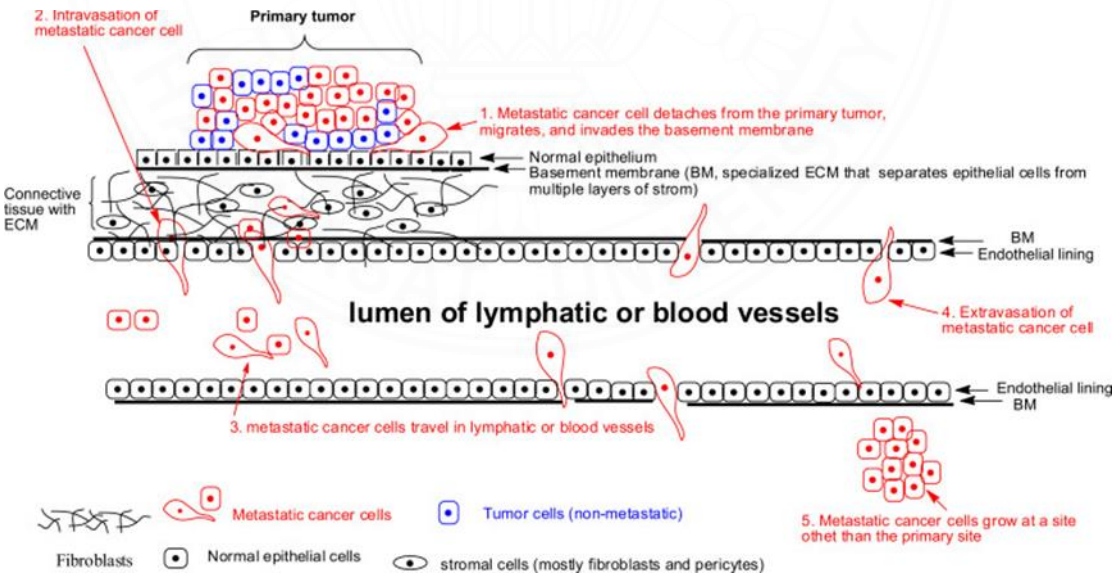

Figure 2-1 Cancer metastasis (Guan, 2015)

Cancer metastasis is the major cause of cancer morbidity and mortality and accounts for about 90% of cancer deaths. Metastasis is facilitated by four essential

steps: detachment, migration, invasion, and adhesion, which begins with a detachment of tumor cells from a primary tumor mass to a different site through blood vessels and lymphatic vessels. It is a complex succession of a series of cell-biological events termed the “invasion–metastasis cascade”. The cascade involves the development of new blood vessels (angiogenesis), departure of metastatic cells from the primary tumor (detachment and migration), invasion through the basement membrane (BM), and metastasis is regulated by various signaling pathways and is affected by the surrounding (ECM) extracellular matrix surrounding the tumor, invasion of the BM supporting the endothelium of local blood and lymphatic vessels, intravasation of the metastatic cells into the blood and/or lymphatic vessels, adhesion of the circulating metastatic cells to the endothelium of capillaries of the target organ site, invasion of the cells through the endothelial cell layer and the surrounding BM (extravasation), and finally the settling and growth of secondary tumors at the target organ site. It is now known that metastasis genes are stress-response genes that physiologically contribute to inflammation, wound healing, and stress-induced angiogenesis; shown provides a brief overview of the process in **Figure 2-1** (Guan, 2015).

2.2 Uterine cancer

Uterine cancer is the sixth most common among females worldwide. The data from World Health Organization (WHO) showed 417,367 new cases diagnosed and 97,370 deaths in 2020.

2.2.1 Risk factors (Felix et al., 2018)

The studies of strong risk factors for uterine cancers have identified numerous risk factors consisting of:

2.2.1.1 Metabolic factors, obesity, and women with obesity-associated diseases such as diabetes, hypertension, and polycystic ovary syndrome are also at elevated risk.

2.2.1.2 Reproductive factors comprise nulliparous women at substantially higher risks than parous women, infertility, young ages at menarche and/or old ages at menopause.

2.2.1.3 The use of combination oral contraceptives has been linked with marked risk reductions, which persist for more than 30 years after discontinuation. Intrauterine devices also appear to reduce endometrial cancer risk.

2.2.1.4 Lifestyle factors such as cigarette smoking and moderate-to-active physical activity levels have been associated with reduced risks.

2.2.1.5 Menopausal hormone therapy, Menopausal hormones have been strongly linked with risk increases, particularly for extended usage of high-dose unopposed estrogens, and progestins cause regression of estrogen-induced endometrial hyperplasia, the presumed precursor of most endometrial cancers.

2.2.1.6 Tamoxifen use by clinical trials has demonstrated increased endometrial cancer risk among tamoxifen-treated breast cancer patients.

2.2.1.7 Familial and genetic factors showed elevated endometrial cancer risks have been noted among women with a first-degree family history of endometrial cancer including familial obesity (genetic or environment) or inherited risk, such as Lynch syndrome, an autosomal dominant cancer predisposition syndrome attributed to germline mutations in one of several mismatch repair genes.

2.2.2 Pathology of Uterine cancer

Uterine cancer is broadly classified consist of endometrial carcinoma, which is the most common type of uterine cancer, and uterine sarcomas.

2.2.2.1 Endometrial carcinoma (Malignant Epithelial Tumors)

The most common symptoms complaint was abnormal uterine bleeding (87.3%). More than half (75.4%) had other medical illnesses or other cancers (10.7%) (Tangjitgamol et al., 2010). There are two different pathogenetic types of endometrial carcinoma

based on epidemiology, conventional histopathology, and clinical behavior (Prat et al., 2007).

(1) The oestrogen-related (endometrial cancer type 1, endometrioid) - grade 1-3

These tumors are endometrioid carcinomas (EECs) that morphologically resemble normal endometrium, and are frequently preceded by endometrial hyperplasia. They are usually confined to the uterus, exhibit low histological grade and most patients are cured by hysterectomy (Prat et al., 2007).

(2) The non-estrogen-related (endometrial cancer type 2, non-endometrioid) - grade 3

These tumors are non-endometrioid carcinomas (NEECs), predominantly high-grade serous or clear cell carcinomas, which are not associated with oestrogen effect and are thought to derive from a malignant lesion designated 'intraepithelial carcinoma'. Frequently, NEECs invade deeply into the myometrium and follow an aggressive clinical course (Prat et al., 2007).

(3) Staging for endometrial cancer on the basis of the FIGO system (Freeman et al., 2012)

Stage I	Tumor confined to the uterus.
IA	Less than half myometrial invasion.
IB	More than or equal to half myometrial invasion.
Stage II	Tumor invades the cervical stroma but does not extend beyond the uterus.
Stage III	Local or regional spread of tumor.
IIIA	Serosal or adnexal invasion.
IIIB	Vaginal or parametrial involvement.
IIIC	Metastasis to pelvic or paraaortic lymph nodes.
IIIC1	Pelvic lymph node involvement.
IIIC2	Paraaortic lymph node involvement (with or without pelvic nodes).

Stage IV	Extension to the pelvic wall, lower one-third of the vagina, or hydronephrosis or nonfunctioning kidney.
IVA	Invasion of bladder or bowel mucosa.
IVB	Distant metastases, including abdominal, or involvement of inguinal lymph nodes.

2.2.2.2 Uterine sarcoma

Uterine sarcomas are uncommon tumors from mesenchymal elements. They are thought to arise primarily from the endometrial stroma and uterine muscle, respectively. When endometrial stroma undergoes malignant transformation, it might be accompanied by a malignant epithelial component. Thus, malignant mesenchymal uterine tumors comprise leiomyosarcoma, endometrial stromal sarcoma, undifferentiated uterine sarcoma, and carcinosarcoma (Wu et al., 2011).

(1) Carcinosarcoma

Carcinosarcoma is an aggressive neoplasm of the female genital tract, which comprises 4% of malignancies of the uterine corpus. The most common symptom was abnormal uterine bleeding in pre-or postmenopausal women.

(2) Leiomyosarcoma

The most common symptoms were abdominal pain, abnormal vaginal bleeding, or palpable abdominal mass. The pain is often uterine colic associated with the passage of clots from the vagina, and the intensity of pain may account for the short interval from onset of symptoms to diagnosis.

(3) Endometrial stromal sarcomas

The most common symptom of ESS is irregular vaginal bleeding. Asymptomatic uterine enlargement, pelvic pain, or palpable mass are also common symptoms (Wu et al., 2011).

(4) Staging for uterine sarcoma on the basis of the FIGO system

(4.1) Leiomyosarcomas and endometrial stromal sarcomas (ESS)

Stage I	Tumor limited to uterus
IA	Less than or equal 5 cm
IB	More than 5 cm
Stage II	Tumor extends to the pelvis
IIA	Adnexal involvement
IIB	Tumor extends to extrauterine pelvic tissue
Stage III	Tumor invades abdominal tissues (not just protruding into the abdomen)
IIIA	One site
IIIB	More than one site
IIIC	Metastasis to pelvic and/or para-aortic lymph nodes IV IVA Tumor invades bladder and/or rectum IVB Distant metastasis

(4.2) Adenosarcomasa

Stage I	Tumor limited to uterus
IA	Tumor limited to endometrium/endocervix with no myometrial invasion
IB	Less than or equal to half myometrial invasion
IC	More than half myometrial invasion
Stage II	Tumor extends to the pelvis
IIA	Adnexal involvement
IIB	Involvement of other pelvic tissues
Stage III	Tumor invades abdominal tissues (not just protruding into the abdomen).
IIIA	One site
IIIB	More than One site
IIIC	Metastasis to pelvic and/or para-aortic lymph nodes
Stage IV	
IVA	Tumor invades bladder and/or rectum

IVB Distant metastasis

(4.3) Carcinosarcomas

Carcinosarcomas should be staged as carcinomas of the endometrium (Wu et al., 2011).

2.2.2.3 Treatment

(1) Surgery

The most important therapy for endometrial cancer is surgery. The procedures include acquisition of peritoneal fluid or washings for cytology, total hysterectomy including the uterine cervix, and bilateral salpingo-oophorectomy; in selected cases, there is a place for omentectomy and a thorough retroperitoneal lymph-node dissection (Amant et al., 2005).

(2) Radiotherapy

Radiation can be delivered externally to the pelvis, as vaginal brachytherapy (colpostats, mould, or cylinder), or as a combination. Treatment can also be directed to the whole abdomen or to an extended field that includes the pelvis and para-aortic region (Amant et al., 2005).

(3) Chemotherapy (National Health Security office, 2018)

(3.1) Chemotherapy regimens of high-risk or advanced diseases of recurrence for endometrial carcinoma consist of:

Multi-agents (preferred)

1) Carboplatin/ Paclitaxel or Carboplatin/ Docetaxel (for stage I)
2) Cisplatin/ Doxorubicin (for stage IIB)
3) Paclitaxel/ Ifosfamide (for carcinosarcoma)
4) Cisplatin/ Ifosfamide (for carcinosarcoma)
5) Paclitaxel

Single agents

1) Carboplatin
2) Cisplatin

3) Doxorubicin

4) Liposomal doxorubicin

5) Ifosfamide (for carcinosarcoma)

(3.2) Chemotherapy regimens for uterine sarcomas consist of:

Combination regimens

1) Docetaxel/ Gemcitabine (preferred for leiomyosarcoma)

2) Doxorubicin/ Ifosfamide

3) Carboplatin/ Paclitaxel (for stage IIB)

Single agents

1) Doxorubicin

2) Gemcitabine

3) Ifosfamide

4) Liposomal doxorubicin

(4) Hormonal treatment

Hormonal therapy, most commonly with progestins (medroxyprogesterone acetate or megestrol acetate, which are the most common hormonal treatments), benefits a small group of patients (Obel et al., 2006).

2.3 Oxidative Stress, Antioxidants and Cancer

Oxidative stress is an imbalance between the systemic manifestation of reactive species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Oxidative stress causes by overproduction of reactive species and/or decreased level or dysfunction of antioxidants. Free radicals are molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals, which the cause by

endogenous or exogenous reactive species. Free radicals that have an important role consist of reactive oxygen species (ROS) (for example superoxide anion radical (O^{2-}), hydroxyl radical ($HO^{\bullet}$), and hydrogen peroxide (H_2O_2)) and reactive nitrogen species (RNS) (e.g. nitric oxide radical ($NO^{\bullet}$), from inducible nitric oxide synthase (iNOS) that found in macrophage cell around of cancer cells, and nitrogen dioxide radical ($NO^{2\bullet}$) are well recognized for biological effects. Cell damage is a result of interaction between reactive species and cellular biological structure, including lipids and membranes, proteins, sugars, and DNA. Reactive species involve carcinogenesis by direct damage to DNA, growth and proliferation, anti-apoptosis, aggressiveness, and metastasis (Wirasorn et al., 2014).

2.4 Anti-inflammatory and cancer

It is well accepted that inflammation is closely associated with cancer growth and metastasis. Inflammation can impact cancer by providing bioactive molecules from cells infiltrating the tumor microenvironment. These bioactive molecules include cytokines, growth factors, chemokines, cell survival signals to avoid apoptosis, proangiogenic factors, and ECM modifying enzymes. Interference with the function of these bioactive molecules has been demonstrated to be effective in the inhibition of cancer growth and/or metastasis. In addition, neutralizing antibodies against CCL2 [chemokine, (C–C motif) ligand 2], CSF-1 (chemokine, colony-stimulating factor-1), IL-6 (Interleukin-6, cytokine), IL-6 receptor, TNF, TGF-β have also been reported. IL-6 plays a key role in promoting proliferation and inhibition of apoptosis by binding to its receptor (IL-6Rα) and co-receptor gp130 (glycoprotein 130). The binding activates the JAK/STAT signaling pathway of the Janus kinases (JAK) and signal transducers and activators of transcription (STATs) STAT1 and STAT3. STATs belong to a family of transcription factors closely associated with the tumorigenic processes. Several studies have highlighted the effect of the IL-6/JAK/STAT signaling pathway on cancer initiation and progression. Current attempts to target the IL-6/JAKSTAT3 pathway include the clinical use of IL-6 and IL-6 receptor blocking antibodies, specific

STAT3 inhibitors, and JAK inhibitors. (Guan, 2015) In addition, Nitric oxide (NO) is lipophilic that regulates a variety of important physiological responses including vasodilation, respiration, cell migration, immune response, and apoptosis that synthesized by three differentially gene-encoded NOS in mammals consists of neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). NO sensitizes tumor cells to chemotherapeutic compounds. The expression of iNOS and eNOS has been found to be increased in a variety of human cancers. The multiple actions of NO in the tumor environment are related to heterogeneous cell responses with particular attention in the regulation of the stress response mediated by the hypoxia-inducible factor-1 and p53 generally leading to growth arrest, apoptosis, or adaptation (Muntane and De la Mata, 2010).

2.5 Antimicrobial and cancer

Outcomes of cancer treatments depend on many factors, many of which are still unknown. Frequent treatment complications include viral, bacterial, and fungal infections, which have roles in the etiology, affect the outcome of the treatment, and may arise as a side effect of the therapy. Antimicrobial agents can benefit cancer patients by killing oncogenic-related microorganisms, protecting them from recurring immunosuppression-induced infection, and their direct antiproliferative/cytotoxic effects. Different mechanisms are involved in the antiproliferative effects of antimicrobials, such as targeting translation initiation factors, degrading tumorigenic proteins by binding to heat shock proteins involved in their folding, having antiangiogenic effects, inhibiting topoisomerases, destabilization of mitochondria, and other effects causing apoptosis. Moreover, antimicrobials could potentially decrease some side effects of currently used therapies against cancer. These include inhibiting proinflammatory cytokine release and the induction of cytotoxic activities by conventional chemotherapy drugs (Taur et al., 2009).

In recent years, evidence has been growing that *Candida albicans* might promote cancer and metastasis through a pro-inflammatory response, mediated by an

increase in cytokine production and in adhesion-molecule expression that is the most prevalent yeast in the human vagina and gives rise to local and systemic infections, particularly in immunocompromised patients where it can result in mortality. The role of *Candida albicans* in tumor genesis by producing carcinogenic substances by *C. albicans* (A), using the enzyme alcohol dehydrogenase (ADH1), which is capable of metabolizing alcohol and other substances, such as carbohydrates, to acetaldehyde, which is carcinogenic. Acetaldehyde (B) is able to induce tumor development through various different pathways. This carcinogen binds to proteins and DNA modifying their structure and functionality and reducing the antioxidant activity of glutathione, increasing the levels of reactive oxygen species (ROS) in the cell. These alterations may produce genome instability, which is linked with an inhibition of the apoptotic machinery, may result in tumor development, and increases the risk of carcinogenesis and metastasis (Ramirez-Garcia et al., 2016).

2.6 Thai Traditional Medicine

Ya-Kae-Ma-Reng-Nai-Mod-Look remedy (MRM) is a Thai traditional remedy recipe recorded in the scripture of Mor-Ya-Sa-Mun-Prai book, which collected Thai herbal medicine of Krom Luang Chumphon Khet Udomsak. The Ya-Kae-Ma-Reng-Nai-Mod-Look remedy (MRM) was used to treat uterine cancer, which consists of *Smilax corbularia* Kunth., *Dioscorea membranacea* Pierre ex Prain & Burkill., *Stemona collinsiae* Craib HC, *Melodorum siamense* (Scheff.) Ban, and *Canna indica* L., one part each, and Sodium Chloride or sea salt used in one handful, and take them into the clay pot to boil with water and drink one of the teacups.

2.6.1 General data of its plant ingredients in Ya-Kae-Ma-Reng-Nai-Mod-Look remedy

2.6.1.1 *Smilax corbularia* Kunth.

Figure 2-2 Dried rhizome of *Smilax corbularia* Kunth.

Scientific name: *Smilax corbularia* Kunth.

Family name: Smilacaceae

Thai name: Khao yen nuea, Khao yen wok, Hua khao yen nuea (Smitinand, 2014)

Part used: Bulb

Chemical composition: Engeletin, Astilbin, Quercetin (Ruangnoo et al., 2012)

General description:

The description of *S. corbularia*, shown in figure 2-2, is a hardwood creeping plant that grows along the ground or climbing plant about up to 5 m long. There is a round or slightly square woody stem and hardy rhizome barbed with dark red flesh. Leaves are alternate, simple leaf and elliptic to ovate shape about 2.5 - 7 cm wide by 5-18 cm long, smooth or entire leaf margin, cuspidate leaf apex, round or obtuse base, and shiny, smooth surface. Petioles are 0.5 - 2 cm long. Umbel inflorescence about 1-3 inflorescences at axillary leaves and dioecious plant. Staminate inflorescences have 20-40 flowers, 6 stamens, and elliptic anthers. Pistillate inflorescences have 15-30 flowers and an ellipsoid ovary about 2 mm long. There are

capped with 3-lobed, and each lobe has 1-2 ovules and 3 needle-like staminodes (Sireeruckhachati Nature Learning Park, n.d.).

Traditional used:

Bulb, drunken-oily taste, used to treat poor lymph disorder, cancer, diuretic (Sireeruckhachati Nature Learning Park, n.d.), and acquired immunological deficiency syndrome, anti-inflammatory, arthritis, and diabetes (Manosroi et al., 2015).

Biological activities:

Cytotoxic activity

The study of cytotoxic activity by using SRB assay was carried out against cervical cancer (HeLa) cell line showed the water and ethanolic extracts of *S. corbularia* showed no cytotoxic activity against HeLa cell line (IC_{50} values of more than 100 µg/mL) (Itharat, 2010). In addition, the study of anti-proliferative by SRB assay of methanolic extract of *S. corbularia* on HeLa and KB cell lines showed the activity with GI_{50} value of more than 100 µg/mL in both cell lines (Manosroi et al., 2015).

Anti-inflammatory activity

The study of 95% ethanol and aqueous extracts from the rhizome of *S. corbularia* on an inflammatory response by using carrageenin-induced paw edema in rats and antinociceptive activity in mice using writhing test, hot plate test, and formalin test and antipyretic activity in yeast-induced fever in rats, and the result showed 95% ethanol extract at oral administration in a dose of 1600 mg/kg significantly suppressed the paw edema induced by carrageenin, and both 95% ethanol and aqueous extract had effect neither antinociceptive activity nor antipyretic activity, and that its actions on the inflammation may be different from those of aspirin (Reanmongkol et al., 2007). In addition, the inhibitory effect of ethanolic, aqueous extract, and isolated compounds, are flavonols from rhizome of *S. corbularia* including Engeletin, Astilbin, and Quercetin on anti-inflammatory activity using lipopolysaccharide-stimulated PGE_2 release, TNF-α, and NO production from RAW 264.7 cells showed Quercetin, the highest inhibitory effect on NO, TNF-α production, and PGE_2 release with IC_{50} values of 11.2±0.6 µg/mL (37.1 µM), 1.25±0.2 µg/mL (4.14 µM), and 14.4±0.9 µg/mL (33.2 µM), respectively. The ethanolic extract inhibited TNF-α and NO production with IC_{50} values

of 61.97±0.9, and 83.90±3.8 μg/mL, respectively but more than 100 μg/ml on PGE_2 release. Meanwhile, the aqueous extract had no activity (IC_{50} > 100 μg/mL). Engeletin and Astilbin had no activity on TNF-α and NO production (IC_{50} > 100 μg/mL) but could inhibit PGE_2 release with IC_{50} values of 19.6±0.01 (43.5 μM), and 19.9±0.01 μg/mL (65.8 μM), respectively (Ruangnoo et al*.,* 2012). Moreover, the inhibitory effect of ethanolic and aqueous extract from rhizome of *S. corbularia* on anti-inflammatory activity using lipopolysaccharide-stimulated NO production from RAW 264.7 cells showed IC_{50} values of 61.2 and 61.0 μg/mL, respectively (Tewtrakul & Itharat, 2007). Furthermore, the ethanolic extract of *S. corbularia* showed significant effect on NK cells activity and lymphocyte proliferation at concentration of 0.01 and 0.1 μg/mL, and could increase IFN-γ production (1-10 μg/mL). The water extract and two isolated compounds from the ethanolic extract including quercetin and astilbin had no significant effect on NK cells activity (Panthong, 2015).

