IN VITRO CYTOTOXIC ACTIVITY AGAINST HUMAN LUNG CELLS OF A-PHAI-SA-LEE REMEDY AND ITS INGREDIENTS

BY

MISS NATHAPAT MAKARATAT

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

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Thesis Title	*IN VITRO* CYTOTOXIC ACTIVITY AGAINST HUMAN LUNG CELLS OF A-PHAI-SA-LEE REMEDY AND ITS INGREDIENTS
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ABSTRACT

A-Phai-Sa-Lee remedy [APSL] is Thai traditional medicine for longevity or rejuvenation and claimed to improve blood circulation. It is listed in NLEM [National List of Essential Medicine, 2013] that recommended to treat indigestion, flatulence and distention. This remedy composed with 19 plant species. It has been used to treat symptoms of COPD and lung care as an inflammatory in lung cancer. The objective of this study is to investigate cytotoxic activity of [APSL] extracts and its ingredient herbs against three different type of cancer cell such as human lung adenocarcinoma cell line [A549], human lung squamous carcinoma cell line [NCI-H226] and human large cell lung carcinoma cell line [COR-L23] and one normal lung cell. APSL and its ingredient plants were extracted by maceration in 95% ethanol and boiling in water. All extracts were tested for cytotoxic activity by sulforhodamine B [SRB] assay. Results of these show that the ethanolic extract of [APSL] exhibited cytotoxic activity against three human lung cancer A549, NCI-H226 and COR-L23 and comparation with lung normal cell (MRC5) by selective index (SI) (IC_{50} values of 34.75±1.93[SI=2.44], 88.72±2.93 [SI=0.95]and 10.16±1.67 [SI=8.34]µg/ml, respectively. The results of

A549 and NCI-H226 were classified as less-active according to the NCI guided line but the result of COR-L23 was classified as active. In ethanolic extract of *Clausena excavate* showed the highest cytotoxic activity against A549, NCI-H226 and COR-L23 with IC_{50} values of 1.14±0.30 [SI=0.70], 10.46±3.93 [SI=0.08], 1.18±0.06 [SI=0.68] μg/ml, respectively. Following the ethanolic extract of *Plumbago indica* showed against A549, NCI-H226 and COR-L23 with IC_{50} values of8.38±0.24 [SI=1.93], 11.36±1.80 [SI=1.42], and 6.18±0.49 [SI=2.61] μg/ml, respectively. However, the water extract of APSL had no cytotoxic against three cancer cells. The water extracts of *Clausena excavate* showed the highest cytotoxic activity against A549 and COR-L23 with IC_{50} values of 28.12±2.25 [SI=0.95] and 26.99±0.14 [SI=0.99] μg/ml, respectively. These results support the use of A-Phai-Sa-Lee remedy in Thai traditional medicine for preventing of lung cancer. *Clausena excavate* and *Plumbago indica* are recommended as active ingredient of this remedy. Its extracts have the potential for developing as new anti-cancer drugs for human health.

Chemical analysis in APSL by HPLC showed that piperine is a main compound in the ethanolic extract of APSL because it showed the highest content [104.64±7.77 mg/g of extract]. The second is eugenol [98.83±6.09 mg/g of extract]. From this result can be used for supporting quality control from chemical fingerprint by using RP-HPLC method.

The 95% ethanolic extract of *C. excavata* which the highest cytotoxic activity was selected for isolation of the active cytotoxic activity compound by bioassay guided fractionation. Sitostenone, β-sitosterol and stigmasterol were isolated and showed less-active results of cytotoxic activity with three cancer cells. The IC_{50} of Sitostenone against A549, NCI-H226 and COR-L23 were 41.11±1.57, 64.87±3.51 and 24.17±3.86 μg/ml, respectively. The IC_{50} of combination of β-sitosterol and stigmasterol with A549, NCI-H226 and COR-L23 were 58.94±1.40, 61.41±5.87 and 25.94±3.51 μg/ml, respectively.

Stability of cytotoxic activity of the 95% ethanolic extracts of APSL remedy showed high activity agianst human lung large cell line [COR-L23] with IC_{50} values in range of 10.16-32.02 μg/ml. For human lung adenocacinoma cell line [A549] showed IC_{50} values in range of 25.07-45.14 μg/ml These results

indicated that the ethanolic extract of APSL under accelerated condition at 40±2^{o}C with 75±5% RH for 6 months [Days 15, 30, 60, 90, 120, 150 and 180] were stable because IC_{50} of day0 and day180 showed no significant different [*p*-value >0.05]. However, the stability of cytotoxic activity of APSL remedy against human lung squamous carcinoma cell line [NCI-H226] showed IC_{50} values in range of 35.68-88.72 µg/ml that unstable because IC_{50} of day0 and day180 showed significant different [*p*-value <0.05].

In conclusion, the 95% ethanolic and water extract of APSL remedy revealed good cytotoxicity for COR-L23. Therefore, these results could support that APSL used for lung cancer treatment. However, this remedy should be continuously developed as cancer product for lung cancer cancer treatment

Keywords: Aphaisalee, Cytotoxicity, Lung cancer

หัวข้อวิทยานิพนธ์	การศึกษาฤทธิ์ความเป็นพิษต่อเซลล์ปอดของสารสกัดตำรับอภัยสาลีและส่วนประกอบของตำรับ
ชื่อผู้เขียน	นางสาวณฐพัชร์ มกรทัต
ชื่อปริญญา	วิทยาศาสตรมหาบัณฑิต
สาขาวิชา/คณะ/มหาวิทยาลัย	การแพทย์แผนไทยประยุกต์ คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์
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บทคัดย่อ

ตำรับอภัยสาลี เป็นตำรับยาไทยที่อยู่ในบัญชียาหลักแห่งชาติ พ.ศ.2556 ใช้บำบัดโรคลม บรรเทาอาการจุกเสียดแน่น ตำรับนี้ประกอบด้วยสมุนไพร 19 ชนิด มีการศึกษาก่อนหน้านี้เกี่ยวกับผลการรักษาตำรับอภัยสาลีใช้ในการรักษาอาการของปอดอุดกั้นเรื้อรัง อาการหายใจลำบาก และโรคหอบหืดเมื่อเทียบกับยาแผนปัจจุบัน นอกจากนี้ยังถูกนำมาใช้เป็นต้านยาต้านอักเสบในโรคมะเร็งปอด ดังนั้นงานวิจัยนี้ มีวัตถุประสงค์คือการศึกษาฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดของสารสกัดตำรับอภัยสาลีและส่วนประกอบของตำรับ การสกัดสารสกัดตำรับอภัยสาลีและส่วนประกอบของตำรับโดยการหมักใน 95 % เอทานอลนำไประเหยแห้ง และสารสกัดตำรับอภัยสาลีและส่วนประกอบของตำรับต้มกับน้ำและทำให้แห้ง นำสารสกัดทั้งหมดไปทดสอบฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดด้วยวิธีการย้อมสี SRB ผลการศึกษาพบว่า สารสกัดชั้น 95% เอทานอลของตำรับ มีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอด 3 ชนิด คือ A549, NCI-H226 และ COR-L23 มีค่า IC_{50} เท่ากับ 34.75±1.93 (SI=2.44), 88.72±2.93 (SI=0.95), 10.16±1.68 (SI=8.34) ไมโครกรัม/มิลลิลิตร ตามลำดับอย่างไรก็ตามหัสคุณเทศ มีฤทธิ์การต้านเซลล์มะเร็งปอดชนิด A549, NCI-H226 และ COR-L23 ดีที่สุด และเปรียบเทียบกับเซลล์ปอดปกติคือเซลล์ MRC5 โดยใช้ค่าความจำเพาะ (SI) โดยมีค่า IC_{50} เท่ากับ 1.14±0.30 (SI=0.70), 10.46±3.93 (SI=0.08), 1.18±0.06 (SI=0.68) รองลงมาคือเจตมูลเพลิงแดงทีค่า IC_{50} เท่ากับ 8.38±0.24 (SI=1.93), 11.36±1.80 (SI=1.42) และ 6.18±0.49 (SI=2.61) ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ผลการศึกษาเหล่านี้ใช้เป็นข้อมูลสนับสนุนการใช้ตำรับอภัยสาลีในทางการแพทย์แผน

ไทย เพื่อป้องกันการเกิดโรคมะเร็งปอด โดยเฉพาะอย่างยิ่งหัสคุณเทศและเจตมูลเพลิงแดง พบว่ามีศักยภาพสูงในการยับยั้งเซลล์มะเร็งปอด

การตรวจหาปริมาณสารสำคัญในตำรับด้วยเทคนิคโครมาโตกราฟีของของเหลวสมรรถนะสูง (HPLC) พบว่า piperine ที่พบในตำรับชั้นเอทานอล มีปริมาณสารมากที่สุด เท่ากับ 104.64±7.77 mg/g ของสารสกัด ตามด้วยสาร eugenol มีปริมาณสารสำคัญที่อยู่ในตำรับเท่ากับ 98.83±6.09 mg/g ของสารสกัดตามลำดับ

หัศคุณเทศมีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดทั้งสามชนิดมากที่สุดจึงถูกนำมาแยกหาสารสำคัญ สารที่แยกได้คือ Sitostenone และ สารผสม β-sitosterol กับ stigmasterol เมื่อนำมาทดสอบฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดทั้งสามชนิด A549, NCI-H226 และ COR-L23 พบว่าเป็นสาร Sitostenone ที่มีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดน้อย IC_{50} เท่ากับ เท่ากับ 41.11±1.57, 64.87±3.51, 24.17±3.86ตามลำดับและสารผสม β-sitosterol กับ stigmasterol มีค่า IC_{50} เท่ากับ 58.94±1.40, 61.41±5.87 และ 25.94±3.51 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ

สารสกัดชั้นเอทานอลของตำรับถูกนำมาทดสอบความคงตัวภายใต้สภาวะเร่งที่อุณหภูมิ 40±2°C ความชื้นสัมพัทธ์ 75±5% RH เป็นเวลา 6 เดือนและเก็บตัวอย่างทุก 0, 15, 30, 60, 90, 120, 150 และ 180 วัน แล้วนำมาทดสอบฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอด พบว่าสารสกัดชั้นเอทานอลของตำรับมีความคงตัวที่ดีเมื่อทดสอบฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดชนิด A549 ผลที่ได้ไม่ได้เปลี่ยนแปลงอย่างมีนัยสำคัญ (p-value> 0.05) เมื่อเปรียบเทียบกับวันที่ 0 สารสกัดชั้นเอทานอลของตำรับมีความคงตัวที่ดีเมื่อทดสอบฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดชนิด NCI-H226 ผลที่ได้เปลี่ยนแปลงอย่างมีนัยสำคัญ (p-value< 0.05) เมื่อเปรียบเทียบกับวันที่ 0 และ สารสกัดชั้นเอทานอลของตำรับมีความคงตัวที่ดีเมื่อทดสอบฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดชนิด COR-L23 ผลที่ได้ไม่ได้เปลี่ยนแปลงอย่างมีนัยสำคัญ (p-value> 0.05) เมื่อเปรียบเทียบกับวันที่ 0

จากผลการศึกษาทั้งหมดพบว่าตำรับอภัยสาลีทั้งชั้นนำและแอลกอออลมีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งโดยเฉพาะมะเร็ง ปอดชนิด COR-L23 จากผลการทดลองสามารถสนับสนุนการใช้ในการรักษามะเร็งปอด อย่างไรก็ตามตำรับยานี้ควรมีการพัฒนาเป็นผลิตภัณฑ์ในการรักษามะเร็งปอดในอนาคต

คำสำคัญ: อภัยสาลี, ฤทธิ์ความเป็นพิษต่อเซลล์, มะเร็งปอด

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Miss Nathapat Makaratat

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
A549	Human lung adenocarcinoma cell line
CAN	Acetonitrile
ATCC	American type culture collection
APSL	Apaisalee remedy
CC	Column chromatography
$CHCl_3$	Chlorofrom
Cm	Centimeter
Cm^3	Cubic centimeter
CO_2	Carbondioxide
COPD	Chronic obstructive pulmonary disease
conc.	Concentration
COR-L23	Human large cell lung carcinoma cell line
DMSO	Dimethy sulfide
e^-	Electron
EC_{50}	Concentration causing 50% effective activity
EDTA	Ethylendiamintetraacetic acid
e.g.	Example gratis, for example
et al	Etalii, and other
Etc	Et cetera, and other things
EtOH	Ethanol
EtOAc	Ethylacetate
G	Gram
FBS	Fetal bovine serum
g/l	Gram per liter
g/ml	Gram per milliliter

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
H	Hour
Hr	Hour
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
H_2O	Water
IC_{50}	Concentration causing 50% inhibition effect
i.e.	Id est, than is
In	Inch
L	Liter
M	Meter
M	Molar [concentration]
MEM	Minimum essential medium eagle
MeOH	Methanol
mg/ml	Milligram per milliliter
Min	Minute
Ml	Milliliter
Mm	Millimeter
Mm	Millimolar
MQ	Milli-Q
MRC-5	Normal human lung myofibroblast cell line
N	Normality
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCI-H226	Human lung squamous carcinoma cell line
NMR	Nuclear magnetic resonance
Ng	Nanogram
Nm	Nanometer
NO	Nitric oxide
O_2	Oxygen
OD	Optical density
P/S	Penicillin-Streptomycin
PBS	Phosphate buffer saline
pg/ml	Picogram per milliliter
pH	Potential of hydrogen ion
R^2	Correlation coefficient
RH	Relative humidity
Rpm	Revolution per minute
RPMI 1640	Roswell Park Memorial Institute 1640
ROS	Reactive oxygen species
SA	Sulfanilamide
SD	Standard deviation
SEM	Standard error of mean
Spp	Species
SRB	Sulforhodamine B
Std	Standrard
TCA	Trichloroacetic acid
TLC	Thin layer chromatography

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
U/ml	Unit per milliliter
UV	Ultraviolet
VLC	Vacuum liquid chromatography
w/v	Weight by volume
w/w	Weight by weight
^{o}C	Degree Celsius
%	Percent
&	And
/	Per
<	Less than
=	Equal
>	More than
Mg	Microgram
Ml	Microliter
µg /ml	Microgram per milliliter
Mm	Micromolar

CHAPTER 1
INTRODUCTION

1.1 General introduction

In 2012, around the world has found an estimated 14.1 million cancer cases, of these 6.7 million cases were in women and 7.4 million in men. This number of cases are expected to increase to 24 million by 2035. This growing cancer burden, within the overall context of non-communicable diseases [NCDs], was a key focus of the September 2011 UN High Level Meeting on NCDs. In both sexs **found lung cancer was the most common cancer worldwide contributing 13 percent of the total number of new cases diagnosed in 2012** [**Ferlay *et al*., 2014**]. Smoking is the principal cause of lung cancer; it is estimated to be responsible for 85% of all types of this cancer [**Ferlay *et al*., 2014**]. Nowadays, the treatments of cancer are several methods such as surgery [19.7%], radiation therapy [21.5%], chemotherapy [3.8%], *etc* [World Health Organization, 2013], but there are many several side effects such as pain, fatigue, infection and bleeding or organ dysfunction [Sohl *et al*., 2009]. However, alternative medicine is now commonly used for cancer patients [Gansler *et al*., 2008] because generally, it is believed that herbal medicine is safe and has less side-effects than surgery, radiation therapy and chemotherapy [Lynch *et al*., 2007].

The Thai traditional medicine called A-Phai-Sa-Lee remedy is used for longevity or rejuvenation and claimed to improve blood circulation. It is found in NLEM [National List of Essential Medicine, 2013] it is recommended to treat indigestion, flatulence and distension. This remedy is composed of 19 species of plants as follows: *Acorus calamus* Linn., *Amomum testaceum* Ridl., *Amorphophallas saraburiensis* (Dennst), *Anethum graveolens* Linn., *Angelica dahurica* Berth., *Atractylodes lancea*, *Ardisia elliptica* Thumb., *Clausena excavate* Burmf., *Cuminum cyminum* L., *Foeniculum valgare* Mill, *Lipidium sativum* Linn., *Myristica fragrans* Houtt, *Piper nigrum* Linn., *Plumbago indigo* L., *Syzygium aromaticum*, *Terminalia chebula*, *Terminalia arjuna* Wight&Arn. A previous study on the effect of A-Phai-Sa-Lee remedy used to treat symtoms of COPD such as dyspnea and asthma were

compared with the modern medicine. The result showed that A-Phai-Sa-Lee remedy was positive impact on the quality of life of COPD patients reduced and quit using inhalers to relieve fatigue [Champasuri *et al*., 2015]. However, there is no report of cytotoxic activity of this preparation against lung cancer cell, but the previous studies reported that some single herb showed cytotoxic activity against lung cancer cell line [A549] was *Piper Nigrum* Linn. [Sen Li *et al*., 2011] Therefore, the objectives of this study are to investigate the cytotoxic activity of A-Phai-Sa-Lee remedy extracts and each of its herb ingredients against three types of human cancer cells from lung such as human lung carcinoma [A549, NCI-H226 and COR-L23]. These results could support the use of this remedy in Thai traditional treatment for lung cancer.

1.2 Objectives

1.2.1 Overall aims

To investigate cytotoxic activity extracts of A-Phai-Sa-Lee remedy and its ingredient against three cancer cell lines such as human lung adenocarcinoma cell lines [A549], human lung squamous carcinoma cell lines [NCI-H226], human large cell lung carcinoma cell lines [COR-L23] and one normal human lung myofibroblast cell lines [MRC-5] and investigate the stability of APSL extracts.

1.2.2 Specific aims

1.2.2.1 To study cytotoxic activity of ethanolic and aqueous extracts of APSL remedy and its plants ingredient against lung carcinoma cells [A549, NCI-H226 and COR-L23].

1.2.2.2 To study cytotoxic activity of ethanolic and aqueous extracts of APSL remedy and its ingredient plants against lung normal cells [MRC-5].

1.2.2.3 To study the chemical fingerprints of ethanolic extract of APSL remedy by using high performance liquid chromatography [HPLC].

1.2.2.4 To study on isolation active compounds from plant ingredients of APSL remedy [95%ethanolic extract of *Clausena excavate* Burmf.]

1.2.2.5 To study the stability of APSL remedy in ethanolic extracts.

CHAPTER 2
REVIEW OF LITERATURE

2.1 The lung

Lungs are a pair of large organs in chest and part of respiratory system. Air enters the body through your nose or mouth run to windpipe [trachea] and through bronchus. Then goes into the lungs .The body gets oxygen when breathe are in the lungs expand with air and goes out of lungs when breathe out that calls the body gets rid of carbon dioxide [National Cancer Institute, 2012].

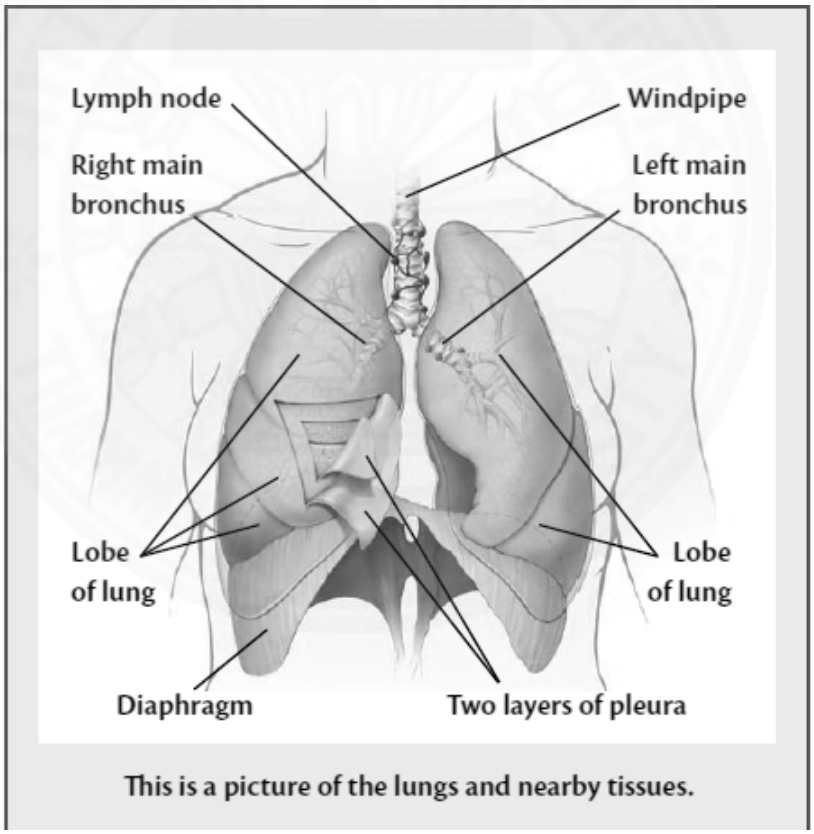


Figure 2.1 Picture of the lungs and nearby tissues.

[National Cancer Institute, 2015]

2.1.1 Lung cancer

Lung cancer is the most of cancer that kill of people in the worldwide. Each year have more than 157k people in the United States and over 1.1 million people worldwide die of lung cancer. This is a major global health concern for many years. There are two majors of cancer such as small cell lung cancer and non-small cell lung cancer. These cancers develop from the epithelial cells that line the airways of the lungs. Non-small and Small cell lung cancers have different treatments and growth patterns. Other rare forms of cancer also occur in the lungs including malignant pleural mesotheliomas and carcinoid tumors [Travis WD *et al*., 2015].

2.1.1.1 Description of lung cancer

Lung cancer develops eventually leads to uncontrolled cell proliferation when normal lung cells sustain genetic damage. Similarly all cancers, lung cancer cells have ability to invade neighboring tissues and spread or metastasize to distant parts of the body. Lung cancer eventually causes death of human. Sometimes it can referred to as bronchiogenic cancer or bronchiogenic carcinoma. The bronchiogenic means originating from the bronchi [the airways of the lungs]. The most of lung cancers begin in the cells lining the bronchi of the lungs. There are two major types of lung cancer and treated different ways [Travis WD *et al*., 2015].

2.1.1.2 Causes of lung cancer

When the cells that line the lungs sustain genetic damage so lung cancer develops in the body. There are several different chemicals and environmental factors that are capable of causing the kind of genetic damage that can lead to lung cancer. Carcinogens are substances capable of producing cancerous changes in cells. More than 85 percent of all lung cancer cases occur among people who are either current or former tobacco smokers. The greater risk of developing lung cancer from smoking is influenced by many factors including the age, sex and genes. The effects of carcinogens accumulate over time. The recent studies suggest that women are more susceptible to the carcinogenic effects of tobacco smoke than men and the younger smoke tobacco more than the past. Second-Hand Smoke is the risk of the lung cancer because of anyone who breathes in air that contains tobacco smoke are exposed to its carcinogens. Therefore, smoking in workplace, the home or in public can increases a person’s risk of lung cancer. The federal Environmental

Protection Agency [EPA] estimates that 3,000 people in the United States die of lung cancer each year because of exposure to second-hand smoke. Moreover, the risk of lung cancer may be the environmental Carcinogens such as asbestos, radon, arsenic, chromium, nickel, polycyclic Aromatic Hydrocarbons [PAHs] and other Environmental Lung Carcinogens include bis[chloromethyl]ether, chloromethyl methyl ether, gamma radiation, ionizing radiation [x-rays], soots, mustard gas, mineral oils, tars, , and vinyl chloride. Suspected lung carcinogens include, cadmium, beryllium, acrylonitrile, ferric oxide dust, and lead. Many other known, suspected, and potential carcinogens could contribute to the development of lung cancer. The genetic factors are the risk that transform of normal cells into cancer cells is a complex, multi-step process. Everyone is exposed to lung carcinogens. Finally, COPD [chronic obstructive pulmonary disease] is the disease of risk for develop to lung cancer. It can makes breathing hard because the lung tissue is damaged or there's too much mucus. The second disease linked to lung cancer is pulmonary fibrosis. Pulmonary fibrosis is major scarring of lung tissue that makes it hard to breathe [Alycia corrigan, 2016].

2.1.1.3 Type of lung cancer

There are 2 major types of lung cancer include non-small cell lung cancer [NSCLC] that about 80% to 85% of lung cancers and small cell lung cancer [SCLC]. There are about 10% to 15%. Subtypes of NSCLC, which start from different types of lung cells such as adenocarcinomas, squamous cell carcinomas and Large cell carcinomas. First, there are about 40% of lung cancers are adenocarcinomas. These cancers start in early versions of the cells that would normally secrete substances such as mucus.This type of lung cancer occurs mainly in current or former smokers, but it is also the most common type of lung cancer seen in non-smokers. It is more common in women than in men, and it is more likely to occur in younger people than other types of lung cancer. Adenocarcinoma is usually found in outer parts of the lung. Though it tends to grow slower than other types of lung cancer and is more likely to be found before it has spread, this varies from patient to patient. Second, There are about 25% to 30% of all lung cancers are squamous cell carcinomas. These cancers start in early versions of squamous cells, which are flat cells that line the inside of the airways in the lungs. They are often linked to a history

of smoking and tend to be found in the bronchus. Third, It's about 10% to 15% of lung cancers called large cell lung cancer that can appear in any part of the lung, tends to grow and spread quickly. A subtype of large cell carcinoma, known as large cell neuroendocrine carcinoma, is a fast-growing cancer that is very similar to small cell lung cancer. [Alycia corrigan, 2016].

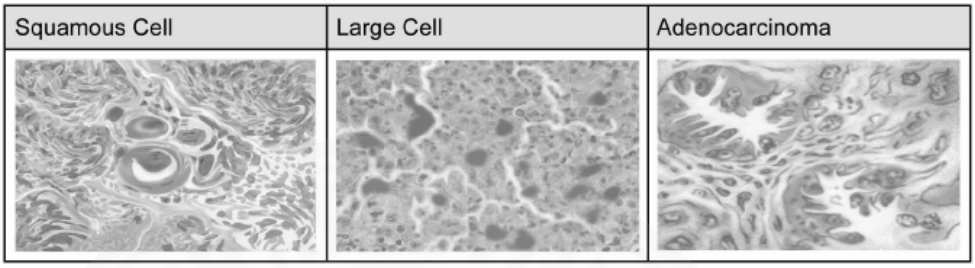


Figure 2.2 subtypes of NSCLC.
[Alycia corrigan, 2016]

Other subtypes of NSCLC such as adenosquamous carcinoma and sarcomatoid carcinoma, are much less common. Other types of lung tumors is lung carcinoid tumors that about fewer than 5% of lung tumors. Most of these grow slowly. Adenoid cystic carcinomas,lymphomas, and sarcomas, as well as benign lung tumors such as hamartomas are rare. These are treated differently from the more common lung cancers [Alycia corrigan, 2016].