Antioxidant activity

The antioxidant study of ethanolic extract from rhizome of *S. corbularia,* was performed by DPPH assay showed EC_{50} value of 4.1±0.2 μg/mL. The supernate of $CHCl_3$: MeOH (1:1) fraction, $CHCl_3$: MeOH (1:1) precipitate, and MeOH fraction showed higher than BHT (EC_{50} values of 2.1±0.2 μg/mL, 11.1±0.9 μg/mL, 8.9±0.1 μg/mL and 11.2±2.4 μg/mL, respectively). In addition, pure compounds including β-sitosterol, β-sitosterol-3-*O*-β-D-glucopyranoside, quercetin, astilbin, and engeletin, isolate from $CHCl_3$: MeOH (1:1) supernate fraction, tested antioxidant activities by using DPPH and lipid peroxidation assay and the result showed quercetin was the highest antioxidant activities with EC_{50} values of 0.6±0.1 μg/mL, and 0.3±0.1 μg/mL, respectively, followed by astilbin (EC_{50} values of 2.5 μg/mL, and 0.8 μg/mL, respectively) and engeletin (EC_{50} values of 3.9 μg/mL, and 1.2 μg/mL, respectively) but less effect than quercetin. For β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside showed EC_{50} values of more than 100 μg/ml on both assays (Kejik, 2008). Moreover, the report of effects of culture periods on antioxidant contents of *S. corbularia* shoots grown under aseptic conditions found that total phenolic, total flavonoid contents, and DPPH radical scavenging activities increased along the culture periods that showed

the 12-week-old regenerated shoots exhibited the highest contents of total phenolic (84.92±5.88 mg GAE/g dry extract), total flavonoid (107.56±7.19 mg CE/g dry extract), and greatest DPPH radical scavenging activity (EC_{50} value of 15.75±0.88 µg/mL) (Jirakiattikul et al., 2019). Besides, the result antioxidant activity of *S. corbularia* by using DPPH assay found that the ethanolic extract of *S. corbularia* exhibited the highest antioxidant activity, followed by the water extract of *S. corbularia* with EC_{50} values 6.40±0.40, and 4.20±0.12 µg/mL, respectively, that more than BHT (EC_{50} value 12.10±1.20 µg/mL) (Ruangnoo & Itharat, 2010). Moreover, the result antioxidant activity of *S. corbularia* by using lipid peroxidation of liposome assay found that the ethanolic extract of *S. corbularia* and the water extract of *S. corbularia* with EC_{50} values 46.87±7.50, and 185.65±0.05 µg/mL, respectively) (Itharat, 2010).

Antifungal activity

The report of antimicrobial of the ethanolic extract of *S. corbularia* showed no inhibition zone of *Candida albican*, and the ethanolic extract of *S. corbularia* showed the highest activity against with *Staphylococcus aureus* and *Escherichia coli* by using minimum inhibitory concentration (MIC) method (MIC less than 2.5 mg/mL), and the extract of *S. corbularia* showed MIC more than 10 mg/mL on *Candida albican* (Itharat, 2010).

2.6.1.2 *Dioscorea membranacea* Pierre ex Prain & Burkill.

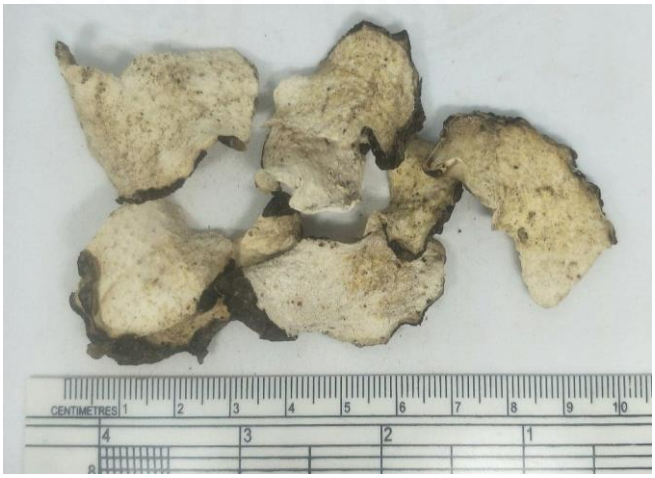

Figure 2-3 Dried rhizome of *Dioscorea membranacea* Pierre ex Prain & Burkill.

Scientific name: *Dioscorea membranacea* Pierre ex Prain & Burkill.

Family name: Dioscoreaceae

Common name: Khruea tao hai, Man mu, Man I yang (Smitinand, 2014), Khao yen tai

Part used: Rhizome

Chemical composition: Dioscorealides A, Dioscorealides B, Dioscoreanone, β-sitosterol, stigmasterol, β-D-sitosterol glucoside, Diosgenin (Itharat et al., 2007)

General description:

The description of *D. membranacea*, shown in figure 2-3, is rhizome wide-running, perhaps even to 2 m, 1/2-1 cm diameter, dark brown, with white flesh. Stem slightly ridged, unarmed, with stipule-like processes at the base of the petioles on the better developed axes. Leaves deeply trifid above a cordate base, shortly acuminate, 9-nerved, two primary nerves reaching the forerunner tip along with the midrib and the second pair reaching the tips of the lateral lobes; petiole 1/2—2/3 the length of the blade. Male flowers in small subsessile cymes with up to 4 flowers, spaced along a leafless conspicuously angled axis; bracts ovate-acuminate, very thin in texture, 1.5 mm long; pedicels exceedingly short. Tube of the flower campanulate or urceolate, 1 mm long, its thin walls strengthened by the vascular bundles which descend in it from the insertion of the filaments; tepals 1 mm long, long-ovate, obtuse, 1-nerved. Stamens all alike, the filaments inserted just below the tepals, incurved, 0,3 mm long; anthers introrse, small. Female flowers on downwardly directed spike-like racemes; axis angled, to 20 cm long or even longer; pedicels I mm; bracts ovate-acute, very thin, 1.5 mm. Tube of the flower absent. Outer tepals obovate, obtuse, just exceeding 1 mm, inner ones lanceolate, a little shorter than the outer. Style short. Capsules 1-2 cm apart and scarcely imbricate, wings a little broader than semicircular and sometimes widest above the middle, retuse at the apex, nearly truncate at the base (Burkill, 1948).

Traditional used:

Rhizomes are used by Thai traditional doctors as ingredients in many medicinal preparations, including those used in the treatment of dermopathy,

lymphopathy, inflammation, cancers, venereal diseases, and leprosy (Tewtrakul & Itharat, 2006).

Biological activities:

Cytotoxic activity

The study of cytotoxic activity of by using SRB assay was carried out against cervical cancer (HeLa) cell line showed the ethanolic extract of *D. membranacea* exhibited high cytotoxic against with IC_{50} value 32.43±3.15 µg/mL. The water extracts of *D. membranacea* showed no cytotoxic activity against HeLa cell line (IC_{50} values of more than 100 µg/mL) (Itharat, 2010). In addition, the study of cytotoxicity of active ingredientsisolated from the ethanolic extract of *D. membranacea*, including dioscorealides A, dioscorealides B, dioscoreanone, β-sitosterol, stigmasterol, diosgenin-(3-O-α-L-rhamnopyranosyl (1⟶2)-β-D-glucopyranoside, glucoside diosgenin 3-O-β-D-glucopyranosyl (1⟶3)-β-D-glucopyranoside, and β-D-sitosterol by using SRB assay showed less active against the two normal cells with IC_{50} values of more than 160, 145.0±1.3, 58.2±1.4, more than 240, more than 240, 3.0±0.8, more than 165, and more than 160 µg/mL, respectively on human keratinocyte cell line (SVK-14) and IC_{50} values of more than 160, 26.3±0.3, 23.5±2.1, more than 240, more than 240, 4.0±0.1, more than 135, and more than 160 µg/mL , respectively on human keratinocyte (HF) (Itharat et al., 2007).

Anti-inflammatory activity

The inhibitory effect of ethanolic and aqueous extract from rhizome of *D. membranacea* on anti-inflammatory activity using lipopolysaccharide-stimulated NO production from RAW 264.7 cells showed IC_{50} values of 23.6 and 57.8 µg/mL, respectively, and furthermore, eight compounds were isolated from ethanolic extract of *D. membranacea* including Naphthofuranoxepins (Dioscorealide A, Dioscorealide B), Phenanthraquinone (Dioscoreanone), Steroids (β-Sitosterol, Stigmasterol, β-Sitosterol-3-O-β-d-glucopyranoside), Diosgenin-3-O-α-L-rhamnopyranosyl(1⟶2)-β-D-glucopyranoside, and Steroidal saponons (Diosgenin-3-O-β-d-glucopyranosyl(1⟶3)-β-D-glucopyranoside, Diosgenin) showed IC_{50} values of 53.7, 24.9, 9.8, 64.7, 58.3, 72.9, 3.5, 43.7, and 55.0 µM respectively. Dioscorealide A, Dioscorealide B, and

Dioscoreanone were also tested for the inhibitory effect on LPS-induced TNF- release in RAW 264.7 cells, the result showed IC_{50} values of 99.9, 93.2, and 17.6 μM, respectively (Tewtrakul & Itharat, 2007). Furthermore, the inhibitory effect of 95% ethanol extract of *D. membranacea* on anti-inflammatory activity using lipopolysaccharide-stimulated in RAW 264.7 cells showed could inhibit IL-1β and IL-6 with IC_{50} values of 46.82±0.80 and 0.90±0.05 μg/mL, respectively. Dioscorealide B, isolated from ethanolic extract of *D. membranacea,* showed could inhibit IL-6 with IC_{50} values of 8.59±2.70 μg/mL, and 2,4 dimethoxy-5,6 dihydroxy-9,10 dihydrophenanthrene, isolated from ethanolic extract of *D. membranacea*, showed could inhibit IL-1β and IL-6 with IC_{50} values of 4.38±2.96 and 9.30±0.1 μg/mL, respectively (Panthong, 2015). Besides, the study of 95% ethanol and aqueous extracts from rhizome of *D. membranacea* on inflammatory response using carrageenin-induced paw edema in rats and antinociceptive activity in mice using writhing test, hot plate test and formalin test and antipyretic activity in yeast-induced fever in rats in the result showed 95% ethanol and aqueous extracts at oral administration in dose of 1600 mg/kg significantly suppressed the paw edema induced by carrageenin at 1, 3 and 4 hr after carrageenin injection though less potent than that of the aspirin (200 mg/kg) and neither ethanol nor aqueous extracts did not show any significantly effect on antinociceptive activity and antipyretic activity (Reanmongkol et al., 2007). Besides, Dioscorealide B and Dioscoreanone, isolated from ethanolic extract of rhizome of *D. membranacea,* the inhibitory effect on LPS-induced NO production in RAW 264.7 cells using Griess's reagent reported IC_{50} values of 2.85±0.62 μM, and 2.50±0.64 μM, respectively and no toxic by using MTT assay. Dioscorealide B and Dioscoreanone could inhibit expression iNOS mRNA as well as IL-1β, and IL-6 mRNA at concentration of 6.0 and 5.0 μM, respectively, and merely Dioscorealide B could inhibit expression IL-10 mRNA. Dioscorealide B inhibited NF-**K**B binding activity in pNF**K**B-Luc-transfected RAW 264.7 macrophages, and inhibited both I**K**B degradation, and ERK1/2 activation by using Western blot analysis (Hiransai, 2010).

Antioxidant activity

The result antioxidant activity of *D. membranacea* by using DPPH assay found that the ethanolic extract of *D. membranacea* exhibited the highest antioxidant activity with EC_{50} values 10.34±1.40 µg/ml, which more than BHT (EC_{50} value 12.10±1.20 µg/mL) and the water extract of *D. membranacea* showed EC_{50} value more than 50 µg/mL (Ruangnoo & Itharat, 2010). Moreover, the result antioxidant activity of *D. membranacea* by using lipid peroxidation of liposome assay found that the ethanolic extract of *D. membranacea* showed highest antioxidant activity, followed by the water extract of *D. membranacea* with EC_{50} values 8.10±1.21, and 742.17±0.05 µg/mL, respectively (Itharat, 2010). In addition, the study of active ingredientsisolated from the ethanolic extract of *D. membranacea*, including dioscorealides A, dioscorealides B, dioscoreanone, β-sitosterol, stigmasterol, diosgenin-(3-O-α-L-rhamnopyranosyl (1⟶2)-β-D-glucopyranoside, glucoside diosgenin 3-O-β-D-glucopyranosyl (1⟶3)-β-D-glucopyranoside, and β-D-sitosterol on antioxidant activity by using DPPH assay showed EC_{50} values 0.8±0.2, 21.7±0.4, 33.1±0.56, 2.81±0.85, 8.05±0.68, 6.24±0.69, 7.135±0.59, and 12.95±1.05 µg/mL, respectively and they showed no activity in the LDH assay (not toxic to cell membranes) for three cancer cell lines (large cell lung carcinoma (COR-L23), human colon adenocarcinoma (LS174T), and human breast adenocarcinoma (MCF-7)) (Itharat et al., 2007).

Antifungal activity

The report of antimicrobial of the ethanolic extract of *D. membranacea* showed the highest activity against with *Staphylococcus aureus* and *Bacillus subtilis* by using disc diffusion method (Inhibition zone diameter = 14±0.1 and 14±0.2 mm, respectively) and the extract of *D. membranacea* showed no inhibition zone of *Candida albican*, and showed the highest activity against with *S. aureus* and *Escherichia coli* by using minimum inhibitory concentration (MIC) method (MIC less than 1.25 and 2.5 mg/ml, respectively), and the extract of *D. membranacea* showed MIC more than 10 mg/ml on *Candida albican* (Itharat, 2010).

2.6.1.3 *Stemona collinsiae* Craib.

Figure 2-4 Dried root of *Stemona collinsiae* Craib

Scientific name: *Stemona collinsiae* Craib.

Family name: Stemonaceae

Thai name: Pong chang, Non-tai yak dok san (Smitinand, 2014)

Part used: Root

Chemical composition: The p-coumaric acid, cinnamic acid, p-Hydroxybenzoic acid, quercetin, luteolin, feruic acid, caffeic acid, Kaempferol (Rutnakornpituk et al., 2018)

General description:

The description of *S. collinsiae*, shown in figure 2-4, is a herbaceous climber or trailing, herb to 60 cm long, glabrous, rarely branched, sterile towards apex when climbing. Leaves alternate; petiole 5–15 cm long; blade broadly or narrowly ovate, 11–15 by 5–11 cm, base cordate. Inflorescences 1–8-flowered, sessile (basal ones) and pedunculate; peduncle 1–4.5 cm long; bracts c. 1 mm long. Flowers: pedicel 5–30 mm long; tepals narrowly ovate, 13–20 by 5–8 mm, the inner ones broadest, to 10 mm broad, and towards the apex rather abruptly narrowed; stamens 11–17 by 2 mm, filaments c. 1 mm long, anthers 5–7 mm long, ridge separating the thecae smooth, c. 1 mm high, petaloid outgrowth of the connective narrowly ovate, 8–9 mm long, only slightly narrower than anthers, with narrow thecal remnants, additional

appendage lacking. Fruit 20–25 by 10–12 mm. Seeds 3–6, pink-red, 10–12 by 3–4 mm; aril finger-like lobed. The tepals are green or pinkish-white, with green veins; the stamens as yellowish-green, the petaloid outgrowth of the connective as yellow; the tuberous roots as 10–40 cm long, 1–1.5 cm thick (Inthachub et al., 2010).

Traditional used:

Root, drunken taste, used to treat dermatitis, poor lymph disorder, hemorrhoids. (Homhual et al., n.d.) The stem is used to treating bronchitis, insect pests, pulmonary tuberculosis, and respiratory disorders (Manosroi et al., 2015).

Biological activities:

Cytotoxic activity

The study of anti-proliferative by SRB assay of methanolic extracts of *S. collinsae* on HeLa and KB cell lines showed the activity with GI_{50} value more than 100 µg/mL in both of cell lines (Manosroi et al., 2015).

Anti-inflammatory activity

No reported on anti-inflammatory activity.

Antioxidant activity

The methanolic extract of *S. collinsae* stem and fraction of methanolic extract in hexane, methanol-water, *n*-butanol, and water solvent on antioxidant activities showed SC_{50} value of more than 100 µg/ml all extracts by using DPPH assay, EC_{50} value of 391.70, more than 500, more than 500, more than 50, and 436.00 µg/mL, respectively by using chelating activity. Total phenolic contents showed 27.45, 24.08, 44.83, 16.82, and 5.83 µg/ml, respectively and total flavonoid contents showed 1.69, not determined (ND), 4.82, ND, and ND µg/mL, respectively (Manosroi et al., 2015). Besides, the study of crude extract from roots, stems and leaves of *S. collinsae* found that the highest total phenolic and flavonoid contents was detected in crude extract from root with 15.58±0.67 mg GAE/g crude extract, and 22.12±0.89 mg QU/g crude extract, respectively, followed by stem with 8.75±0.33 mg GAE/g crude extract, and 13.26±0.45 mg QU/g crude extract, respectively, and leave with 6.21±0.15 mg GAE/g crude extract, and 5.92±0.75 mg QU/g crude extract, respectively. Moreover, they

showed higher DPPH radical scavenging activity with EC_{50} values of 118.7±1.12, 167.8±1.33, and 282.0±0.85 ppm, respectively (Rutnakornpituk et al., 2018).

Antifungal activity

The diclolomethan-methanol, 95% ethanol and aqueous extracts from root of *S. collinsae* were investigated for antiviral activity by using a plaque reduction assay found that the diclolomethan-methanol extract showed moderate activity against herpes simplex virus (HSV) type 1 and type 2 with IC_{50} values of 105±3.5 and 107±6.2 µg/mL, respectively and other extracts showed IC_{50} values of more than 300 µg/mL. They were investigated for antimicrobial activity by using disc diffusion assay showed all extracts displayed no activity against *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Candida albicans* and, *Aspergillus niger* (Akanitapichat et al., 2005).

2.6.1.4 *Rauwenhoffia siamensis* Scheff.

Figure 2-5 Dried root of *Rauwenhoffia siamensis* Scheff.

Scientific name: *Rauwenhoffia siamensis* Scheff. or *Uvaria siamensis* (Scheff.) L. L. Zhou, Y. C. F. Su & R. M. K. Saunders *C* or *Melodorum siamense* (Scheff.) Ban

Family name: Annonaceae

Thai name: Nom maeo (Smitinand, 2014)

Part used: Root

Chemical composition: Toussaintine C, N- (4- hydroxyphenethyl) cinnamamide, melodamide A, 2′ ,4′ - dihydroxy- 4,6′ - dimethoxychalcone, 4,4′ - dihydroxy2′ ,6′ - dimethoxychalcone, helichrysetin, 2′,4,4′-trihydroxy-6′-methoxy-3′(3″-hydroxybenzyl) dihydrochalcone, 2′ ,4′ - dihydroxy- 4,6′ - dimethoxy- 3′ (3″ - hydroxybenzyl) - dihydrochalcone, 4′,4-dihydroxy-2′,6′-dimethoxydihydrochalcone, uvangoletin, 2′,4′-dihydroxy4′ ,6′ - dimethoxydihydrochalcone, 2′ ,4,4′ - trihydroxy- 6′ - methoxydihydrochalcone, Flavokawain- A, Tsugafolin, Naringenin trimethyl ether, Alpinetin, Kaempferol, Kaempferide, N-cinnamoyl-(2-phenylethyl)amine, 3-hydroxy-3-methoxy-1- phenyl-2-(3′, 4′, 5′- trimethoxy- phenyl)- propenone and 3-hydroxy-3-methoxy-1-phenyl-2-(2′, 3′, 4′-trimethoxy-phenyl)-propenone, oleic acid, palmitic acid, capric acid, linoleic acid, and stearidonic acid (Jaidee et al., 2019 ; Intaranongpai, 2006)

General description:

The description of R. *siamensis*, shown in figure 2-5, is a shrub or small tree up to 2 m, or climber up to 20 m. older wood becoming striate with prominent lenticels, subglabrous. Leaves (bluntly) lanceolate to elliptical, base retuse, apex narrowly–bluntly acute about 90–150 x 30–40 mm, thinly coriaceous, waxy texture above; veins 8–11; indument glabrous above, except for sparse spreading stellate hairs on upper midrib. Inflorescence leaf-opposed or subopposed, flowers solitary or rarely 2 or 3, shortly and densely tomentose, basal and medial bracts ovate. Calyx sepals 3, valvate, opening early, very broadly ovate about 3 x 6 mm, thickened and remaining incurved, base concave, outer surface indument as for pedicel, inner surface glabrous. Corolla 2 whorls of 3 petals, petals yellow to white in color, outer petals broadly ovate, inner petals broadly elliptical with constricted base, outer surface of both whorls densely tomentose, hairs fine, pale, inner surface of outer whorl tomentose at apex and on all margins, base glabrous, inner whorl glabrous at base, apex and apical margins tomentose. Stamens numerous, cuneate, apex flat or shallowly convex, obscuring locules, rusty brown or orange in color with waxy texture, covered with minute blunt papillae. Ovaries covered with blunt stallate hair-scales, giving very finely warted appearance, neck between ovary and stigma covered with long stellate hairs,

stigmas prominent, lobed, U-shaped, hairy about 1 mm long, forming distinct dome above stamens. Fruit monocarps 2–10 (usually 3–6), globose or shortly oblong, very minutely tubercled with subtomentose indument of blunt stellate scales, outline of seeds often clear within pericarp (Zhou et al., 2010).

Traditional used:

In traditional Isan medical, root is drunk for abnormal menstruation, and nasal polyps (Homhual et al., n.d.).

Biological activities:

No reported on biological activities.

2.6.1.5 *Canna indica* L.

Figure 2-6 Dried rhizome of *Canna indica* L.

Scientific name: *Canna indica* L.

Family name: Cannaceae

Common name: Bua lawong, Pla-ya, Phuttha raksa, Phuttha raksa kin hua, Phuttha son, Sa khu mon, Sa khu hua kha, A-ta-lut, Australian arrowroot, Edible Canna, Indian shot (Smitinand, 2014)

Part used: Rhizome

Chemical composition: m-Salicylic acid, Phenyl acetic acid, Ellagic acid, p-Coumaric acid, 4-Hydroxystyrene, Triamcinolone, Rosmarinic acid, (–)-Usnic acid, Coumarin, Isoeugenitol, Pantothenic acid, Acetylsalicylic acid , m-Hydroxyphenyl-pyruvic acid, Psoromic acid, o-Hydroxyphenylpyruvic acid lactone, Rosmarinic acid, Usnic acid, Acetylsalicylic acid, Lipoamide, Pseudohypericin, 3-(4-hydroxyphenyl) pyruvic acid, 3,4-Dihydroxyphenyl-propionic acid, Choline, Metaraminol, Fendiline, and Swietenine (Ayusman et al*.,* 2020).

General description:

The description of C. *indica*, shown in figure 2-6, is a long-lived, large, perennial herb, growing up to 2 m tall, spreading laterally by means of fleshy underground stems. Leaves simple, spirally arranged, greenish-maroon or green, ovate-lanceolate, entire, base attenuate, pinnately veined with a distinct midrib, petiole 7-11 cm long, with a short open sheath gradually passing into the petiole, upper surface glabrous. Inflorescence racemose, raceme simple. Flowers complete, bisexual, zygomorphic, epigynous, upper portion maroon chocolate or green, lower portion maroon or light-green, ovate-lanceolate; bracteoles 3, ovate, pedicel short. Sepals 3, maroon, not petaloid, polysepalous, unequal, imbricate, persistent in fruits, glabrous. Petals 3, connate in a cylindrical corolla tube, lanceolate, red, equal in each flower, imbricate, glabrous. Stamens 5, 1 partially perfect, all petaloid, connate below in a cylindrical (Sultana et al*.,* 2019).

Traditional used:

Many parts of *C. indica are* used in traditional medicine as diaphoretic and diuretic in fevers and dropsy, as a demulcent, to stimulate menstruation, treat suppuration, rheumatism, and regain energy (Woradulayapinij et al., 2005). Folklore medicine in tropical and subtropical areas with beneficial effects in numerous diseases, including infection, rheumatism, and hepatitis (Chen et al.,2013).

Biological activities:

Cytotoxic activity

The study of dichloromethane and ethanolic extracts from leave of *C. indica* showed LC_{50} value 273.9 (non-toxic), and more than 1000 µg/mL (non-toxic), respectively by brine shrimp test (Moshi et al., 2010).

Anti-inflammatory activity

The report of 80% ethanol extract from rhizome of *C. indica* on anti-inflammatory activity using lipopolysaccharide-stimulated PGE_2, NO, and IL-1β from RAW 264.7 macrophages by using ELISA assay showed the ethanolic extracts at concentration of 10, 50, 100, and 500 µg/mL could inhibit PGE_2, NO, and IL-1β (concentration of 500 µg/ml was the highest of inhibit the production of inflammatory mediators) and In this study was found the increases in HG-induced mRNA expressions of IL-8 and MCP-1 were also significantly inhibited by ethanolic extracts. Stimulation of HG in U937 monocytes resulted in activation of p38 MAPK, ERK1/2, and JNK. However, ethanolic extracts treatment significantly decreased phosphorylation of p38 MAPK, ERK1/2, and JNK. Moreover, total contents of phenolic acid, Flavonoid, and Anthocyanin compounds showed values of 25.14±0.54 mg/g of GAE of total phenolics, 11.39±0.46 mg/g of QE of total flavonoids, and 0.71±0.02 mg/g of CGE of anthocyanins, respectively (Chen et al., 2013).

Antioxidant activity

The report of methanolic extract from aerial parts of *C. indica* on antioxidant activity showed good activity in Hydroxyl assay with EC_{50} value of 53.91 µg/mL, followed by DPPH assay with EC_{50} value of 55.21 µg/mL, Nitric oxide assay with EC_{50} value of 75.33 µg/mL, and Hydrogen peroxide assay with EC_{50} value of 82.70 µg/mL, respectively (Joshi et al., 2009). Moreover, the study of antioxidant activities of acetone, ethyl acetate, methanol, and water extracts from rhizome of *C. Indica* showed good antioxidant, in DPPH assay showed EC_{50} values of 21±3, 48±4, 34±2, 61±4 µg/mL, respectively, in ABTS assay showed EC_{50} values of 23±3, 53±10, 42±3, and 114±5 µg/mL, respectively, in Superoxide assay showed EC_{50} values of 170±10 µg/mL, no activity showed, 210±10 µg/mL, and no activity showed, respectively, in CUPRAC assay

showed values of 640±10, 230±10, 590±10, and 50±4 µg BHAE/mg of RE, respectively, in FRAP assay showed 180±10, 91±10, 122±10, and 50±2 µg/mL, respectively, in Metal chelating activity showed 2.9±0.2, 1.3±0.2, and 4.1±0.4 µg/mL, respectively. They showed contents of total phenolics 334±10, 230±10, 250±10, and 110±10 µg GAE/mg extract, respectively, and contents of total flavonoid 220±10, 31±4, 122±4, and 0 µg QCTE/mg extract, respectively (Ayusman et al., 2020).

Antifungal activity

The antifungal activity of essential oil extracts from *C. indica* rhizome showed inhibition zone of 12 mm each *against A. niger, C. albican, F. oxysporum, Rhodotorula species*, and *T. virideae*, followed by *A. aculeatus* (10 mm), and *A. awomori* (7 mm) (George, 2014). Furthermore, the antifungal activity study of aerial part of *C. indica* extracts with ethanol solvents was determined by agar well-diffusion method that showed diameter of inhibition zone in 16±0.02, 12±0.01, 14±0.02, and 10±0.01 mm for *Aspegillus niger, Aspergillus flavus, Fusarium solani,* and *Nigrospora oryzae* strains, respectively (Singh et al., 2016).

2.6.1.6 Sodium Chloride (sea salt)

Figure 2-7 Sea salt

Scientific name: Sodium chloride

General description:

It has white crystals and salty taste.

Traditional used:

In Thai traditional medicine used salt to treat constipation, eye disease, nourish the body, and lymphatic system (Foundation of Thai Traditional Medicine & Siriraj Applied Thai Traditional Medicine, 2007).

Biological activities:

No reported on biological activities.

2.6.2 The result of biological activities of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy (MRM)

Table 2-1 The result of biological activities of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy (MRM)

Herb / Activities	MRM	*S. corbularia*	*D. membranacea*	*S. collinsiae*	*R. siamensis*	*C. indica*	Sodium chloride
Cytotoxicity	NR	/	/	/	NR	NR	NR
Antioxidant	NR	/	/	/	NR	/	NR
Anti-inflammatory	NR	/	/	NR	NR	/	NR
Antimicrobial	NR	/	/	/	NR	/	NR

* NR = No report

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Chemicals and reagents

The chemicals and reagents used in this study are summarized in **Table 3-1.**

Table 3-1 List of chemicals and reagents used in this study

Activities	Name	Source
Extraction	95% ethanol (commercial grade)	RCI Labscan, Thailand
Quality control	Chloroform ($CHCl_3$) Analytical grade	RCI Labscan, Thailand
	Hydrochloric acid (HCl) fuming 37%	Merck, Germany
HPLC	Acetonitrile (C_2H_3N)	RCI Labscan, Thailand
	Methanol	RCI Labscan, Thailand
	Acetic acid (CH_3COOH)	RCI Labscan, Thailand
Cytotoxic activity (SRB assay)	Minimum essential medium (MEM)	Gibco, USA
	Dulbecco's modified eagle medium (DMEM)	Gibco, USA
	RPMI-1640 medium	Gibco, USA
	McCoy's 5A medium	ATCC, USA
	Penicillin-Streptomycin (P/S)	Gibco, USA
	Phosphate-buffered saline (PBS)	Gibco, United Kingdom
	Fetal bovine serum (FBS)	Gibco, United Kingdom
	Sodium bicarbonate, ($NaHCO_3$)	BHD, England
	Dimethylsulfoxide, ($(CH_3)_2SO$) (DMSO)	RCI Labscan, Thailand
	Sulforhodamine B sodium salt ($C_{27}H_{29}N_2NaO_7S_2$)	Sigma-Aldrich, USA
	Trypsin-EDTA	Gibco, Canada

Table 3-1 List of chemicals and reagents used in this study (continued)

Activities	Name	Source
Cytotoxic activity (SRB assay)	Tris(hydroxymethyl) aminoethane ($NH_2C(CH_2OH)_3$)	Sigma-Aldrich, USA
	Trichloroacetic acid (Cl_3CCOOH) (TCA)	Merck, Germany
	Trypan blue stain 0.4%	Gibco, USA
Antioxidant activities (FRAP assay)	Sodium acetate ($CH_3COONa.3H_2O$)	Sigma-Aldrich, Germany
	Iron (lll) chloride hexahydrate ($FeCl_3.6H_2O$)	Qrec, Newzealand
	2,4,6-Tripyridyl-s-triazine (TPTZ) ($C_{18}H_{12}N_6$)	Sigma-Aldrich, India
	Glacial acetic acid	RCI Labscan, Thailand
Antioxidant activities (NBT assay)	Iscove's Modified Dulbecco's Medium (IMDM)	Gibco, USA
	Penicillin-Streptomycin (P/S)	Gibco, USA
	Phosphate-buffered saline (PBS)	Gibco, United Kingdom
	Fetal bovine serum (FBS)	Gibco, United Kingdom
	Phorbol 12- myristate 13-acetate (PMA)	Sigma, USA
	Lipopolysaccharide (LPS), form *Escherichia coli*	Sigma, USA
	Trypan blue stain 0.4%	Gibco, USA
	3-(4,5- dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT)	TCI, Japan
	Nitro blue tetrazolium (NBT)	TCI, Japan
Total flavonoid	Sodium nitrite ($NaNO_2$)	Suksapan, Thailand
	Aluminium chloride 6-hydrate ($AlCl_3.6H_2O$)	Kemaus, Australia

Table 3-1 List of chemicals and reagents used in this study (continued)

Activities	Name	Source
Total flavonoid content	Quercetin	Sigma-Aldrich, Germany
	Sodium hydroxide (NaOH)	BDH, England
Anti-inflammatory (NO assay)	Dulbecco's modified eagle medium (DMEM)	Gibco, USA
	Penicillin-Streptomycin (P/S)	Gibco, USA
	Phosphate-buffered saline (PBS)	Gibco, United Kingdom
	Fetal bovine serum (FBS)	Gibco, United Kingdom
	Trypan blue stain 0.4%	Gibco, USA
	Lipopolysaccharide (LPS), form *Escherichia coli*	Sigma, USA
	Trypsin-EDTA	Gibco, Canada
	Sulfanilamide	Sigma-Aldrich, China
	N-(1-Naphthy) ethylenediamine dihydrochloride	Sigma-Aldrich, USA
	Phosphoric acid	TCI, Japan
	Dimethylsulfoxide, ($(CH_3)_2SO$) (DMSO)	RCI Labscan, Thailand
	Isopropanol (C_3H_8O)	RCI Labscan, Thailand
	Hydrochloric acid (HCI) fuming37%	Merck, Germany
	3-(4,5- dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT)	TCI, Japan
Anti-inflammatory (Inhibition of IL-6)	Dulbecco's modified eagle medium (DMEM)	Gibco, USA
	Penicillin-Streptomycin (P/S)	Gibco, USA
	Phosphate-buffered saline (PBS)	Gibco, United Kingdom
	Fetal bovine serum (FBS)	Gibco, United Kingdom
	Trypan blue stain 0.4%	Gibco, USA
	Trypsin-EDTA	Gibco, Canada

Table 3-1 List of chemicals and reagents used in this study (continued)

Activities	Name	Source
Anti-inflammatory (Inhibition of IL-6)	Lipopolysaccharide (LPS), form *Escherichia coli*	Sigma, USA
	Mouse IL-6 capture antibody	R&D systems, USA
	Tween 20	TCI, Japan
	Bovine serum albumin (BSA)	Sigma-Aldrich, USA
	Mouse IL-6 standard	R&D systems, USA
	Mouse IL-6 detection antibody	R&D systems, USA
	Streptavidin-HRP	R&D systems, USA
	Tetramethylbenzidine (TMB)	TCI, Japan
	Sulfuric acid (H_2SO_4)	RCI Labscan, Thailand
Anti-inflammatory (Inhibition of TNF-α)	Dulbecco's modified eagle medium (DMEM)	Gibco, USA
	Penicillin-Streptomycin (P/S)	Gibco, USA
	Phosphate-buffered saline (PBS)	Gibco, United Kingdom
	Fetal bovine serum (FBS)	Gibco, UK
	Trypan blue stain 0.4%	Gibco, USA
	Lipopolysaccharide (LPS), form *E. coli*	Sigma, USA
	Trypsin-EDTA	Gibco, Canada
	Mouse TNF-alpha capture antibody	R&D systems, USA
	Tween 20	TCI, Japan
	Bovine serum albumin (BSA)	Sigma-Aldrich, USA
	Mouse TNF-alpha standard	R&D systems, USA
	Mouse TNF-alpha detection antibody	R&D systems, USA
	Streptavidin-HRP	R&D systems, USA
	Tetramethylbenzidine (TMB)	TCI, Japan
	Sulfuric acid (H_2SO_4)	RCI Labscan, Thailand

Table 3-1 List of chemicals and reagents used in this study (continued)

Activities	Name	Source
Antifungal activities	Mueller Hinton Broth (MHB)	Difco, France
(MIC, MFC assay)	Sabouraud Dextrose Agar (SDA)	BD, France
	Resazurin sodium salt	Sigma-Aldrich, USA

3.2 Instruments, plastics and glassware

The Instruments, plastics and glassware used in this study are summarized in **Table 3-2.**

Table 3-2 List of Instruments, plastics and glassware used in this study

Name	Source
Rotary evaporator	Heidolph, Germany
Hot plate	Thermolyne cimarec, USA
Filter paper No.1, 4 (125mm, diameter)	Whatman, USA
Hot air oven	Memmert, Germany
Lyophilizer	Labconco, USA
Freezer (-20°C)	Sanyo, Japan
Freezer (-4°C)	Sharp, Japan
Autoclave	Hirayama, Japan
pH buffer	Thermo Scientific, Singapore
pH meter	Thermo Scientific, USA
CO_2 humidified incubator	ESCO, Singapore
Inverted microscope	Leica, China
Haemocytometer	HBG, Germany
Purifier Logic+ Biosafety Cabinets	Labconco, USA
Biosafety Cabinets faster model BHA48	Megafil, Thailand
Centrifuge machine	Boeco, Germany

Table 3-2 List of Instruments, plastics and glassware used in this study (continued)

Name	Source
Microplate reader	Biotek, USA
Sonicator	Elma, Germany
Vortex mixer	Scientific Industries, USA
Incubated table top orbital shaker	Heidolph, Germany
Water bath	Memmert, Germany
Analytical Balance	Mettler Toledo, Taiwan
	Precisa, Switzerland
Moisture analyzer	A&D Company, Japan
McFarland Densitometer	Grant-bio, United Kingdom
Muffle furnace	Nabertherm, Germany
Atomic Absorption Spectrophotometer	Hitachi, Japan
Cryogenic tube 2 mL (Sterile)	Corning, USA
25 cm^2 plastic tissue culture flask	Corning, USA
75 cm^2 plastic tissue culture flask	Corning, USA
96-well plate flat, bottom with lid	Costar corning, China
96-well plate flat, bottom without lid	Costar corning, China
Filter unit (0.22 µm, radio-sterilized)	Millipore, Ireland
Eppendorf	Axygen, China
Syringe 5,10 mL	Nipro, Thailand
Centrifuge tube 15, 50 mL	Costar corning, China
Disposable pipette 25 mL	Costar corning, USA
Disposable pipette 5, 10 mL	SPL Life Sciences, Korea
Graduated Pipette 5, 10 mL	HBG, Germany
Pipette tips	Axygen, China
Pipette boy	Accu-jet pro, Germany
Micropipettes 2-20 µL, 20-200 µL, 100-1000 µL	Brand GMBH, Germany
Multi-Channels pipette 20-200 µL	Corning, Porland

Table 3-2 List of Instruments, plastics and glassware used in this study (continued)

Name	Source
Reservoir	Costar corning, USA
Tips 200 µL, 1000 µL	Costar corning, USA
Petri dish	Biomed, Thailand
Buchner funnel	Schott Duran, Germany
Erlenmeyer flask	Schott Duran, Germany
Evaporating flask 1000 mL	Schott Duran, Germany
Cylinder	Isolab, Germany
Crucible	Coostex, USA
Glass bottles	Schott Duran, Germany

3.3 Preparation of MRM remedy and plant ingredients extracts

3.3.1 Plant Materials

Smilax corbularia Kunth. was collected from Loei Province, Thailand. *Stemona collinsiae* Craib HC was collected from Phetchabun Province, Thailand. *Rauwenhoffia siamense* Scheff. was collected from Lampang Province, Thailand. *Canna indica* L. was purchased from Suphan Buri, Thailand, while salt was purchased from local markets in Samut Prakan, Thailand. *Dioscorea membranacea* Pierre ex Prain & Burkill., was obtained from Asst.Prof.Dr. Panumart Rithichai, Faculty of Science and Technology, Thammasat University. *R. siamense* was defined by identifying voucher specimens at Thai Traditional Medicine Herbarium, Thai Traditional Medicine Research Institute, Department of Thai Traditional and Alternative Medicine, Bangkok, Thailand. Three plant samples, including *S. corbularia*, *C. indica,* and *S. collinsiae* were identified by a taxonomist at Sirinthon Plant Herbarium Museum, Department of Agriculture, Kasetsart University, Bangkok, Thailand, where a voucher specimen of this collection was deposited. The voucher specimen numbers are shown in **Table 3-3.**

Table 3-3 Plants and part of plants component in Ya-Kae-Ma-Reng-Nai-Mod-Look remedy

Thai name	Scientific name	Part of used	Voucher specimen number
Khao Yen Nua	*Smilax corbularia* Kunth.	Bulb	BK No.082275
Khao Yen Tai	*Dioscorea membranacea* Pierre ex Prain & Burkill.	Bulb	SKP A062041305
Nom maeo	*Rauwenhoffia siamense* Scheff.	Root	TTM No.0006005
Phuttha raksa Dok Khao	*Canna indica* L.	Rhizome	Bk No.083535
Non-Tai yak	*Stemona collinsiae* Craib *HC*	Root	Bk No.083534
Sea salt	Sodium chloride	-	-

3.3.2 Preparation of crude extracts

Plant materials were cleaned, cut into small pieces, dried at 50°C, and then ground to a coarse powder. Ya-Kae-Ma-Reng-Nai-Mod-Look remedy (MRM) was prepared by mixing each plant and sea salt in equal proportions, as shown in **Table 3-4**. Then, each plant and MRM were divided into two extraction methods. The first part of MRM was macerated with 95% ethanol (E) for three days. After that, the extracts were filtered through Whatman No.1 paper, then concentrated by the Rotary Vacuum Evaporator. The residue was then reextracted twice following the same procedure. Finally, all extracts were dried at 45°C in a hot air oven. Another part of MRM was boiled with water (W) for 15 minutes and filtered through Whatman No.1 paper. The residue was then re-boiled twice, following the same procedure. The total volume of water extract was then brought down to one-third of its original volume by boiling. After that, the water extracts were dried with a lyophilizer. All dried extracts were stored at -20°C until use. The percentage of yield was calculated using the following formula.

$$\%\text{Yield} = \frac{\text{Weight of the extract (g) x100}}{\text{Weight of dried powder (g)}}$$

Table 3-4 Weight of dried each plant in Ya-Kae-Ma-Reng-Nai-Mod-Look remedy

Scientific name	Dried plant weight (grams)
Smilax corbularia Kunth.	60
Dioscorea membranacea Pierre ex Prain & Burkill.	60
Rauwenhoffia siamense Scheff.	60
Canna indica L.	60
Stemona collinsiae Craib	60
Sodium chloride	25

3.4 Physiochemical properties assay (Thai Herbal Pharmacopeia, 2020)

3.4.1 Loss on drying

Moisture content was determined using loss on drying. Two grams of Ma-Reng-Nai-Mod-Luke (MRM) remedy powder and each plant ingredients powder were put onto the aluminum tray that was operated at 105°C until stable weight. The percentage of loss on drying was calculated using the following equation below.

$$\%\text{Loss on drying} = \frac{\text{weight of plant powder before test (g) - stable weight after test (g)}}{\text{weight of plant powder before test (g) x 100}}$$

3.4.2 Extractive value

The extractive value was recorded in ethanol-soluble and water-soluble extractive values. First, five grams of coarsely powdered MRM and each plant ingredient were macerated with 100 mL of ethanol (ethanol-soluble extractive) or 100 mL of 0.25% chloroform in water (water-soluble extractive) in a closed flask with foil for 24 hours. Flask was frequently shaken during the first 6 hours and then allowed to

stand for 18 hours. Then, filter rapidly, evaporate 20 mL of the filtrate to dryness in a tared, flat-bottomed, shallow dish, and dry at 105°C to constant weight. Next, the percentage of extractives concerning the air-dried drug was calculated using the following equation.

$$\%\text{Extractive value} = \frac{\text{weight of extracts (g) x 100 x5}}{\text{weight of coarsely powder (g)}}$$

3.4.3 Total ash

Two grams of MRM remedy powder and each plant ingredients powder were weighed into the crucible and burned in the muffle furnace at 450°C for 5 hours until free from carbon. Crucible was allowed to stand for cooling, and then it was weighed after burning. The percentage of total ash was calculated using the following equation below.

$$\%\text{Total ash} = \frac{\text{stable weight after burning (g) x 100}}{\text{weight before burning (g)}}$$

3.4.4 Acid-insoluble ash

The ash after total ash testing was analyzed in the acid-insoluble ash. It was boiled with 25 mL of 10% hydrochloric acid for 5 minutes. Then, the insoluble matter will be collected by ashless filter paper and washed with hot water until neutral (pH 7). The insoluble matter with ashless filter paper will be burned at 500°C in a muffle furnace. The percentage of acid-insoluble ash was calculated using the following equation below.

$$\% \text{ Acid-insoluble ash} = \frac{\text{stable weight after burning (g)} \times 100}{\text{weight before burning (g)}}$$

3.4.5 Heavy metals by atomic absorption spectrophotometer (AAS)

Firstly, the stock solution of the heavy metal standard, including arsenic (As), cadmium (Cd), and lead (Pb), was prepared and diluted with 0.2% HNO_3. All standard solutions were analyzed by atomic absorption spectrophotometer model Z-8200 and repeated three times. Linear detector responses were observed for the calibration curve standard in the range of 0-4 ppb for Cd and 0-100 ppb for As and Pb. Calculation of linear correlation coefficient and correlation analysis was carried out using MS Office Excel. The linear regression equation for a straight line is, $Y = mx + c$ where, Y = absorbance of standard solution, m = slope of the calibration curve, x = concentration of standard solution, c = intercept. R^2 of the calibration curve should be greater than 0.99. The sample solution from acid-insoluble ash was used to detect heavy metal contamination in plant ingredients. After that, the heavy metal contamination in samples were analyzed using calibration curve of heavy metal standard and expressed as ppm.