2.1.1.4 Signs and symtoms of lung cancer

Signs and symtoms of lung cancer patients showed with progressive shortness of breath, lost of voice, cough, haemoptysis and chest pain. Patients with small cell lung cancer [SCLC] differ in many ways from those with Non-small cell lung cancer [NSCLC] [Travis *et al*., 2004].

2.1.1.5 Treatment of lung cancer

People with lung cancer have many treatment options. The treatments of cancer are several methods such as surgery [19.7%], radiation therapy [21.5%], chemotherapy [3.8%], *etc* [World Health Organization, 2013]. First, surgery may be an option for people with early-stage lung cancer that calls lobectomy. Second, radiation therapy for people with any stage of lung cancer such as people with early lung cancer may choose radiation therapy instead of surgery. But after surgery there are side effects to the body. Third, Chemotherapy uses drugs to kill cancer cells that usually given

directly into a vein [intravenous] through a thin needle but there are side effects depend mainly on which drugs are given and how much. Chemotherapy kills fast-growing cancer cells, but the drugs can also harm normal cells that divide rapidly. In addition, nutrition is alternative for treatment the lung cancer. Eating well is important such as vegetables and herbs [Alycia corrigan, 2016].

2.2 Cancer in Thai traditional medicine

2.2.1 Pathogenesis cancer in Thai traditional medicine.

The principle theory of Thai traditional medicine is the knowledge of four elements namely earth or Pathavi [consisting of 20 components], water or Apo [Semha][consisting 12 of components], wind or Wayo [Wata] [consisting of 6 types] and fire or Techo [consisting of 4 components]. Disease occurs when there is an imbalance of elements in the body and involves many factors, for example season, environment and behavior all bring about the imbalance. The concept of cancer in Thai traditional medicine is imbalance from pita, wata, semha disorder [Old medical texts of Thailand, 2007].

There are many factors affecting lung cancer such as cigarette, pollution and COPD. The beginning of lung is an increase of Pitha from various risk factors. These effects result in increase of Pathavi in the lung, the Semha [disorder of blood and Wata, bubble is blood] resolting inincrease of Wata and Pittha. First, the effects result in increases of Pita moving upward by cause of cigarette, pollution, emotion, stress, foods and sleepless. Second, the effects result in decreases of Semha drying of blood producing, loss weight and cough. Third, Wata increase there are effect of blood circulation not flow, weak and can’t take a deep breath that caused abnormal of lung function, chest and part of respiratory system or the imbalance of body elements. The Thai traditional remedy which treats these symptoms is hot and mild for decreasing Wata and Pittha. APSL remedy controls abnormalities of the wind and fire elements in the body. The mechanism is shown in **Figure 2.3.**

The concept of cancer as seen in Thai traditional medicine terms

Causes : cigarette, pollution, COPD, emotion, stress, foods and sleepless.

Effects : drying of blood producing, loss weight and cough.

Effects : blood circulation not flow, weak and can’t take a deep breath.

Effects : abnormal of lung function, chest and part of respiratory system or the imbalance of body elements.

Figure 2.3 The concept of cancer as seen in Thai traditional medicine terms

2.3 A-Phai-Sa-Lee remedy and plant ingredients

2.3.1 A-Phai-Sa-Lee remedy in Thai traditional medicine

The Thai traditional medicine called A-Phai-Sa-Lee remedy is used for longevity or rejuvenation and claimed to improve blood circulation. It is found in NLEM [National List of Essential Medicine, 2013] it recommended to treat indigestion, flatulence and distension. This remedy is composed of 19 species of plants as follows: *Acorus calamus* Linn., *Amomum testaceum* Ridl., *Amorphophallas saraburiensis* (Dennst), *Anethum graveolens* Linn., *Angelicadahurica* Berth., *Atractylodes lancea*, *Ardisia elliptica* Thumb., *Clausena excavate* Burmf., *Cuminum cyminum* L., *Foeniculum valgare* Mill, *Lipidium sativum* Linn., *Myristica fragrans* Houtt, *Piper nigrum* Linn., *Plumbago indigo* L., *Syzygium aromaticum, Terminalia chebula*, *Terminalia arjuna* Wight&Arn. A previous study on the effect of A-Phai-Sa-Lee remedy used to treat symtoms of COPD such as dyspnea and asthma were compare with the modern medicine. The result showed A-Phai-Sa-Lee remedy was positive impact on the quality of life of COPD patients reduced and quit using inhalers to relieve fatigue [Champasuri *et al*., 2015].

The classification of ingredients in this remedy are shown in **Figure2.4** and the biological activities data shown in **Table 2.1**

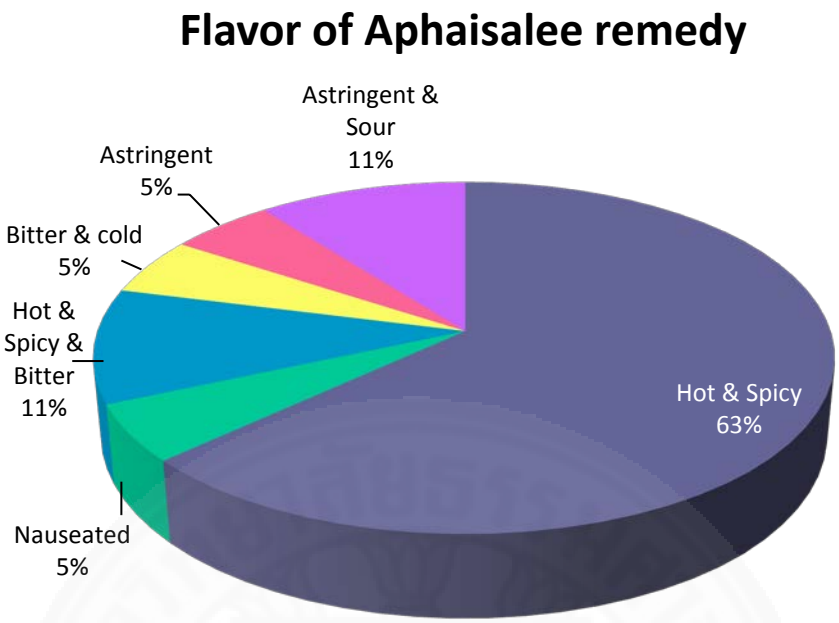


Figure 2.4 The classification of ingredients in this remedy in Thai traditional medicine

Table 2.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Aphaisalee remedy	-	-	Tumrab a phai sa lee	100	-	Hot & Spicy	Longevity or rejuvenation and is claimed to improve blood circulation
Acorus calamus Linn.	ARACEAE	SKP 015 01 30 01	Wan num	3.74	Rhizome	Hot & Spicy	Gastrointestinal agent,carminative
Amomum testaceum Ridl.	ZINGIBERACEAE	SKP 206 01 20 01	Krawan	1.6	Fruit	Hot & Spicy	Promote blood circulation,carminative
Amorphophallas saraburiensis (Dennst)	ARACEAE	SKP 015 01 19 01	Book ror	8.02	Rhizome	Nauseated	Promote lymphatic system
Anethum graveolens Linn.	UMBELLIFERAE	SKP 199 01 07 01	Thien tatakkataen	4.81	Seed	Hot & Spicy & Bitter	Carminative
Angelicadahurica Berth.	UMBELLIFERAE	SKP 199 01 04 01	Kot sor	4.28	Rhizome	Bitter & cold	Antipyretic, tonic, expectorant, anti-asthma and cough

Table 2.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation (continued)

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Atractylodes lancea	COMPOSITAE	SKP 051 01 12 01	Kot khamoa	4.81	Rhizome	Hot & Spicy	Tonic, antipyretic,paregoric, diuretic agents, relieve common cold, relieve asthma, oral prophylaxis
Ardisia elliptica Thumb.	MYRSINACEAE	SKP 122 01 16 01	Philangkasa	3.21	Fruit	Astringent	Anti-diarrhea, relieve urticarial
Clausena excavate Burmf.	RUTACEAE	SKP 166 03 05 01	Hasakhun thet	27.59	Stem	Hot & Spicy	Promote blood circulation, anti-nasal polyp,expectorant
Cuminum cyminum L.	UMBELLIFERAE	SKP 199 03 03 01	Thien kao	4.28	Seed	Hot & Spicy & Bitter	Carminative, promote blood circulation
Foeniculum valgare Mill	UMBELLIFERAE	SKP 199 06 22 01	Thien kaopeug	5.35	Seed	Hot & Spicy	Carminative, promote blood circulation

Table 2.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation (continued)

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Lipidium sativum Linn.	CRUCIFERAE	SKP 057 12 19 01	Thien daeng	5.88	Seed	Hot & Spicy	Gastrointestinal agent, carminative, expectorant
Myristica fragrans Houtt	MYRISTICACEAE	SKP 121 13 06 01	Chanthet (Kaen chan)	8.56	Stem	Hot & Spicy	Tonic, cadio-tonic, carminative
Myristica fragrans Houtt	MYRISTICACEAE	SKP 121 13 06 01	Chanthet (Dok chan)	1.07	Mace	Hot & Spicy	Tonic, cadio-tonic, carminative
Myristica fragrans Houtt	MYRISTICACEAE	SKP 121 13 06 01	Chanthet (Look chan)	0.53	Nutmeg	Hot & Spicy	Tonic, cadio-tonic, carminative
Piper nigrum Linn.	PIPERACEAE	SKP 146 16 14 01	Prikthai	8.56	Seed	Hot & Spicy	Carminative, promote blood circulation, tonic for longevity

Table 2.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation (continued)

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Plumbago indigo L.	PLUMBAGINACEAE	SKP 148 16 09 01	Chetamoolplerng	6.42	Root	Hot & Spicy	Promote blood circulation, carminative, gynecology
Syzygium aromaticum (L.) Merr. Et Perry.	MYRISTICACEAE	SKP 123 19 01 01	Kan phlu	2.14	Flower	Hot & Spicy	Carminative, paregoric of tooth and pyorrhea, expectorant
Terminalia chebula Rez var Chebula.	COMBRETACEAE	SKP 049 20 03 01	Samor thai	6.95	Fruit	Astringent & Sour	Laxative, anti-diarrhea,expectorant
Terminalia arjuna Wight& Arn.	COMBRETACEAE	SKP 049 20 01 01	Samor thet	6.95	Fruit	Astringent & Sour	Laxative, anti-diarrhea,expectorant

2.3.2 General data of the ingredients in A-Phai-Sa-Lee remedy

2.3.2.1 *Acorus calamus* Linn. [ARACEAE]

Figure 2.5 *Acorus calamus* Linn.
https://innovareacademics.in

Common names: Kalmus, Acore aromatique, Acore calame, Calamus
Thai common name: Wan nam, Wan nam lek, Hang khao pha [Chiang Mai], Som chuen.

Traditional uses for indigestion, ague, gastritis and tonic dyspepsia. Dried rhizome used to relieve dyspepsia. Rhizome used to tonic, masticatory for toothache and stimulant. Oil is carminative used to digestive and increase the appetite. [Sandeep *et al*., 2014].

In Ayuraveda used for memory loss, hysteria, psychoneurosis, insomnia, cough, epilepsy, fever, bronchitis, inflammatory, depression, general debility and tumors [Sandeep *et al*., 2014].

In Chinese medicine used to regulate gastrointestinal fermentation and aid digestion. Ancient Chinese medicine used to relieve swelling [Sandeep *et al*., 2014].

Chemical constituents of *Acorus calamus* Linn.

A. calamus have constituents such as phenols, alkaloids, gums, flavonoids, lectins, quinone, mucilage, saponins, tannins sugars, and triterpenes [steroids] [Chit *et al*., 2001]. A new study found sesquiterpene, named neo-acorane A (1), and two known ones, acoric acid (2) and calamusin D (3) [Li *et al*., 2017].

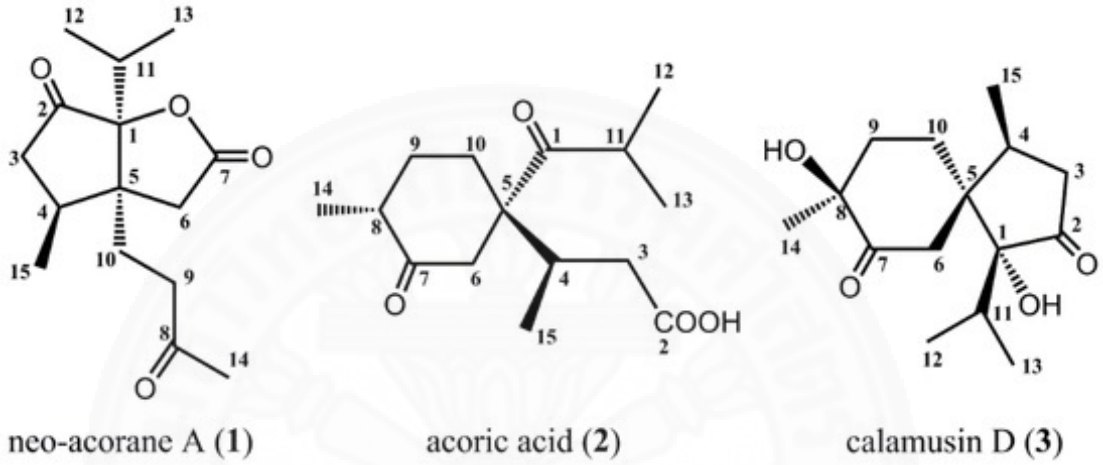


Figure 2.6 Some chemical constituents of *Acorus calamus* Linn.

2.3.2.2 *Amomum testaceum* Ridl. [ZINGIBERACEAE]

Figure 2.7 *Amomum testaceum* Ridl.
www.pharmacy.mahidol.ac.th

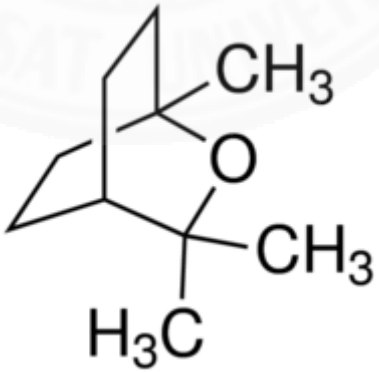
Common name: Cardamon

Thai common name: Krawan

Traditional uses: The fruit of *A. testaceum* Ridl. And *A. kravanh* are commonly used as a culinary spice especially in Asia. The fruit are used to promote blood circulation, expectorant and carminative [Prapaspong *et al*., 1999].

Chemical constituents of *Amomum testaceum* Ridl.

There is constituents such as essential oil, limonene, camphor, linalool, bornyl acetate, myrcene, cineol, 1,8-cineol, safrole, cis-laceol and etc. [Sirat , Hong , 2001].



1,8-cineol

Figure 2.8 Some chemical constituents of *Amomum testaceum* Ridl.

2.3.3.3 *Amorphophallas saraburiensis* (Dennst) [ARACEAE]

Figure 2.9 *Amorphophallas saraburiensis* (Dennst)

http://www.wikiwand.com

Common names: Shade palm, Umbrella arum

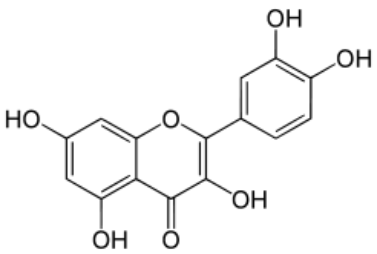
Thai common name: Book ror

In Thai traditional medicine used rhizomes to promote lymphatic system, anti-blood coagulation. The decocted rhizomes used to treat hepatopathy, abdominal edema and expectorant [Prapaspong *et al*., 1999].

In Ayurveda used in tumors, arthralgia, inflammations, elephantiasis, hemorrhages, hemorrhoids, bronchitis, vomiting, cough asthma, colic, dyspepsia, constipation, amenorrhea, hepato-splenopathies, dysmenorrhea, general debility, fatigue and seminal weakness [Anuradha, 2014)].

Chemical constituents of *Amorphophallas saraburiensis* (Dennst)

There is constituents such as sugar, starch, proteins minerals [Singh *et al*., 2014] flavonoid [Quercetin] [Nataraj *et al*., 2009].



Quercetin

Figure 2.10 Some chemical constituents of *Amorphophallas saraburiensis* (Dennst)

2.3.3.4 *Anethum graveolens* Linn. [UMBELLIFEARE]

Figure 2.11 *Anethum graveolens* Linn.
http://hashmidawakhana.co.in

Common name: Dill

Thai common name: Thian tatakkataen

Traditional uses: In Thailand seed is used for carminative, restorative, expectorant and relieves nausea [Chanwithesuk *et al*., 2005].

Dill is used for everything in worldwide. Essential oil used as carminative in many countries. Seed is used for insomnia, stomach acidity, dyspepsia, flatulence and colic.

Nursing mothers use the dill to increase milk flow and chewing of the seeds is helpful in halitosis when decoction this herb is used for colic in babies.

Chemical constituents of *Anethum graveolens* Linn.

There is constituents such as essential oil, fatty oil, moisture, proteins, carbohydrates, fiber, mineral elements [Ishikawa *et al*., 2002].

2.3.3.5 *Angelica dahurica* Benth. [UMBELLIFERAE]

Figure 2.12 *Angelica dahurica* Benth.

www.mdidea.com

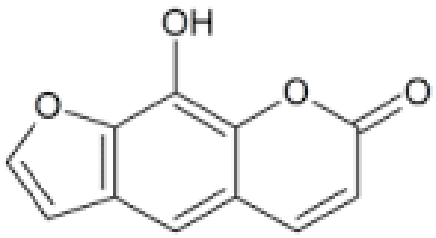
Common name: Dahurian angelica

Thai common name: Kot sor

Traditional uses: The roots are commonly used in Chinese and Thai medicine. In Thailand, used to antipyretic, treat to asthma, tonic and cough. In China used to treat cold, rhinitis, headache, and toothache [Prapaspong *et al*., 1999].

Chemical constituents of *Angelica dahurica* Benth.

There is constituents such as imparation, isoimparation, alloisoimparation, alloimparation, oxypeucedanin, xypeucedanin hydrate, isooxypeucedanin**,** byakangelicol, phellopterin, neo byakangelicol, bergapten, 5-methoxy-8-hydroxypsoralen, xanthotoxol, cnidilin, pabulenol, cumarins, sitosterol, palmitic acid, calcium, iron zinc, copper, potassium, magnesium, and etc. [Zhao *et al*., 2012].



xanthotoxol

Figure 2.13 Some chemical constituents of *Angelica dahurica* Benth.

2.3.3.6 *Ardisia elliptica* Thumb. [MYRSINACEAE]

Figure 2.14 *Ardisia elliptica* Thumb.

www.pharm.su.ac.th

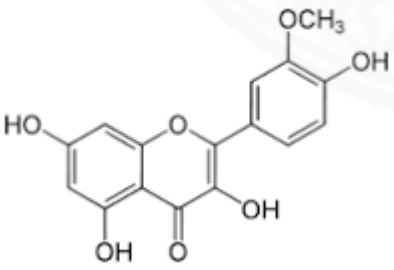
Common name: Shoebutton

Thai common name: Pilungkasa

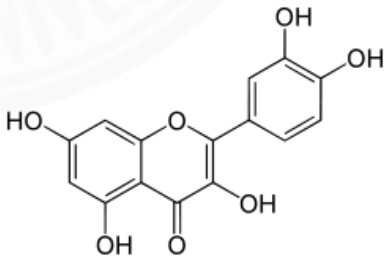
Traditional uses: In Thailand, Pilungkasa is mainly used for treatment of the diseases such as diarrhea, fever, liver disease, and leprosy disease [Yukongphan *et al*., 2012]. In many countries, it is used for complications of parturition, liver poisoning, fever, and diarrhea.

Chemical constituents of *Ardisia elliptica* Thumb.

There is constituents such as syringic acid, isorhamnetin and quercetin [Phadungkit *et al*., 2006].



isorhamnetin



quercetin

Figure 2.15 Some chemical constituents of *Ardisia elliptica* Thumb.

2.3.3.7 *Atractylodes lancea* (Thunb) D.C. [COMPOSITAE]

Figure 2.16 *Atractylodes lancea* (Thunb) D.C.
http://www.yomeishu.co.jp

Common name: Atracylodes

Thai common name: Kot khamoa

Traditional uses: The rhizome is common in Chinese, Korean, Japan and Thai traditional medicine. In Thailand used the dried rhizome has been to treat fever, tonic, common cold carminative, diuretic and asthma [Prapaspong *et al*., 1999].

In Chinese medicine, it is used for rheumatic diseases, digestive disorders, night blindness and influenza [Koonrungsesomboon *et al*., 2014].

Chemical constituents of *Atractylodes lancea* (Thunb) D.C.

There is constituents such as high essential oil, atratylenlide, hinesol, beta-eudesmol, 1H-cyclopropa (a) naphthalene and gamma-eudesmol [Chiaki *et al*., 2014].

2.3.3.8 *Clausena excavate* Burmf. [RUTACEAE]

Figure 2.17 *Clausena excavate* Burmf.
http://www.bansamunpaiosot.com

Common name: Clausena

Thai common names: Chamat, Hatsa khun, Hastsa khun khok, Mui yai, Oi chang, Phia fan, Rui, Saen so, Samui, San sok, Samat bai yai

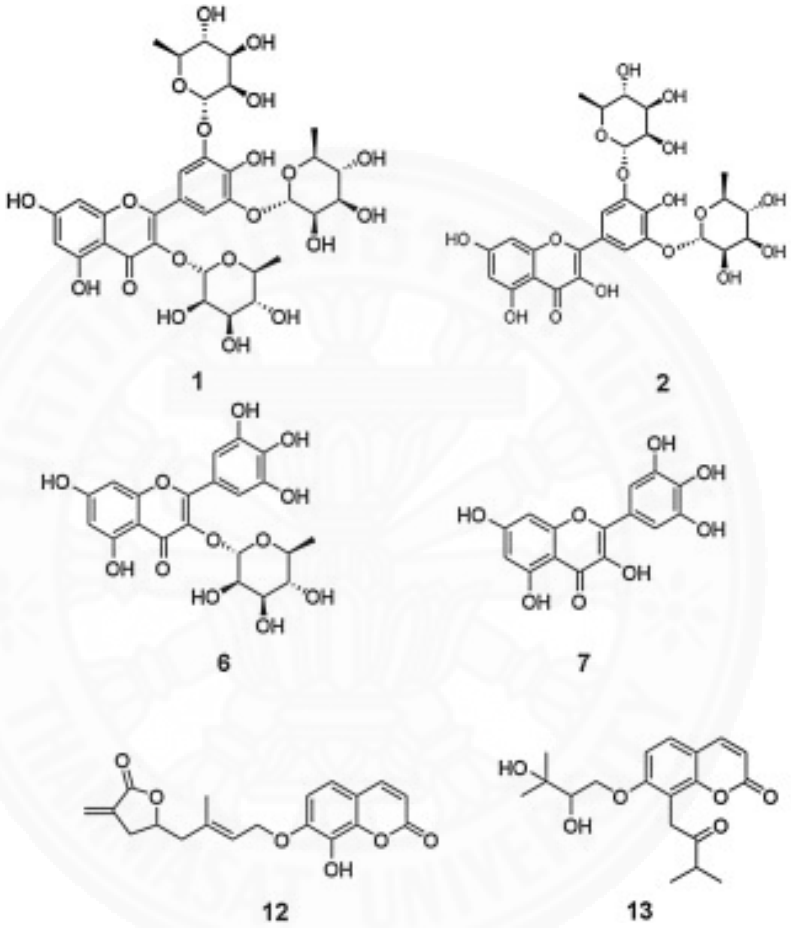
In many countries it is used for especially colic, bowel problems. Malays drink a decoction of roots for treatment of snakebites, abdominal pain, and for detoxification. Fumigation from burning leaves and bark can treat ulcerations of the nose. Pounded root used to poultice for sores including ulcerations of the nose. Poultice of pounded leaves applied to the head for headaches [Rahman *et al*., 2002].

Chemical constituents of *Clausena excavate* Burmf.

There is constituents such as carbazole alkaloids [excavatine A, excavatine B, excavatine C] [Wen *et al*., 2013], coumarins [binorponcitrin, xanthoxyletin, dentatin, nordentatin, clausenidin, scopoletin, dictamine, clausine D, clausine F, murrayafoline A, murrayanine, clauszoline I, 2-hydroxy-3-formyl-7-methoxycarbazole, 3-formyl-2, 7-dimethoxy-carbazole, clauszoline J, clausine H, murrayacine and heptaphyline] [Sripisut *et al*., 2012] The stem bark has a limonoid called clausenarin [Sharifand, 2009] and Clausenolide-1-metylether [Cordell *et al*., 1991].

Phytochemical investigation on leaves, stem bark and roots of Malaysian *Clausena excavata* has led to the isolation and identification of limonoid compounds, clausenolide-1-methyl ether, clausenarin, carbazole alkaloids, 3-formyl-2,7-dimethoxycarbazole, clausine-K, together with coumarins, xanthyletin, dentatin

and nordentatin.[N. W. Muhd Sharif *et al*., 2011] In this study showed two new flavonoid glycosides, excavaside A (**1**) and excavaside B (**2**), along with six flavonoids compounds (**3**–**8**) and eight coumarin derivatives (**9**–**16**), were isolated from the EtOH extract of the leaves and twigs of *C. excavate* [Seo *et al*., 2017].