3.5 Cytotoxicity activity by Sulforhodamine B (SRB) Assay (Skehan et al.,1990; Itharat et al., 2004)

3.5.1 Human cell lines

The ovarian cancer cell line (SKOV-3) ATCC HTB-77 was grown on RPMI-1640 Medium containing 10% fetal bovine serum and 1% of 2mM l-glutamine and 50 IU/ml penicillin and 50 µg/ml streptomycin. The cervical cancer cell line (HeLa) ATCC CCL-2 was grown on Minimum Essential Medium (MEM) containing 10% fetal bovine serum and 1% of 2mM l-glutamine and 50 IU/mL penicillin and 50 µg/mL streptomycin. The human endometrial adenocarcinoma cell line (HEC-1-A) ATCC HTB112 was grown on McCoy's 5a medium supplemented with 10% fetal bovine serum and 50 IU/mL penicillin and 50 µg/mL streptomycin. The normal human lung

tissue cell line (MRC5) ATCC CCL-171 and the immortalized human keratinocyte cell line (HaCaT) 300493-SF were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplement with 10% fetal bovine serum, and 1% of 2mM l-glutamine and 50 IU/mL penicillin and 50 µg/mL streptomycin. All cells were maintained at 37°C in 5% CO_2 with 95% humidity.

3.5.2 Preparation of sample solution

The ethanolic and aqueous extracts of MRM, its plant ingredients, and marker compound were investigated cytotoxic activity against cancer cell lines and normal cell lines. The ethanolic extracts and marker compound were dissolved in sterile dimethylsulfoxide (DMSO) and adjusted to a concentration of 50 mg/mL. In contrast, the water extracts were dissolved in sterile water (10 mg/mL) and then filtered through a 0.22 µm sterile syringe filtered. Samples were diluted at various concentrations with cell culture media.

3.5.3 Cytotoxic activity

According to cell growth profiles, the optimal plating densities of each cell line were calculated in the number of cells per well. SKOV-3 was seeded at a cell density of $1x10^4$ cells/well. HeLa and HEC-1-A were seeded at a cell density of $3x10^3$ and $4x10^3$ cells/well, respectively. MRC5 and HaCaT were seeded at a cell density of $5x10^3$ cells/well and $8x10^3$ cells/well, respectively.

In the first step (Day 1), cells were grown as a monolayer in a 75 cm^3 flask. After each cell line reached an 80-90% confluency, cells were washed twice with sterile PBS. Then, PBS was removed, and cells were trypsinized by 0.125% trypsin-EDTA at 37°C in a 5% CO_2 atmosphere with 95% humidity for 5 minutes or until cells detached. After that, cell culture media were added to the flask to inactivate trypsin-EDTA activity. Then cells were centrifuged at 1500 rpm for 5 minutes. The supernatant was removed by pipetting. Cells were resuspended in media and mixed with 0.4% trypan blue to count using a hematocytometer. After that, each cell line was adjusted to optimal cell density and seeded into a 96-well plate. The plate was incubated at 37°C in a 5% CO_2 atmosphere with 95% humidity for 24 hours. After 24 hours (Day 2), the cells were treated with various concentrations of crude extracts and DMSO as a

controlled solvent. The plate was incubated again to continue cell proliferation with the same condition for 72 hours. After incubation (Day 5), the supernatant was removed and washed with sterile PBS (200 µL/well). Then, the cells were added 200 µL/well of media into each well and incubated for 72 hours.

After incubation (Day 8), cells were fixed by 100 µL/well of ice-cold 40% trichloroacetic acid (TCA) and incubated at 4°C for 1 hour. After that, plates were washed five times with tap water to drain off the non-viable cells. The viable cells were fixed in each well. After air-dry, the plate was stained with 0.4% SRB solution in 1% acetic acid (50 µL/well) for 30 minutes. Then, the plates were washed four times with 1% acetic acid. The protein-bound dye in each well was dissolved by 10 mM Tris base (100 µL/well). The plates were shaken gently for about 20 minutes. The optical density (OD) was determined using a microplate reader at 492 nm. Cytotoxicity was expressed as the percentage of inhibition. The IC_{50} values were calculated using the Prism program. According to National Cancer Institute guidelines, plant extracts that show IC_{50} values less than 20 µg/mL are considered active (Boyd, 1997). The percentage of inhibition was calculated using the following equation below.

$$\%\text{inhibition} = \frac{(\text{OD control} - \text{OD sample}) \times 100}{\text{OD control}}$$

3.6 Antioxidant activity

3.6.1 Ferric Reducing Antioxidant Power (FRAP) Assay (Benzie and Strain, 1996)

The FRAP assay is a direct method to determine the antioxidant power of samples. This assay measures the reduction of the ferric iron (Fe^{3+}) to the ferrous ion (Fe^{2+}) colored form in the presence of antioxidants.

3.6.1.1 Preparation of FRAP reagent solution

The FRAP reagent was prepared freshly before the experiment. It consists of 300 mmol/L acetate buffer, 10 mmol/L TPTZ solution in 40

mmol/L HCL, and 20 mmol/L $FeCl_3$. $6H_2O$ in ratio 10:1:1, respectively. FRAP reagent was warmed at 37°C for 4 minutes before use.

3.6.1.2 Preparation of sample solution

The ethanolic and aqueous extracts of MRM, its plant ingredients, and astilbin (the main compound of MRM extract) were investigated the antioxidant power by FRAP assay. Crude extracts and astilbin were dissolved in absolute ethanol for ethanolic extract and water for water extract. The sample solution was adjusted to 1 mg/mL. Calibration curves were prepared using concentrations of Trolox in the range of 5 to 300 µg/mL and Fe^{2+} ($FeSO_4$. $7H_2O$) in the range of 5 to 300 µg/mL and 500 to 800 µg/mL.

3.6.1.3 Ferric reducing antioxidant power (FRAP) test

One hundred eighty microlitres of fresh FRAP reagent was added to 96-well plates. Then sample solution, Trolox, or Fe^{2+} (20 µL/well) were added and incubated for 8 minutes at room temperature. The optical density was determined at 593 nm. All experiments were carried out in triplicate. The antioxidant capacity of samples was determined from the calibration curve plotted using Trolox and Fe^{2+} as the reference standard.

3.6.2 Nitroblue tetrazolium (NBT) reduction assay (Mosmann, 1983; Srisawat et al., 2010)

3.6.2.1 Human cell line

This experiment used the human promyelocytic leukemia cell line (HL-60) ATCC CCL-240. It was cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplement with 20% fetal bovine serum and 1% of 2mM l-glutamine and 50 IU/mL penicillin, and 50 µg/mL streptomycin. The cell line was maintained in an incubator at 37°C with a 5% CO_2 to 95% humidity. Cells were passaged every 4 days.

3.6.2.2 Preparation of sample solution

The ethanolic and water extracts of MRM, its plant ingredients, and astilbin, the main compound of MRM extract, were investigated antioxidant activity by NBT assay. The ethanolic extracts and astilbin were dissolved in sterile dimethylsulfoxide (DMSO) and adjusted to 50 mg/mL. The water extracts were

dissolved in sterile water (10mg/mL) and filtrated through 0.22 µm of the sterile filter. The sample solution was diluted to a final concentration of 1,000 µg/mL with Hank's balanced salt solution (HBSS) for the screening step. The samples showing more than 50% inhibition effect examined the antioxidant effect at various concentrations (1,000, 500, 250, and 125 µg/mL).

3.6.2.3 Preparation of NBT and PMA solution

The NBT and PMA solution was prepared freshly at a concentration of 1.25 mg/mL and 2 mg/mL (final concentration 500 ng/mL), respectively. Both of them were diluted with HBSS. After that, the NBT solution was kept in the dark by covering it with foil.

3.6.2.4 Procedure of NBT assay

Firstly, HL-60 cells (750,000 cells/mL) were induced in 15 mL of IMDM containing 1.3% DMSO and incubated at 37°C in 5% CO_2 with 95% humidity for seven days. After that, cells were centrifuged at 4000 rpm for 10 minutes. The supernatant was removed, and the new media (2.5 mL) was replaced. The hematocytometer counted the viable cells by 0.4% trypan blue exclusion. Then, the single-cell suspension was diluted and adjusted to $5x10^6$ cells/mL in HBSS. After that, 200 µL/tube of differentiated HL-60 cells in HBSS were incubated with 500 µL/tube of various dilutions of samples or propyl gallate (positive control) for 15 minutes. After incubation, cells were added 250 µL/tube of NBT solution and 50 µL/tube of PMA solution to each tube except for the blank tube. The blank was added to 250 µL/tube of HBSS and 50 µL/tube of PMA solution. All samples were incubated for 1 hour. Next, 2 mL of 1N HCl was added to stop the reaction and centrifuged at 4,000 rpm for 10 minutes. Discard the supernatant, and allow tubes to dry at room temperature. Then, the formazan product was dissolved in 300 µL of DMSO and transferred to a 96-well plate of the triplicate wells (100 µL/well). The absorbance was determined at 572 nm. The following formula was used to calculate the percentage of inhibition:

$$\%\text{inhibition} = \frac{(\text{OD control} - \text{OD sample}) \times 100}{\text{OD control}}$$

The IC_{50} value was calculated by using the GraphPad Prism5 program. All experiments were performed in triplicate.

3.6.2.5 Cytotoxic activity by MTT assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on converting MTT into formazan crystals by living cells, which determines mitochondrial activity. For most cell populations, the total mitochondrial activity is related to the number of viable cells (Meerloo et al., 2011).

In the first step, 200 µL/tube of differentiated HL-60 cells in HBSS was incubated with 500 µL/tube of various dilutions of samples or propyl gallate (positive control) for 15 minutes. After incubation, cells were added 250 µL/tube of HBSS and 50 µL/tube of PMA solution to each tube and incubated for 1 hour. After incubation, cells were centrifuged at 4,000 rpm for 10 minutes to discard the supernatant. HBSS (1 mL/tube) was replaced. MTT solution (100 µL/tube) was added to each tube and incubated for 2 hours at 37°C in 5% CO_2. After that, cells were centrifuged at 4,000 rpm for 10 minutes and kept cell pellet. Then, the formazan product was dissolved in 300 mL of DMSO and transferred into a 96-well plate. The absorbance was determined at 570 nm. The below formula was used to calculate the percentage of survival:

$$\%\text{Survival} = \frac{\text{OD sample} \times 100}{\text{OD control}}$$

3.7 Anti-inflammatory activity

3.7.1 NO• inhibition assay in LPS-stimulated RAW 264.7 macrophages

The biological activities of Nitric Oxide (NO) have been recognized as a pleiotropic biological mediator, regulating diverse activities ranging from neuronal function to immune system regulation. It is a gaseous free radical with a short half-life in vivo of a few seconds or less. Therefore, the levels of the more stable NO

metabolites, nitrite (NO_2^-) and nitrate (NO_3^-) have been used to measure NO indirectly in biological fluids. Altered NO levels have been shown to be associated with sepsis, reproduction, infection, hypertension, exercise, type 2 diabetes, hypoxia, and cancer. This assay determines nitric oxide concentrations based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess Reaction. The Griess reaction is based on the two-step diazotization reaction in which acidified NO_2^- produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion as shown in **Figure 3-1** (Bryan & Grisham, 2007).

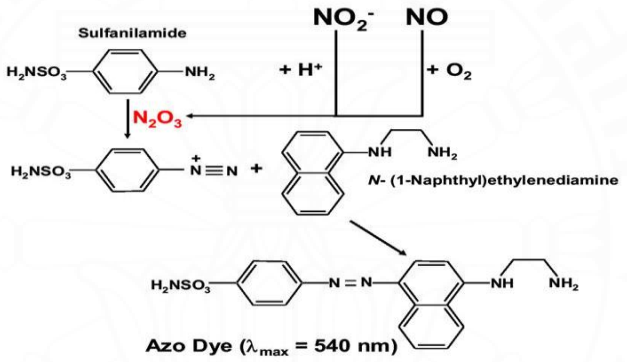

Figure 3-1 The Griess Reaction (Bryan & Grisham, 2007)

3.7.1.1 Human cell line

In this study, the Murine macrophage leukemia (RAW 264.7) cell line was used in this experiment. It was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplement with 10% fetal bovine serum and 1% of 2mM l-glutamine and 50 IU/mL penicillin, and 50 µg/mL streptomycin. The cell line was maintained in an incubator at 37°C with 5% CO_2 and 95% humidity.

3.7.1.2 Preparation of sample solution

The ethanolic and water extracts of MRM, its plant ingredients, and astilbin, the main compound of MRM extract, were investigated antioxidant activity by nitric oxide assay. The ethanolic extracts were dissolved in sterile dimethylsulfoxide (DMSO) and adjusted to a concentration of 50 mg/mL. The water extracts were

dissolved in sterile water (10 mg/mL) and then filtered through a 0.22 µm sterile syringe filter.

3.7.1.3 Inhibition of nitric oxide production on LPS-stimulated RAW 264.7 cell

RAW264.7 cells that grow to 70-80% confluence were washed with sterile PBS. Then, 0.125% trypsin was added to detach cells from the flask for 10 minutes. After that, growth media (7 mL) was added to inactivated trypsin-EDTA. The cell suspension was transferred to a sterile tube and centrifuge at 1,500 rpm for 5 minutes to wash trypsin and resuspend it in a growth medium. The viable cells were stained with 0.4% trypan blue and added to a hematocytometer to count cells. The cell suspension was adjusted to $1x10^6$ cells/mL and seeded in 96-well plates (100 µL/well). The plate was incubated at 37°C in a 5% CO_2 atmosphere with 95% humidity for 24 hours. After that, the old medium was replaced with a fresh medium (100 µL/well) containing LPS (10 ng/mL). The sample was tested at various concentrations and added to each well (100 µL/well). The medium with LPS and medium without LPS (200 µL/well) was used as a control. The plate was incubated at 37°C in a 5% CO_2 atmosphere with 95% humidity for 24 hours. Finally, the supernatant was transferred into the new 96-well plate (100 µL/well). Griess reagent was added to each well, and the optical density was determined at 570 nm. Prednisolone was used as a positive control. The experiment was performed in triplicate. The percentage of inhibition was calculated using the below formula:

$$\%\text{inhibition} = \frac{(\text{OD control} - \text{OD sample}) \times 100}{\text{OD control}}$$

3.7.1.4 Cytotoxicity of sample on RAW264.7 cells

MTT assay was used to test the cytotoxic activity. After the supernate transfer, MTT solution (10 µL, 5 mg/ml in PBS) was added to each well and incubated at 37°C in a 5% CO_2 atmosphere with 95% humidity for 2 hours. After that, supernate was removed and replaced with 0.04 M HCl in isopropanol for dissolving the

formazan crystals. The absorbance was measured at 570 nm, and the survival percentage was calculated using the formula below.

$$\%\text{Survival} = \frac{\text{OD sample} \times 100}{\text{OD control}}$$

3.7.2 Measurement of IL-6 cytokine production in LPS-stimulated RAW264.7 (Cho et al., 2014)

Interleukin-6 (IL-6) is a cytokine with multiple biological activities produced by various cell types and exerts pleiotropic effects on different organ systems. The diversity of IL-6-dependent effects is prevalent. Elevated IL-6 serum levels have been detected in acute and chronic inflammation. IL-6 was measured in supernatants using a specific enzyme-linked immunosorbent assay (ELISA) (Mudter & Neurath, 2007). The sandwich ELISA technique measured the production of interleukin 6 (IL-6) in RAW264.7 cells.

3.7.2.1 Cell line

RAW264.7 cells were used in this experiment. It was cultured in a growth medium, Dulbecco's Modified Eagle Medium (DMEM) supplement with 10% fetal bovine serum, 50 IU/mL penicillin, and 50 µg/mL streptomycin. The cell line was maintained in an incubator at 37°C with a 5% CO_2 to 95% humidity.

3.7.2.2 Preparation of sample solution

The ethanolic and water extracts of MRM, its plant ingredients, and astilbin, the main compound of MRM extract, were investigated in this study. The ethanolic extracts and astilbin were dissolved in sterile dimethylsulfoxide (DMSO) to 50 mg/mL. The extracts were dissolved in sterile water to a 10 mg/mL concentration and then filtered through a sterile syringe at 0.22 µm. The extracts were diluted to various concentrations (12.5-100 µg/mL) in the growth medium to test IL-6 inhibition.

3.7.2.3 Procedure of inhibitory effects on LPS-induce IL-6 release from RAW 264.7 cells line assay

Briefly, the cells were seeded in 96-well plates with $1x10^6$ cells/mL (100 µL/well) and incubated for 24 hours at 37°C with a 5% CO_2 to 95%

humidity. After that, the medium was replaced with a growth medium containing 100 ng/ml of LPS and testing samples at various concentrations and then incubated at 37°C for 24 hours. After incubation, the supernatant was collected and kept at -20°C until use. Next, the mouse IL-6 capture antibody was coated in a 96-well plate (100 µL/well) and incubated overnight at 4°C. After that, the plate was washed five times with wash buffer and blocked with 5% BSA in PBS for 2 hours at room temperature. After blocking, a supernatant of samples or recombinant protein mouse IL-6 was added to each well (100 µL/well) and incubated for 2 hours at room temperature. Next, the plate was washed five times with wash buffer, then Streptavidin-HRP was added. The plate was wrapped with foil to avoid the light and incubated for 20 minutes. The plate was washed five times with a wash buffer. Next step, the TMB substrate solution was added and incubated for 20 minutes. Finally, the stop solution (2N H_2SO_4) was added (50 µL/well) to stop the reaction. The optical density of each sample was determined at 450 nm by spectrophotometer. The standard curve was developed using the recombinant IL-6 protein to calculate the IL-6 production of each sample. The inhibition of IL-6 production was calculated using the following equation, and IC_{50} values were calculated by using Prism Program.

$$\%\text{Inhibition of IL-6} = \frac{(\text{IL-6 production of control} - \text{IL-6 production of sample}) \times 100}{\text{IL-6 production of control}}$$

3.7.3 Assay for inhibiting extract on TNF- α releasing from RAW 264.7 cell line.

Inflammation is essential to innate immunity and is regulated in many steps. One such regulating step is the cytokine network, where tumor necrosis factor α (TNF-α) plays one of the most critical roles and influences the typical inflammatory features in the organism. TNF-α participates in vasodilatation, edema formation, and leukocyte adhesion to epithelium through the expression of adhesion molecules; it regulates blood coagulation and contributes to oxidative stress in inflammation sites, indirectly inducing fever. The connection between TNF-a and cancer is also mentioned (Zelová & Hošek, 2013).

3.7.3.1 Cell line

RAW264.7 was used in this experiment. It was cultured in a growth medium, Dulbecco's Modified Eagle Medium (DMEM) supplement with 10% fetal bovine serum, 50 IU/mL penicillin, and 50 µg/mL streptomycin. The cell line was maintained in an incubator at 37°C with a 5% CO_2 to 95% humidity.

3.7.3.2 Preparation of sample solution

The ethanolic and water extracts of MRM, its plant ingredients, and astilbin, the main compound of MRM extract, were investigated in this study. The ethanolic extracts and astilbin were dissolved in sterile dimethylsulfoxide (DMSO) to 50 mg/mL. The extracts were dissolved in sterile water to a 10 mg/mL concentration and then filtered through a sterile syringe at 0.22 µm. The extracts were diluted to various concentrations (12.5-100 µg/mL) in the growth medium to test TNF-α inhibition.

3.7.3.3 Procedure of inhibitory effects on LPS-induce TNF-α release from RAW 264.7 cells line assay

Briefly, the cells were seeded in 96-well plates with $1x10^6$ cells/mL (100 µL/well) and incubated for 24 hours at 37°C with a 5% CO_2 to 95% humidity. After that, the medium was replaced with a growth medium containing 10 ng/ml of LPS and testing samples at various concentrations and then incubated at 37°C for 24 hours. After incubation, the supernatant was collected and kept at -20°C until use. Next, the mouse TNF-α capture antibody was coated in a 96-well plate (100 µL/well) and incubated overnight at 4°C. After that, the plate was washed five times with wash buffer and blocked with 5% BSA in PBS for 2 hours at room temperature. After blocking, a supernatant of samples or recombinant protein mouse TNF-α was added to each well (100 µL/well) and incubated for 2 hours at room temperature. Next, the plate was washed five times with wash buffer, then Streptavidin-HRP was added. The plate was wrapped with foil to avoid the light and incubated for 20 minutes. The plate was washed five times with a wash buffer. Next step, the TMB substrate solution was added and incubated for 20 minutes. Finally, the stop solution (1N HCl) was added (50 µL/well) to stop the reaction. The optical density of each

sample was determined at 450 nm by spectrophotometer. The standard curve was developed using the recombinant TNF-α protein to calculate the TNF-α production of each sample. The inhibition of TNF-α production was calculated using the following equation, and IC_{50} values were calculated by using Prism Program.

$$\%\text{Inhibition of TNF-}\alpha = \frac{(\text{TNF-}\alpha \text{ production of control - TNF-}\alpha \text{ production of sample})}{\text{TNF-}\alpha \text{ production of control x100}}$$

3.8 Antifungal activity

3.8.1 Determination of minimal inhibitory concentrations (MIC) by Microtiter Plate-based Method (Sarker et al., 2007)

The minimal inhibitory concentration (MIC) is a key indicator of an antimicrobial agent's potency. The principle of this test is based on the visual detection of the reduction of the resazurin reagent. Resazurin is evaluated by active microbial cells to reduce the non-fluorescent resazurin (blue) to the fluorescent resorufin (pink), as shown in **Figure 3-2**. The MIC value is defined as the lowest concentration of drug showing no visible growth.

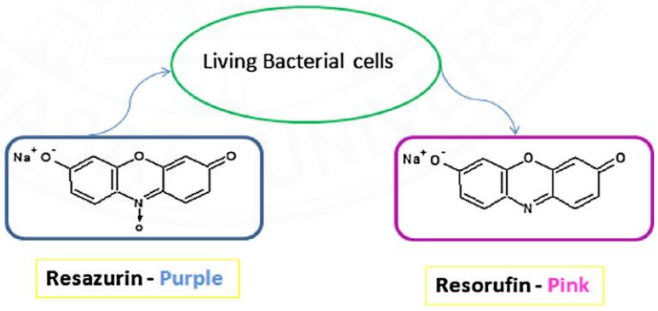

Figure 3-2 Active living cells cause reduction of resazurin (purpleblue) to resorufin (pink-colorless) (Elshikh et al., 2016)

3.8.1.1 Microorganism test

All samples were determined the antifungal activity against *Candida albicans* ATCC 9028. *C. albican* was cultured on Sabouraud's dextrose agar plates and incubated at 37°C for 48 hours.

3.8.1.2 Preparation of sample solution

The ethanolic and water extracts of MRM and its plant ingredients were investigated in this study. The ethanolic extracts were dissolved in sterile dimethylsulfoxide (DMSO) to a concentration of 500 mg/mL. The ethanolic extracts were diluted using Mueller Hinton Broth (MHB) and adjusted to 10 mg/mL. The water extracts were dissolved in sterile water to 10 mg/mL concentration and filtered through a sterile syringe filter of 0.22 µm.

3.8.1.3 Procedure of antifungal activity by Microtiter Plate-based Method

The sample solutions were diluted two-fold and added to 50 µL/well into a 96-well plate. Next, the inoculum of *C. albicans* was prepared in MHB and adjusted to 0.5 McFarland standards using a densitometer. Then, the inoculum was added to 96-well each well (50 µL/well), and the plate was covered with plastic wrap. The plate was incubated at 37°C for 48 hours in the incubator shaker. After incubation, resazurin solution (1 mg/mL) was pipetted to each well (10 µL/well) and continued incubated at 37°C for 3 hours. The MIC value was defined as the lowest concentration of the crude extract that showed no changing in resazurin color in the 96-well plate.

3.8.2 Minimum fungicidal concentration (MFC)

The MFC is defined as the lowest concentration of the drug, which kills a fungal and reduces the viability of the initial fungal inoculum by ≥99.9% (Kumar & Jha, 2017).

The sample solution from MIC value was transferred onto Sabouraud Dextrose Agar and incubated for 48 hours at 37°C. The minimal fungicidal concentration was the lowest concentration that showed no colony on the surface of SDA. All experiments were repeated in triplicate.

3.9 Quantitative analysis of the chemical marker in MRM remedy using HPLC technique

The main compound in the 95% ethanolic extract of MRM remedy was analyzed by a high-performance liquid chromatography (HPLC) system. The analytical process was conducted on Autosampler, Thermo Separation Product (TSP) HPLC system comprising Spectra system UV2000 UV-vis detector, Spectra System P4000 gradient pump, and Spectra System AS3500 automatic injector. The instrument was controlled using ChromQuest 5.0 software. The separation was performed using a reversed-phase Phenomenex Luna C_{18} column (250 x 4.60 mm, particle size 5.0 µm) with a guard column. The mobile phase used consisted of 0.1% acetic acid (solvent A) and acetonitrile (solvent B) in gradient elution as follows: 84% to 80% A in 15 min, 80% to 60% in 25 min, 60% to 0% in 5 min, held for 7 min, 0% to 84% in 1 min, held for 4 min. The flow rate was 1 mL/min with UV absorbance detection at 280 nm. The operating temperature was maintained at room temperature. Ya-Kae-Ma-Reng-Nai-Mod-Look extract and astilbin (Standard maker) were prepared at a concentration of 10 mg/mL with methanol and then filtered through a 0.45 µm membrane filter before analysis. Standard and sample solutions were injected with a 10 µL.

3.10 Stability test of Ma-Reng-Nai-Mod-Look (MRM) extract

Stability testing was carried out in triplicate using transparent vials. All samples were put in each vial and exposed under 40±2°C with 75±5% RH as an accelerated condition for six months period. The sample was collected on days 0, 30, 60, 90, 120, 150, and 180. All samples were tested for antioxidant activity by FRAP assay, anti-inflammatory activity by NO assay, and cytotoxic activity against the HeLa cell line. Finally, all extracts were analyzed with the amount of astilbin by HPLC.

3.11 Statistical analysis

All data were presented as means±standard error of the mean from at least three separate experiments. The GraphPad Prism 5 program was used to calculate TEAC, FRAP, and IC_{50} values. Then, the statistical significance between the two groups was analyzed by paired t-test. Statistical significance was indicated when $p < 0.05$.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Preparation of plant extracts

The ethanolic extracts are obtained by maceration of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy (MRM) and its plant ingredients with 95% ethanol. At the same time, the water extracts of MRM and its plant ingredients are obtained by decoction. As a result, all extracts' percentage yield ranged from 3.02% to 49.37%, as summarized in **Table 4-1**.

Table 4-1 The yield of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients

Sample	Solvent	Code	Yield (%w/w)
Ya-Kae-Ma-Reng-Nai-	95% Ethanol	MRME	11.09
Mod-Look remedy	Water	MRMW	47.70
S. corbularia	95% Ethanol	SCE	17.01
	Water	SCW	33.80
D. membranacea	95% Ethanol	DME	4.51
	Water	DMW	34.03
S. collinsiae	95% Ethanol	SCLE	7.36
	Water	SCLW	41.12
R. siamense	95% Ethanol	RSE	13.03
	Water	RSW	10.19
C. indica	95% Ethanol	CIE	3.02
	Water	CIW	49.37

The water extract of *C. indica* was the highest yield, with a percent yield of 49.37%, while the ethanolic extract of *C. indica* showed the lowest yield of 3.02%. The extractive yield of all samples, except *R. siamense*, was higher by decoction than by the maceration method. The solvent polarity effect phytochemical extraction. The

various solvents could provide different chemical compounds, and it has been found that polar solvents (water or ethanol) are general solvents for the extraction of phenolic compounds. In contrast, nonpolar solvents (hexane or chloroform) are used to extract oil and fats (Muhamad et al., 2017). Another reason the yield of aqueous extracts is so high is that many hydrophilic substances, such as carbohydrates, glycosides, and phenolic compounds, become more water-soluble, which improves their yield. Furthermore, sodium chloride, an ingredient in MRM remedy, may cause a high yield of the water extract (Alpert, 1990; Lefebvre et al., 2021).

Moreover, the application of temperature in the decoction method affects the extraction of chemical compounds (Kim, 2017). Thus, high temperatures may cause a high yield of the aqueous extract. A similar result has been found in Indian medicinal plant extracts (Kaneria, Kanani, and Chanda, 2012).

4.2 Physicochemical properties of plant extracts

The physicochemical properties include loss on drying, extractive value, total ash, acid-insoluble ash, and heavy metal. The results found that the loss on drying of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients ranges from 5.60 to 8.77%. The percentage of all samples' ethanol-soluble and water-soluble extractive values ranges from 0.46 to1.74 and 1.59 to 5.51, respectively. In addition, the percentage of total ash ranged from 2.15 to 6.70, and acid-soluble ash ranged from 0.01 to 1.53, as shown in **Table 4-2**.

Table 4-2 The percentage of the loss on drying, extractive value, total ash, and acid-insoluble ash of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients (Mean±SEM, N=3)

Sample	Loss on drying	Extractive value		Total ash	Acid-insoluble ash
		Ethanol	Water		
Ya-Kae-Ma-Reng-Nai-Mod-Look remedy	8.77±0.50	1.10±0.04	3.77±0.02	6.45±0.06	1.48±0.07
S. corbularia	6.13±0.09	1.74±0.09	2.74±0.09	2.15±0.05	0.08±0.00
D. membranacea	5.60±0.06	0.57±0.04	3.32±0.02	6.70±0.001	1.53±0.04
S. collinsiae	5.83±0.09	1.10±0.01	5.51±0.04	4.20±0.03	1.01±0.72
R. siamense	6.47±0.19	1.71±0.04	1.59±0.08	4.13±0.04	0.58±0.02
C. indica	6.13±0.03	0.46±0.01	2.93±0.03	6.64±0.05	0.01±0.00

Moreover, the Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients were examined the heavy metal contamination, including arsenic, cadmium, and lead. The acceptance criteria are arsenic must be less than 4 ppm, cadmium less than 0.3 ppm, and lead less than 10 ppm (Thai Herbal Pharmacopoeia, 2021). The results showed that all samples passed acceptance criteria and contained heavy metal at a low level, as shown in **Table 4-3**.

Table 4-3 The heavy metals contamination of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients (Mean±SEM, N=3).

Sample	Heavy metal contamination (ppm)		
	Arsenic	Cadmium	Lead
Ya-Kae-Ma-Reng-Nai-Mod-Look remedy	-0.0142±0.0007	0.0094±0.0010	-0.0053±0.0007
S. corbularia	-0.0219±0.0046	0.0013±0.0003	-0.0049±0.0005
D. membranacea	-0.0027±0.0139	0.0068±0.0002	-0.0009±0.0008
S. collinsiae	0.0142±0.0220	0.0050±0.0001	-0.0018±0.0019
R. siamense	-0.0124±0.0031	0.0179±0.0010	0.0049±0.0023
C. indica	-0.0159±0.0089	0.0103±0.0003	0.0093±0.0069

4.3 Cytotoxicity activity by Sulforhodamine B (SRB) Assay

All extracts were investigated cytotoxic activity against three cancer cell lines, including endometrioid adenocarcinoma cell line (HEC-1-A), cervical cancer cell line (HeLa), ovarian cancer cell line (SKOV-3), normal lung fibroblast line (MRC-5) and human keratinocyte cell line (HaCaT).

The results showed that MRME and MRMW have no cytotoxic effect on HEC-1-A and SKOV-3. On the other hand, MRME was highly toxic to HeLa cells with an IC_{50} value of 0.1 µg/mL, while MRMW has no toxicity to this cell line.

The results of plant ingredients of MRME showed that only three ingredients, including DME, SCLE, and RSE showed significant cytotoxicity against cancer cell lines. The DME, SCLE, and RSE significantly inhibited the growth of HEC-1-A with IC_{50} values of 48.26, 69.29, and 30.06 µg/mL, respectively. Moreover, RSE had the highest significant cytotoxic activity with IC_{50} of 0.01±0.00 µg/mL, followed by SCLE and DME with IC_{50} of 12.59±0.88 µg/mL and 35.80±1.45 µg/mL, respectively. The RSE also showed significant cytotoxicity against SKOV-3 with IC_{50} of 54.46 µg/mL. Only RSW showed significant cytotoxicity against Hela with an IC_{50} value of 84.48 µg/mL. However,

the cytotoxicity activity of all samples was less than paclitaxel which was used as a positive control, as shown in **Table 4-4 to 4-6**.

Table 4-4 Cytotoxic activity of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients against endometrioid adenocarcinoma cell (HEC-1-A) (Mean±SEM, N=3)

Sample	Concentration (µg/mL)	Percentage of Inhibition (%)	IC_{50} (µg/mL)
MRME	100	36.41±2.26	>100
MRMW	100	1.82±0.99	>100
SCE	100	8.70±1.22	>100
SCW	100	-9.62±0.66	>100
DME	100	93.56±2.30	48.26±4.88*
	50	56.28±7.56	
	25	14.43±1.60	
	12.5	5.80±1.15	
DMW	100	-9.82±0.59	>100
SCLE	100	57.56±1.17	69.29±4.47*
	50	47.04±1.34	
	25	43.04±1.34	
	12.5	38.97±2.51	
SCLW	100	-8.04±0.77	>100
RSE	100	99.19±0.08	30.06±0.79*
	50	89.43±4.83	
	25	42.63±0.57	
	12.5	33.53±1.59	

* Significant difference at the p-value < 0.05 compared with paclitaxel

Table 4-4 Cytotoxic activity of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients against endometrioid adenocarcinoma cell (HEC-1-A) (Mean±SEM, N=3) (Cont.)

Sample	Concentration (µg/mL)	Percentage of Inhibition (%)	IC_{50} (µg/mL)
RSW	100	17.69±2.22	>100
CIE	100	40.19±0.42	>100
CIW	100	-4.57±0.68	>100
Paclitaxel	0.01 pg/mL	94.94±1.04	0.00006±0.00 pg/mL
	0.001 pg/mL	88.67±1.53	
	0.0001 pg/mL	78.78±2.84	
	0.00001 pg/mL	14.75±0.65	

* Significant difference at the p-value < 0.05 compared with paclitaxel

Table 4-5 Cytotoxic activity of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients against cervical cancer cell (HeLa) (Mean±SEM, N=3)

Sample	Concentration (µg/mL)	Percentage of Inhibition (%)	IC_{50} (µg/mL)
MRME	0.391	70.98±2.00	0.10±0.00*
	0.195	64.75±2.07	
	0.097	50.39±1.73	
	0.049	28.58±0.93	
MRMW	100	8.86±0.68	>100
SCE	100	9.75±0.91	>100
SCW	100	5.63±0.45	>100
DME	100	99.31±0.12	35.80±1.45*
	50	84.99±4.79	
	25	27.35±2.18	
	12.5	15.83±1.79	
DMW	100	7.79±0.53	>100

Table 4-5 Cytotoxic activity of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients against cervical cancer cell (HeLa) (Mean±SEM, N=3) (Cont.)

Sample	Concentration (µg/mL)	Percentage of Inhibition (%)	IC_{50} (µg/mL)
SCLE	50	65.92±2.72	12.59±0.88*
	25	58.59±1.97	
	12.5	50.62±1.94	
	6.25	27.10±1.68	
SCLW	100	2.70±0.04	>100
RSE	0.024	67.35±2.07	0.01±0.00*
	0.012	56.54±0.63	
	0.006	48.36±1.18	
	0.003	36.15±3.07	
RSW	100	63.85±1.52	84.48±2.24*
	50	23.96±2.50	
	25	12.59±1.47	
	12.5	6.83±1.67	
CIE	100	41.34±0.97	>100
CIW	100	4.63±0.04	>100
Astilbin	100	3.04±0.28	>100
Paclitaxel	0.01 pg/mL	84.92±1.66	0.0001±0.00 pg/mL
	0.001 pg/mL	73.69±2.34	
	0.0001 pg/mL	48.65±0.62	
	0.00001 pg/mL	33.33±2.31	

* Significant difference at the p-value < 0.05 compared with paclitaxel

Table 4-6 Cytotoxic activity of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients against ovarian cancer cell (SKOV-3) (Mean±SEM, N=3)

Sample	Concentration (µg/mL)	Percentage of Inhibition (%)	IC_{50} (µg/mL)
MRME	100	12.62±0.46	>100
MRMW	100	-2.72±0.30	>100
SCE	100	4.05±0.66	>100
SCW	100	-11.55±2.73	>100
DME	100	42.33±1.37	>100
DMW	100	-8.95±0.14	>100
SCLE	100	33.45±0.17	>100
SCLW	100	-3.55±0.40	>100
RSE	100	89.02±0.16	54.46±0.67*
	50	43.28±0.80	
	25	3.13±1.43	
	12.5	-5.79±1.19	
RSW	100	-3.82±0.88	>100
CIE	100	27.83±0.59	>100
CIW	100	0.74±0.00	>100
Paclitaxel	10 ng/mL	68.78±1.16	0.1±0.00 ng/mL
	1 ng/mL	61.91±3.12	
	0.1 ng/mL	46.91±0.89	
	0.01 ng/mL	36.15±0.64	

* Significant difference at the p-value < 0.05 compared with paclitaxel

In addition, all samples were investigated cytotoxic activity against normal lung fibroblast (MRC5) and the immortalized human keratinocyte (HaCaT). RSE showed the highest toxicity to MRC-5 with IC_{50} of 0.004 µg/mL, followed by MRME, SCLE, DME, and RSW with IC_{50} of 0.10, 36.84, 71.06, and 79.37 µg/mL, respectively, as shown in **Table 4-7**. Moreover, the results of cytotoxic activity against HaCaT showed that only

RSE and DME was a moderate cytotoxic activity against HaCaT with IC_{50} of 24.12 and 90.57 µg/mL, while the other extracts had non-toxic with IC_{50} values of more than 100 µg/mL, as shown in **Table 4-8**.

Table 4-7 Cytotoxic activity of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients against normal lung fibroblast (MRC-5) (Mean±SEM, N=3)

Sample	Concentration (µg/mL)	Percentage of Inhibition (%)	IC_{50} (µg/mL)
MRME	0.195	67.21±4.37	0.10±0.02
	0.097	50.72±1.62	
	0.049	37.67±1.46	
	0.024	18.19±0.60	
MRMW	100	4.14±0.21	>100
SCE	100	13.80±1.51	>100
SCW	100	-0.62±0.35	>100
DME	100	89.92±1.46	71.06±0.13
	50	23.43±0.40	
	25	0.45±0.11	
	12.5	-3.66±0.21	
DMW	100	-4.23±0.18	>100
SCLE	100	78.21±4.03	36.84±4.07
	50	58.17±1.97	
	25	40.06±2.66	
	12.5	29.61±3.08	
SCLW	100	-2.49±0.00	>100
RSE	0.024	72.71±2.91	0.004±0.00
	0.012	66.85±3.40	
	0.006	56.83±1.85	
	0.003	46.85±0.59	

Table 4-7 Cytotoxic activity of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients against normal lung fibroblast (MRC-5) (Mean±SEM, N=3) (Cont.)

Sample	Concentration (µg/mL)	Percentage of Inhibition (%)	IC_{50} (µg/mL)
RSW	100	56.79±5.31	79.37±1.75
	50	30.82±3.27	
	25	6.34±0.54	
	12.5	-0.61±0.82	
CIE	100	24.89±0.52	>100
CIW	100	2.51±0.17	>100
Astilbin	100	0.19±0.01	>100

Table 4-8 Cytotoxic activity of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy and its plant ingredients against the immortalized human keratinocytes cell (HaCaT) (Mean±SEM, N=3)

Crude extracts	Concentration (µg/mL)	Percentage of Inhibition (%)	IC_{50} (µg/mL)
MRME	100	44.96±2.12	>100
MRMW	100	-18.79±0.94	>100
SCE	100	-5.57±0.23	>100
SCW	100	-17.17±0.01	>100
DME	100	66.91±0.41	90.57±0.13
	50	0.84±0.58	
	25	-2.60±1.07	
	12.5	-11.93±0.94	
DMW	100	-14.28±0.78	>100
SCLE	100	28.15±0.88	>100
SCLW	100	-9.95±1.55	>100
RSE	100	98.95±0.17	24.12±0.64
	50	89.34±1.37	
	25	51.21±0.82	
	12.5	41.00±0.95	
RSW	100	5.79±0.12	>100
CIE	100	-2.33±0.44	>100
CIW	100	-11.95±0.60	>100
Astilbin	100	-2.55±1.31	>100

Based on NCI guidelines, a crude extract and a pure compound are generally considered to have high cytotoxic activity when the IC_{50} value of the crude extract and the pure compound is not more than 20 and 4 µg/mL, respectively (Alabsi et al., 2016). Moreover, the extract showing an IC_{50} range between 21-200 µg/mL is moderately cytotoxic. The weak cytotoxic activity was defined when IC_{50} of extract

ranged between 201-500 µg/mL, and there was no cytotoxic activity when IC_{50} was more than 500 µg/mL (Damasuri & Sholikhah, 2020; Sajjadi et al., 2015). In this study, MRME was high cytotoxicity on HeLa. However, it also showed high cytotoxicity on MRC-5 but had no toxicity on HaCaT. The selective index of MRME was 1 when compared with MRC-5, but it was more than 1,000 when compared with HaCaT, as shown in **Table 4-9.** MRC-5 cells are fibroblast, while HaCaT cells are immortalized human keratinocyte lines or spontaneously transformed human epithelial cells (Boukamp et al., 1988; Das et al., 2014). The cell type of HaCaT is similar to HeLa, SKOV-3, and HEC-1A cells. Thus, the selectivity index of MRME and its ingredients should be compared to HaCat cells. The results demonstrated that MRME is high cytotoxicity on HeLa and without toxicity in the HaCaT cells.

The previous study reported that re-evaluating other biosystems is needed for confirmation when the selective index is 1 (Indrayanto et al., 2021). Thus, the cytotoxicity of MRME against MRC-5 should be confirmed in different environments or methods.

Plant ingredients of MRM showed cytotoxicity against cancer cell lines, especially for HeLa. RSE was the most potent anticancer activity against three cancer cell lines and MRC-5 except HaCaT. In addition, *R. siamense* belongs to the Annonaceae family. The various plants in the Annonaceae family showed high cytotoxic activity, such as *Annona muricata, and Goniothalamus macrophyllus. A. muricata* inhibited the proliferation of HeLa, PC-3, HL-60, and MDA-MB-435S cancer cell lines (Gavamukulya et al., 2019; George et al., 2012; Pieme et al., 2014). Its active compounds were acetogenins, alkaloids, phenols, and other compounds (Gavamukulya et al., 2017). Moreover, *G. macrophyllus* showed high cytotoxic activity against lung, colon, and breast cancer cells and moderate toxicity against skin fibroblast (ST3) and human fibroblast (HF) and HeLa cell lines (Alabsi et al., 2013; Wattanapiromsakul et al., 2005). RSE showed the highest cytotoxic activity against cancer cell lines in this study. Therefore, *R. siamense* may contain similar chemical compounds to other plants in the Annonaceae family. Moreover, *R. siamense* has found benzoyl groups such as toussaintine C, N-(4-hydroxyphenethyl) cinnamamide,

melodamide A, and uvangoletin. Moreover, chalcone derivatives such as 2′,4′-dihydroxy-4,6′-dimethoxychalcone, 4,4′-dihydroxy2′,6′-dimethoxychalcone, helichrysetin, 2′,4,4′-trihydroxy-6′-methoxy-3′(3″-hydroxybenzyl) dihydrochalcone, 2′,4′-dihydroxy-4,6′-dimethoxy-3′(3″-hydroxybenzyl)-dihydrochalcone, 4′,4-dihydroxy-2′,6′-dimethoxydihydrochalcone, 2′,4′-dihydroxy4′,6′-dimethoxydihydrochalcone, and 2′,4,4′-trihydroxy-6′-methoxydihydrochalcone have been found in *R. siamense*. In addition, the chalcone derivatives induced the cell cycle arrest at the G2/M phase and triggered apoptosis via caspases-3 and -9 in human breast cancer MCF-7 cells (Jaidee et al., 2019; Intaranongpai, 2006; Hongnak et al., 2015; Modzelewska et al., 2006; Gao et al., 2020). Thus, chalcone derivatives may be active compounds of *R. siamense* to inhibit other cancer cell lines.