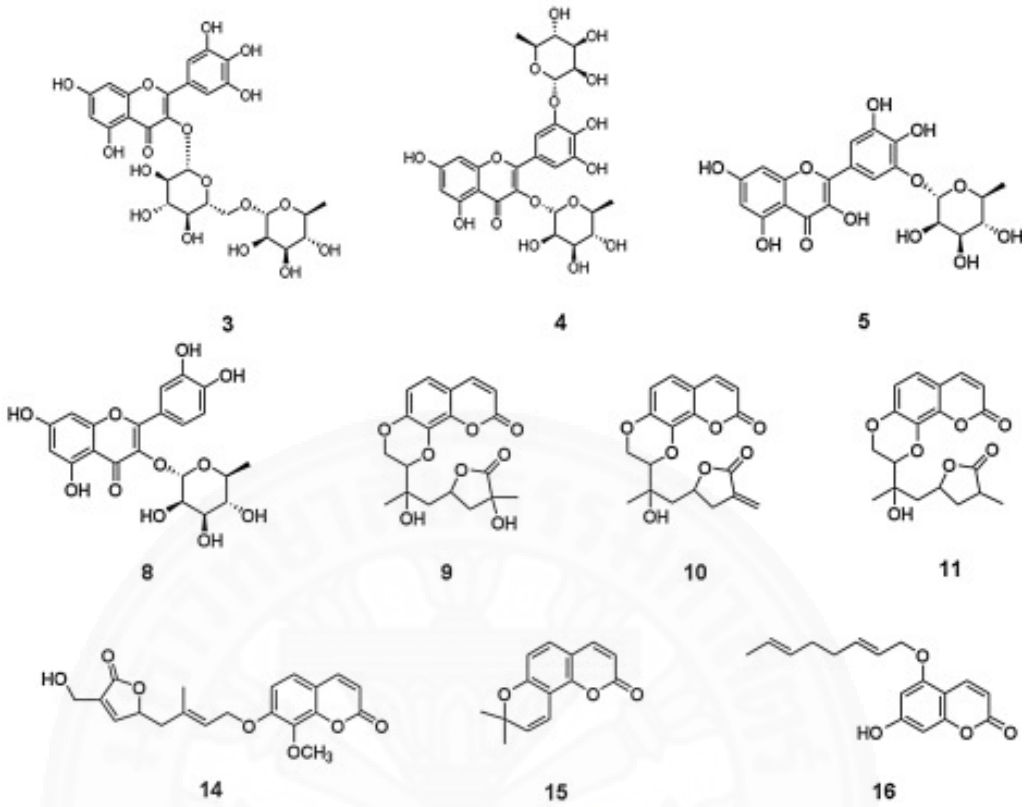


Figure 2.18 Some chemical constituents of *Clausena excavate* Burmf.

2.3.3.9 *Cuminum cyminum* L. [UMBELLIFERAE]

Figure 2.19 *Cuminum cyminum* L.

www.organicfacts.net

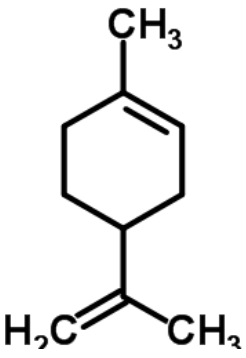
Common name: Cumin

Thai common name: Thian kao

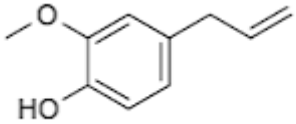
Cumin is most widely used for culinary and medicinal purposes. It is generally used as a popular spice for food additive and flavoring agent in many cuisines. In traditional medicine to treat a variety of diseases including hypolipidemia, cancer, and diabetes. In Thailand, seed is used as restorative, carminative, expectorant and to relieve nausea [Chanwithesuk *et al*., 2005].

Chemical constituents of *Cuminum cyminum* L.

There is constituents such as cumin that is the main active compound, [Derakhshan *et al*., 2008] limonene, eugenol, α-and β-pinenes [Dorman *et al*., 2000].



limonene



eugenol

Figure 2.20 Some chemical constituents of *Cuminum cyminum* L.

2.3.3.10 *Foeniculum vulgare* Miller subsp. var. vulgare. [UMBELLIFERAE]

Figure 2.21 *Foeniculum vulgare* Miller subsp. var. vulgare. http://www.reherb.eu

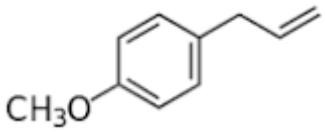
Common name: Fennel

Thai common name: Tian kaopeug

In traditional medicine for a wide range of ailments related to reproductive, digestive, endocrine and respiratory systems. Fennel is an important medicinal and aromatic plant widely used as carminative, digestive, diuretic, treating respiratory and gastrointestinal disorders. Its seeds are used as flavorings in ice cream, baked goods, meat, alcoholic beverages, fish dishes and herb mixtures [Shamkant *et al*., 2014].

Chemical constituents of *Foeniculum vulgare* Miller subsp. var. vulgare.

There is constituents such as essential oil, trans-anethole, estragole, d-limonene. [Zhaoa *et al*., 2012].



stragole

Figure 2.22 Some chemical constituents of *Foeniculum vulgare* Miller subsp. var. vulgare.

2.3.3.11 *Lipidium sativum* Linn. [CRUCIFERAE]

Figure 2.23 *Lipidium sativum* Linn.

http://boliviachef.blogspot.com

Common name: Garden cress

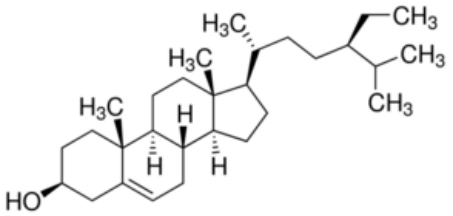
Thai common name: Thian dang

Cress has been widely used to treat a number of ailments in traditional systems of medicine throughout India. Cold infusions of seeds are used to relieve hiccough, chronic enlargement of liver and spleen and also used as carminative adjunct to purgatives, seed mixed with lime juice are used to local application for the relief of inflammatory and rheumatic pains.

The seeds are bitter depurative, themogenic, galactagogue, rubefacient, tonic, aphrodisiac, emmenagogue, and diuretic. They are useful as poultices for sprains, and in leprosy, skin diseases, dysentery, diarrhea, splenomegaly and asthma [Manohar *et al*., 2012].

Chemical constituents of *Lipidium sativum* Linn.

There is constituents such as palmitic, stearic, behenic, lignoceric, oleic, linoleic, β-sitosterol, α-tocopherol, stigmast-5-en-3 and β 27-diol 27-benzoate [Ravindra *et al*., 2007].



β-sitosterol

Figure 2.24 Some chemical constituents of *Lipidium sativum* Linn.

2.3.3.12 *Myristica fragrans* Houtt. [MYRISTICACEAE]

Figure 2.25 *Myristica fragrans* Houtt.
http://hashmidawakhana.co.in

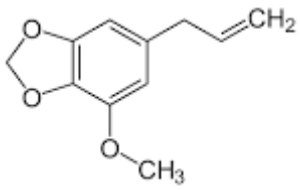
Common name: Nutmeg & Mace

Thai common names: Look chan [Nutmeg], Dok chan [Mace]

Traditional uses: *M. fragrans* is a unique spice as it produces two economically important spices, nutmeg and mace. It is such a wonderful traditional Ayurvedic herbal medicine explained by acharyas for the treatment of various disorders. *M. fragrans* has been used for centuries for diarrhea, dysentery, insomnia, inflammation, heart diseases and impotency [Sonali *et al*., 2014]. In Thai used *M. fragrans* for carminative and cardio-tonic [Prapaspong *et al*., 1999].

Chemical constituents of *Myristica fragrans* Houtt.

There is constituents such as fixed oil that contains myristic acid, trymiristin, glycocides, tridecanoic, stearic and palmitic acid. [Spricigo *et al*., 1999] Nutmeg has essential oil such as myristicin, elemicin, eugenol and safrole [Dorman *et al*., 2000]. Mace has higher myristicin than nutmeg [Hallstrom *et al*., 1997].



myristicin

Figure 2.26 Some chemical constituents of *Myristica fragrans* Houtt.

2.3.3.13 *Piper nigrum* Linn. [PIPERACEAE]

Figure 2.27 *Piper nigrum* Linn.

www.th.newstyle-live.com

Common names: Pepper, White pepper

Thai common name: Prikthailon

In Thai traditional medicine used the fruit for promoting body elements [earth, water, wind and fire], carminative, and expectorant [Prapaspong *et al*., 1999].

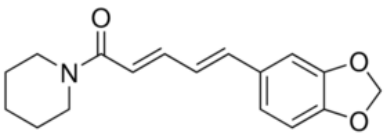
In Ayurveda used paste of black pepper for piles, boils, headache, rheumatic pains, toothaches, prolapsed rectum.

Pepper used for dyspepsia, diarrhea, flatulence, gonorrhea, cholera, cough and malarial fever [Nisar *et al*., 2012].

In India used in traditional medicine for diarrhea, constipation, gangrene, heartburn, earache, hoarseness, hernia, insect bites, indigestion, liver, lung problems, sunburm, dental caries and toothache [Nisar *et al.*, 2012].

Chemical constituents of *Piper nigrum* Linn.

There is constituents such as volatile oil, alkaloid, piperine, [piperyline, piperoleines and piperamine] α- and β-pinene, limonene, myrcene, linalool, α-phellandrene, sabinene, β-caryophyllene, germacrene-D, etc. [Sruthi *et al*., 2012].



piperine

Figure 2.28 Some chemical constituents of *Piper nigrum* Linn.

2.3.3.14 *Plumbago indica* L. [PLUMBAGINACEAE]

Figure 2.29 *Plumbago indica* L.
http://database.prota.org

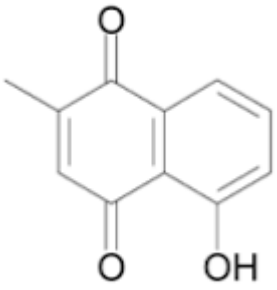
Common names: Fire plant, Scarlet Leadwort

Thai common name: Chetamoolplerng

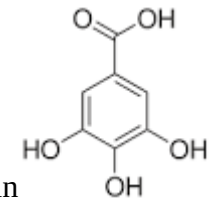
In Thai traditional used roots for a part of Benjakul remedy that helps to promote and balance body elements. Moreover, it is used as diarrhea, carminative, to promote blood circulation, dermatophytosis, skin disease and menstruation disorder but it can lead to abortion [Prapaspong *et al*., 1999].

Chemical constituents of *Plumbago indica* L.

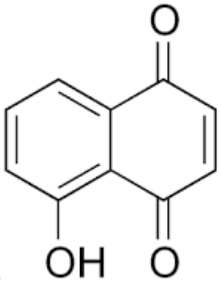
There is constituents such as plumbagin, tannin, napthoquinone, sitosterol glycoside, glucose and organic acids. Aerial parts contain 6-hydroxyplumbagin, plumbagin, stigmasterol, sitosterol, campesterol, roseanone, bi-naphthaquinone, elliptinone, droserone and zucylanone [Ghani, 2003].



napthoquinone



plumbagin



tannin

Figure 2.30 Some chemical constituents of *Plumbago indica* L.

2.3.3.15 *Syzygium aromaticum* (L.) Merr. Et Perry. [MYRTACEAE]

Figure 2.31 *Syzygium aromaticum* (L.) Merr. Et Perry.
www.powo.science.kew.org

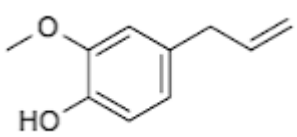
Common name: Clove

Thai common name: Kanphlu

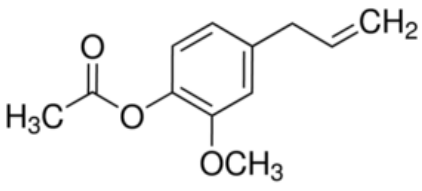
Clove is a spice commonly and appears to have some traditional usage as an aphrodisiac as well as for the medicinal purposes of dental disorders, respiratory disorders, headache and sore throat. The part used is the flower, buds of the clove. The aromatic oils of the clove have a stimulant and irritant effect. Cloves can raise a person's temperature slightly and increase blood circulation. The oils of the cloves have been known to stimulate and disinfect as it travels through the body. Clove can be used to promote the flow of saliva gastric juices [Prapaspong *et al*., 1999].

Chemical constituents of *Syzygium aromaticum* (L.) Merr. Et Perry.

There is constituents such as eugenol, acetoeugenol, β-caryophyllene as major constituent. α-Copaene, α-humulene, methyl salicylate were other constituents [Pal *et al*., 2008].



eugenol



acetoeugenol

Figure 2.32 Some chemical constituents of *Syzygium aromaticum* (L.) Merr. Et Perry.

2.3.3.16 *Terminalia chebula* Rez var Chebula. [COMBRETACEAE]

Figure 2.33 *Terminalia chebula* Rez var Chebula.

www.thaicrudedrug.com

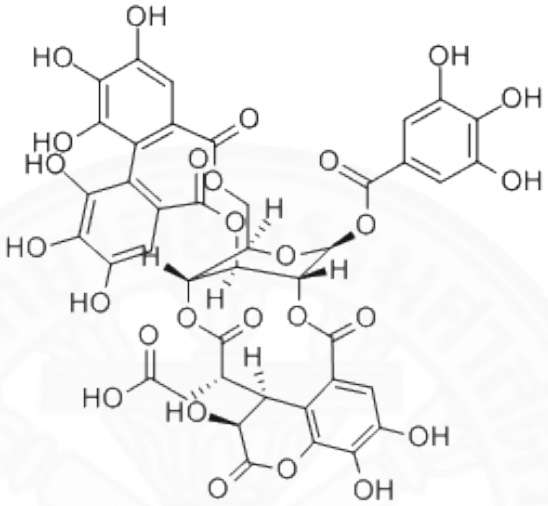
Common names: Myrobalan, Chebulic myrobalan

Thai common name: Samor thai

It is given as adjuvant herb in chronic fever and on long term use it is helpful in gaining weight in emaciated persons and in losing weight in obese persons. Myrobalan is used for curing swellings, skin and eye disease. It can be used as a home remedy against fever, cough, asthma and urinary disease. There is the ability to stop bleeding and prevent hemorrhage. Its powder is used as toothplate that will make your teeth stronger and healthy. The paste of dried fruit is used for chronic ulcers, wound and scalds [Prakash *et al*., 2012].

Chemical constituents of *Terminalia chebula* Rez var Chebula.

There is constituents such as tannins, gallic acid, ellagic acid, chebulagic acid, pyrogallol, punicalagin, corilagin,chebulanin, neochebulinic acid, chebulinic acid, 1,2,3,4,6-penta-O-galloyl-β-D-glucose, 1-6-di-o-galloyl-D-glucose, casuarinin, 3,4,6-tri-o-glloyl-D-glucose, terchebulin [Juang *et al*., 2004].



chebulagic acid

Figure 2.34 Some chemical constituents of *Terminalia chebula* Rez var *Chebula*.

2.3.3.17 *Terminalia arjuna* Wight& Arn. [COMBRETACEAE]

Figure 2.35 *Terminalia arjuna* Wight& Arn.

www.wallawit-herb.plazathai.com

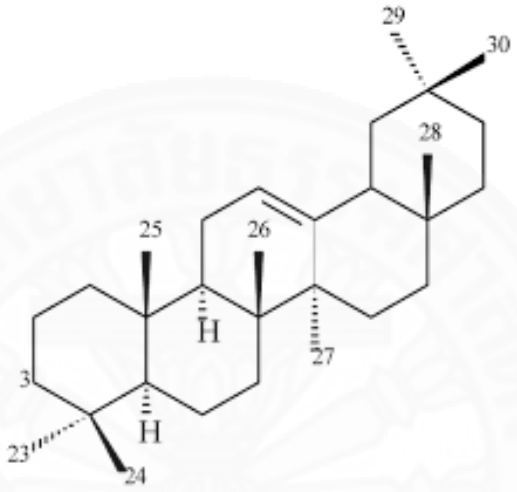
Common names: Arjun, Arjuna

Thai common name: Samorthet

Arjuna has been widely used in Ayurveda medicine for the treatment of cancer, heart diseases, dematological, gynecological and complaintsand urinary disorders. The bark used for astringent and tonic, high blood pressure and ulcers. The cancer cell growth inhibitory constituent [luteolin] has been isolated from bark, stem and leaves of *T. arjuna*. Luteolin has also been shown to have specific anti-bacterial activity against *Neisseria gonorrhea*. It can also be used as styptic, alexiteric, tonic and anthelmintic and it is useful in fractures, inflammation and wounds and ulcers [Orwa *et al*., 2009].

Chemical constituents of *Terminalia arjuna* Wight& Arn.

There is constituents such as triterpenoid glycosides ie, arjunetosides I, II, III, IV, arjunine, arjunetein, Co-enzyme2 Q10, tannins ie., ellagic acid, gallic acid, beta-sitosterol; flavonoids viz., arjunone, arjunolone, luteolin, triterpenoid saponins viz., arjunolic acid, arjunic acid, arjungennin, elements ie., calcium, magnesium, zinc and copper [Giri *et al*., 2012].



triterpenoid saponins

Figure 2.36 Some chemical constituents of *Terminalia arjuna* Wight& Arn.

2.3.3 Biological studies of ingredients of Aphaisalee remedy

Table 2.2 Biological studies of ingredients of Aphaisalee remedy(continued)

Scientific name	Activities	Biological activities	References
Acorus calamus Linn. [ARACEAE]	Cytotoxicity	The cytotoxicity of *Acorus calamus* against human colorectal adenocarcinoma [HT–29], human epidermoid carcinoma [KB] and human cervical cancer [SiHa] cells at 24, 48 and 72 h. Both accessions at 300 µg/mL and 72 h showed the highest activity against all the tumoral cells.	Rakesh *et* al., 2016
Amomum testaceum Ridl. [ZINGIBERACEAE]	Cytotoxicity	The ethanolic extract of *Amomum testaceum* Ridl. at conc.100 µg/ml with MTT assay inhibited SMMC-7721 and conc. 50 µg/ml inhibited SMMC-7721 with 40% C.-L.	Lu *et al*., 2013
Anethum graveolens Linn. [UMBELLIFERAE]	Anti-cancer	*Anethum graveolens* exhibited non-cytotoxicity in normal cell. On the other hand, it promoted anticamcer activity on KB-Oral cavity cancer and MCF-7-Breast cancer.This activity provides an important role for clinical studies and may be advantageous in other directions about the use of medicinal herbs that are grown in Thailand to high advantage.	Peerakam *et al*., 2014
Atractylodes lancea [COMPOSITAE]	Cytotoxicity	The extract from *A. lancea* showed the most potent and most selective against cholangiocarcinoma [IC_{50} = 24.09 µg/ml, SI = 8.6].	Mahavorasirikul *et al*., 2010

Table 2.2 Biological studies of ingredients of Aphaisalee remedy(continued)

Scientific name	Activities	Biological activities	References
Clausena excavate Burmf. [RUTACEAE]	Cytotoxicity	All roots extracts except methanol showed strong activity against HL-60 and MCF-7 cancer cell lines with IC_{50} values 4-6 μg/ml. Dentatin was found to be the most cytotoxic constituent against all cancer cell lines with IC_{50} values 5-10 μg/ml.	Sharif *et al*., 2011
Cuminum cyminum L. [UMBELLIFERAE]	Cytotoxicity	The cytotoxic activity of *C. cyminum* in ethanolic extract with seven human cancer cell lines Colon 502713, Colo-205, Hep-2, A-549, OVCAR-5, PC-5, SF-295 those were determined using SRB assay found to be 25%, 61%, 40%, 31%, 31%, 28%, 27% against SF-295, Colon 502713, Colo-205, Hep-2, A-549, OVCAR-5, PC-5 human cancer cell lines respectively. *C. cyminum* extract showed 61% maximum activity against Colon 502713 cell line.	Ekta *et al*., 2014
Foeniculum valgare Mill [UMBELLIFERAE]	Cytotoxicity	*F. vulgare* showed low cytotoxicity by using SRB assay against V79 cells of the essential oil.	Wakabayashi *et al*., 2015
Lipidium sativum Linn. [CRUCIFERAE]	Cytotoxicity	Cytotoxic effect of aqueous extract *of L. sativum* seeds on human breat camcer cells [MCF-7] showed a significant cytotoxic effect on MCF-7 cells.	Sawsan *et al*., 2013

Table 2.2 Biological studies of ingredients of Aphaisalee remedy(continued)

Scientific name	Activities	Biological activities	References
Myristica fragrans Houtt [MYRISTICACEAE]	Cytotoxicity	*M. fragrans* against seven human cancer cell lines ascribed to central nervous system, Colon cell [Colon502713,Colo205], Liver[Hep-2], Lung [A-549], Ovary OVCAR-5 and Prostrate [PC-5]. The SRB assay was used to test cytotoxic activity for all the cell lines. The result of OVCAR-5 [Ovary] cell line showed highest cytotoxic activity [97%] Growth inhibition of colon 502713 and PC-5 [Prostrate] cell lines was 86%. SF-295[CNS] and Colo205 showed 73% growth inhibition, while Hep-2 [Liver] showed 72%. A-549 [Lung] showed no significant activity [50%].	Ekta *et al*., 2013
Piper nigrum Linn. [PIPERACEAE]	Cytotoxicity	The ethanolic of root by MTT assay inhibited COLO-205 with IC_{50} values 3.10 mg/ml and 0.018 mg/ml	Rao *et al*., 2011

Table 2.2 Biological studies of ingredients of Aphaisalee remedy(continued)

Scientific name	Activities	Biological activities	References
Piper nigrum Linn. [PIPERACEAE]	Cytotoxicity	Piperidine in *Piper nigurm* inhibited potentiate in sublines MCF-7/DOX and A-549/DDP from MCF-7 and A-549 at conc. 50%	Sen *et al*., 2011
	Cytotoxicity	Test by MTT assay with HCT-8 ,MDA-MB-435 and SF-295 can inhibited.	Mesquita *et al*., 2009
Plumbago indigo L. [PLUMBAGINACEAE]	Cytotoxicity	The methanolic extract of *P. indica* showed the activity against brine shrimp nauplii and calculated LC_{50} and LC_{90} value was 5.0µg/ml and 12 µg/ml respectively.	Dibyajyoti *et al*., 2012
	Cytotoxicity	Cytotoxic activity of detoxified root ethanol extract of *P. auriculata, P. indica* and *P. zeylanica* were against *H. pylori* and cytotoxicity activity with MTT assay in HGE-17 cell lines with IC_{50} ranging about 50-250 µg/ml. These have dose dependent cytotoxicity activity in HGE-17 cell lines. Zone of inhibition test of these Plumbaginales plants ethanol extract against H. pylori have significant activity. *P. indica* [10 mg] have more activity compared to other two plants. Three Plumbaginales detoxified plants root have cytotoxicity in HGE-17 cell lines.	Ann *et al*., 2013

Table 2.2 Biological studies of ingredients of Aphaisalee remedy(continued)

Scientific name	Activities	Biological activities	References
Syzygium aromaticum [MYRISTICACEAE]	Cytotoxicity	Clove oil and Eugenol from *Syzygium aromaticum* test by SRB assay was found IC_{50} = 0.162 and 0.167 mg/mL	Khunkitti *et al*., 2012
Syzygium aromaticum [MYRISTICACEAE] *Terminalia chebula* [COMBRETACEAE]	Cytotoxicity	The water extract of *Syzygium aromaticum* can anti pro-apoptosis with first of lung carcinoma.	Banegee *et al*., 2006
	Cytotoxicity	The ethanolic 50% of *Syzygium aromaticum* at conc.0.5-2 mg/mL inhibited at 1.5 mg/mL with cervical cancer	Hussain *et al*., 2009
	Cytotoxicity	Chebulagic acid of *Syzygium aromaticum* inhibited COX-2 and 5-LOX and COLO-205	Reddy *et al*., 2009
Terminalia chebula [COMBRETACEAE]	Cytotoxicity	*Terminalia chebula* inhibiting COLO-205,HCT-15, MDA-MB-231, DU-145 and K562 cell lines with IC_{50} values of 18±0.2186, 20.3±0.23, 26.2±0.472, 28.54±0.389 and 30.66±0.36 µM	Reddy *et al*., 2009

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Materials and Methodology

3.1.1 Plant materials

Plant materials were purchased from Charernsook Osot pharmacy [Nakornpathom, Thailand]. The voucher specimens were referenced from the herbarium of Southern Center of Thai Medicinal Plant, Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand. Aphaisalee remedy consists of 19 herbs and each plant material was weighed in the ratio specified in NLEM [Thai Nathional List of Essential Medicine, 2013] used for longevity or rejuvenation and is claimed to improve blood circulation and shown in **Table 3.1**

Table 3.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Aphaisalee remedy	-	-	Tumrab a phai sa lee	100	-	Hot & Spicy	Longevity or rejuvenation and is claimed to improve blood circulation
Acorus calamus Linn.	ARACEAE	SKP 015 01 30 01	Wan num	3.74	Rhizome	Hot & Spicy	Gastrointestinal agent,carminative
Amomum testaceum Ridl.	ZINGIBERACEAE	SKP 206 01 20 01	Krawan	1.6	Fruit	Hot & Spicy	Promote blood circulation,carminative
Amorphophallas saraburiensis (Dennst)	ARACEAE	SKP 015 01 19 01	Book ror	8.02	Rhizome	Nauseated	Promote lymphatic system
Anethum graveolens Linn.	UMBELLIFERAE	SKP 199 01 07 01	Thien tatakkataen	4.81	Seed	Hot & Spicy & Bitter	Carminative

Table 3.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation (continued)

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Angelicadahurica Berth.	UMBELLIFERAE	SKP 199 01 04 01	Kot sor	4.28	Rhizome	Bitter & cold	Antipyretic, tonic, expectorant, anti-asthma and cough
Atractylodes lancea	COMPOSITAE	SKP 051 01 12 01	Kot khamoa	4.81	Rhizome	Hot & Spicy	Tonic, antipyretic,paregoric, diuretic agents, relieve common cold, relieve asthma, oral prophylaxis
Ardisia elliptica Thumb.	MYRSINACEAE	SKP 122 01 16 01	Philangkasa	3.21	Fruit	Astringent	Anti-diarrhea, relieve urticarial
Clausena excavate Burmf.	RUTACEAE	SKP 166 03 05 01	Hasakhun thet	27.59	Stem	Hot & Spicy	Promote blood circulation, anti-nasal polyp,expectorant
Cuminum cyminum L.	UMBELLIFERAE	SKP 199 03 03 01	Thien kao	4.28	Seed	Hot & Spicy & Bitter	Carminative, promote blood circulation