Table 4-9 The IC_{50} values of cytotoxic activity and selective index (SI) of MRM extract, its plant ingredients extract, and astilbin against ovarian cancer cell (SKOV-3), uterine cancer cell (HEC-1-A), cervical cancer cell (HeLa), normal lung fibroblast (MRC-5), and the immortalized human keratinocytes cell (HaCaT) (Mean±SEM, N=3)

Crude extracts	**IC_{50} of Cancer cell lines (μg/mL)** (SI value compared to MRC-5 and HaCaT)			**IC_{50} of Normal cell lines (μg/mL)**	
	HEC-1-A	HeLa	SKOV-3	MRC-5	HaCaT
MRME	>100	**0.10±0.00*** (1.00, >1,000)	>100	0.10±0.02	>100
MRMW	>100	>100	>100	>100	>100
SCE	>100	>100	>100	>100	>100
SCW	>100	>100	>100	>100	>100
DME	**48.26±4.88*** (1.47, 1.88)	**35.80±1.45*** (1.98, 2.53)	>100	71.06±0.13	90.57±0.13
DMW	>100	>100	>100	>100	>100
SCLE	**69.29±4.47*** (0.53, >1.44)	**12.59±0.88*** (2.93, >7.94)	>100	36.84±4.07	>100
SCLW	>100	>100	>100	>100	>100
RSE	**30.06±0.79*** ($1.33x10^{-4}$, 0.80)	**0.01±0.00*** (0.57, 3,445.71)	**54.46±0.67*** ($7.40x10^{-5}$, 0.44)	0.004±0.00	24.12±0.64
RSW	>100	**84.48±2.24*** (0.94, >1.18)	>100	79.37±1.75	>100
CIE	>100	>100	>100	>100	>100
CIW	>100	>100	>100	>100	>100
Astilbin	NT	>100	NT	>100	>100
Paclitaxel	0.00006±0.00 pg/mL	0.0001±0.00 pg/mL	0.1±0.00 ng/mL	NT	NT

NT=Not tested, * Significant difference at the p-value < 0.05 compared with paclitaxel

DME showed cytotoxicity against HEC-1-A and Hela cells. DME inhibited the growth of the HeLa cell line with an IC_{50} value of 35.80 µg/mL, similar to the inhibition previously reported for DME (Itharat et al., 2014). The isolated compounds of *D. membranaceae* consisted of 5,6-dihydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene, 5-trihydroxy-2,4,6-dimethoxy-9,10-dihydrophenanthrene, dioscorealide A, dioscorealide B, panthogenin B, and epipanthogenin B (Itharat et al., 2014; Thongdeeying, 2016). In addition, two dihydrophenanthrene compounds have exhibited cytotoxic activity against the HeLa cell line with IC_{50} values of less than 30 µg/mL (Itharat et al., 2014). Moreover, other isolated compounds from *D. membranacea,* including dioscorealide B and dioscoreanone, inhibited the growth of large cell lung carcinoma COR-L23, colon adenocarcinoma LS174T, and breast adenocarcinoma MCF-7 (Itharat et al., 2003). The previous report found that Hua-Khao-Yen has various species from at least three genera and five species, *D. membranacea* Pierre. (Dioscoreaceae), *D. burmanica* Prain ex Burkill. (Dioscoreaceae), *S. corbularia* Kunth. (Smilacaceae), *S. glabra* Roxb. (Smilacaceae), and *Pygmeopremna herbacea* Prain et Burkill. (Verbenaceae). However, the highest cytotoxic activity was found in *D. membranacea*. The ethanolic extract of *D. membranacea* showed cytotoxic activity against various cancer cell lines, including COR-L23, LS-174T, MCF-7, SVK-141, HeLa, and HepG2 (Itharat et al., 2004). Furthermore, it has antioxidant and antimicrobial activity against *S. aureus*, *B.subtilis* and *E. floccosum*. Moreover, the best extract from the genera Smilax was *S. corbularia*, which showed cytotoxic against HepG2 and antioxidant activity and antimicrobial activity against *S. aureus* and *B.subtilis* (Itharat, 2010). Therefore, these reports supported the selection of *D. membranacea* and *S. corbularia* to be avtive ingredients in MRM remedy. In the present study, SCLE exhibited cytotoxicity against HEC-1-A and Hela cells. *S. collinsae* consisted of p-coumaric acid, ferulic acid, p-hydroxybenzoic acid, didehydrostemofoline, and stemofoline (Kongkiatpaiboon&Gritsanapan, 2012; Rutnakornpituk, 2018). Previously report indicated that co-administration of stemofoline and doxorubicin significantly inhibited the growth rate of drug resistance leukemic cells K562 in a dose-dependent manner (Umsumarng et al., 2018). However, the cytotoxic activity of *S. collinsae* extract

and its isolated compounds on other cancer cells have been reported. Thai traditional scripture refers Non-tai yak, a medicinal plant, as an anti-cancer drug. Non-tai yak has two species, including *Stemona collinsae* and *S. tuberosa* (Smitinand, 2014). However, they have different morphological characteristics. *S. tuberosa* has a long tuberous root, while *S. collinsae* has a short tuberous root (Homhual et al., n.d.). The previous report found that *S. tuberosa* had moderate cytotoxic activity against four cancer cell lines, including KB (a human epidermal carcinoma), MCF7 (human breast carcinoma), SK-LU-1 (human lung carcinoma), HepG2 (hepatocellular carcinoma), and human medullary thyroid carcinomas (Khamko et al., 2013; Li et al., 2007). Thus, *S. tuberosa* may be used as an ingredient of MRM to compare cytotoxic activity with *S. collinsae.*

Moreover, this study used the root of the white flower *C. indica* as an ingredient of MRM. However, CIE showed no cytotoxicity against HEC-1-A, SKOV-3, and HeLa cells. In contrast, the previous study of the root extract of red flower *C. indica* showed cytotoxicity against P388 leukemia cells with ED_{50} of 64.50 µg/mL (hexane extract) and 133.50 µg/mL (ethanol extract) (Chainakul et al., 2001). In addition, the previous study compared the cytotoxic activity of two cultivar groups, including green and red cultivar groups, of the red flower *Canna* species against colon cancer cells. The previous report found the highest cytotoxicity in the dichloromethane extract of the red cultivar group of *Canna* species. Moreover, the phytochemical contents of the red cultivar group differed from the green cultivar group (Widyarini et al., 2020). The previous report showed different cultivar groups lead to different biological effects. However, the red flower *Canna* species showed more active in cytotoxic activity. Thus, these reports support the concept of Thai folk medicine, which believed red flowers were more cvtotoxic activity than white flowers.

MRME inhibited the proliferation of HeLa cells, while MRMW showed no cytotoxic activity on all tested cell lines. However, astilbin, the analytical marker of MRM, showed no cytotoxic activity on HeLa cells. RSE was the most potent anticancer cell, followed by SCLE and DME, as shown in **Table 4-9**. In addition, they contained isolated compounds that showed cytotoxic activity against cancer cell lines (Modzelewska et al., 2006; Gao et al., 2020; Itharat et al., 2014; Thongdeeying, 2016;

Umsumarng et al., 2018). Thus, active compounds of MRME may be found in *R. siamense*, *S. collinsae*, and *D. membranaceae*.

4.4 Antioxidant activities

In this study, there are two methods for measuring the antioxidant power of samples, including a chemical-based assay and a cell-based assay. Ferric Antioxidant Power (FRAP) assay is used to measure the reduction of the ferric iron (Fe^{3+}) to the ferrous ion (Fe^{2+}) colored form in the presence of antioxidants. The reduction of superoxide anion in HL-60 cell lines is used to detect antioxidant activity in cells.

4.4.1 Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidant activity of MRM and its plant ingredients using ferric reducing antioxidant power assay was shown in **Table 4-10.** MRME was higher ferric-reducing power than MRMW, with FRAP and TEAC values of 384.15 mgFe^{2+}/g extracts (1.38±0.03 mM Fe^{2+}/g extracts) and 144.59 mgTrolox/g, respectively. For the ethanolic extract of plant ingredients, SCE was the highest antioxidant activity with a FRAP value of 636.44 mgFe^{2+}/g extracts (2.28±0.08 mM Fe^{2+}/g extracts), followed by RSE, CIE, DME, and SCLE. On the other hand, MRMW showed a FRAP value of 79.12 mgFe^{2+}/g of extract. RSW, its plant ingredient extract, showed the highest FRAP value with 881.40 mgFe^{2+}/g extracts (3.17±0.09 mM Fe^{2+}/g extracts, followed by SCW, CIW, DMW, and SCLW. Moreover, astilbin, the analytical marker of MRM and isolated compound in SCE, showed the highest FRAP value and TEAC value of 1,227 mgFe^{2+}/g extracts and 572.98 mgTrolox/g, respectively.

The previous report found that green tea (*Camellia sinesis*) is widely known to have high antioxidants, such as phenolic catechins (Musial et al., 2020). The study found that water extract of *C. sinesis* leaves by freeze-dried method showed the highest ferric-reducing power (FRAP value) than the superheated steam-dried extract and oven-dried extract with FRAP values of 5.07± 0.01, 2.75±0.01, and 2.07±0.01 mM Fe^{2+}/g, respectively (Roslan et al., 2020). It could be higher the FRAP value, the better the antioxidant activity. In addition, Ma-Kham-Pom (*Phyllanthus emblica*) is well known

high potent antioxidant properties due to high vitamin C and polyphenol compounds derived from nature such as rutin, quercetin, and kaemferol (Jatiyananda & Thapphasaraphong, 2017). The previous report showed the ethanolic extract of *P. emblica* represented a TEAC value of 392.98 mgTrolox/g of extract (Chaiyasut et al., 2017). MRME and CIE had moderate antioxidant activity compared with *C. sinesis* and *P. emblica*. On the other hand, RSE, RSW, and SCE had high antioxidant activity because their FRAP values were similar to *C. sinesis*. At the same time, the RSW was more antioxidant power than *P. emblica.*

4.4.2 Nitroblue tetrazolium (NBT) reduction assay

In this study, the cytotoxicity of all samples on HL-60 cells was measured by MTT assay before testing for the superoxide radical scavenging activity. The extract that showed the toxic effect on HL-60 was not determined antioxidant activity. The results showed that MRME had high antioxidant activity to reduce superoxide radicals with IC_{50} of 45.06 µg/mL, while MRMW had no antioxidant effect. Interestingly, the RSE had the highest anti-superoxide radical activity with IC_{50} of 21.30 µg/mL, followed by RSW, SCE, CIE, SCW, and CIW, respectively. However, all samples showed less antioxidant effect than the positive control (propyl gallate), as shown in **Table 4-11**.

Table 4-10 The result of antioxidant activity by ferric reducing antioxidant power (FRAP) assay of MRM, its plant ingredients, and astilbin (Mean±SEM, N=3)

Sample	**FRAP value (mgFe^{2+}/g)** *(FRAP value, mMFe^{2+}/g)*	**TEAC value (mgTrolox/g)** *(TEAC value, mMTrolox/g)*
MRME	384.15±9.18 *(1.38±0.03)*	144.59±3.54 *(0.58±0.01)*
MRMW	79.12±2.10 *(0.28±0.01)*	27.05±0.81 *(0.11±0.00)*
SCE	636.44±22.91 *(2.28±0.08)*	241.80±8.83 *(0.96±0.03)*
SCW	280.31±23.56 *(1.01±0.08)*	104.58±9.08 *(0.42±0.04)*
DME	230.63±11.45 *(0.83±0.04)*	85.43±4.41 *(0.34±0.02)*
DMW	26.14±1.21 *(0.09±0.00)*	6.64±0.46 *(0.03±0.00)*
SCLE	47.21±4.89 *(0.17±0.02)*	14.76±1.89 *(0.06±0.01)*
SCLW	19.49±1.13 (0.07*±0.00)*	4.08±0.43 *(0.06±0.01)*
RSE	589.05±20.63 *(2.11±0.07)*	223.54±7.95 *(0.89±0.03)*
RSW	881.40±26.03 *(3.17±0.09)*	336.19±10.03 *(1.34±0.04)*
CIE	424.63±13.77 *(1.52±0.05)*	160.19±5.30 *(0.64±0.02)*
CIW	109.91±2.49 *(0.40±0.01)*	38.92±0.96 *(0.15±0.00)*
Astilbin	1,227.82±17.51 *(2.20±0.06)*	572.98±9.25 *(0.93±0.03)*

Table 4-11 The survival rate and inhibition effect of MRM and its plant ingredients on superoxide radical scavenging assay (Mean±SEM, N=3)

Crude extracts	Concentration (µg/mL)	Survival rate (%)	Inhibition effect (%)	IC_{50} (µg/mL)
MRME	250	93.58±3.55	72.66±1.88	45.06±7.80
	125	90.67±5.63	62.07±3.22	
	62.50	108.02±8.78	53.72±1.44	
	31.25	104.63±6.61	45.97±2.29	
MRMW	1000	105.38±4.30	35.45±4.93	>1000
SCE	125	107.94±5.44	90.67±2.37	33.22±5.78
	62.50	107.73±5.44	79.07±2.97	
	31.25	107.73±8.93	65.72±3.44	
	15.62	87.37±3.69	34.76±0.24	
SCW	500	103.91±2.43	62.54±0.63	117.46±2.20*
	250	101.93±0.55	53.28±1.40	
	125	91.49±2.80	51.32±0.40	
	62.50	102.18±1.13	36.36±1.50	
DME	250	61.39±3.82	Not Tested	>125
	125	75.58±1.36	18.41±5.76	
DMW	1000	83.70±0.22	14.61±0.03	>1000
SCLE	250	29.94±8.83	Not Tested	>125
	125	84.14±5.87	-4.00±3.89	
SCLW	1000	90.95±5.95	23.01±0.95	>1000
RSE	125	110.55±4.59	88.03±5.48	21.30±1.34*
	62.50	100.78±6.21	64.98±1.88	
	31.25	104.71±5.69	59.54±1.59	
	15.62	106.03±2.93	43.53±1.38	

* Significant difference at the p-value < 0.05 compared with propyl gallate

Table 4-11 The survival rate and inhibition effect of MRM and its plant ingredients on superoxide radical scavenging assay (Mean±SEM, N=3) (Cont.)

Crude extracts	Concentration (μg/mL)	Survival rate (%)	Inhibition effect (%)	IC_{50} (μg/mL)
RSW	125	98.96±3.57	82.97±2.74	27.68±1.95*
	62.50	96.63±3.27	69.03±3.38	
	31.25	98.61±1.36	53.96±2.22	
	15.62	97.30±3.59	34.58±4.11	
CIE	250	47.46±0.31	Not Tested	
	125	82.89±2.85	55.22±1.84	96.41±9.50*
	62.50	112.20±8.14	42.70±2.99	
	31.25	116.06±7.80	31.10±1.81	
	15.62	100.30±3.74	18.59±5.71	
CIW	1000	89.53±5.11	53.71±0.61	759.86±45.09*
	500	92.60±1.48	45.73±1.57	
	250	102.95±3.53	38.01±3.43	
	125	103.32±2.25	29.95±0.77	
Astilbin	250	108.61±8.82	81.72±5.19	61.73±1.99*
	125	111.78±2.31	63.07±4.59	
	62.50	116.08±4.03	50.35±1.16	
	31.25	108.17±6.03	26.39±4.40	
Propyl Gallate	31.25	95.26±2.59	71.19±2.23	11.32±0.38
	15.62	91.46±1.96	58.27±1.15	
	7.81	103.64±0.92	41.62±1.04	
	3.91	85.79±4.36	31.29±2.74	

* Significant difference at the p-value < 0.05 compared with propyl gallate

A free radical contains an unpaired electron in an atomic orbital. There are many free radicals, such as hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical

(Lobo et al., 2010). They can damage DNA leading to mutation and cancer (Čipak Gašparović, 2020). MRME had high antioxidant activity, including ferric reducing ability and superoxide anion reduction. Astilbin also showed similar results to MRME. The ingredients of MRME that showed high antioxidant activity were RSE and SCE. The previous report showed that total phenolic, flavonoid contents, and DPPH radical scavenging activity of *S. corbularia* regenerated shoots increased along the culture periods. The ethanolic extract of the 12-week-old regenerated shoots was the highest contents of total phenolic (84.92±5.88 mgGAE/g dry extract), total flavonoid (107.56±7.19 mg CE/g dry extract) and DPPH radical scavenging with EC_{50} 15.75±0.88 µg/mL (Jirakiattikul et al., 2019). In addition, the previous report found that quercetin, the pure compound of SCE, was the highest antioxidant activities using the DPPH assay with EC_{50} values of 0.6±0.1 µg/ml, followed by astilbin (EC_{50} values of 2.5 µg/ml) and engeletin (EC_{50} values of 3.9 µg/ml) (Kejik, 2008). For *R. siamense*, it has not been reported as having antioxidant activity. However, its roots contained dimeric chalcone derivatives, welwitschin E, flavonoids, a cyclohexane oxide derivative, and aromatic aldehyde (Salae et al., 2017). The previous report showed that DME was the highest antioxidant activity using the DPPH assay with EC_{50} values of 10.34±1.40 µg/mL compared with BHT and DMW (Ruangnoo & Itharat, 2010). Moreover, DME inhibited lipid peroxidation with EC_{50} values of 8.10±1.21 µg/mL (Itharat, 2010). The isolated compounds of DME were dioscorealides A, β-sitosterol, diosgenin-(3-O-α-L-rhamnopyranosyl (1⟶2)-β-D-glucopyranoside, glucoside diosgenin 3-O-β-D-glucopyranosyl (1⟶3)-β-D-glucopyranoside, stigmasterol, β-D-sitosterol, dioscorealides B, and dioscoreanone. They also showed high antioxidant activity by using the DPPH assay with EC_{50} values of 0.8±0.2, 2.81±0.85, 6.24±0.69, 7.13±0.59, 8.05±0.68, 12.95±1.05, 21.7±0.4, and 33.1±0.56 µg/mL, respectively (Itharat et al., 2007). In this study, CIE inhibited superoxide radicals with an IC_{50} value of 96.41 µg/ml. The acetone extract of the rhizome of *C. indica* has been reported to scavenge DPPH radical and ABTS radical (Ayusman et al., 2020). However, its antioxidant compounds are unknown.

4.5 Anti-inflammatory activity

4.5.1 Nitric oxide inhibition assay in LPS-stimulated RAW 264.7 macrophages

MRM and its plant ingredient extracts were investigated to inhibit nitric oxide production in LPS-stimulated RAW264.7 cells. The results showed that MRME had a better inhibition effect than MRMW and prednisolone with IC_{50} of 45.06, >100, and 64.16 µg/mL, respectively. Moreover, the results of plant ingredient extracts showed that DME had the highest effect on inhibiting nitric oxide production with IC_{50} of 15.75 µg/mL, followed by RSE, SCLE, and CIE (IC_{50} values = 44.82, 71.41, and 91.79 µg/mL), as shown in **Table 4-12**.

Table 4-12 The inhibition effect of nitric oxide production and cytotoxic activity of MRM and its plant ingredients on RAW264.7 cells (Mean±SEM, N=3)

Sample	Inhibition effect at various concentrations (%) *(Survival rate, %)*				IC_{50} (μg/mL)
	100 μg/mL	50 μg/mL	25 μg/mL	12.50 μg/mL	
MRME	72.43±4.77 *(99.85±5.27)*	53.57±2.61 *(102.47±3.43)*	30.10±1.99 *(100.81±3.64)*	16.59±2.60 *(96.74±5.81)*	45.93±2.70
MRMW	11.25±4.15 *(105.48±3.53)*	NT	NT	NT	>100
SCE	21.79±7.05 *(85.71±15.27)*	NT	NT	NT	>100
SCW	6.83±0.43 *96.56±0.39*	NT	NT	NT	>100
DME	94.76±0.52 *(79.88±6.28)*	83.71±2.65 *(104.10±6.48)*	68.68±3.67 *(112.32±6.10)*	42.35±8.41 *(114.50±8.60)*	15.75±2.45*
DMW	4.10±0.55 *(91.99±0.79)*	NT	NT	NT	>100
SCLE	57.38±1.89 *(104.92±13.52)*	40.25±4.01 *(94.16±5.39)*	25.67±2.66 *(91.15±5.40)*	15.71±5.88 *(92.20±12.08)*	71.41±8.93
SCLW	18.33±0.12 *(100.08±0.01)*	NT	NT	NT	>100
RSE	71.45±0.19 *(71.91±4.86)*	55.65±2.16 *(72.66±3.31)*	26.61±2.83 *(78.23±2.78)*	9.34±2.34 *(85.81±7.48)*	44.82±2.22*
RSW	9.43±0.15 *(103.33±0.87)*	NT	NT	NT	>100

* Significant difference at the p-value < 0.05 compared with prednisolone

Table 4-12 The inhibition effect of nitric oxide production and cytotoxic activity of MRM and its plant ingredients on RAW264.7 cells (Mean±SEM, N=3) (Cont.)

Sample	Inhibition effect at various concentrations (%) *(Survival rate, %)*				IC_{50} (µg/mL)
	100 µg/mL	50 µg/mL	25 µg/mL	12.50 µg/mL	
CIE	52.38±0.62 *(71.48±2.12)*	34.09±2.57 *(80.88±3.44)*	20.24±3.86 *(80.16±5.46)*	9.73±3.38 *(85.16±4.25)*	91.79±2.29*
CIW	5.99±0.38 *(102.89±0.83)*	NT	NT	NT	>100
Astilbin	6.31±2.92 *(104.25±5.05)*	NT	NT	NT	>100
Prednisolone	62.64±3.06 *(92.75±2.91)*	44.06±2.37 *(93.21±1.16)*	33.43±1.00 *(85.44±2.34)*	29.78±3.53 *(100.29±4.36)*	64.16±6.04

* Significant difference at the p-value < 0.05 compared with prednisolone

4.5.2 Measurement of IL-6 cytokine production in LPS-stimulated RAW264.7

MRM and its plant ingredients extracts were investigated for inhibition of IL-6 production in RAW 264.7 cells using sandwich ELISA, as shown in **Table 4-13**. The result showed that MRME had the highest inhibition effect of IL-6 cytokine with IC_{50} of 35.47 µg/mL, but MRMW showed no inhibition effect on IL-6. Thus, the plant ingredient extracts of MRME were investigated to inhibit IL-6 production. The results showed that DME and RSE inhibited IL-6 production with IC_{50} values of 39.57 and 99.39 µg/mL, respectively. However, the other plant ingredient extracts, including SCE, SCLE, and CIE, had no inhibitory effect on IL-6 cytokine with IC_{50} more than 100 µg/mL.

Table 4-13 The inhibition effect of IL-6 cytokine production in LPS-stimulated RAW 264.7 cell (Mean±SEM, N=3)

Sample	%Inhibition at various concentrations (µg/mL)				IC_{50}
	100	50	25	12.50	(µg/mL)
MRME	90.11±0.91	65.14±1.98	39.44±4.69	36.63±2.08	35.47±3.29
MRMW	34.23±0.37	NT	NT	NT	>100
SCE	18.84±3.40	NT	NT	NT	>100
DME	98.94±0.22	71.24±2.69	17.84±5.94	3.16±3.63	39.57±2.34
SCLE	44.32±0.73	NT	NT	NT	>100
RSE	50.30±0.79	26.82±0.49	13.93±3.86	2.16±1.55	99.39±0.09
CIE	31.74±1.64	NT	NT	NT	>100
Astilbin	-13.75±1.96	NT	NT	NT	>100
Prednisolone	52.86±1.99	29.14±1.74	21.75±11.68	-9.89±1.26	62.29±1.21

NT = Not tested, * Significant difference at the p-value < 0.05 compared with prednisolone

4.5.3 Assay for inhibition of extract on TNF- α releasing from RAW 264.7 cell line

MRME, MRMW, and astilbin were investigated to inhibit TNF- α releasing from RAW 264.7 cells. The results found that all samples had no inhibition effect on TNF- α releasing with IC_{50} more than 100 µg/mL, as shown in **Table 4-14**.

Table 4-14 The inhibition effect of TNF- α releasing in LPS-stimulated RAW 264.7 cell (Mean±SEM, N=3)

Crude extracts	%Inhibition at various concentrations (µg/mL)				IC_{50}
	100	50	25	12.50	(µg/mL)
MRME	-10.52±3.69	NT	NT	NT	>100
MRMW	20.55±1.87	NT	NT	NT	>100
Astilbin	-13.75±1.96	NT	NT	NT	>100
Prednisolone	35.25±2.44	NT	NT	NT	>100

NT = Not tested, * Significant difference at the p-value < 0.05 compared with prednisolone

The nitric oxide (NO), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) can induce acute and chronic inflammation, which have shown essential roles in signaling in tumor cell growth (Zhou et al., 2018; Taher et al., 2018). This study showed that MRME could be an anti-inflammatory agent through inhibited nitric oxide and IL-6 production from RAW 264.7 cells. However, astilbin was not an active compound for the anti-inflammatory activity of MRME because it showed no inhibition effect on nitric oxide and inflammatory cytokines production. The plant ingredients of MRME that showed good anti-inflammatory activity were DME and RSE. The previous study indicated that DME could inhibit nitric oxide production with IC_{50} values of 23.6 μg/ml. The active compounds that were isolated from DME were naphthofuranoxepins (dioscorealide A, dioscorealide B), phenanthraquinone (dioscoreanone), steroids (β-sitosterol, stigmasterol, β-sitosterol-3-O-β-d-glucopyranoside), diosgenin-3-O-α-L-rhamnopyranosyl(1⟶2)-β-D-glucopyranoside, and steroidal saponins (diosgenin-3-O-β-d-glucopyranosyl(1⟶3)-β-D-glucopyranoside, diosgenin) (IC_{50} values = 3.5-72.9 μM) (Tewtrakul & Itharat, 2007; Hiransai, 2010). Furthermore, the extracts of *R. siamense* had high flavonoid compounds such as 2′,4′-dihydroxy-4,6′-dimethoxychalcone, 4,4′-dihydroxy2′,6′-dimethoxychalcone, 2′,4,4′-trihydroxy-6′-methoxy-3′(3″-hydroxybenzyl) dihydrochalcone,2′,4′-dihydroxy-4,6′-dimethoxy-3′(3″-hydroxybenzyl)-dihydrochalcone, 4′,4-dihydroxy-2′,6′-dimethoxydihydrochalcone, uvangoletin, 2′,4′-dihydroxy4′,6′-dimethoxydihydrochalcone, 2′,4,4′-trihydroxy-6′-methoxydihydrochalcone, flavokawain-A, tsugafolin, and kaempferol. The previous report found that the chalcone showed good anti-inflammatory activity. (Jaidee et al., 2019; Intaranongpai, 2006; Mahapatra et al., 2017)

Although SCE showed no anti-inflammatory activity in the present study, the previous report showed that it inhibited TNF-α and nitric oxide production with IC_{50} values of 61.97±0.9 and 83.90±3.8 μg/mL, respectively (Ruangnoo et al., 2012). The authors explained that *S. corbularia,* which was collected in January, showed the highest inhibition effect on nitric oxide production. On the other hand, *S. corbularia,* collected in June, showed less inhibition effect on nitric oxide. Thus, collecting *S. corbularia* in the winter could get high-quality plant material. This previous report also

related to the principle of Thai traditional medicine, which refers to collecting the rhizome in the winter. The rhizome stopped growing, collected secondary metabolites in winter, and showed high active ingredients this season (Ruangnoo et al., 2012). Another previous study found that the culture periods of *S. corbularia* affected its total phenolic and total flavonoid contents (Jirakiattikul et al., 2018). Furthermore, the quality of soil affected the active compounds in plants (Angioni et al., 2006; Appiah et al., 2022). Thus, the seasonal change and quality of the soil may be used to explain the different biological activities of plants in this study.

4.6 The study of antifungal activities by determination of minimal inhibitory concentrations (MIC) and Minimum fungicidal concentration (MFC)

The results of the MIC and MFC value of MRM and its plant ingredient extracts against *C. albicans* are shown in **Table 4-15**. In this study, MRME inhibited *C. albicans* with a MIC value of 2.5 mg/mL and MFC value of more than 5 mg/mL, while MRMW showed no antifungal activity. Furthermore, RSE, the plant ingredient of MRME, had good antifungal activity against *C. albicans* with a MIC value of 0.625 mg/mL. Another plant ingredient that showed antifungal activity was DME. DME inhibited *C. albicans* with a MIC value of 2.5 mg/mL.

The result of this study is consistent with the previous research, which found that the SCE showed no inhibition effect against *C. albicans* (Itharat, 2010). The dichloromethane-methanol, 95% ethanol, and aqueous extracts from the root of *S. collinsae* also showed no antifungal activity against *C. albicans* (Akanitapichat et al., 2005). However, the essential oil extracts from *C. indica* rhizome showed an inhibition zone of 12 mm against *C. albicans* (George, 2014). In contrast, the previous study found that DME showed no antifungal activity against *C. albicans* (Itharat, 2010). However, the previous study reported that some plant ingredients of MRM had high flavonoid and anthraquinones, including flavonoids, phenols, and phenolic glycosides, and showed good antifungal activity (Webster et al., 2008). In addition, the anthraquinones

with an OH group at the para position inhibited *C. albicans* biofilm formation (Manoharan et al., 2017).

Table 4-15 The result of MIC and MFC value of Ya-Kae-Ma-Reng-Nai-Mod-Look remedy (MRM) and its plant ingredients extracts against *C. albicans* ATCC 9028 (N=3)

Sample	MIC (mg/mL)	MFC (mg/mL)
MRME	2.5	>5
MRMW	>5	>5
SCE	>5	>5
SCW	>5	>5
DME	2.5	5
DMW	5	>5
SCLE	>5	>5
SCLW	>5	>5
RSE	0.625	>5
RSW	5	>5
CIE	>5	>5
CIW	>5	>5
Amphotericin B	0.125	0.5

All biological activity of MRM and its plant ingredient extracts were presented in **Table 4-16**. The results showed that MRME had higher biological activity than MRMW, especially cytotoxic activity against HeLa cells. The active compound of MRME that showed anticancer and anti-inflammatory activities is not astilbin. However, astilbin can be used as an active marker for the antioxidant activity of MRME. Moreover, the critical plant ingredient that showed good biological activity was RSE and DME. The other plant ingredient also showed some biological activity, such as SCE was a high antioxidant activity. Therefore, MRME was determined by the analytical marker, astilbin, and stability testing.

Table 4-16 Biological activity of MRM and its plant ingredient extracts (N=3)

Sample	Cytotoxic activity (IC_{50}, µg/mL)					Antioxidant activity			Anti-inflammatory activity (IC_{50}, µg/mL)			MIC (mg/mL)
	Cancer cell lines			Normal cell lines		FRAP Value ($mgFe^{2+}/g$)	TEAC Value (mgTrolox/g)	Superoxide anion (IC_{50}, µg/mL)	Nitric oxide	IL-6	TNF-α	
	HEC-1-A (SI)	HeLa (SI)	SKOV-3 (SI)	MRC-5	HaCaT							
MRME	>100	0.10±0.00* (>1,000)	>100	0.10±0.02	>100	384.15±9.18	144.59±3.54	45.06±7.80	45.93±2.70	35.47±3.29	>100	2.5
MRMW	>100	>100	>100	>100	>100	79.12±2.10	27.05±0.81	>1000	>100	>100	>100	>5
SCE	>100	>100	>100	>100	>100	636.44±22.91	241.80±8.83	33.22±5.78	>100	>100	NT	>5
SCW	>100	>100	>100	>100	>100	280.31±23.56	104.58±9.08	117.46±2.20*	>100	NT	NT	>5
DME	48.26±4.88* (1.88)	35.80±1.45* (2.53)	>100	71.06±0.13	90.57±0.13	230.63±11.45	85.43±4.41	>125	15.75±2.45*	39.57±2.34	NT	2.5
DMW	>100	>100	>100	>100	>100	26.14±1.21	6.64±0.46	>1000	>100	NT	NT	5
SCLE	69.29±4.47* (>1.44)	12.59±0.88* (>7.94)	>100	36.84±4.07	>100	47.21±4.89	14.76±1.89	>125	71.41±8.93	>100	NT	>5
SCLW	>100	>100	>100	>100	>100	19.49±1.13	4.08±0.43	>1000	>100	NT	NT	>5
RSE	30.06±0.79* (0.80)	0.01±0.00* (>3,445.71)	54.46±0.67* (0.44)	0.004±0.00	24.12±0.64	589.05±20.63	223.54±7.95	21.30±1.34*	44.82±2.22*	99.39±0.09	NT	0.625
RSW	>100	84.48±2.24* (>1.18)	>100	79.37±1.75	>100	881.40±26.03	336.19±10.03	27.68±1.95*	>100	NT	NT	5
CIE	>100	>100	>100	>100	>100	424.63±13.77	160.19±5.30	96.41±9.50*	91.79±2.29*	>100	NT	>5
CIW	>100	>100	>100	>100	>100	109.91±2.49	38.92±0.96	759.86±45.09*	>100	NT	NT	>5
Astilbin	NT	>100	NT	>100	>100	1,227.82±17.51	572.98±9.25	61.73±1.99*	>100	>100	>100	NT

SI = Selective index when compared with HaCaT cells, NT = Not tested, * Significant difference at the p-value < 0.05 compared with positive control

4.7 Quantitative analysis of the chemical marker in MRME using the HPLC technique

Ultraviolet-visible (UV-vis) spectroscopy is used to obtain the absorbance spectra of MRME. The pattern of UV-vis spectrum of MRME showed high absorption at 280 nm, as shown in **Figure 4-1**. Based on the previous result, It was familiar to UV-vis spectrum of astilbin, as shown in **Figure 4-2** (Jaicharoensub, 2020). Moreover, astilbin is an isolated compound in *S. corbularia*, one of the ingredients in the MRME. There has been no report of astilbin on cytotoxicity testing against ovarian cancer and endometrial adenocarcinoma. Thus, astilbin was used as an analytical marker of MRME. The astilbin content in MRME was confirmed by using the HPLC technique. Furthermore, astilbin was investigated for cytotoxic activity and biological activities.

The results showed that MRME extract expressed high antioxidant activity. Thus, the main compound of flavonoid in MRME was confirmed by using the HPLC technique. After analysis, astilbin was found as the main pure compound in MRME with a retention time of 20.39 minutes, as shown in **Figure 4-3**. The content of astilbin in MRME extracts was 32.54 ± 0.90 mg/g of extract. SCE also contained high astilbin with 159.66±3.87 mg/g of extract, as shown in **Table 4-17**.

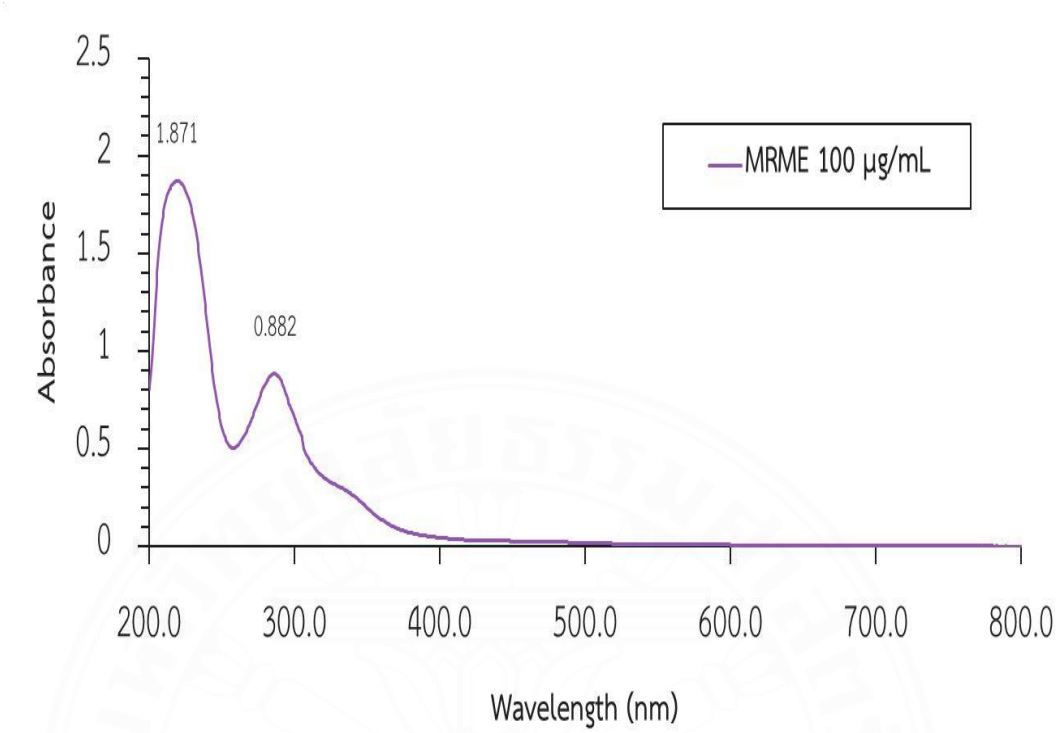

Figure 4-1 Scanning spectrophotometers UV-vis wavelength of MRME 100 µg/mL

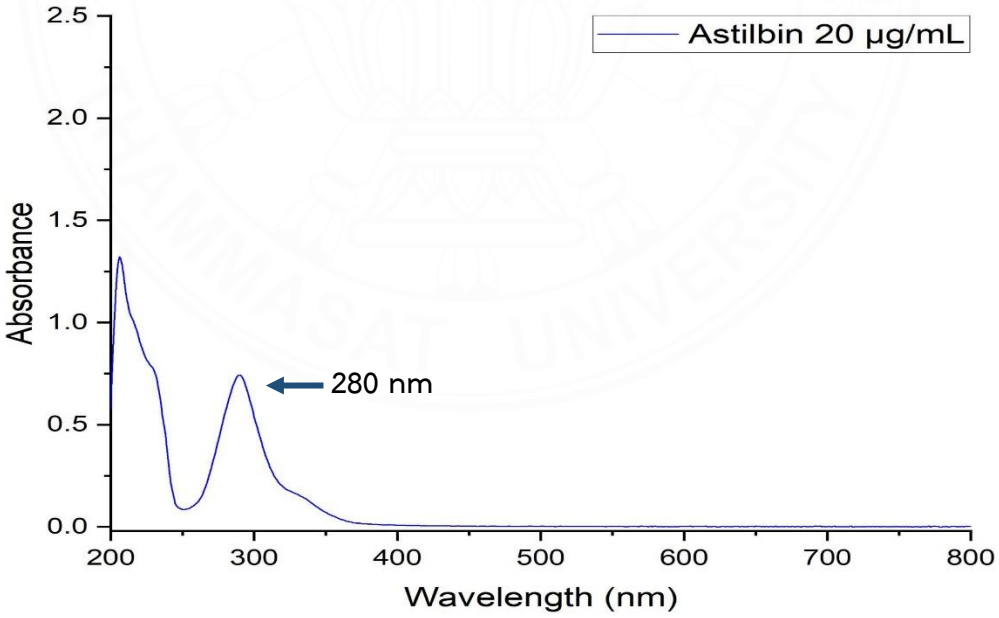

Figure 4-2 Scanning spectrophotometers UV-vis wavelength of astilbin 20 µg/mL (Jaicharoensub, 2020)

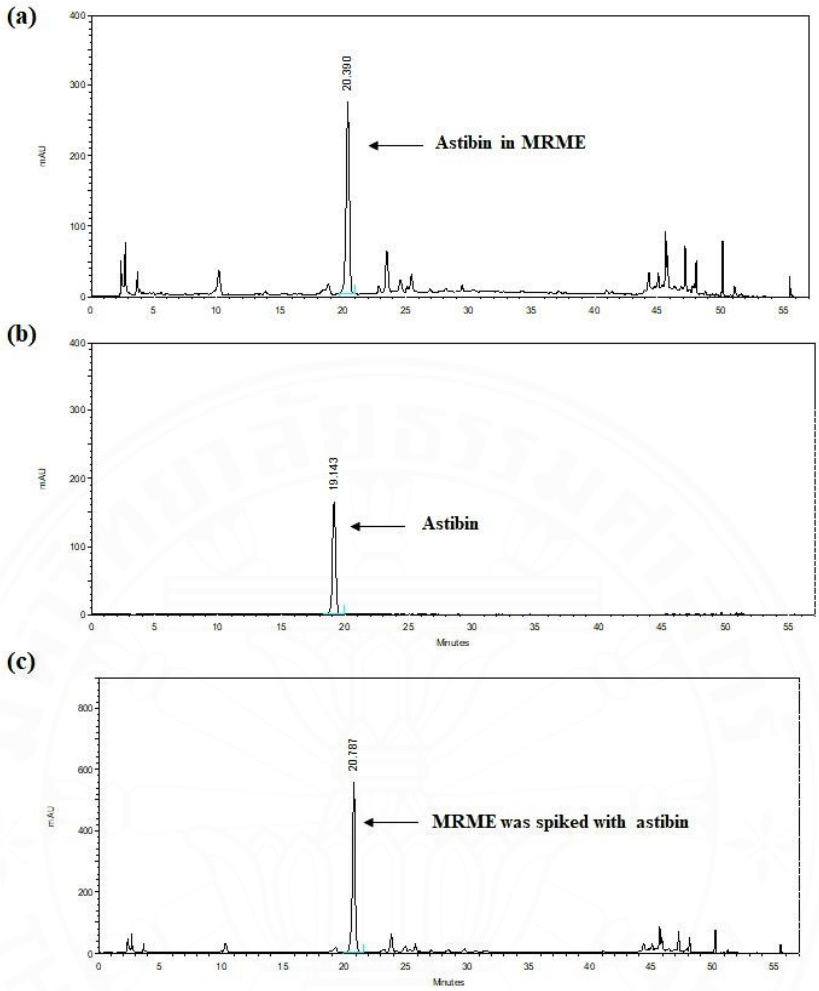

Figure 4-3 HPLC chromatograms of MRME 10 mg/mL (a), astilbin 200 µg/mL (b), and MRME 10 mg/mL was spiked with astilbin 200 µg/mL (c).

Table 4-17 The astilbin content in MRME and its plant ingredient extracts (Mean±SEM; N=3)

Sample	Astilbin content (mg/g)
MRME	32.54±0.90
SCE	159.66±3.87
DME	Not detectable
SCLE	Not detectable
RSE	Not detectable
CIE	Not detectable

4.8 Stability test of MRME

MRME was investigated through stability testing under the accelerated condition at 40±2°C with 75±5% RH for six months. The sample was collected on days 0, 30, 60, 90, 120, 150, and 180. After that, all samples were determined for cytotoxic activity against cervical cancer cells (HeLa) by SRB assay, antioxidant activity by FRAP assay, and anti-inflammatory activity by inhibiting nitric oxide production assay. The cytotoxic activity of MRME against HeLa after accelerated conditions is shown in **Table 4-18** and **Figure 4-4**.