Table 3.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation (continued)

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Foeniculum valgare Mill	UMBELLIFERAE	SKP 199 06 22 01	Thien kaopeug	5.35	Seed	Hot & Spicy	Carminative, promote blood circulation
Lipidium sativum Linn.	CRUCIFERAE	SKP 057 12 19 01	Thien daeng	5.88	Seed	Hot & Spicy	Gastrointestinal agent, carminative, expectorant
Myristica fragrans Houtt	MYRISTICACEAE	SKP 121 13 06 01	Chanthet (Kaen chan)	8.56	Stem	Hot & Spicy	Tonic, cadio-tonic, carminative
Myristica fragrans Houtt	MYRISTICACEAE	SKP 121 13 06 01	Chanthet (Dok chan)	1.07	Mace	Hot & Spicy	Tonic, cadio-tonic, carminative
Myristica fragrans Houtt	MYRISTICACEAE	SKP 121 13 06 01	Chanthet (Look chan)	0.53	Nutmeg	Hot & Spicy	Tonic, cadio-tonic, carminative

Table 3.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation (continued)

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Piper nigrum Linn.	PIPERACEAE	SKP 146 16 14 01	Prikthai	8.56	Seed	Hot & Spicy	Carminative, promote blood circulation, toni longevity
Plumbago indigo L.	PLUMBAGINACEAE	SKP 148 16 09 01	Chetamoolplerng	6.42	Root	Hot & Spicy	Promote blood circulation, carminative, gynecology
Syzygium aromaticum (L.) Merr. Et Perry.	MYRISTICACEAE	SKP 123 19 01 01	Kan phlu	2.14	Flower	Hot & Spicy	Carminative, paregoric of tooth and pyorrhea, expectorant
Terminalia chebula Rez var Chebula.	COMBRETACEAE	SKP 049 20 03 01	Samor thai	6.95	Fruit	Astringent & Sour	Laxative, anti-diarrhea,expectorant

Conceptual framework of this research

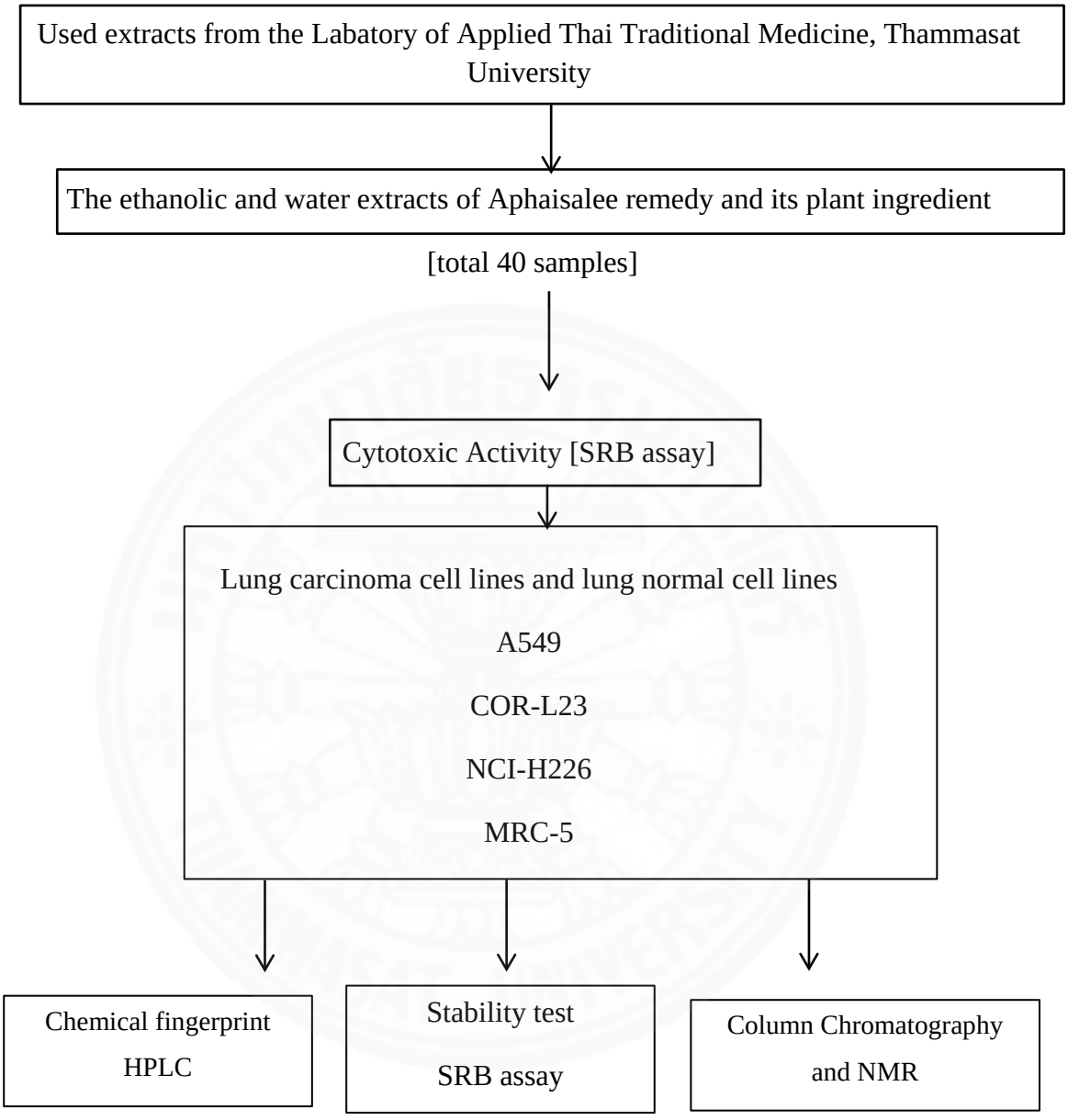


Figure 3.1 Conceptual framework of this research

3.2 Chemicals, reagents, instruments, plastics and glasswares

Table 3.2 Chemicals and reagents for cytotoxic activity [SRB assay]

Name	Source
Dimethyl sulfoxide [$(CH_3)_2SO$)][DMSO]	RCI Labscan, Thailand
Fetalbovineserum [FBS]	Biochem, Germany
Hydrochloric acid [HCl]	Univar, Australia
Sodium hydroxide [NaOH]	Univar, Australia
Minimum Essential Medium [MEM]	Gibco, USA
Penicillin-Streptomycin [P/S]	Gibco, USA
RPMI medium 1640	Gibco, USA
Trypan blue stain 0.4%	Gibco, USA
Trypsin-EDTA	Gibco, USA
Phosphate buffered saline [PBS]	Amresco, USA
Sodium bicarbonate [$NaHCO_3$]	BHD, England
Sulforhodamine B sodium salt [$C_{27}H_{29}N_2NaO_7S_2$]	Sigma-Aldrich, USA
Tris [hydroxymethyl] aminomethane [$(HOCH_2)_3CNH_2$]	Sigma-Aldrich, USA
Trichloroacetic acid [Cl_3CCOOH] [TCA]	Merck, Germany

Phytochemical investigantion of compound isolated

Table 3.3 Column chromatography

Name	Source
Dichloromethame, [CH2Cl2] Analytical grade	RCI Labscan, Thailand
Hexane, [CH3[CH2]4CH3] Analytical grade	RCI Labscan, Thailand
Chloroform, [CHCl3] Analytical grade	RCI Labscan, Thailand
Methanol, [CH3OH] Analytical grade	RCI Labscan, Thailand
Ethyl acetate, [CH3COOC2H5] Analytical grade	RCI Labscan, Thailand
Acetone, [CH3COCH3]	RCI Labscan, Thailand
Silica Gel 60 [0.063-0.200 mm]	Merck, Germany
Silica Gel 60 [0.040-0.063 mm]	Merck, Germany
TLC silica gel 60 F254	Merck, Germany
Anisaldehyde, [C8H8O2]	Fluka, Switzerland
Sulfuric acid, [H2SO4]	Merck, Germany

Table 3.4 List of instruments

Name	Source
96-well plate flat, bottom with lid	Costar Corning, USA
96-well plate flat, bottom without lid	Costar Corning, USA
Autoclave	Hirayama, Japan
Balance 0.01 mg - 41 g	Mettler-Toledo, Swizerland
Balance 0.01 g - 220 g	Precica, Swizerland
Balance 0.5 mg - 3100 g	Mettler-Toledo, Swizerland
Cell culture flask, canted neck 25, 75 cm^3	Corning, USA
Centrifuge machine	Boeco, Germany
CO_2 humidified incubator	Forma, USA
Crucibles	Coorstex, USA
Cryogenic tube 2 ml	Corning, USA
Disposable pipette 2,5,10 and 25 ml	Corning, USA
Eppendorf	Costar Corning, USA
Filter paper no.1 [125 mmØ]	Whatman, USA
Filter paper no.40 [125 mmØ]	Whatman, USA
Freezer	Sanyo, Japan
Glass bottles	Schott Duran, Germany
Glasswares	Schott Duran, Germany
Hematocytometer	Boeco, Germany
High Performance Liquid Chromatography [HPLC]	Algelent technologies, USA
Hot air oven	Memmert, Germany
Hot plate	Thermolyne, USA
HPLC column	Phenomenex, USA
Inverted microscope	Nikon, Japan
Laminar air flow	Faster, Italy
Lyophilizer	Telster, Spain
Micropipettes	Eppendrof, Germany
Microplate reader	Bio Tek, USA

Table 3.5 List of equipments, plastics and glasswares used in the studies

Name	Source
Microplate washer	Bio Tek, USA
Moisture analyzer	Scaltec instrument, Germany
Muffle furnace	Nabertherm, Germany
Multi-channels pipette	Costar Corning, USA
PGE_2 Enzyme Immuno-Assay Kit	Cayman, USA
pH meter	WTW inolab, Germany
Pipette tips	Costar Corning, USA
Pipette boy	Brand, USA
Quantikine mouse TNF- α ELISA test kit	R&D systems, USA
Reagent reservoir [Sterile]	Costar Corning, USA
Refrigerrator [-20 ^{o}C]	Sanyo, Japan
Rotary evaporator	Buchi, Swizerland
Shaking incubator	Vision Scientific, Korea
Sonicator	Elma, Germany
Syringes	Nipro, Thailand
Vacuum Desiccator	Simax, USA
Vacuum pump	Rocker, Taiwan
Vortex	Scientific industries, USA
Water bath	Memmert, Germany
Water purification machine	Elga, UK

3.3 Preparation of crude extracts

The ethanolic and water extracts of Aphaisalee remedy and its plant ingredient brought from the Labatory of Applied Thai Traditional Medicine ,Thammasat University [Champasuri *et al*., 2015].

Plant parts, of APSL remedy were collected and purchased from several regions of Thailand. These were washed, sliced thinly, dried in an oven at 50^{o}C and powdered. After that, these plants were weighed and mixed to be APSL remedy. The extracts would be obtained by two methods, namely maceration and decoction.

3.3.1 Maceration Plants of APSL remedy were macerated with 95% ethanol for 3 days. Next, filtered and concentrated to dryness under pressure. The maceration was repeated. 2 times [total 3 times] and dried by using an evaporator. Percentages of yield were calculated.

3.3.2 Decoction Plants of APSL remedy were boiled in distilled water for 15 minutes. Then filtered using Whatman NO.1 filter paper and concentrated to dryness by lyophilizer. This was repeated 2 times [total 3]. Percentages of yields from all extracts were calculated.

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

3.4 *In vitro* assay for Cytotoxic activity by SRB assay

Preparation of sample solution

Each sample was initially dissolved in sterile dimethylsulfoxide [DMSO] for ethanolic extracts and aqueous extracts was initially dissolved in DI water. Then, filtered using 0.22 micron filter and prepared sample at concentration 10 mg/ml. The first screening, final concentration of sample solution was prepared at 100 µg/ml. If the percent inhibition of cancer cell by the extract is more than 50%, the extract was further tested for IC_{50} value. IC_{50} values were determined by preparing as four serial dilutions [200, 100, 20 and 2 µg/ml, respectively] to give the final concentration of 100, 50, 10, 1 µg/ml, respectively.

Human cell lines

Human Lung Carcinoma [A549, ATT CCL-185 and NCI-H226] and Human Caucasian lung large cell carcinoma [COR-L23, ECCACC 92031919] Cell Lines were purchased from the American Type Culture Collection [ATCC, USA]. All cell lines were cultured in RPMI-1640 medium [BIOCHROMAG]. Lung Normal [MRC-5, ATCC CCL-171] Cell Lines were purchased from the American Type Culture Collection [ATCC, USA] what were cultured in Minimum Essential Medium [MEM]. These supplemented with 10% heated fetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin. All cell lines were maintained at 37 °C in an incubator with 5% CO_2 and 95% humidity.

Cytotoxicity assay

Cells growing as monolayer in 75 cm^3 flask were washed with Phosphate-buffered saline [PBS] [Biochem, Germany] and cells detached with 0.025% trypsin-EDTA [Gibco, USA] to make a single cell suspension.3 ml medium was then added to flask to inactivate trypsin-EDTA.The viable cells were counted in haemocytometerusing Trypan Blue Exclusion Test of cell viability [Freshney., 1994]. Single cell suspension was diluted with medium to give a final concentration of 3×10^5cells/ml for each of NCI-H226 and 10^5 cells/ml for A549 and COR-L23, respectively. One hundred microl itres per well of these cells suspensions were added in 96-well microtiter plates and incubated at 37 °C in 5% CO_2 atmosphere with 95% humidity for 24 hours. Cells were then treated with extracts. 100 µl sample solution

was added and mixed where 2% DMSO solution was used as control solvent. Cells in 96-well plate were incubated in CO_2 incubator for 72 hours. Medium was removed and washed with PBS. 200 µl fresh medium was added, incubated for recovery period of 72 hours and the survival percentage was measured calorimetrically using SRB assay and IC_{50} values were calculated using Prism program.

Sulphorhodamine B [SRB] assay

The anti-proliferative assay, SRB [Sulphorhodamine B] assay was performed essentially according to the method of Skehan [Skehan *et al* ., 1990]. This assay is used for cell density determination, which performed to assess growth inhibition by a colorimetric assay by staining total cellular protein with the dye SRB. Cell cultures were fixed with 100 µl of ice-cold 40% Trichloroacetic acid [TCA] per well, incubated at 4 °C for 1 hour in refrigerator and washed with distilled water to wash non viable cells, 50 µl of SRB solution [0.4% w/v in 1% acetic acid, Sigma] was added per well andallowed contact with cells for 30 minutes and washed with 1% acetic acid. The plate was dried and 100 µl of 10 mMTris Base pH 10.5 [Tris [hydroxyl methyl] aminomethane] was added. The absorbance [OD] of each well was read at 492 nm. The IC_{50} values were calculated using Prism program by plotting the percentage of survival versus the concentrations, interpolated by cubic spine. According to National Cancer Institute guidelines [Boyed *et al*., 1997] extracts with IC_{50} values < 20 µg/ml were considered active.

3.5 Column Chromatography

The plants ingredient witch showed the highest cytotoxic activity was chosen to separate by column chromatography. Dried extract of *Clausena excavate* Burmf. was mixed with silica gel as stationary phase. The silica gel column was stationary phase it was eluted with low to high polarity solvents as followed: Hexane:Dichloromethane [9:1, 2,000 ml], Dichloromethane [1,000 ml], Dichloromethane:Methanol [9:1, 500 ml], Dichloromethane:Methanol [7:3, 500 ml], Dichloromethane:Methanol [5:5, 500 ml], Dichloromethane:Methanol [3:7, 500 ml], and Methanol [500 ml], Respectively. The solvent was collected for 10 mL/tube. The collected fractions were examined by TLC chromatogram and detected with

anisaldehyde spray. The structure of isolated pure compound was indentified by TLC chromatogram characteristics and protron Nuclear Magnetic Resonance spectrum.

Bioassay-guided fractionation of the 95%ethanolic extract of *C. excavate* Burmf.

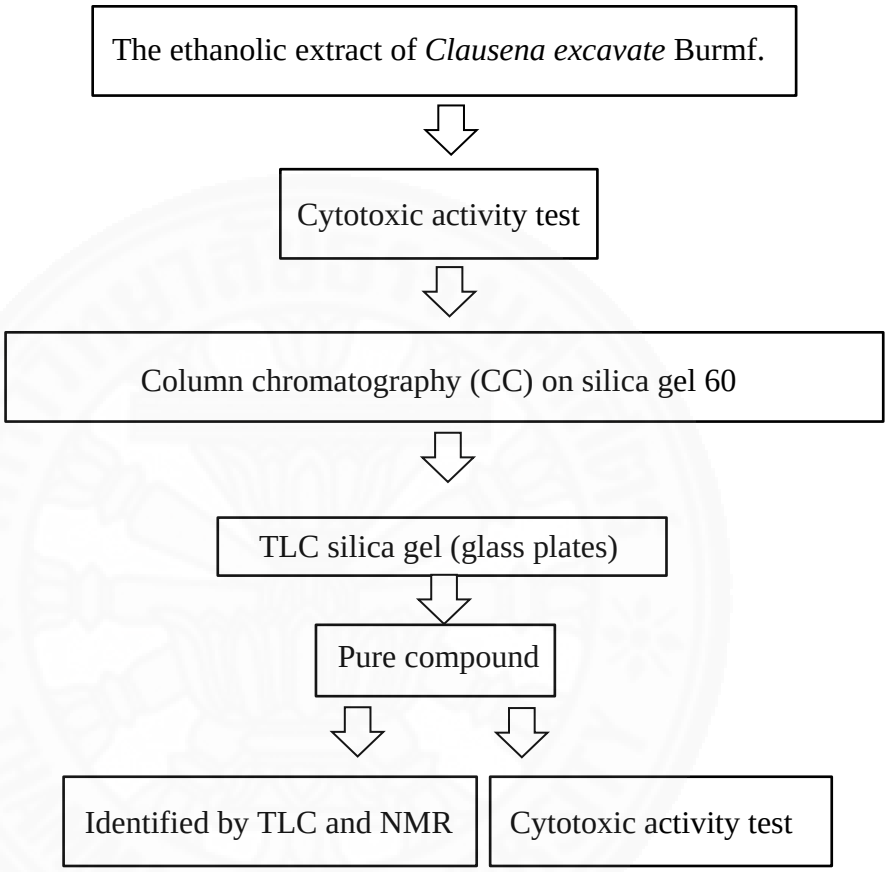


Figure 3.2 Bioassay-guided of isolation

3.6 Study on chemical fingerprint of APSL extract

Chemicals and Reagents

Authentic compounds expected from HPLC chromatogram and cytotoxic assay will be purchased. Acetonitrile, methanol and purified water [HPLC grade] from Labscan [Bangkok, Thailand].

Apparatus and chromatographic conditions

RP-HPLC analysis

The chemical fingerprint study will carry out using high performance liquid chromatography [HPLC] system [Agilent 1200] which consist of a solvent degasser [G1322A], aquaternary solvent pump [G1311A], an autosampler [G1329A], column oven [G1316A] and a photodiode array detector [G1315D]. The chromatosraphic data will be processed by Chemstation software revision B.04.01SP1.

Separation of compounds in A-Phai-Sa-Lee remedy will be conducted along a c18 column [250x4.6 mm, 5 micron] with guard column using gradient mobile phase composing of water and acetonitrile. The chromatographic condition will be modified from Thongmee. [Thongmee, 2015] for optimum separation of peaks found in the remedy.

Preparation of Aphaisalee for HPLC analysis

Ten milligrams of A-Phai-Sa-Lee ethanolic extract was dissolved in 1 ml methanol, and then sonicated for 15 minutes. This solution was filtered through a membrane filter [pore size 0.45 μm] prior to analysis.

Preparation of standard solutions

A stock solution of expected compounds were prepared at a concentration of 1.0 mg/ml in appropriated solvent.

3.7 The stability test on APSL extract

The stability of ethanolic extracts of APSL remedy brought from the Labatory of Applied Thai Traditional Medicine ,Thammasat University**.**

Stability testing was done using transparent vials. APSL extract was put in these vials and exposed under 40±2^{o}C with 75±5% RH as accelerated testing for 6 a periods. All samples were tested for nitric oxide inhibition effect and cytotoxicity activities on days 0, 15, 30, 60, 90, 120, 150, 180 [Champasuri *et al*., 2015].

3.8 Statistical analysis

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

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Extraction of A-Phai-Sa-Lee [APSL] remedy and its ingredients

4.1.1 Plant materials

Plant materials were purchased from Charernsook Osot pharmacy [Nakornpathom, Thailand]. The voucher specimens were referenced from the herbarium of Southern Center of Thai Medicinal Plant, Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand. Aphaisalee remedy consists of 19 herbs and each plant material was weighed in the ratio specified in NLEM [Thai Nathional List of Essential Medicine, 2013] used for longevity or rejuvenation and is claimed to improve blood circulation and shown in **Table 4.1**

Table 4.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Aphaisalee remedy	-	-	Tumrab a phai sa lee	100	-	Hot & Spicy	Longevity or rejuvenation and is claimed to improve blood circulation
Acorus calamus Linn.	ARACEAE	SKP 015 01 30 01	Wan num	3.74	Rhizome	Hot & Spicy	Gastrointestinal agent,carminative
Amomum testaceum Ridl.	ZINGIBERACEAE	SKP 206 01 20 01	Krawan	1.6	Fruit	Hot & Spicy	Promote blood circulation,carminative
Amorphophallas saraburiensis (Dennst)	ARACEAE	SKP 015 01 19 01	Book ror	8.02	Rhizome	Nauseated	Promote lymphatic system
Anethum graveolens Linn.	UMBELLIFERAE	SKP 199 01 07 01	Thien tatakkataen	4.81	Seed	Hot & Spicy & Bitter	Carminative
Angelicadahurica Berth.	UMBELLIFERAE	SKP 199 01 04 01	Kot sor	4.28	Rhizome	Bitter & cold	Antipyretic, tonic, expectorant, anti-asthma and cough

Table 4.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation (continued)

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Atractylodes lancea	COMPOSITAE	SKP 051 01 12 01	Kot khamoa	4.81	Rhizome	Hot & Spicy	Tonic, antipyretic,paregoric, diuretic agents, relieve common cold, relieve asthma, oral prophylaxis
Ardisia elliptica Thumb.	MYRSINACEAE	SKP 122 01 16 01	Philangkasa	3.21	Fruit	Astringent	Anti-diarrhea, relieve urticarial
Clausena excavate Burmf.	RUTACEAE	SKP 166 03 05 01	Hasakhun thet	27.59	Stem	Hot & Spicy	Promote blood circulation, anti-nasal polyp,expectorant
Cuminum cyminum L.	UMBELLIFERAE	SKP 199 03 03 01	Thien kao	4.28	Seed	Hot & Spicy & Bitter	Carminative, promote blood circulation
Foeniculum valgare Mill	UMBELLIFERAE	SKP 199 06 22 01	Thien kaopeug	5.35	Seed	Hot & Spicy	Carminative, promote blood circulation

Table 4.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation (continued)

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Lipidium sativum Linn.	CRUCIFERAE	SKP 057 12 19 01	Thien daeng	5.88	Seed	Hot & Spicy	Gastrointestinal agent, carminative, expectorant
Myristica fragrans Houtt	MYRISTICACEAE	SKP 121 13 06 01	Chanthet (Kaen chan)	8.56	Stem	Hot & Spicy	Tonic, cadio-tonic, carminative
Myristica fragrans Houtt	MYRISTICACEAE	SKP 121 13 06 01	Chanthet (Dok chan)	1.07	Mace	Hot & Spicy	Tonic, cadio-tonic, carminative
Myristica fragrans Houtt	MYRISTICACEAE	SKP 121 13 06 01	Chanthet (Look chan)	0.53	Nutmeg	Hot & Spicy	Tonic, cadio-tonic, carminative
Piper nigrum Linn.	PIPERACEAE	SKP 146 16 14 01	Prikthai	8.56	Seed	Hot & Spicy	Carminative, promote blood circulation, tonic for longevity

Table 4.1 Plants and parts of plants comprising A-Phai-Sa-Lee preparation (continued)

Botanical name	Family	Voucher specimen	Thai name	% in remedy	Part of used	Flavor	Thai traditional medicine used
Plumbago indigo L.	PLUMBAGINACEAE	SKP 148 16 09 01	Chetamoolplerng	6.42	Root	Hot & Spicy	Promote blood circulation, carminative, gynecology
Syzygium aromaticum (L.) Merr. Et Perry.	MYRISTICACEAE	SKP 123 19 01 01	Kan phlu	2.14	Flower	Hot & Spicy	Carminative, paregoric of tooth and pyorrhea, expectorant
Terminalia chebula Rez var Chebula.	COMBRETACEAE	SKP 049 20 03 01	Samor thai	6.95	Fruit	Astringent & Sour	Laxative, anti-diarrhea,expectorant
Terminalia arjuna Wight& Arn.	COMBRETACEAE	SKP 049 20 01 01	Samor thet	6.95	Fruit	Astringent & Sour	Laxative, anti-diarrhea,expectorant

4.2 *In vitro* cytotoxic activity

4.2.1 Sulforhodamine B colorimetric assay

The results of the 95% ethanolic and water extracts of APSL remedy and its plants ingredient against three different types of lung cancer cell lines and one normal such as human lung adenocarcinoma cell line [A549], human large cell lung carcinoma cell line [COR-L23], human lung squamous carcinoma cell line [NCI-H226] and nomal human lung myofibroblast cell line [MRC-5]. The results of IC_{50} values are calculated and summarized in **Table 4.2**, **Figure 4.1 and 4.2**

The results of this study showed that the 95% ethanolic extracts and water extracts of APSL remedy and it plant ingredients exhibited higher than the all water extracts. The 95% ethanolic extracts of APSL remedy show high cytotoxic activity against on three different lung cancer cell lines such as human lung adenocarcinoma cell line [A549], human large cell lung carcinoma cell line [COR-L23], human lung squamous carcinoma cell line [NCI-H226] with IC_{50} values of 34.75±1.93 [SI=2.44], 10.16±1.68 [SI=8.34] and 88.72±2.93 [SI=0.95], μg/ml, respectively. However, the 95% ethanolic extracts had no cytotoxic against nomal lung myofibroblast cell line [MRC-5] with IC_{50} values of 84.68±3.72 μg/ml. In addition, the water extract of APSL had no cytotoxic activity against three lung carcinoma cell lines. Furthermore, all the water extract of APSL remedy and its plant ingredients showed no effective against on three different cancer cell lines.