Table 4-18 Cytotoxic activity of MRME against cervical cancer cell (HeLa) after stability testing for 180 days (Mean±SEM, N=3)

Day	Inhibition effect at various concentrations (%)				IC_{50} (µg/mL)
	0.391 µg/mL	0.195 µg/mL	0.098 µg/mL	0.049 µg/mL	
0	70.98±2.00	64.75±2.07	50.39±1.73	28.58±0.93	0.10±0.00
30	70.63±2.36	61.43±1.87	3394±0.60	20.90±1.80	0.15±0.00
60	66.37±1.16	51.80±2.06	29.16±2.29	15.16±2.22	0.18±0.01*
90	71.43±4.36	54.81±3.58	29.87±1.65	18.73±1.32	0.18±0.01
120	67.27±2.48	53.99±2.63	33.66±3.18	20.07±1.83	0.17±0.02
150	72.02±3.84	57.68±1.70	39.54±2.04	20.97±1.30	0.14±0.01
180	69.98±3.42	59.27±3.30	42.63±4.97	27.04±2.65	0.13±0.02

*p-value < 0.05 when compared with Day 0

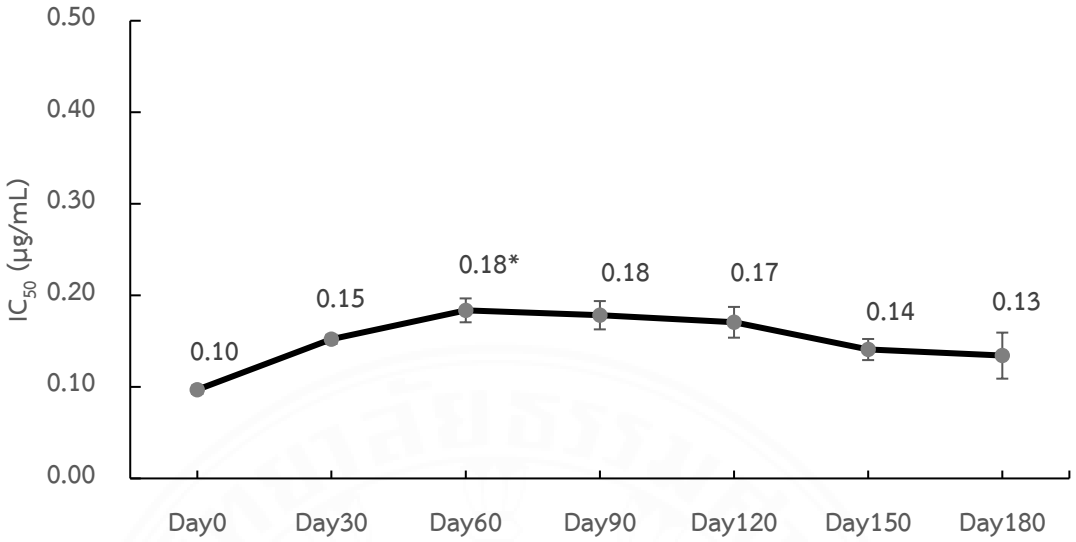

Figure 4-4 Cytotoxic activity of MRME against Hela cells after stability testing for 180 days. Data are mean±SEM (n=3). *p-value < 0.05 when compared with day 0.

The results indicated that MRME showed a significantly decreased cytotoxic activity against Hela cells after 60 days. However, the cytotoxic activity of MRME had not significantly changed after 180 days.

The antioxidant activity of MRME under accelerated conditions was investigated using FRAP assay at various times after stability testing, as shown in **Table 4-19** and **Figure 4-5**. The results showed that FRAP values and TEAC values of MRME had less change after 180 days. Moreover, the inhibition of nitric oxide production of MRME was investigated. The results showed that the inhibition effect of MRME tends to decrease after 30 days, then it showed no inhibition effect on nitric oxide production with an IC_{50} value was more than 100 µg/mL, as shown in **Table 4-20** and **Figure 4-6**.

Table 4-19 Antioxidant activity of MRME by FRAP assay after stability testing for 180 days (Mean±SEM, n=3)

Day	FRAP value (mgFe^{2+}/g)	TEAC value (mgTrolox/g)
0	384.15±9.18	144.59±3.54
30	397.53±17.55	149.75±6.76
60	396.68±18.53	149.42±7.14
90	390.34±15.20	146.97±5.86
120	381.79±14.18	143.68±5.47
150	371.05±21.87	139.54±8.43
180	370.86±3.56	139.47±1.37

*p-value < 0.05 when compared with day 0

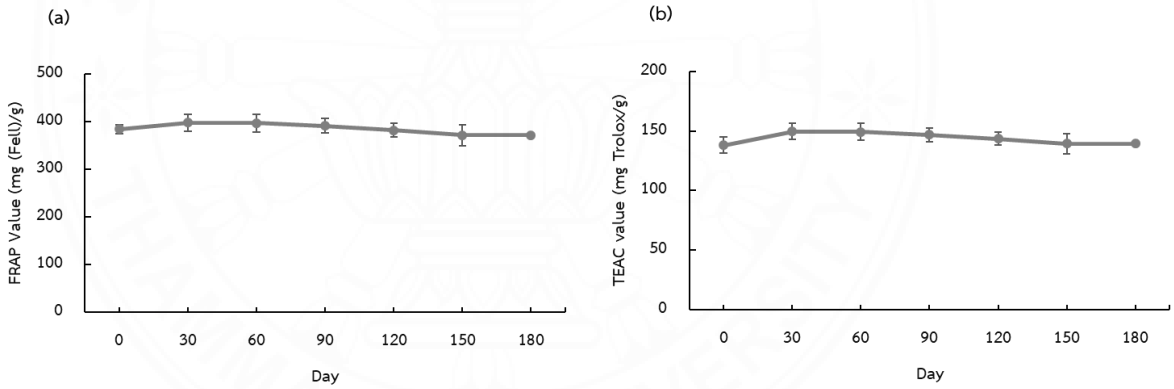

Figure 4-5 Antioxidant activity of MRME by FRAP assay after stability testing for 180 days. Data are mean±SEM (n=3). *p-value < 0.05 when compared with day 0.

Table 4-20 The inhibition of nitric oxide production and survival rate of MRME on RAW264.7 cells after stability testing for 180 days (Mean±SEM, n=3)

Day	Inhibition effect at various concentrations (%) (Survival rate, %)				IC_{50} (µg/mL)
	100 µg/mL	50 µg/mL	25 µg/mL	12.50 µg/mL	
0	72.43±4.77 (99.85±5.27)	53.57±2.61 (102.47±3.43)	30.10±1.99 (100.81±3.64)	16.59±2.60 (96.74±5.81)	45.93±2.70
30	70.58±3.00 (81.01±0.53)	37.30±3.16 (89.19±2.22)	20.50±1.99 (82.05±3.43)	9.47±4.70 (77.16±2.51)	68.53±3.98*
60	66.85±4.51 (89.16±2.51)	40.78±3.69 (82.36±0.40)	19.82±5.27 (84.52±4.69)	9.31±6.14 (81.25±4.08)	62.60±5.12*
90	73.61±2.20 (92.38±1.36)	38.09±5.02 (84.49±3.26)	19.18±5.10 (83.18±1.29)	3.45±2.78 (71.97±0.22)	66.04±5.15*
120	46.40±1.17 (83.15±5.21)	21.74±1.20 (79.61±1.04)	16.92±1.26 (82.05±2.46)	11.92±1.12 (79.51±1.36)	>100
150	42.57±1.53 (88.51±4.91)	22.96±1.82 (83.25±4.55)	12.83±1.76 (83.61±6.29)	11.31±2.14 (86.85±3.28)	>100
180	46.30±0.60 (86.96±1.45)	17.98±1.87 (83.46±1.61)	12.22±0.04 (83.17±3.87)	5.85±0.35 (90.26±1.76)	>100

*p-value < 0.05 when compared with day 0

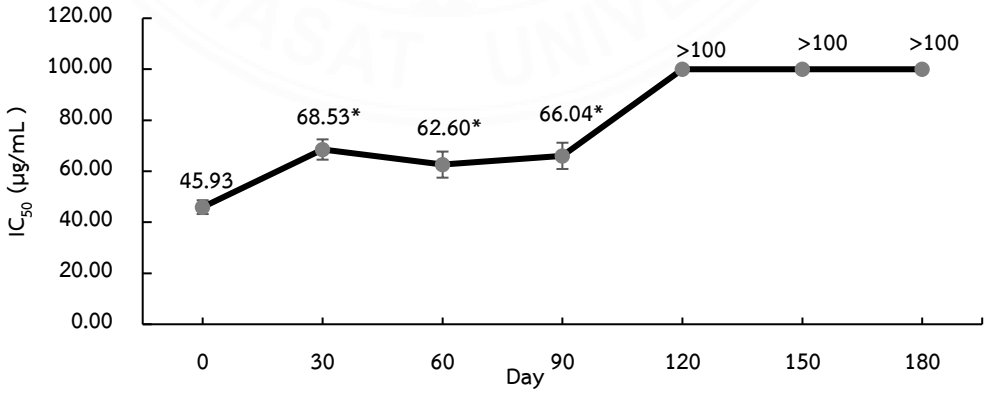

Figure 4-6 The inhibition effect of MRME on nitric oxide production in RAW264.7 cells after stability testing for 180 days. Data are mean±SEM (n=3). *p-value < 0.05 when compared with day 0.

Finally, MRME was analyzed as astilbin content after stability testing using the HPLC technique. The analysis of astilbin in MRME showed a significant decrease in the amount of astilbin after 60 days under accelerated conditions. Moreover, the remaining astilbin was 16.38% after 180 days, as shown in **Table 4-21** and **Figure 4-6**.

Table 4-21 The astilbin content of MRME after stability testing for 180 days (Mean±SEM, n=3)

Day	Astilbin content (mg/g)	Astilbin remaining (% w/w)
0	32.54±0.90	100.00±0.00
30	35.60±3.02	109.74±10.30
60	22.43±1.41*	69.29±6.36*
90	20.02±5.06	62.45±17.63
120	11.08±0.64*	34.18±2.74*
150	11.48±1.73*	35.49±5.98*
180	5.39±1.22*	16.38±3.41*

*p-value < 0.05 when compared with day 0

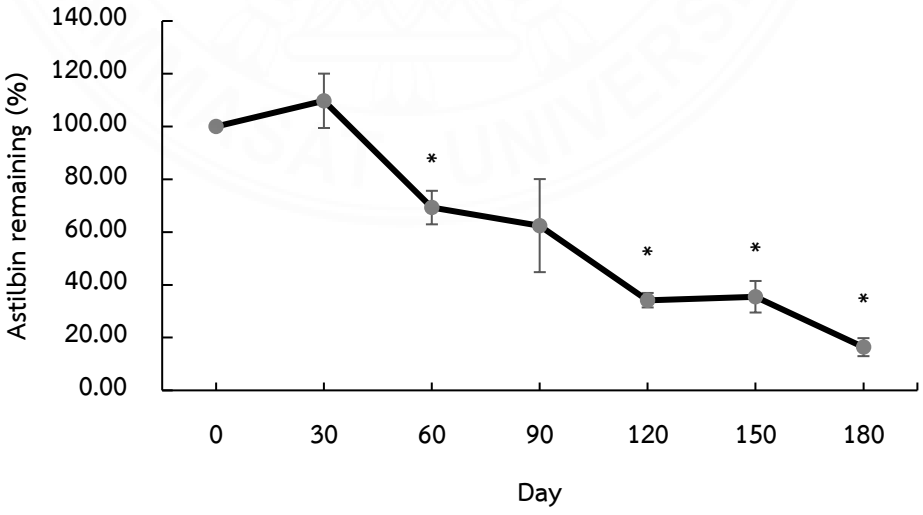

Figure 4-7 The remaining astilbin in MRME after stability testing for 180 days. Data are mean±SEM (n=3). *p-value < 0.05 when compared with day 0.

The result of stability testing under accelerated conditions showed that MRME had no change in cytotoxic activity against Hela cells and FRAP value after 180 days. On the other hand, MRME exhibited decreased change in inhibition of nitric oxide production after 30 days and showed no inhibitory effect after 120 days. Moreover, the amount of astilbin in MRME decreased after 60 days under accelerated conditions. The previous study found that the degradation rate of astilbin increased with the rise of temperature. Astilbin showed percentage of remaining values in the aqueous solution as 50% at 45ºC after 2 hours (Zhang et al., 2013). Thus, the degradation of astilbin in MRME may cause by high temperatures under accelerated conditions. Furthermore, the compounds isolated from the ethanolic extract of *D. membranacea,* such as dioscoreanone, dioscorealide B, dioscorealide A, diosgenin, stigmasterol, and β-sitosterol showed a high inhibitory effect on NO production from RAW 264.7 cells (Tewtrakul & Itharat, 2007). Moreover, kaempferol which is found in *R. siamensis* could inhibit nitric oxide (NO) production (Jaidee et al., 2019; Rho et al., 2011). The previous study found that kaempferol in *Centella asiatica* extract decreased gradually in the stability samples over six months under accelerated stability conditions. In addition, the stability of phytosterol in cocoa butter and soybean oil, including β-sitosterol and stigmasterol, decreased during storage after 28 days (Mohamad et al., 2019). Plant ingredients of MRM consist of many compounds, including kaempferol, β-Sitosterol, and stigmasterol, that have been reported as being unstable under accelerated conditions. Therefore, the anti-inflammatory activity of MRME extract exhibited a decreased change, possibly because this extract may be unstable of active compound anti-inflammatory activity such as kaempferol, β-sitosterol, and stigmasterol. On the other hand, the FRAP value and cytotoxic activity of MRME were stable. One possibility is that the active compounds as cytotoxic and antioxidant agents in MRME may differ from the antiinflammatory agents in MRME.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Gynecological cancers include ovarian, cervical, and uterine cancer, which were common diseases from the past to the present and are the leading cause of death among the female world population, including Thailand. Moreover, the incidence rate continues to increase continuously. In Thai traditional medicine, Ya-Kae-Ma-Reng-Nai-Mod-Look remedy (MRM) has been used for uterus cancer treatment, which consists of rhizome of *Smilax corbularia* Kunth., rhizome of *Dioscorea membranacea* Pierre ex Prain & Burkill., whole plant of *Stemona collinsiae* Craib, root of *Rauwenhoffia siamensis* Scheff, and rhizome of *Canna indica* L., and sea salt (Sodium chloride). However, there was no scientific research on cytotoxic activity and biological activities related to the anti-cancer of MRM remedy. Thus, this research aimed to study the cytotoxic activity of MRM extracts and their plant component against three types of female reproductive cancer and normal cell lines. Moreover, biological activities related to anti-cancer, such as antioxidant, anti-inflammation, and antifungal activities, were also investigated in this study.

The results can be concluded that the MRM ethanolic extract (MRME) was better cytotoxic effect than the water extract of MRM (MRMW). Hence, sea salt that becomes more water-soluble in MRMW did not affect biological activities. It may be used as a preservative of MRM remedy in traditional use. Thus, sea salt may be excluded from the ethanolic extract of MRM in the future to reduce the adverse renal effects. Besides, MRME can induce cytotoxicity in a cervical cancer cell line (HeLa cells) in a dose-dependent manner (IC_{50} value = 0.10±0.00 µg/mL). At the same time, it showed no cytotoxicity on keratinocyte cells (HaCaT). However, it is toxic to normal lung fibroblast cells (MRC-5) with an IC_{50} value of 0.10±0.02 µg/mL. These results showed MRME had cytotoxic activity on cervical cancer cells (HeLa) but it had toxic on normal lung fibroblast cells (MRC-5). Thus, the development of MRME for treating cancer patients should be concerned with the toxic effect on fibroblast cells.

Furthermore, the MRM extract should be studied on other types of cancer cell lines contaminated by HeLa cells, such as KB cells, which may have a good inhibitory effect on these cells. Moreover, the extracts showed cytotoxic activity against HeLa should be tested for cytotoxic activity on normal cervical epithelial cells to confirm the toxicity of extracts. In addition, the MRME showed high ferric-reducing antioxidant power. Furthermore, it was observed that the superoxide radical scavenging activity was positively correlated to the concentration of the extracts. Moreover, MRME reduced the production of inflammatory markers such as nitric oxide (NO) and interleukin-6 (IL-6) with IC_{50} of 45.93±2.70 and 35.47±3.29 µg/mL, respectively.

The results of plant components of MRM showed that RSE might be an active component of MRM. RSE showed the highest cytotoxic activity against all tested cancer cell lines. Furthermore, it is toxic to normal cell lines. Other plant components, SCLE and DME, exhibited cytotoxic activity against HeLa cells and no toxic effect on normal cell lines. Plant component of MRM that showed good anti-inflammatory activity was DME and RSE. The high antioxidant activity was found in all plant components except *S. collinsiae* and should be studied of the biological activities which related to cancer treatment of Non-tai Yak compared between *S. tuberosa* and *S. collinsae* when this remedy is changed species of plant in the ingredient of MRME remedy to confirm and select the best species to use in remedy for treating cancer.

In addition, astilbin was the main pure compound of MRM extract (32.54±0.90 mg/g). It showed good antioxidant activity but low cytotoxic activity against cancer cell lines. Thus, astilbin may not be an active marker of MRME for anti-cancer. The stability study of the MRME extract under accelerated conditions showed that its cytotoxic and antioxidant activities were stable. However, the astilbin content and anti-inflammatory of MRME were unstable. Thus, the MRME extract is unstable at high temperatures. Therefore, MRME should be kept at low temperatures or tested to confirm its activity.

Other biological activities related to cancer treatment, such as the cytotoxic activity of normal cervical cell lines and immunomodulatory activity, should be performed in the future. Furthermore, pure compounds for the anticancer activity

of MRME should be isolated and identified as active markers. The active marker may be quercetin and pure compounds in *R. siamensis* that showed high cytotoxicity on cancer cell in this study. The safety study of MRME in the animal model should be tested before product development.

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APPENDIX

CHEMICAL REAGENTS

1. Reagents for Cytotoxic activity

1.1 Minimum essential medium (MEM)

9.7 g of Minimum essential medium powder dissolve with distilled water and add 2.2 g of sodium bicarbonate and dilute to 1,000 mL with distilled water. Adjust pH to 7.2-7.4 with 10% HCl, filter through 0.2-micron membrane filter and keep in sterile bottle and store at 4ºC.

The complete media is mixture of 10% FBS (inactivated) and 1% P/S into basic MEM medium.

1.2 Dulbeco's modified eagle medium (DMEM)

13.4 g of Minimum essential medium powder dissolve with distilled water, add 3.7 g of sodium bicarbonate, and dilute to 1,000 mL with distilled water. Adjust pH to 7.2-7.4 with 10% HCl, filter through 0.2-micron membrane filter and keep in sterile bottle and store at 4ºC.

The complete media is mixture of 10% FBS (inactivated) and 1% P/S into basic DMEM medium.

1.4 RPMI-1640 medium

10.4 g of Minimum essential medium powder dissolve with distilled water and add 2.0 g of sodium bicarbonate and dilute to 1,000 mL with distilled water. Adjust pH to 7.2-7.4 with 10% HCl, filter through 0.2-micron membrane filter and keep in sterile bottle and store at 4ºC.

The complete media is mixture of 10% FBS (inactivated) and 1% P/S into basic RPMI medium 1640.

1.5 10% Hydrochloric acid (HCl)

Concentrated hydrochloric (37%) 27 mL dissolve with distilled water 100 mL.

1.6 Fetal bovineserum (FBS)

FBS (inactivate), warm in water bath at 37 ºC for 15 minutes before use.

1.7 Penicillin-Streptomycin (P/S)

Warm in water bath at 37 ºC for 15 minutes before use.

1.8 Phosphate buffer saline (PBS)

1 tablet of PBS dissolve with 500 mL of DI water.

1.9 0.4% Sulforhodamine B (SRB)

2 g sulforhodamine B dissolve with 500 mL of 1% acetic acid.

1.10 40% Trichloroacetic acid (TCA)

250 g trichloroacetic acid dissolves with 625 mL of DI water.

1.11 1% Acetic acid

200 mL of glacial acetic acid mixed with 19.80 L of water.

2. Reagents for Antioxidant activity

2.1 Iscove's Modified Dulbecco's Medium (IMDM)

10.4 g of Minimum essential medium powder dissolve with distilled water and add 2.0 g of sodium bicarbonate and dilute to 1,000 mL with distilled water. Adjust pH to 7.2-7.4 with 10% HCl, filter through 0.2-micron membrane filter and keep in sterile bottle and store at 4ºC.

The complete media is mixture of 20% FBS (inactivated) and 1% P/S into basic IMDM medium.

2.2 NBT solutuion (1.25 mg/mL)

Dissolve NBT powder with Hank's buffer until completely dissolve and covering with foil.

3. Reagents for Anti-inflammatory activity idant activity

3.1 Griess reagent

Sulfanilamide	1.0 g
N-(1-Naphthyl) ethylenediamine dihydrochloride	0.1 g

Phosphoric acid	2.5 g

Dissolve all reagents in 100 mL of DI water, kept in the dark by covering with foil and store at 4°C.

3.2 MTT solution

Thiazolyl blue tetrazolium bromide	200 mg
Phosphate-buffered saline (PBS)	40 mL

Dissolve Thiazolyl blue tetrazolium bromide in 200 mL of sterile PBS, kept in the dark by covering with foil and store at 4°C.

3.3 0.04 M HCl in isopropanol

Hydrochloric acid (37%)	0.83 mL
Isopropanol	249.17 mL

Adjust volume to 250 ml with isopropanol.

3.4 Wash buffer solution

0.5 mL of 0.05% Tween mix with 950 mL of sterile PBS.

3.5 Reagent diluent

1% Bovine serum albumin (BSA) in sterile PBS.

4. Reagents for Antifungal activity

4.1 Mueller Hinton Broth (MHB)

Dissolve 21 g of MHB powder in 1,000 mL of DI water. Divide 5 mL of MHB into tube and autoclave at 121°C for 15 minutes. Place MHB tube at room temperature until cool and kept at 2-8°C.

4.2 Sabouraud Dextrose Agar (SDA)

Boil 65 g of SDA powder in 1,000 mL of DI water, mix well until completely dissolve the medium and autoclave at 121°C for 15 minutes. Pour SDA which cool but not solidify into each sterile plastic petri dish and place SDA agar at room temperature until set and cool. Kept at 2-8°C.

4.3 Resazurin solution (1 mg/mL)

Dissolve 1 mg of resazurin sodium salt in 10 mL of sterile DI water, filter through 0.2 µm membrane filter, kept in the dark by covering with foil and store at 4°C.

BIOGRAPHY

Name	Duangpacharaporn Kwanchian
Date of Birth	January 16, 1995
Educational Attainment	2018: Bachelor of Applied Thai Traditional Medicine

Publications

Duangpacharaporn Kwanchian, Sumalee Panthong, Arunporn Itharat. (2021, July). Antioxidant activity and total flavonoid contents of the ethanolic extract of Canna indica L., [Poster presentation]. Academic Conference in 2021, Thammasat university, Pathum Thani, Thailand.

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