Table 4.2 Cytotoxic activity of 95% ethanolic extracts and water extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of Human Lung Carcinoma Cell lines [A549, NCI-H226 and COR-L23] by SRB assay. [n=3]

Botanical name	% yield of ethanolic extract	IC_{50} (µg/ml ±SEM) of ethanolic extract			% yield of water extract	IC_{50} (µg/ml ±SEM) of water extract		
		A549	NCI-H226	COR-L23		A549	NCI-H226	COR-L23
Aphaisalee remedy	4.68	34.75±1.93	88.72±2.93	10.16±1.68	4.04	>100	>100	>100
Acorus calamus Linn.	5.97	>100	>100	>100	3.65	>100	>100	>100
Amomum testaceum Ridl.	1.12	59.72±2.44	>100	>100	17.36	>100	>100	>100
Amorphophallas saraburiensis (Dennst)	1.62	>100	>100	>100	1.36	>100	>100	>100
Anethum graveolens Linn.	2.03	>100	>100	>100	6.50	>100	>100	>100
Angelicadahurica Berth.	2.58	53.85±3.82	>100	>100	18.92	>100	>100	>100
Atractylodes lancea	6.90	47.50±4.07	82.33±2.709	38.77±4.65	8.70	>100	>100	>100

Table 4.2 Cytotoxic activity of 95% ethanolic extracts and water extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of Human Lung Carcinoma Cell lines [A549, NCI-H226 and COR-L23] by SRB assay. [n=3] (continued)

Botanical name	% yield of ethanolic extract	IC_{50} (µg/ml ±SEM) of ethanolic extract			% yield of water extract	IC_{50} (µg/ml ±SEM) of water extract		
		A549	NCI-H226	COR-L23		A549	NCI-H226	COR-L23
Clausena excavate Burmf.	1.29	1.14±0.30	10.46±3.93	1.18±0.06	3.16	28.12±2.25	>100	26.99±0.14
Cuminum cyminum L.	5.00	37.60±0.28	34.79±1.34	34.62±1.18	13.07	>100	>100	>100
Foeniculum valgare Mill	3.15	49.44±4.36	>100	>100	8.93	>100	>100	>100
Lipidium sativum Linn.	0.87	>100	>100	>100	0.85	>100	>100	>100
Myristica fragrans Houtt (stem)	1.57	58.56±5.36	>100	40.11±0.49	1.58	>100	>100	>100
Myristica fragrans Houtt (mace)	10.01	83.81±0.74	94.22±4.45	>100	0.00	>100	>100	>100
Myristica fragrans Houtt (nutmeg)	9.06	55.11±1.77	75.30±4.89	59.23±3.69	9.51	>100	>100	>100

Table 4.2 Cytotoxic activity of 95% ethanolic extracts and water extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of Human Lung Carcinoma Cell lines [A549, NCI-H226 and COR-L23] by SRB assay. [n=3] (continued)

Botanical name	% yield of ethanolic extract	IC_{50} (µg/ml ±SEM) of ethanolic extract			% yield of water extract	IC50 (µg/ml ±SEM) of water extract		
		A549	NCI-H226	COR-L23		A549	NCI-H226	COR-L23
Piper nigrum Linn.	4.39	35.43±0.64	39.10±1.28	32.84±1.20	3.51	>100	>100	>100
Plumbago indigo L.	5.39	8.38±0.24	11.36±1.80	6.18±0.49	15.82	>100	>100	>100
Syzygium aromaticum (L.) Merr. Et Perry.	22.85	>100	>100	>100	5.09	30.67±0.60	28.39±1.30	27.17±3.21
Terminalia chebula Rez var Chebula.	30.15	53.22±5.86	52.31±3.92	>100	5.74	39.50±1.78	85.17±4.92	64.69±2.52
Terminalia arjuna Wight& Arn	25.92	32.13±1.77	40.68±1.19	31.11±0.88	18.23	20.37±2.07	75.170±0.67	31.44±0.44

Table 4.3 Selectivity index [SI] of IC_{50} of extracts against normal lung cell [MRC-5] were compared with IC_{50} of extracts against lung carcinoma cell lines [A549, NCI-H226, COR-L23]

Botanical name	IC_{50} (µg/ml ±SEM) of ethanolic extract				IC_{50} (µg/ml ±SEM) of water extract			
	MRC-5	**A549**	**NCI-H226**	**COR-L23**	**MRC-5**	**A549**	**NCI-H226**	**COR-L23**
Aphaisalee remedy	84.68±3.72	34.75±1.93 [SI=2.44]	88.72±2.93 [SI=0.95]	10.16±1.68 [SI=8.34]	NT	>100	>100	>100
Acorus calamus Linn.	NT	>100	>100	>100	NT	>100	>100	>100
Amomum testaceum Ridl.	11.48±2.50	59.72±2.44 [SI=0.19]	>100	>100	NT	>100	>100	>100
Amorphophallas saraburiensis (Dennst)	NT	>100	>100	>100	NT	>100	>100	>100
Anethum graveolens Linn.	NT	>100	>100	>100	NT	>100	>100	>100
Angelicadahurica Berth.	68.31±3.44	53.85±3.82 [SI=0.23]	>100	>100	NT	>100	>100	>100
Atractylodes lancea	10.69±1.952	47.50±4.07 [SI=1.27]	82.33±2.71 [SI=0.13]	38.77±4.65 [SI=0.28]	NT	>100	>100	>100

Table 4.3 Selectivity index [SI] of IC_{50} of extracts against normal lung cell [MRC-5] were compared with IC_{50} of extracts against lung carcinoma cell lines [A549, NCI-H226, COR-L23] (continued)

Botanical name	IC_{50} (µg/ml ±SEM) of ethanolic extract				IC_{50} (µg/ml ±SEM) of water extract			
	MRC-5	**A549**	**NCI-H226**	**COR-L23**	**MRC-5**	**A549**	**NCI-H226**	**COR-L23**
Ardisia elliptica Thumb.	NT	>100	>100	>100	NT	>100	>100	>100
Clausena excavate Burmf.	0.80±0.25	1.14±0.30 [SI=0.70]	10.46±3.93 [SI=0.08]	1.18±0.06 [SI=0.68]	26.62±16.13	28.12±2.25 [SI=0.95]	>100	26.99±0.14 [SI=0.99]
Cuminum cyminum L.	7.55±3.00	37.60±0.28 [SI=1.20]	34.79±1.34 [SI=0.22]	34.62±1.18 [SI=0.22]	NT	>100	>100	>100
Foeniculum valgare Mill	6.97±1.24	49.44±4.36 [SI=0.14]	>100	>100	NT	>100	>100	>100
Lipidium sativum Linn.	NT	>100	>100	>100	NT	>100	>100	>100
Myristica fragrans Houtt (stem)	11.18±1.16	58.56±5.36 [SI=0.19]	>100	40.11±0.49 [SI=0.28]	NT	>100	>100	>100
Myristica fragrans Houtt (mace)	5.90±3.09	83.81±0.75 [SI=0.07]	94.22±4.45	>100	NT	>100	>100	>100

Table 4.3 Selectivity index [SI] of IC_{50} of extracts against normal lung cell [MRC-5] were compared with IC_{50} of extracts against lung carcinoma cell lines [A549, NCI-H226, COR-L23] (continued)

Botanical name	IC_{50} (µg/ml ±SEM) of ethanolic extract				IC_{50} (µg/ml ±SEM) of water extract			
	MRC-5	A549	NCI-H226	COR-L23	MRC-5	A549	NCI-H226	COR-L23
Myristica fragrans Houtt (nutmeg)	4.21±0.78	55.11±1.77 [SI=0.08]	75.30±4.89 [SI=0.06]	59.23±3.69 [SI=0.07]	NT	>100	>100	>100
Piper nigrum Linn.	15.19±1.70	35.43±0.64 [SI=0.43]	39.10±1.28 [SI=0.39]	32.84±1.20 [SI=0.46]	NT	>100	>100	>100
Plumbago indigo L.	16.14±2.92	8.38±0.24 [SI=1.93]	11.36±1.80 [SI=1.42]	6.18±0.49 [SI=2.61]	NT	>100	>100	>100
Syzygium aromaticum (L.) Merr. Et Perry.	NT	>100	>100	>100	68.50±3.47	30.67±0.60 [SI=2.23]	28.39±1.30 [SI=2.14]	27.17±3.21 [SI=2.52]
Terminalia chebula Rez var Chebula.	67.22±4.79	53.22±5.86 [SI=1.26]	52.31±3.92 [SI=1.29]	>100	39.35±1.60	39.50±1.78 [SI=0.99]	85.17±4.92 [SI=0.99]	64.69±2.52 [SI=0.61]
Terminalia arjuna Wight& Arn	46.67±0.80	32.13±1.77 [SI=1.45]	40.68±1.19 [SI=1.15]	31.11±0.88 [SI=1.50]	4.60±0.30	20.37±2.07 [SI=0.23]	75.17±0.67 [SI=0.06]	31.44±0.44 [SI=0.15]

***NT = not test , SI = Selectivity index [**IC_{50} of extracts against normal lung cell/ IC_{50} of extracts against lung carcinoma cell lines**]**

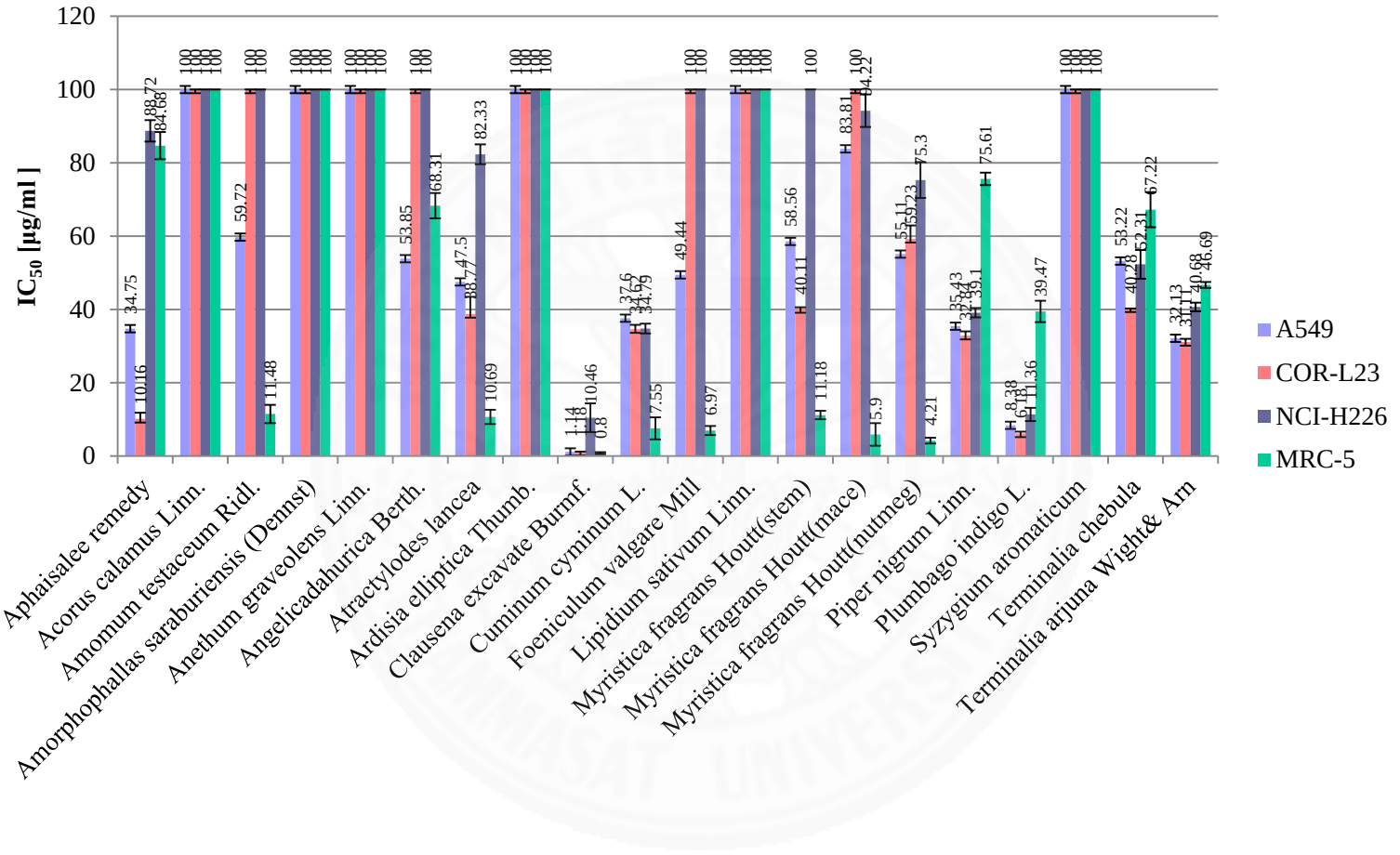


Figure 4.1 Cytotoxic activity of 95% ethanolic extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of Human Lung Carcinoma Cell lines [A549, NCI-H226 and COR-L23] by SRB assay. [n=3]

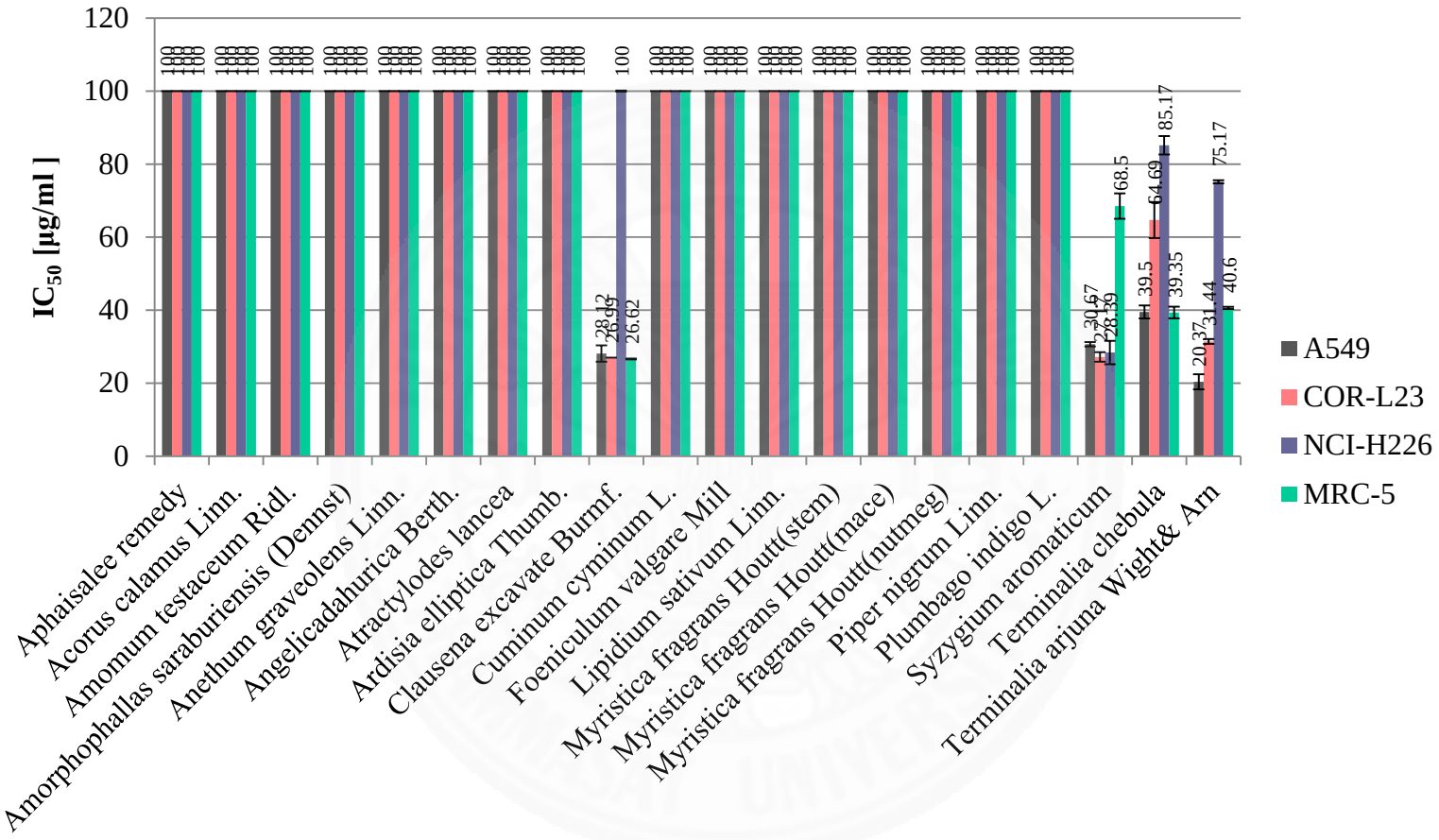


Figure 4.2 Cytotoxic activity of water extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of Human Lung Carcinoma Cell lines [A549, NCI-H226, COR-L23] by SRB assay. [n=3]

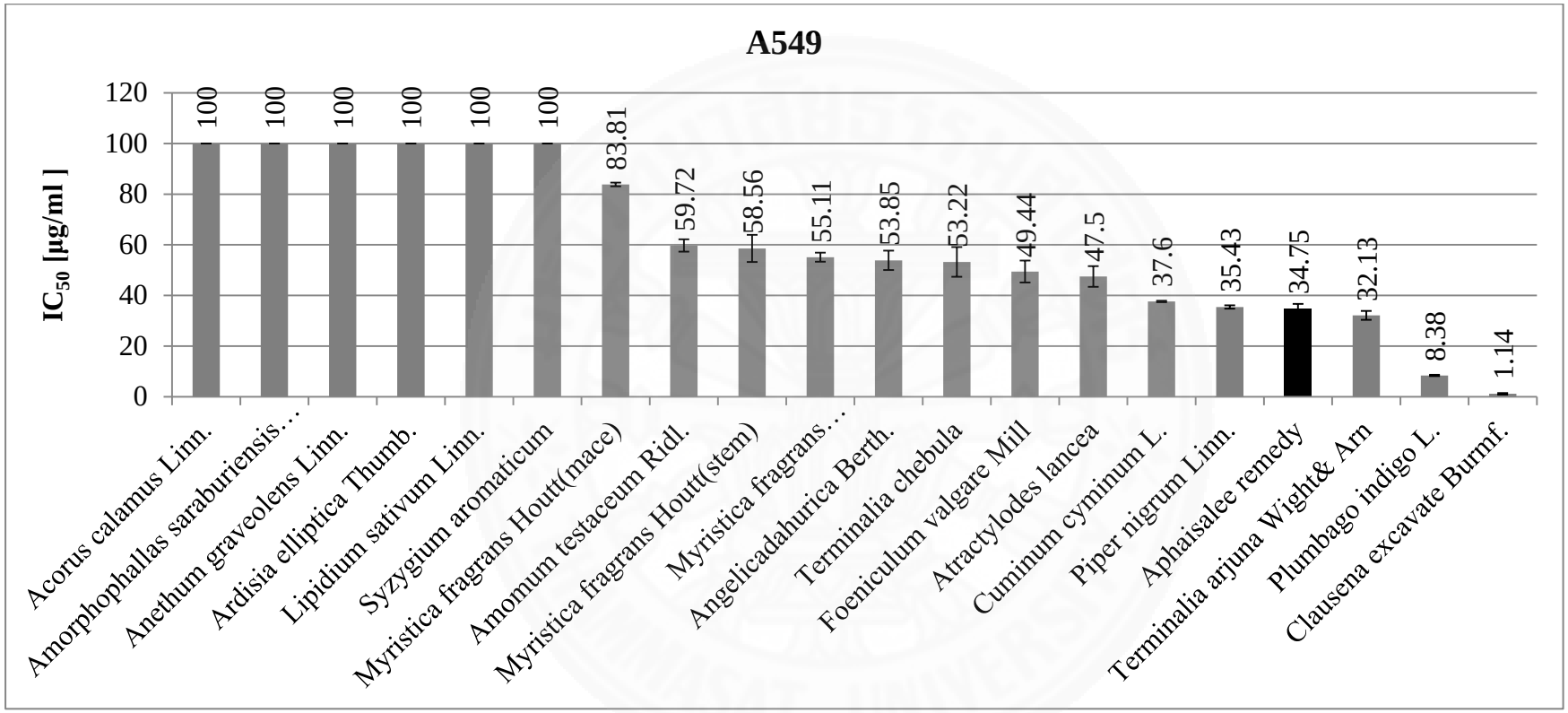


Figure 4.3 Cytotoxic activity of 95% ethanolic extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of Human Lung Carcinoma Cell lines [A549] by SRB assay. [n=3]

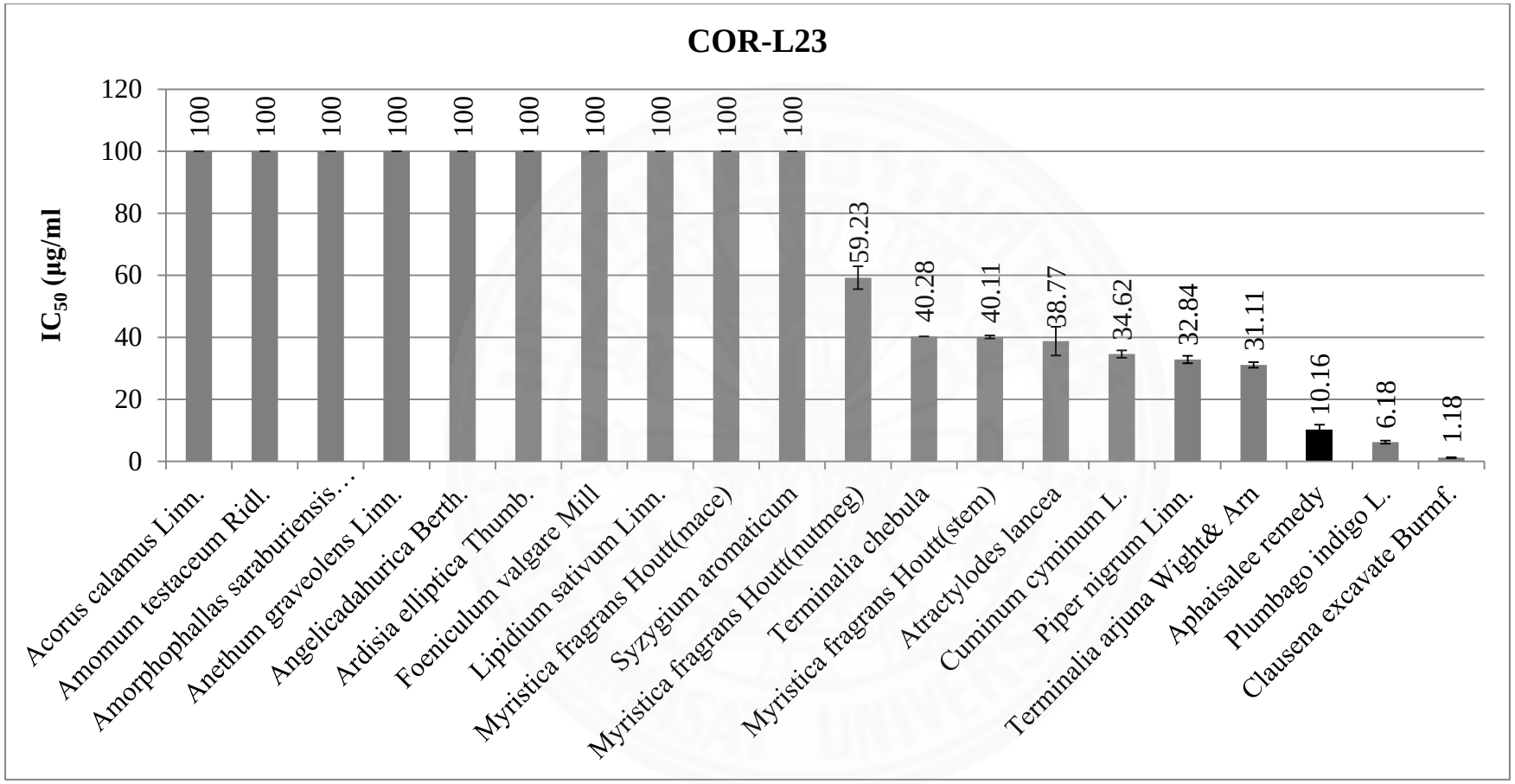


Figure 4.4 Cytotoxic activity of 95% ethanolic extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of Human Lung Carcinoma Cell lines [COR-L23] by SRB assay. [n=3]

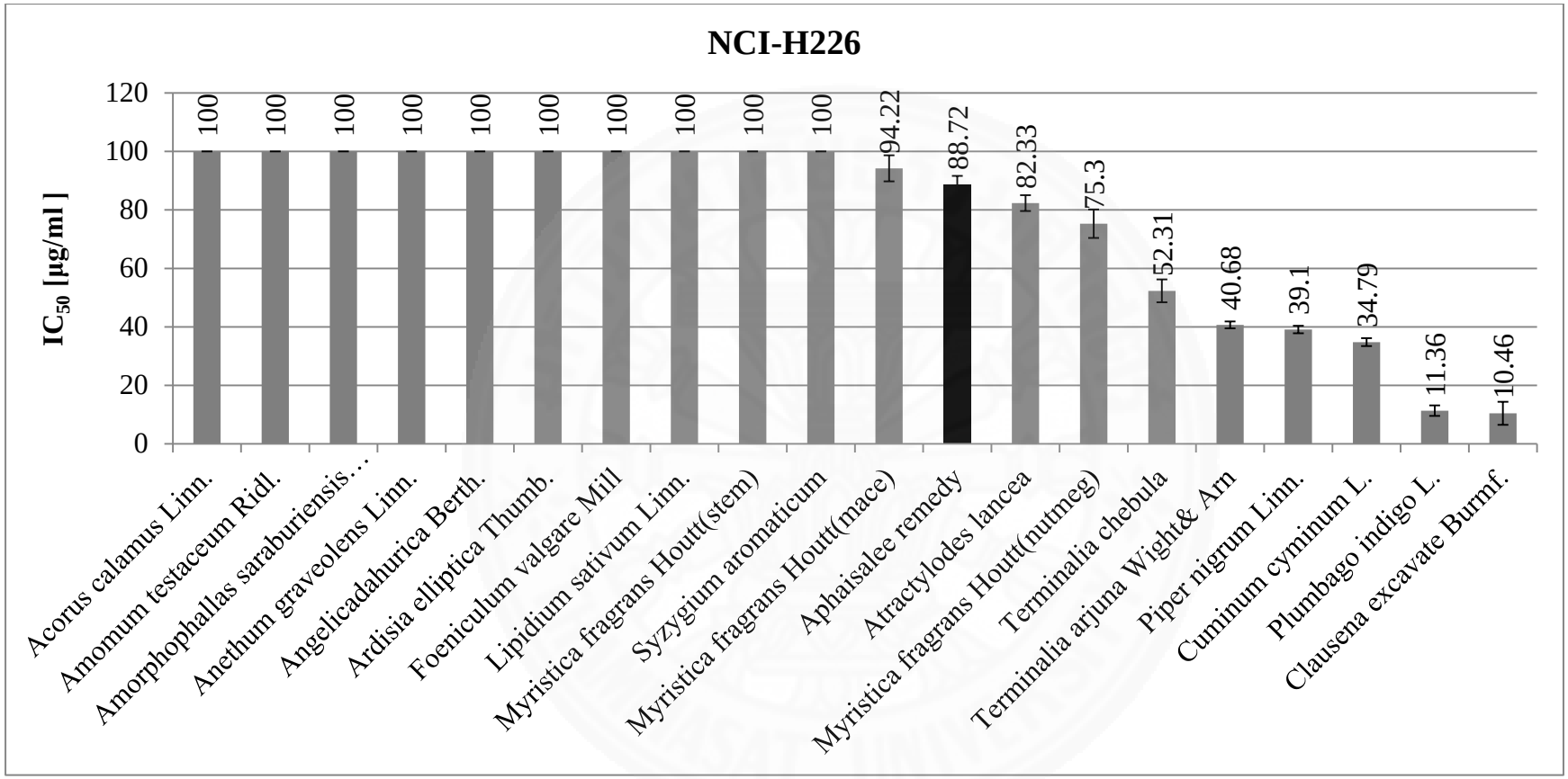


Figure 4.5 Cytotoxic activity of 95% ethanolic extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of Human Lung Carcinoma Cell lines [NCI-H226] by SRB assay. [n=3]

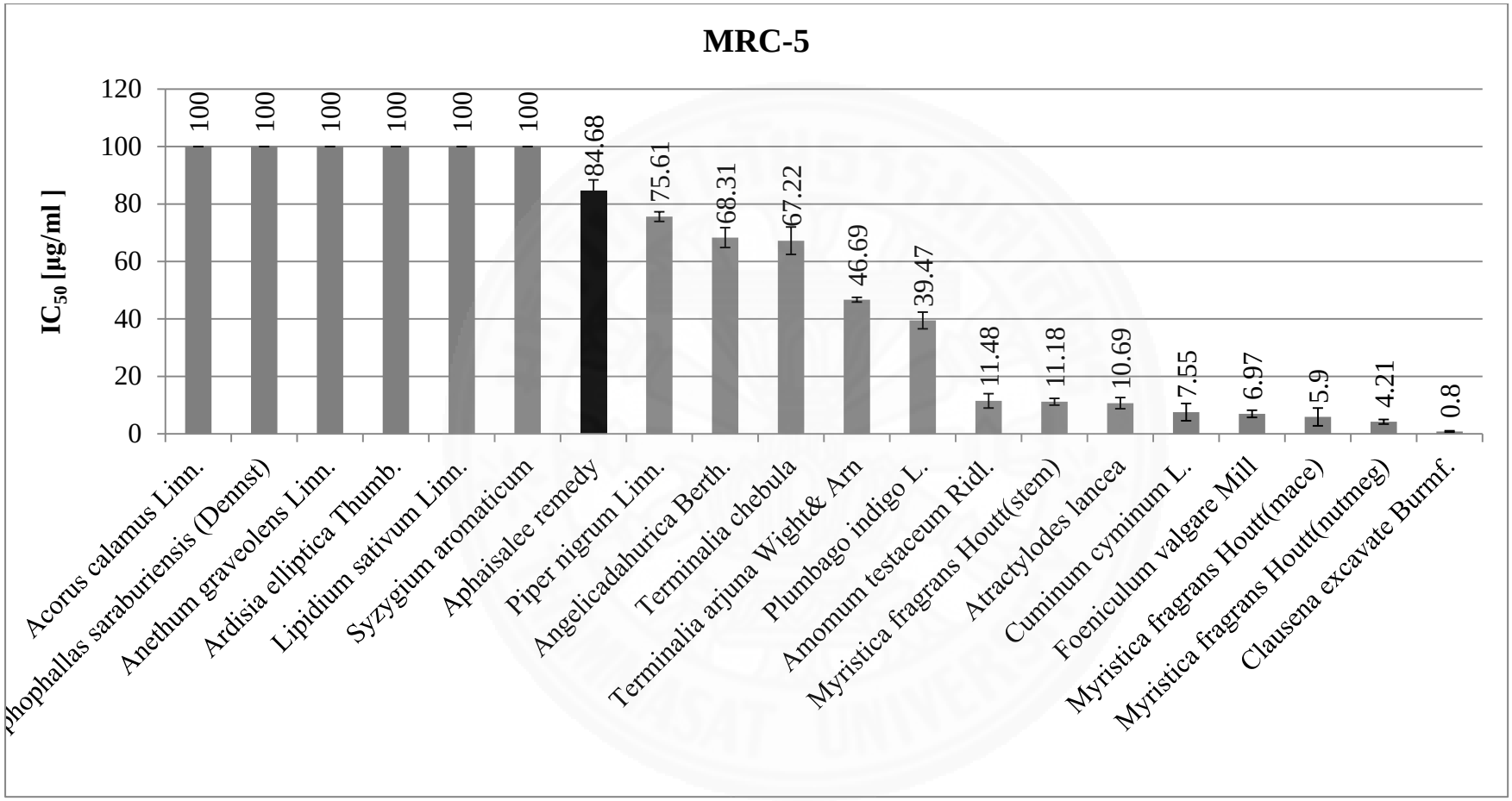


Figure 4.6 Cytotoxic activity of 95% ethanolic extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of normal lung Cell [MRC-5] by SRB assay. [n=3]

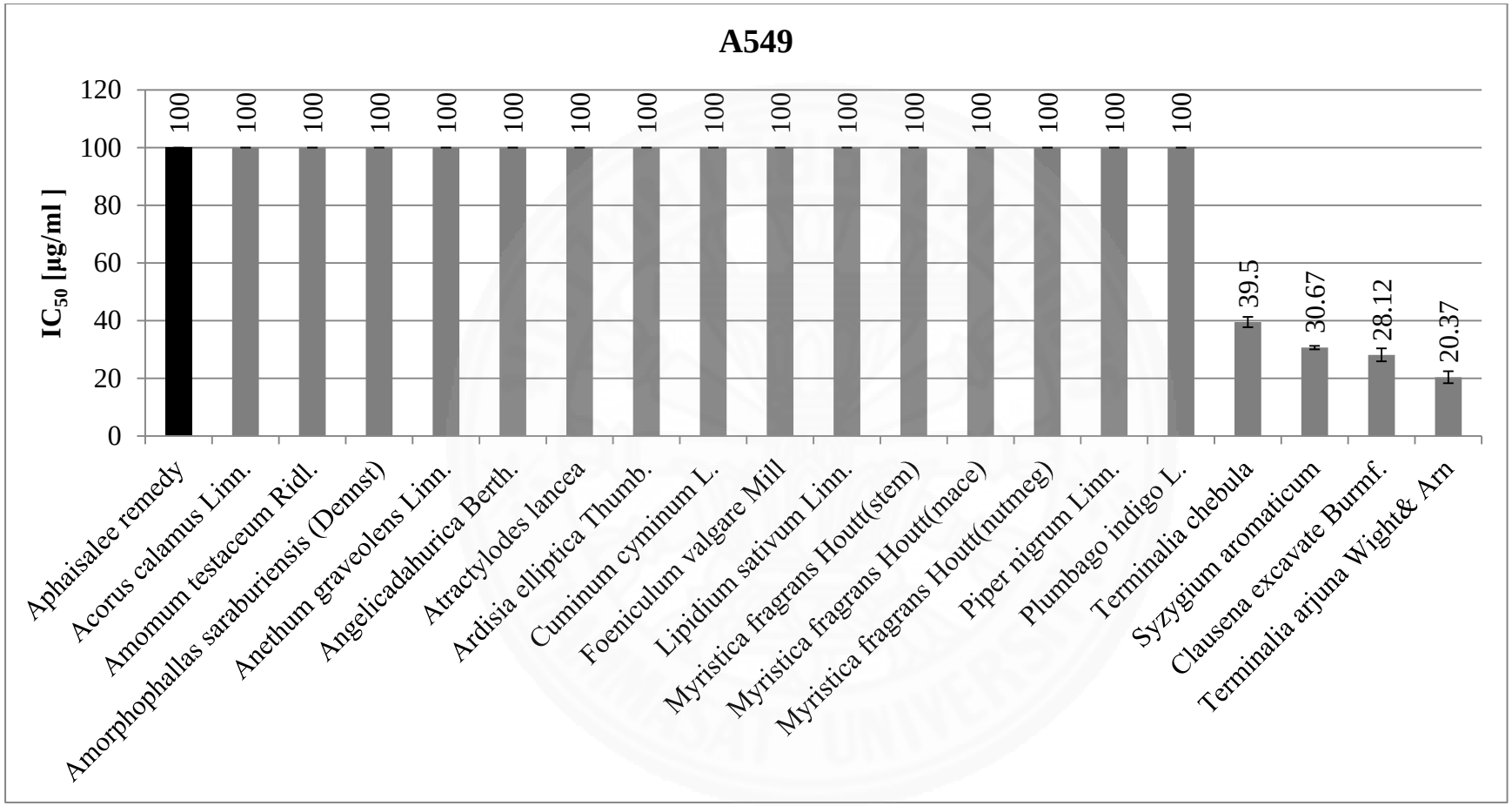


Figure 4.7 Cytotoxic activity of water extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of Human Lung Carcinoma Cell lines [A549] by SRB assay. [n=3]

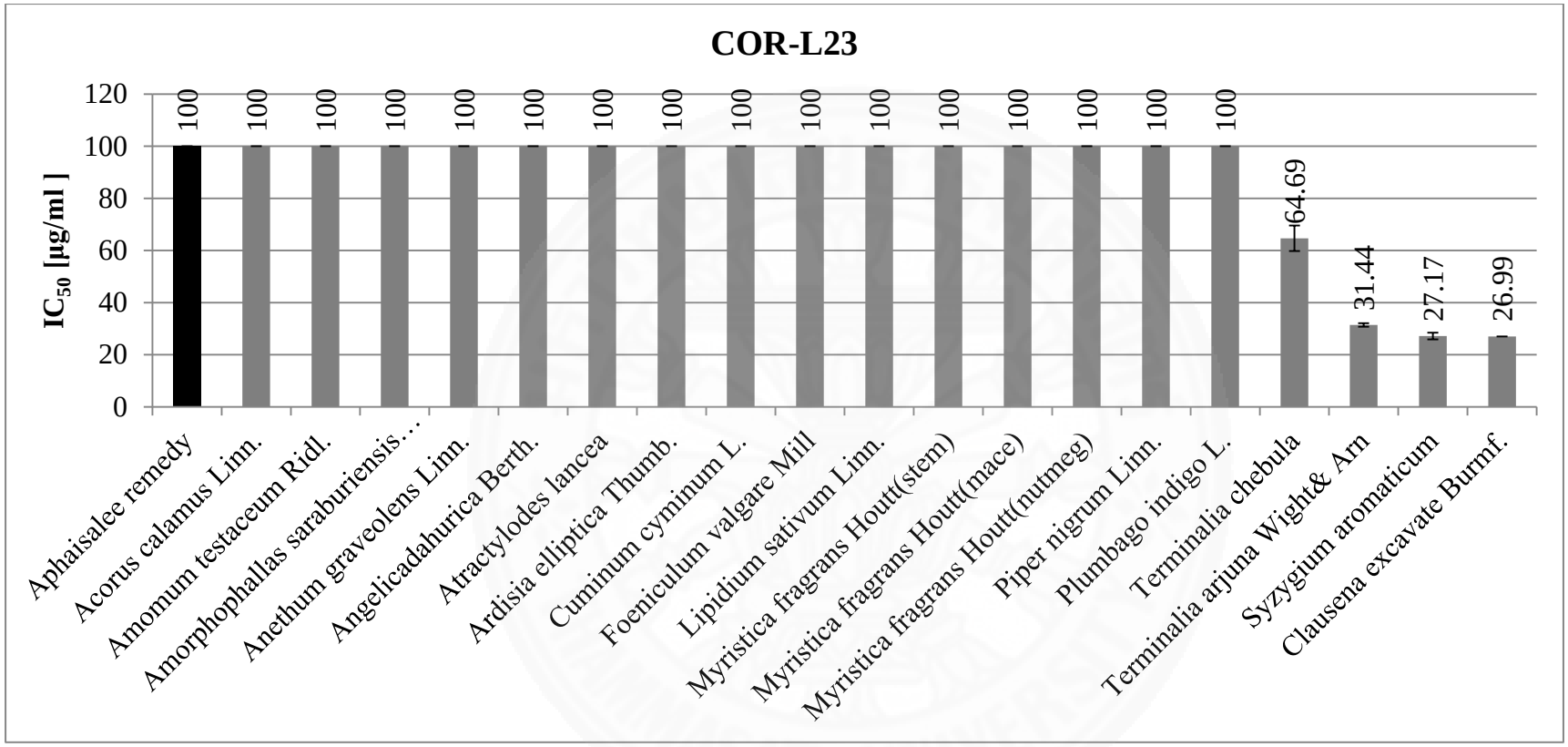


Figure 4.8 Cytotoxic activity of water extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of Human Lung Carcinoma Cell lines [COR-L23] by SRB assay. [n=3]

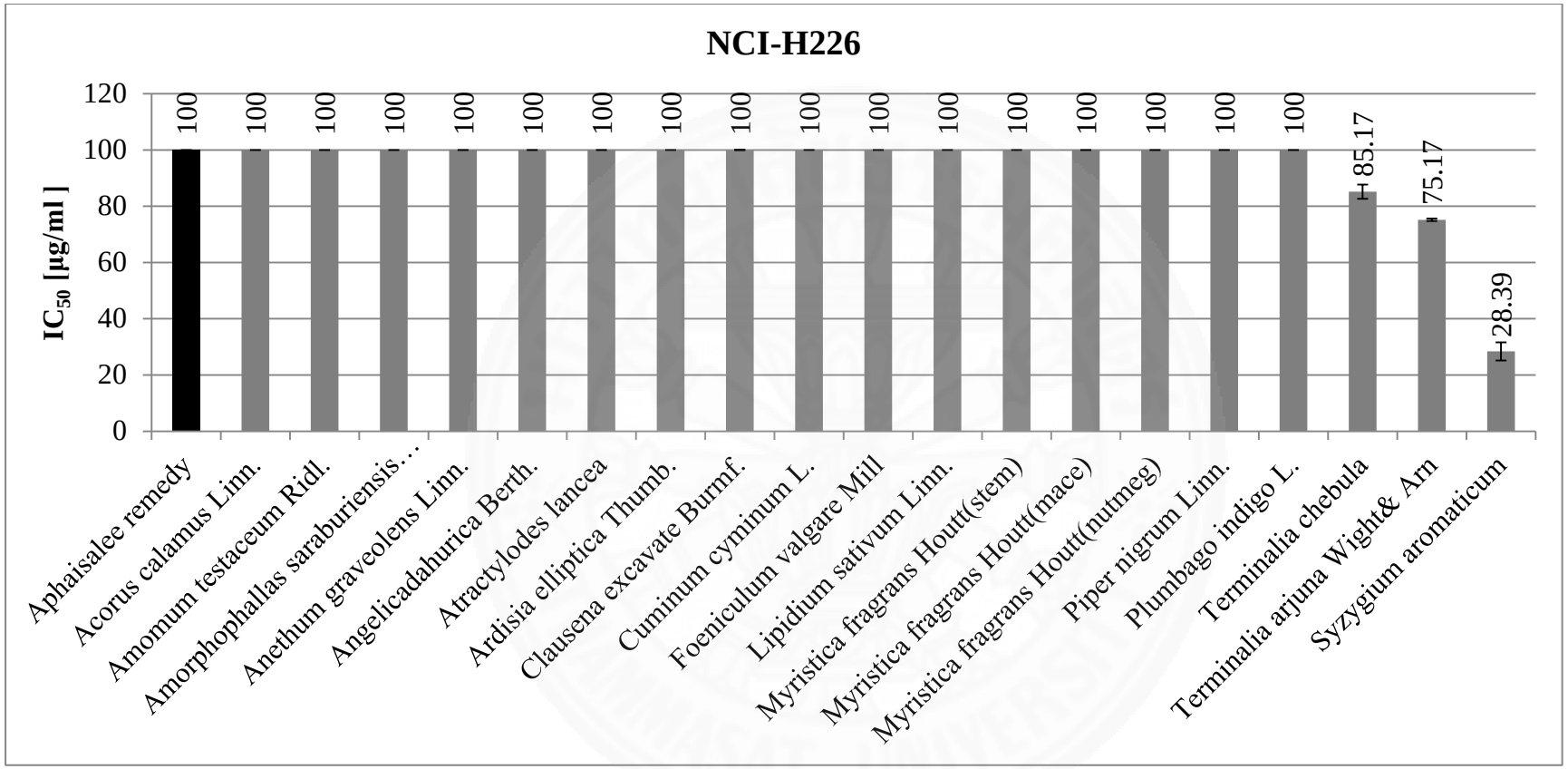


Figure 4.9 Cytotoxic activity of water extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of Human Lung Carcinoma Cell lines [NCI-H226] by SRB assay. [n=3]

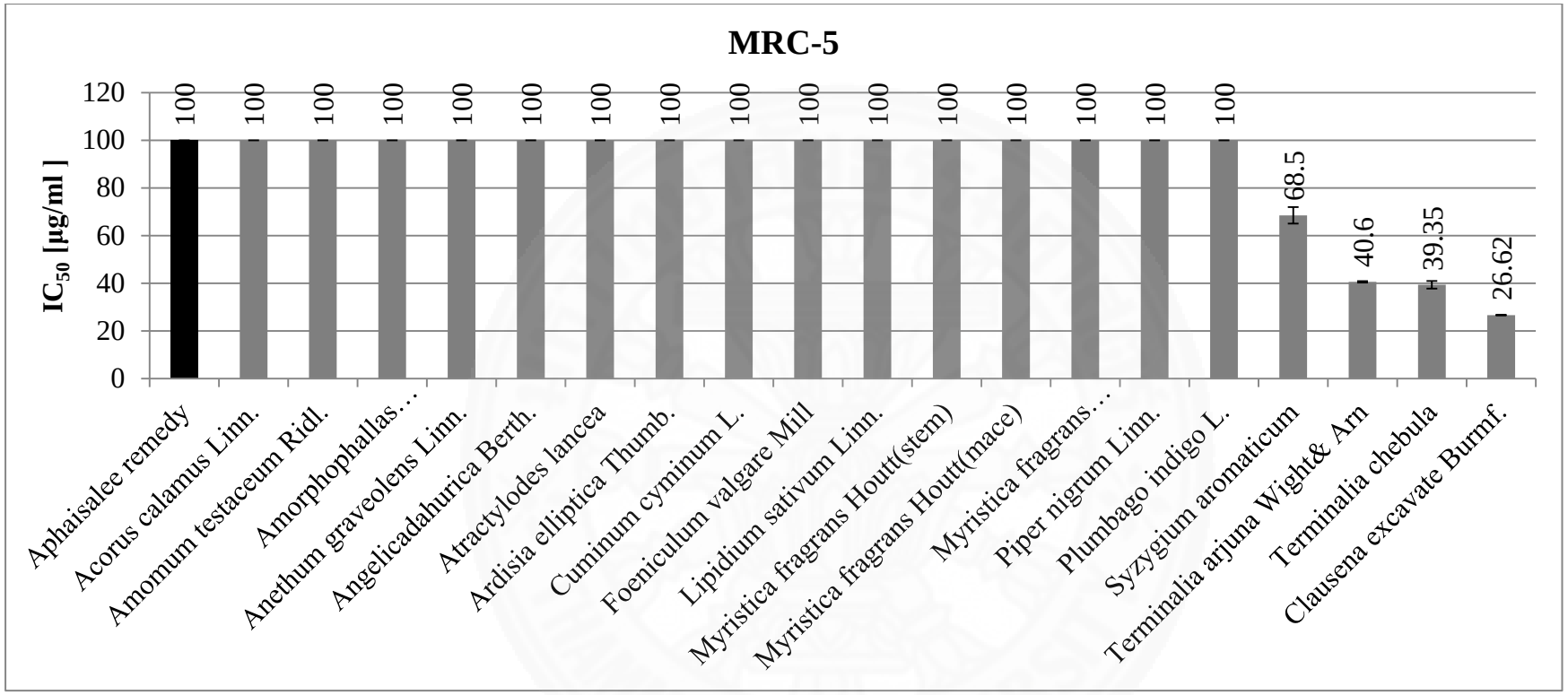


Figure 4.10 Cytotoxic activity of water extracts from A-Phai-Sa-Lee remedy and its herbal ingredients against of normal lung Cell [MRC-5] by SRB assay. [n=3]

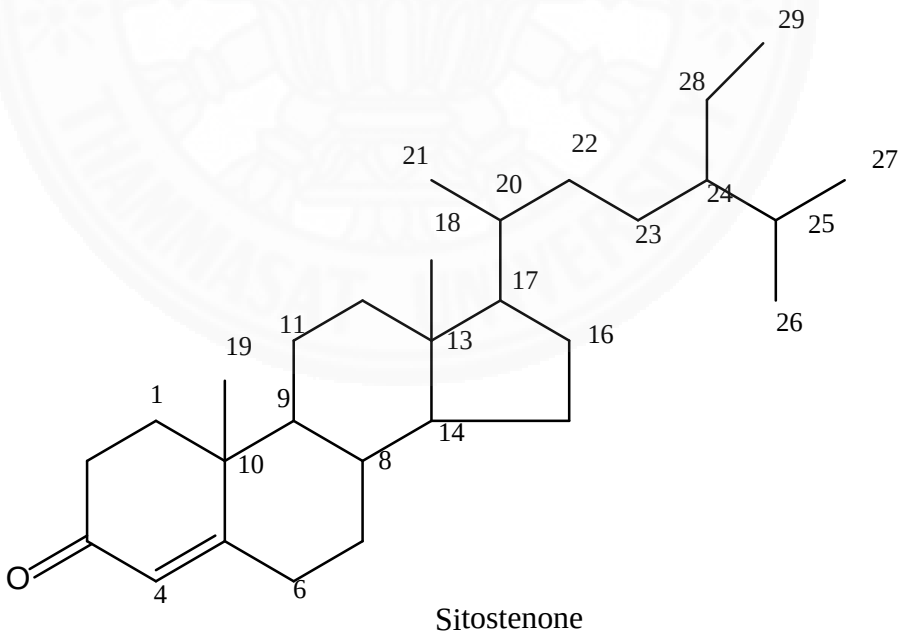
4.3 Bioassay-gudided fractionation

The plants ingredient witch showed the highest cytotoxic activity was chosen to separate by column chromatography. Dried extract of *Clausena excavate* Burmf. was mixed with silica gel as stationary phase. The silica gel column was stationary phase it was eluted with low to high polarity solvents as followed: Hexane:Dichloromethane [9:1, 2,000 ml], Dichloromethane [1,000 ml], Dichloromethane:Methanol [9:1, 500 ml], Dichloromethane:Methanol [7:3, 500 ml], Dichloromethane:Methanol [5:5, 500 ml], Dichloromethane:Methanol [3:7, 500 ml], and Methanol [500 ml], Respectively. The solvent was collected for 10 mL/tube. The collected fractions were examined by TLC chromatogram and detected with anisaldehyde spray. The structure of isolated pure compound was indentified by TLC chromatogram characteristics and protron Nuclear Magnetic Resonance spectrum. Finally, the results found compound A that obtained as a white powder, absorbed UV at wavelength 254 nm, compound B that obtained as a powder, no absorbed UV and gave pink-orange anisaldehyde test. The Nuclear Magnetic Resonance [NMR] were record in $CDCL_3$ and shown in **Table 4.4, Figure 4.11 - 4.19**

The results of Compound A and B showed cytotoxic activity against three different lung cancer cell with IC_{50} values ranging in 24-64 μg/ml. The results of compound A against human lung carcinoma cell line [A549], human lung large cell [COR-L23] and lung squamous cell line [NCI-H226] showed IC_{50} values 41.11±1.57, 24.17±3.86 and 64.87±3.51 μg/ml, respectively. The results of compound A against human lung carcinoma cell line [A549], human lung large cell [COR-L23] and lung squamous cell line [NCI-H226] showed IC_{50} values 58.94±1.40, 25.94±3.51 and 61.41±5.87 μg/ml, respectively. That of all showed in **Table 4.5.**

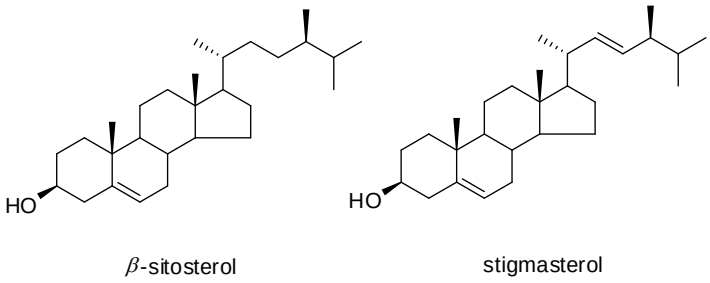
Table 4.4 compared ^{1}H NMR between β-sitostenone with A

Position	β-sitostenone	Compound A
4	5.74 [1H, s]	5.72 [1H, s]
18	0.71 [3H, s]	0.71 [3H, s]
19	1.18 [3H, s]	1.18 [3H, s]
21	0.80-1.10 [m]	0.91 [3H, d, J=6.5 Hz]
26	0.80-1.10 [m]	0.80 [3H, d,J= 6.9 Hz]
27	0.80-1.10 [m]	0.80 [3H, d, J=6.9 Hz]
29	0.80-1.10 [m]	0.84 [3H, m]



Sitostenone

Figure 4.11 Structure of Sitostenone



β-sitosterol stigmasterol

Figure 4.12 Structure of β –sitosterol and stigmasterol

The ^{1}H NMR [δ_H, $CDCl_3$, 400 MHz] of compound B revealed signals for two singlet methyl at δ 1.05 [H-19] and 0.67 [H-18], three doublet methyl at δ 0.95 [*d*, *J* = 6.5 Hz, H-21], 0.83 [*d*, *J* = 7.1 Hz, H-26] and 0.81 [*d*, *J* = 6.9 Hz, H-27] and one triplet methyl at δ 0.84 [*t*, *J* = 7.6 Hz, H-29].

There were oxymethine proton at δ 3.52 [*m*], three olefinic proton at δ 5.35 [*brd*, *J* = 5.1 Hz, H-6], 5.14 [*dd*, *J* = 15.1, 8.6 Hz, H-22] and 5.01 [*dd*, *J* = 15.1, 8.6 Hz, H-23]. The ^{1}H NMR data was corresponded to previous report data of β-sitosterol and stigmasterol [Jin-yan *et al*., 2007]. Thus the mixture was assigned as β-sitosterol and stigmasterol.

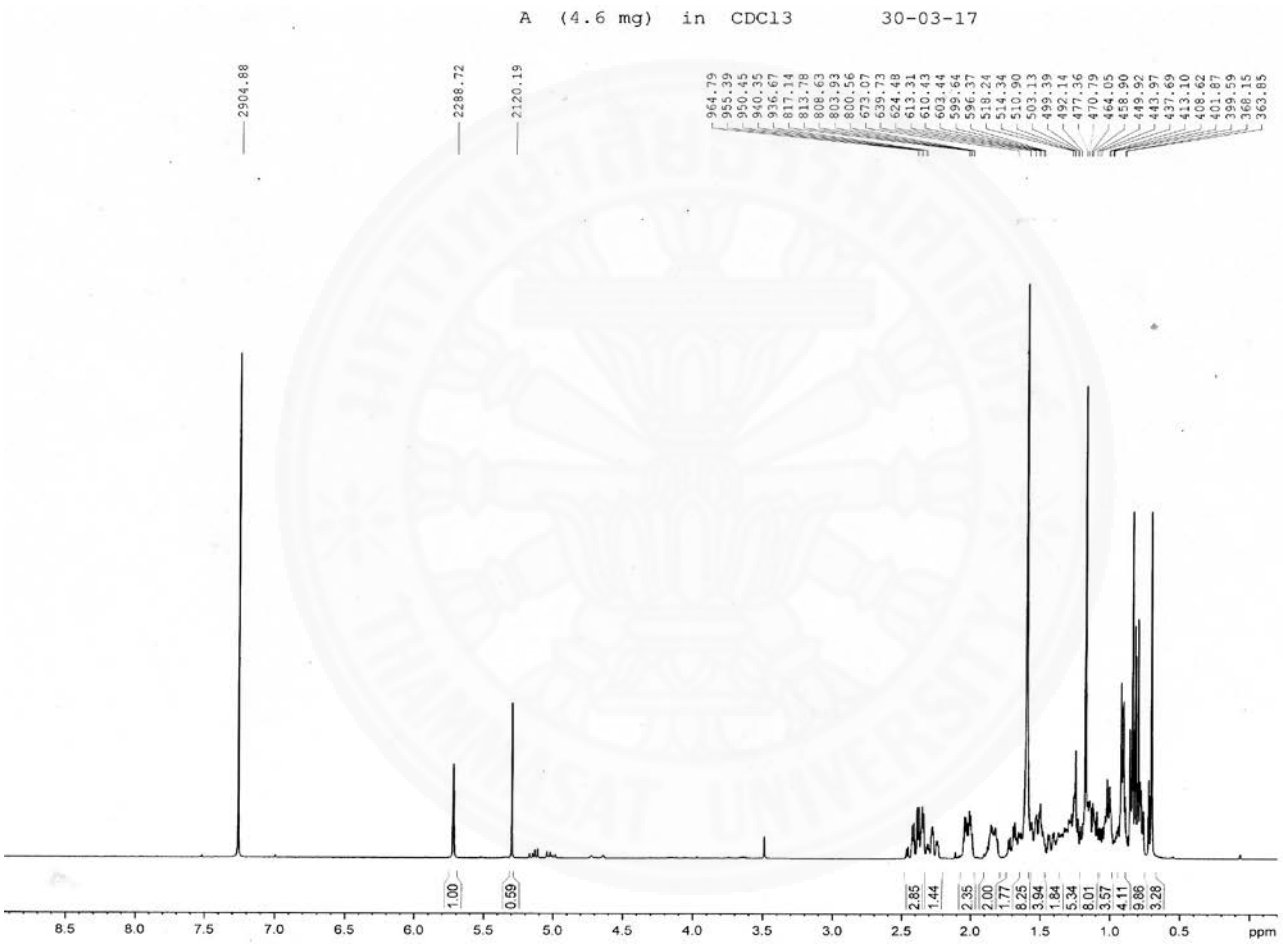


Figure 4.13 ^{1}H NMR Spectrum of Sitostenone in $CDCL_3$ [A], δ_H, $CDCl_3$, 400 MHz

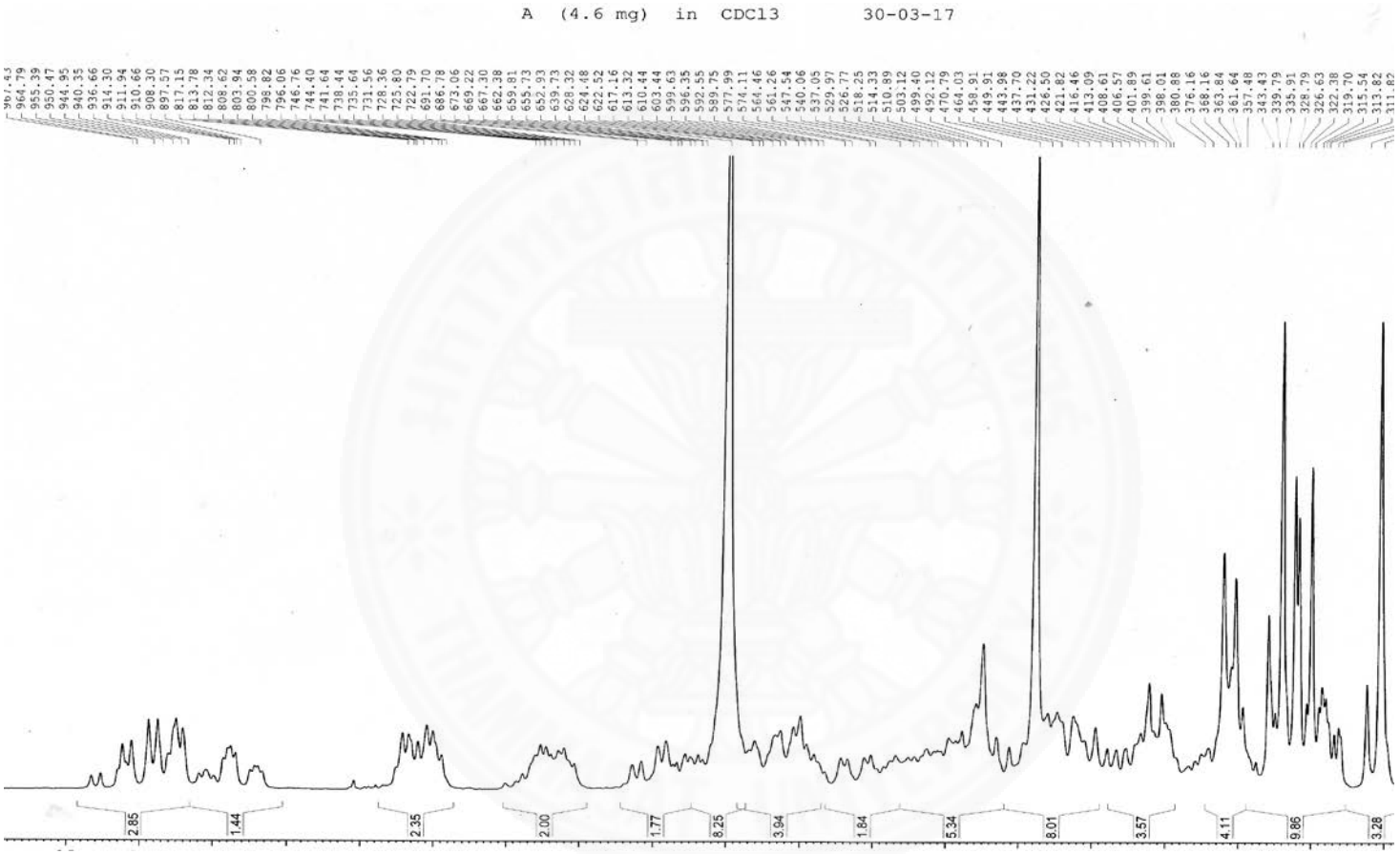


Figure 4.14 ^{1}H NMR Spectrum of Sitostenone in $CDCL_3$ [A], δ_H, $CDCl_3$, 400 MHz

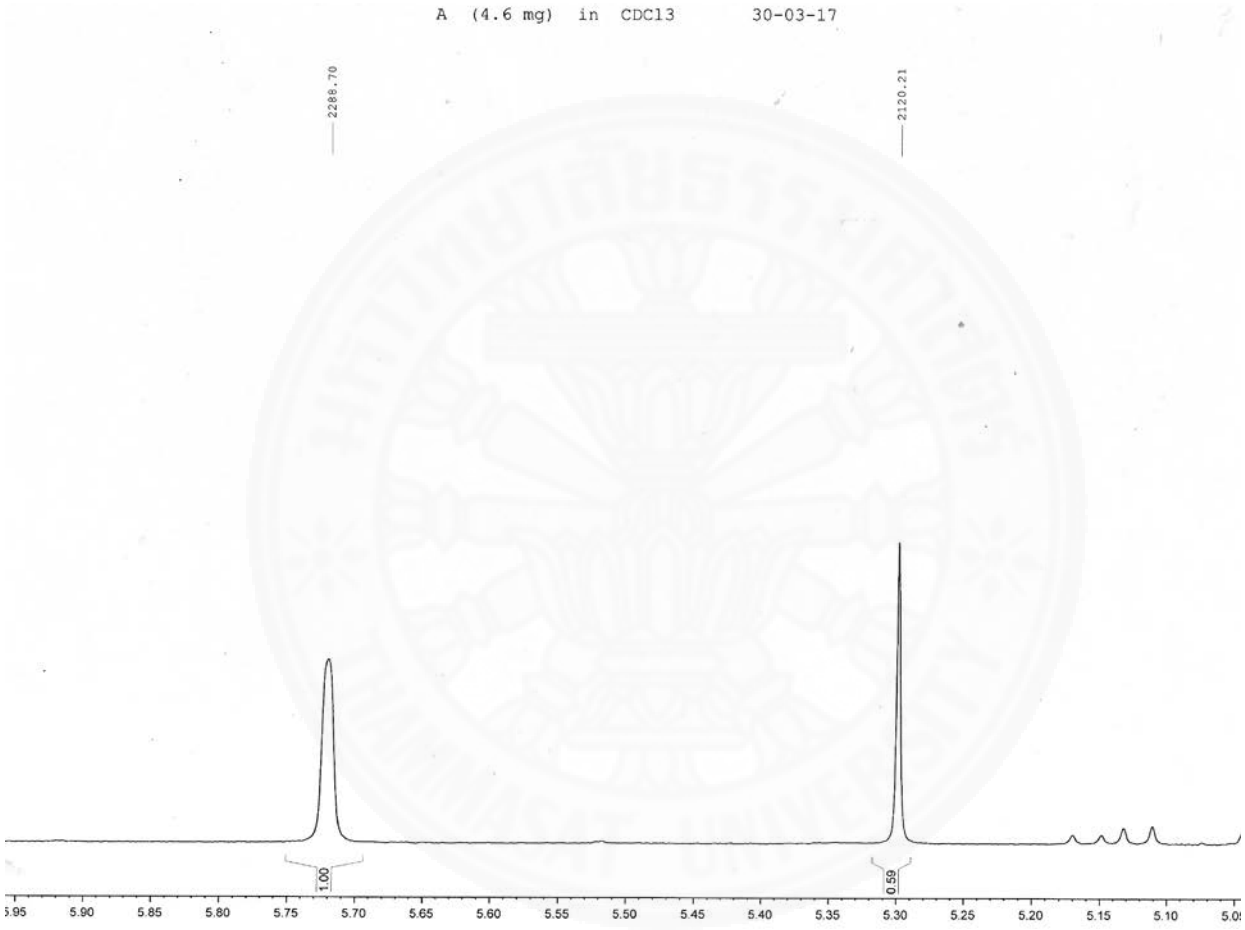


Figure 4.15 ^{1}H NMR Spectrum of Sitostenone in $CDCL_3$ [A], δ_H, $CDCl_3$, 400 MHz

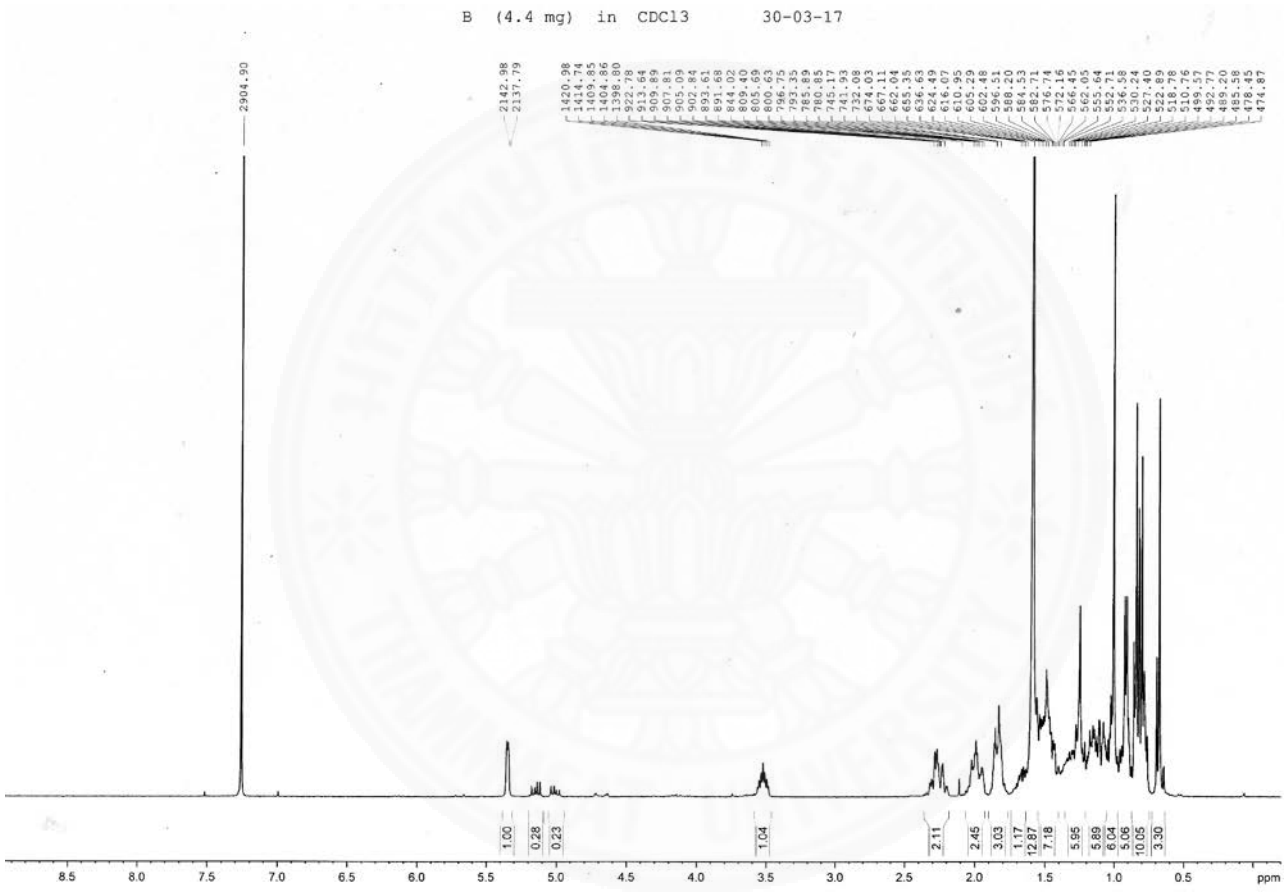


Figure 4.16 ^{1}H NMR Spectrum of Sitostenone in $CDCL_3$ [B], δ_H, $CDCl_3$, 400 MHz

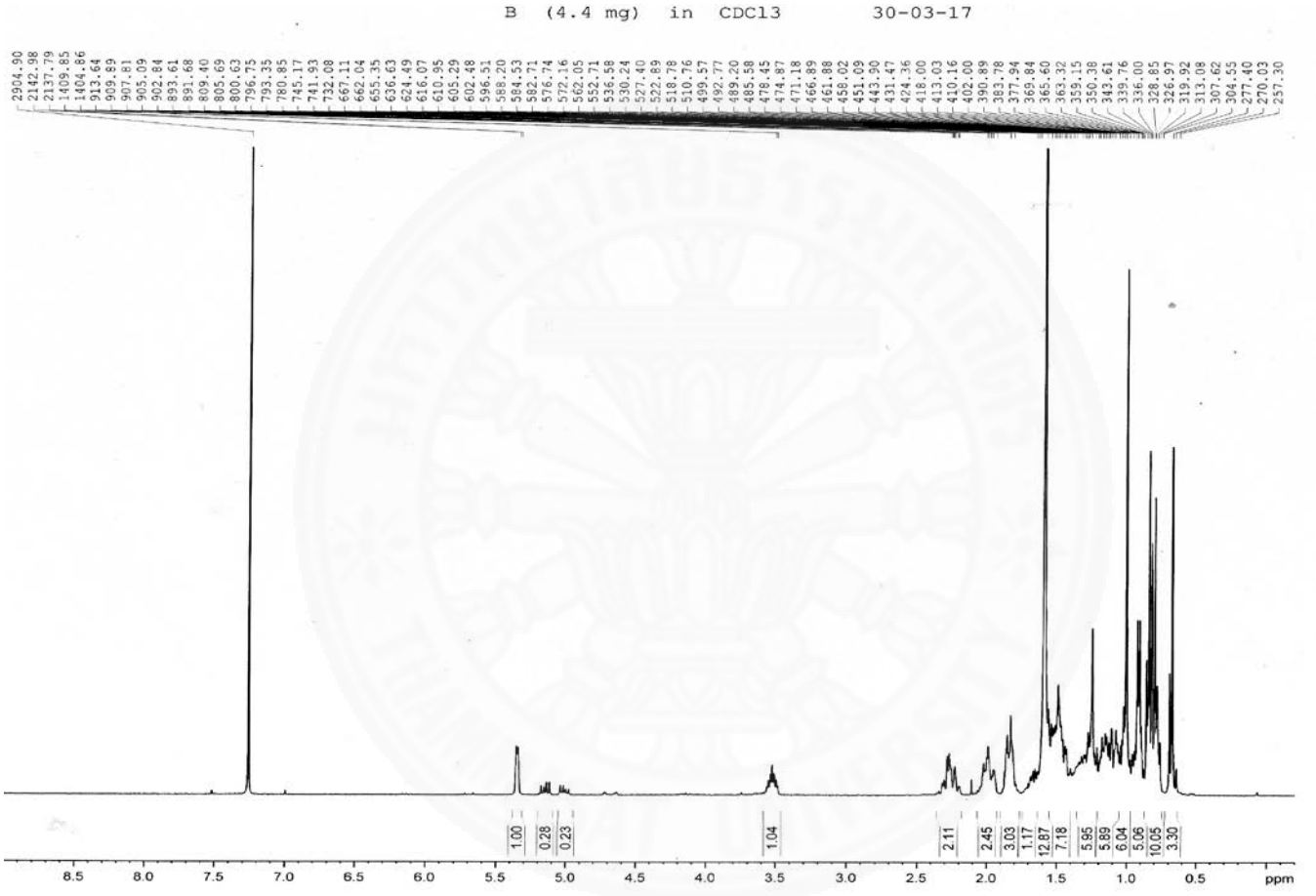


Figure 4.17 ^{1}H NMR Spectrum of Sitostenone in $CDCL_3$ [B], δ_H, $CDCl_3$, 400 MHz

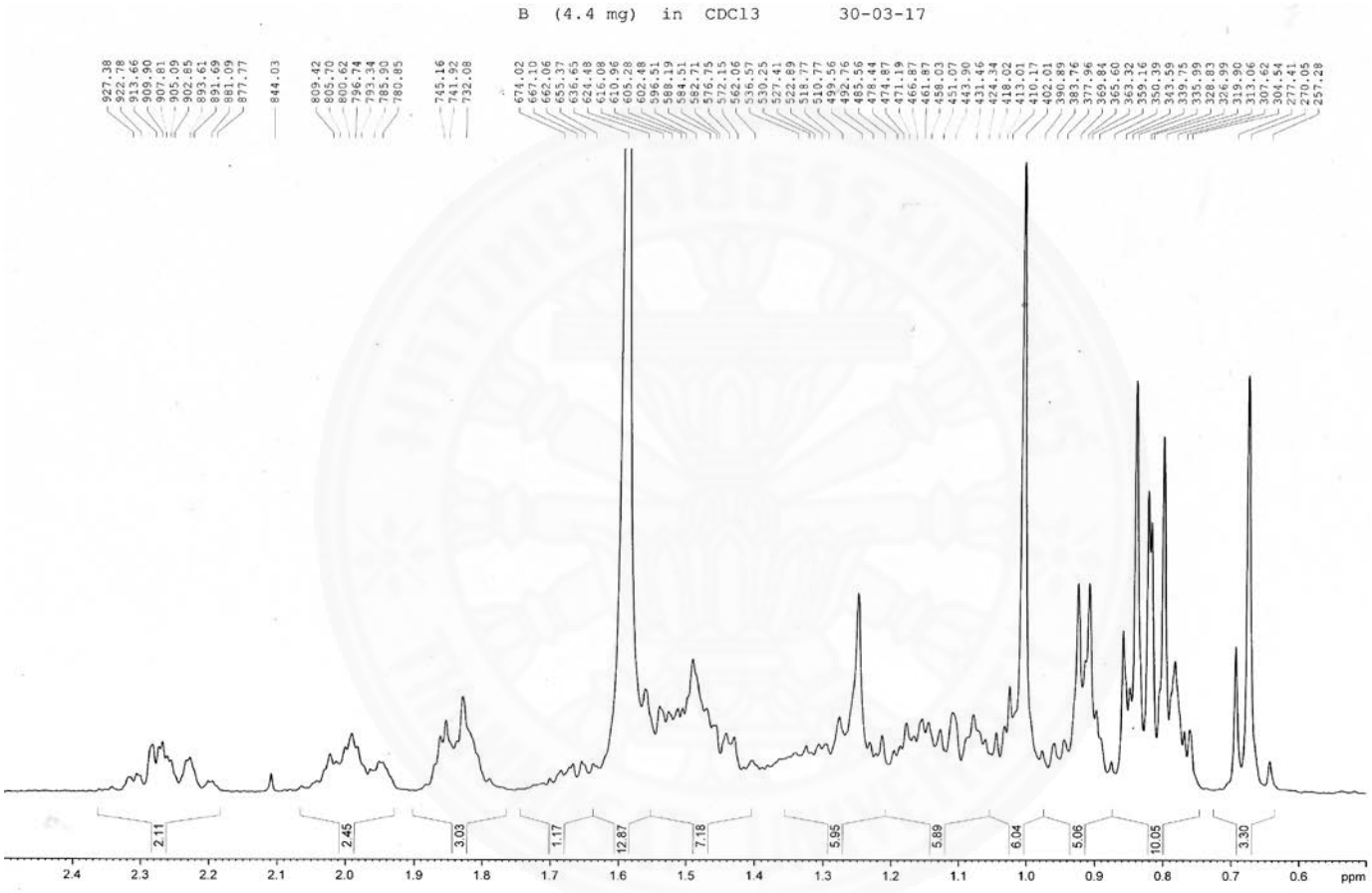


Figure 4.18 ^{1}H NMR Spectrum of Sitostenone in $CDCL_3$ [B], δ_H, $CDCl_3$, 400 MHz

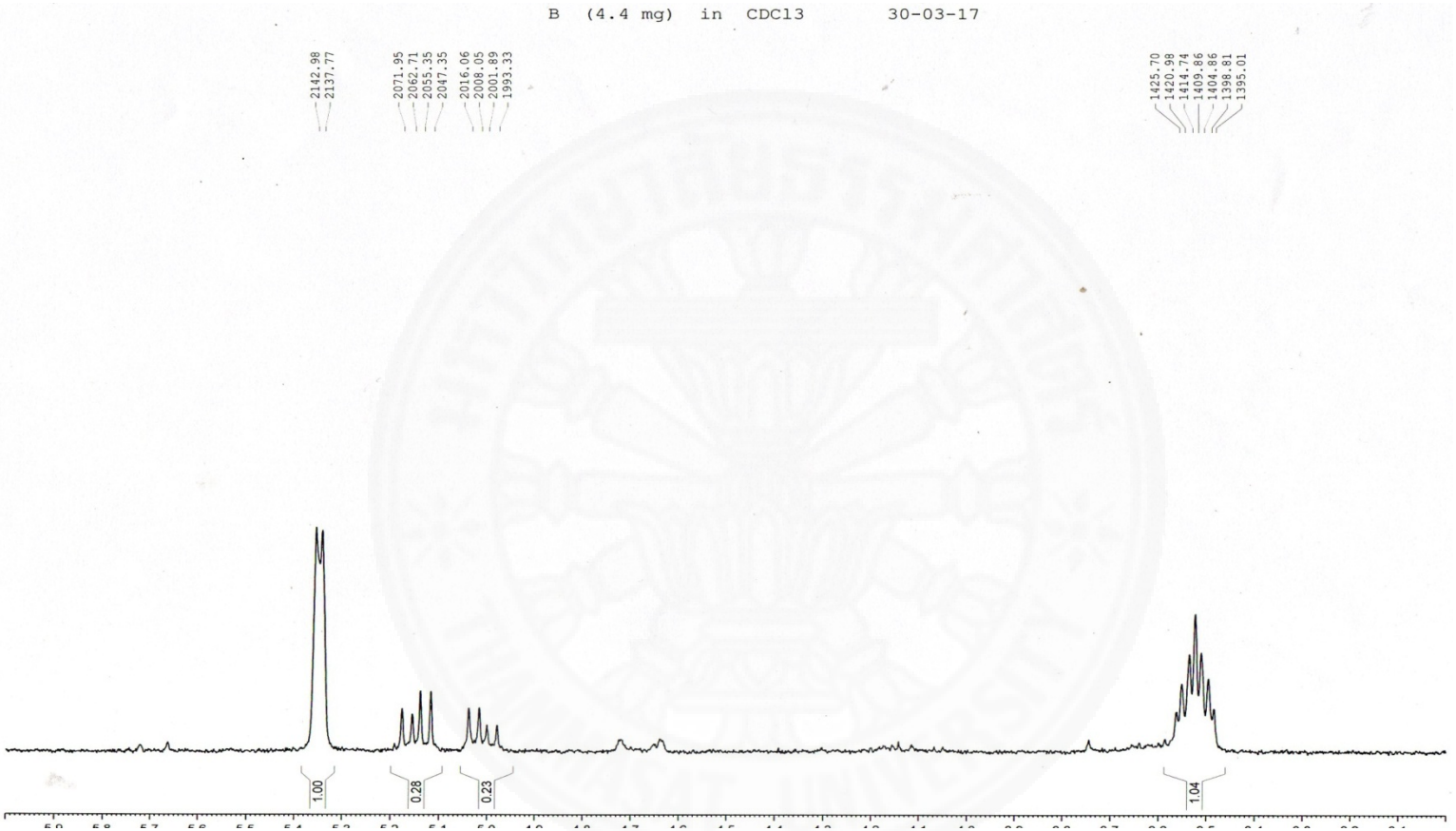


Figure 4.19 ^{1}H NMR Spectrum of Sitostenone in $CDCL_3$ [B], δ_H, $CDCl_3$, 400 MHz

Table 4.5 IC_{50} [µg/ml] of cytotoxic activity of APSL remedy against human lung carcinoma cell line [A549, NCI-H226 and COR-L23] using SRB assay [n=3] on A and B

Type of cells	IC_{50} [µg/ml] ±SEM of cytotoxic activity	
	Compound A [Sitostenone]	**Compound B [β –sitosterol and stigmasterol]**
A549	41.11±1.57	58.94±1.40
CORL-23	24.17±3.86	25.94±3.51
NCI-H226	64.87±3.51	61.41±5.87

4.4 Study on chemical fingerprint of APSL remedy preparation using high performance liquid chromatography

The aim was to develop a reversed phase high performance liquid chromatography [RP-HPLC] method to control quality of APSL remedy in two aspects namely chemical fingerprint and quantification. In this method, piperine, plumbagin, and eugenol as the compoundshaving the most potent inhibition of cytotoxic activity were used to be markers because this method has good sensitivity, precision, and accuracy. Preparation of APSL remedy sample and pure compounds is shown in.

The result showed that piperine in the ethanolic extract of APSL was highest content [104.64±7.77 mg/g of extract], followed by eugenol with content of 98.83±6.09 mg/g of extract, respectively. From this result, new knowledge and scientific dataof the chemical fingerprint and quantification of APSL remedy and RP-HPLC method may be considered for quality control of APSL extract.

Table 4.6 HPLC condition for analysis of ethanolic extract of APSL remedy

Operating parameters	**Conditions**
Stationary Phase	Phenomenex Luna 5 µ C18[2] 100A analytical column [150 x 4.60 mm. 5 micron]
Mobile Phase	Water-acetronitrile with gradient elution as follows: 0 min, 90:10; 30 min, 50:50; 40 min, 5:95; 45 min, 5:95; 45.1 min, 90:10; 50 min 90:10.
Flow Rate	1.0 ml/min
Wavelength	210 and 256 nm.
Injection Volume	10 µl

Table 4.7 The concentration range, linearity [r^2], retention time [RT], and content [mg/g of extract] of bioactive markers from APSL extract analyzed by using HPLC.

Compounds	Wavelength [nm]	Concentration range [µg/ml]	Linearity [r^2]	RT [min]		Content [mg/g of extract]
				Standard	APSL extract	HPLC
Eugenol	210	5-25	0.9985	26.505	30.321	98.828±6.09
Piperine	256	5-25	0.9921	32.321	37.260	104.636±7.77

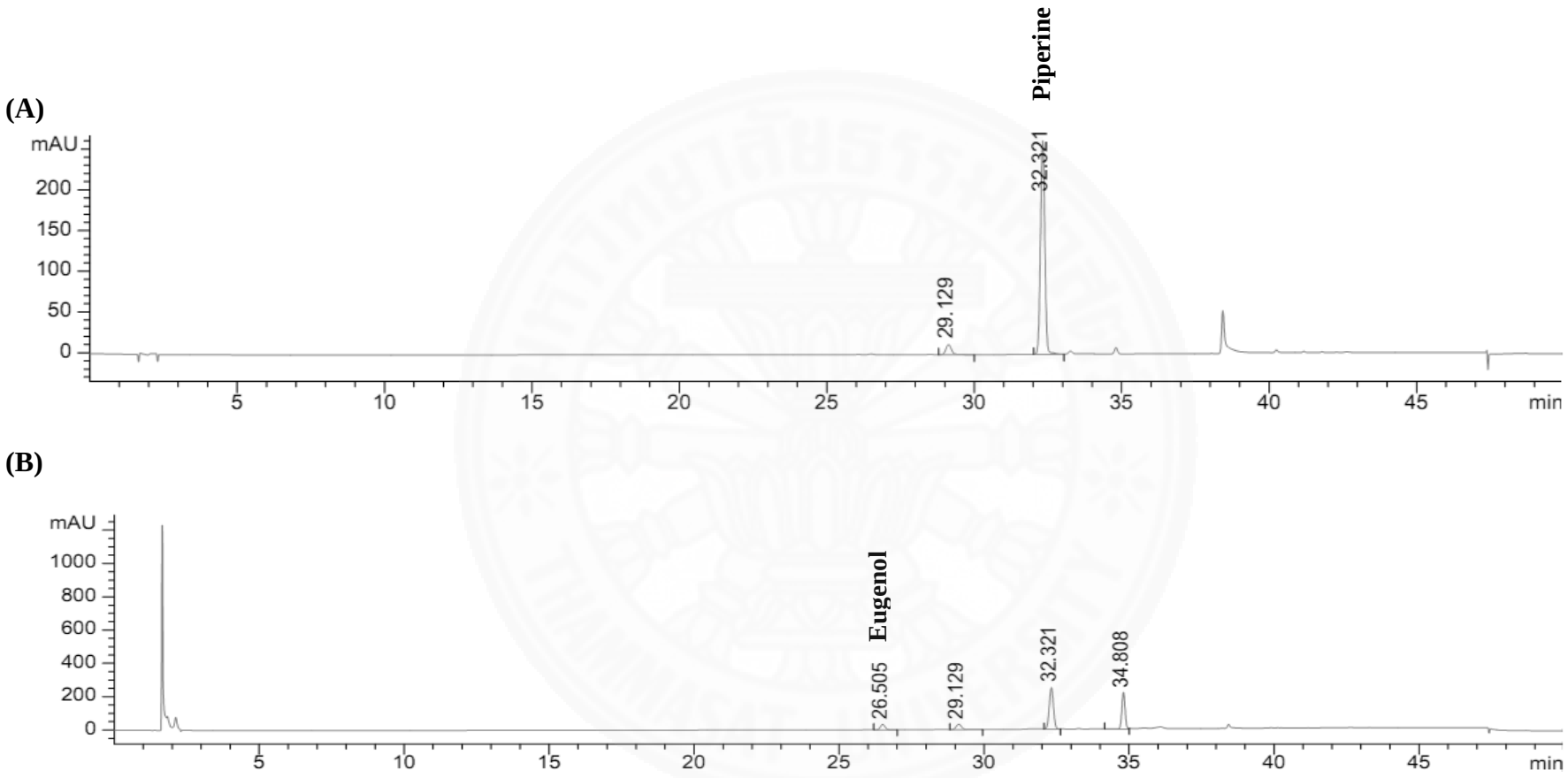
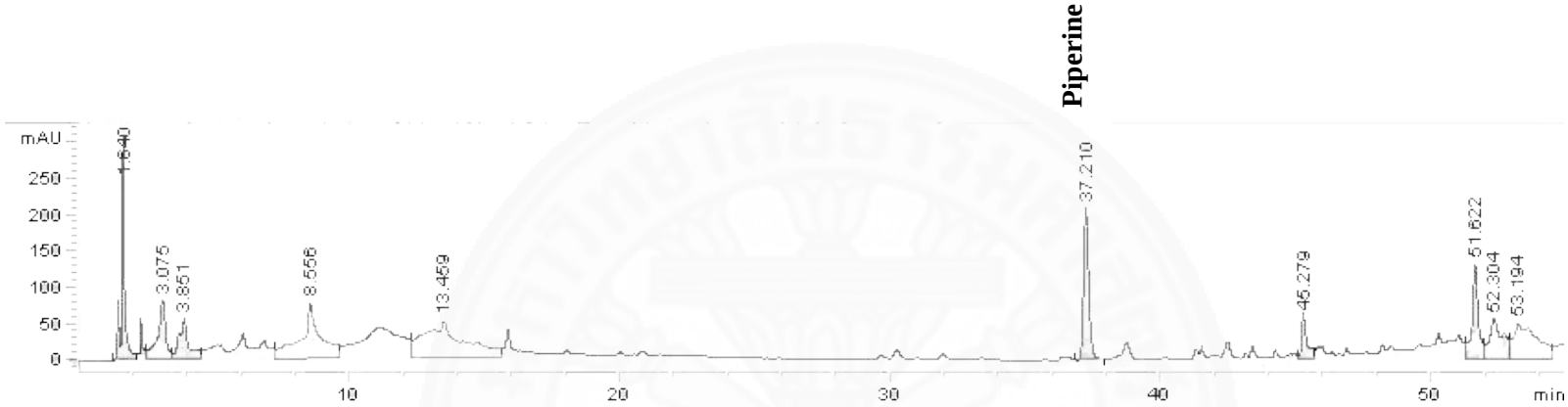


Figure 4.20 HPLC chromatogram of standard (A) piperine in wavelength at 256 nm and (B) eugenol in absorption at 210 nm

(A)



(B)

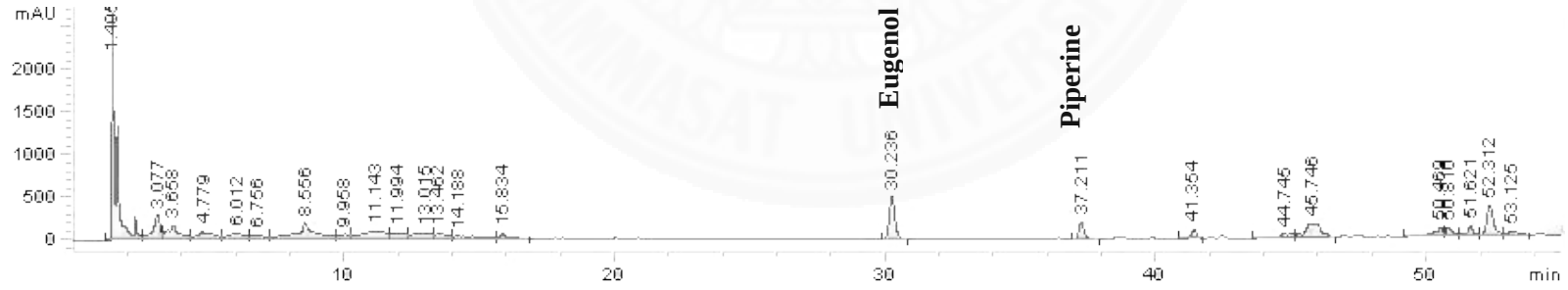


Figure 4.21 Chromatogram of ethanolic extract of APSL (A) wavelength at 256 nm. (B) wavelength at 210 nm.

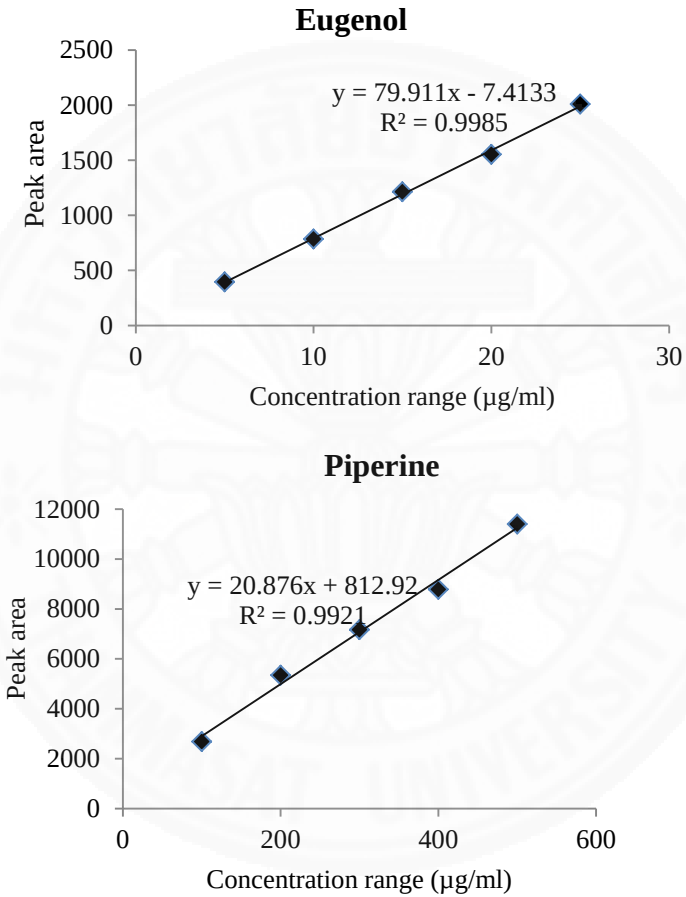


Figure 4.22 Calibration curve (HPLC pattern of standard)

Table 4.8 IC_{50} [µg/ml] of cytotoxic activity of APSL remedy against human lung cell line [A549, NCI-H226, COR-L23 and MRC-5] using SRB assay [n=3] on eugenol, piperine and plumbagin

Type of cells	IC_{50} [µg/ml] ±SEM of cytotoxic activity	
	Eugenol	**Piperine**
A549	>100	4.69±0.19 [SI=1.99]
CORL-23	>100	5.22±0.03 [SI=1.79]
NCI-H226	>100	5.79±0.06 [SI=1.61]
MRC-5	>100	9.33±0.36

4.5 Stability test

4.5.1 *In vitro* cytotoxic activity by SRB assay of APSL remedy from stability test

The result revealed that all APSL ethanolic extracts from the stability testing under accelerated condition at 40±2^{o}C with 75±5% RH for 6 months [Days 15, 30, 60, 90, 120, 150 and 180]. All data are the mean of three replications. Values of different parameters are expressed as the mean ± standard error of mean. Statistical analysis was performed using SPSS [SPSS 13 for windows] statistical software.

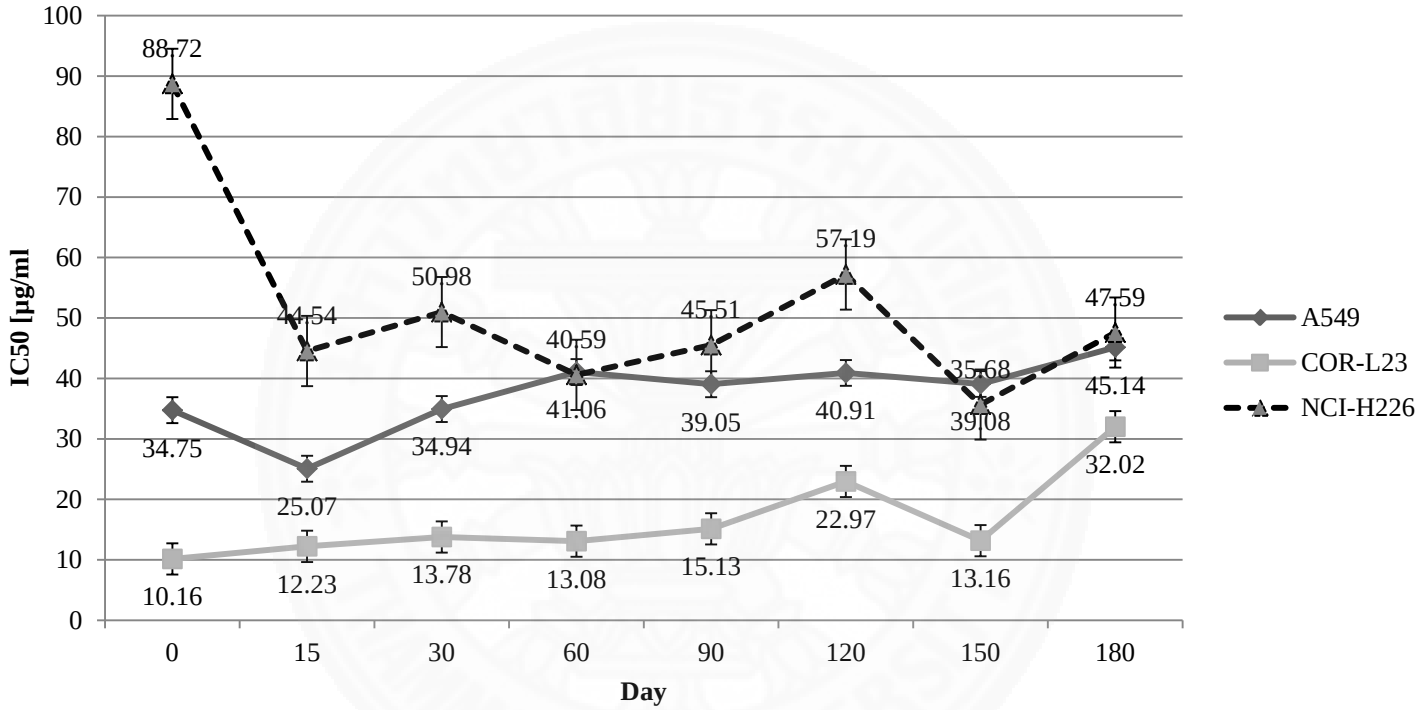
Stability on *in vitro* assay for cytotoxic activity by SRB assay of the APSL remedy with A549 showed results with IC_{50} values 34.75±1.93, 25.07±0.78, 34.94±4.95, 41.06±4.76, 39.05±3.58, 40.91±4.80, 39.08±9.57 and 45.14±3.34 μg/ml, respectively these are stable because between day 0, 15, 30, 90, 120, 150 and 180 were no significant different [p value > 0.05].

Stability of the APSL remedy with COR-L23 showed results of day 0, 15, 30, 90, 120, 150 and 180 with IC_{50} values 10.16±1.68, 12.23±6.76, 13.78±5.69, 13.08±5.69, 13.08±3.61, 15.13±6.53, 22.97±5.88, 13.16±8.24 and 32.02±6.25 μg/ml, respectively . The activity on each day were no significant different with day 0 [p value > 0.05].

Stability of the APSL remedy with NCI-H226 showed results of day 0, 15, 30, 90, 120, 150 and 180 with IC_{50} values 88.72±2.93, 44.54±5.93, 50.98±4.27, 40.59±3.50, 45.51±0.65, 57.19±9.01, 35.68±5.32 and 47.59±8.87 μg/ml, respectively these are unstable compared with day 0 which had significant different [p value < 0.05]. Surprisingly, the cytotoxic activity against NCI-H226 showed better activity when keep APSL at more than 15 days. This results can conclude that APSL keep in high temperature , some chemical may be change to be active cytotoxic against NCI-H226.

Table 4.9 IC_{50} [µg/ml] of cytotoxic activity of APSL remedy against human lung carcinoma cell line [A549, NCI-H226 and COR-L23] using SRB assay [n=3] on stability testing under accelerated condition at 40±2°C with 75±5% RH for 6 months.

Botanical name	Type of cells	Staybility of cytotoxic activity [IC_{50} [µg/ml] ±SEM]in under accelerated condition at 40±2°C with 75±5% RH for 6 months.							
		Day0	**Day15**	**Day30**	**Day60**	**Day90**	**Day120**	**Day150**	**Day180**
95%APSL remedy	A549	34.75 ±1.93	25.07 ±0.78	34.94 ±4.95	41.06 ±4.76	39.05 ±3.58	40.91 ±4.80	39.08 ±9.57	45.14 ±3.34
	COR-L23	10.16 ±1.68	12.23 ±6.76	13.78 ±5.69	13.08 ±3.61	15.13 ±6.53	22.97 ±5.88	13.16 ±8.24	32.02 ±6.25
	NCI-H226	88.72 ±2.93	44.54 ±5.93	50.98 ±4.27	40.59 ±3.50	45.51 ±0.65	57.19 ±9.01	35.68 ±5.32	47.59 ±8.87



A549 and COR-L23 showed *p*-value > 0.05 but NCI-H226 showed *p*-value < 0.05

Figure 4.23 IC_{50} [µg/ml] of cytotoxic activity of APSL remedy against human lung carcinoma cell line [A549, NCI-H226 and COR-L23] using SRB assay [n=3] on stability testing under accelerated condition at 40±2°C with 75±5% RH for 6 months.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

The APSL remedy recommended for the treatment of longevity or rejuvenation and is claimed to improve blood circulation. It has been used to treat the symptoms of COPD and lung diseases such as the inflammation in lung cancer but there is no report of cytotoxic activity against lung cancer cell of this preparation. However, some plant ingredients of APSL have report of cytotoxic activity against other cancer as follows *Acorus calamus* Linn. can against human colorectal adenocarcinoma [HT–29], human epidermoid carcinoma [KB] and human cervical cancer [SiHa] cells [Rakesh *et al*., 2016], *Atractylodes lancea* can against cholangiocarcinoma [Wiratchanee *et al*., 2010], *Clausena excavate* Burmf. can against HL-60 and MCF-7 cancer cell lines [Sharif *et al*., 2011], *Cuminum cyminum* L. can against Colon 502713, Colo-205, Hep-2, A-549, OVCAR-5, PC-5 and SF-295 [Ekta *et al*., 2014], *Foeniculum valgare* Mill can against V79 cells [Wakabayashi *et al*., 2015], *Lipidium sativum* Linn. can against human breat camcer cells [MCF-7] [Sawsan *et al*., 2013] , *Myristica fragrans* Houtt can against seven human cancer cell lines ascribed to central nervous system, Colon cell [Colon502713,Colo205], Liver[Hep-2], Lung [A-549], Ovary OVCAR-5 and Prostrate [PC-5] [Ekta *et al*., 2013], *Piper nigrum* Linn. can against COLO-205 [Rao *et al*., 2011], MCF-7 and A-549 [Sen *et al*., 2011], *Plumbago indigo* L. can against HCT-8 ,MDA-MB-435 and SF-295 [Mariana *et al*., 2009], *Syzygium aromaticum* can against COLO-205 [Reddy *et al*., 2009] and *Terminalia chebula* can against COLO-205, HCT-15, MDA-MB-231, DU-145 and K562 cell lines [Reddy *et al*., 2009].

The ethanolic extract of APSL showed high cytotoxic activity against CORL-23 with IC_{50} =10.16±1.68 [SI=8.34] µg/ml but water extract had no activity. The ethanolic extract of its plant ingredients which showed the highest cytotoxic activity against COR-L23 are *Clauseana excavata* and *Plumbago indica* with IC_{50} values 1.18±0.06 [SI=0.68] and 6.18±0.49 [SI=2.61] µg/ml and were accepted by criteria of NCI [active extract have to show IC_{50} less than 20 µg/ml]. The ethanolic of *Terminaria arjuna, Piper nigrum, Nigella sativa and Atractylodes lancea* also showed

cytotoxic activity against lung cancer cell with IC_{50} value as 30-40µg/ml. The water extract of *Clauseana excavate*, *Syzygium aromaticum and Terminaria arjuna* also showed high cytotoxic activity but they had the IC_{50} values more than 20 µg/ml [IC_{50}= 20-30 µg/ml respectively].

The results of cytotoxic activity in this study had no previous report and revealed that sitostenone and mixture of β –sitosterol and stigmasterol from stem of *C. excuvata* exhibited cytotoxic activity against human lung carcinoma cell lines [COR-L23, A549 and NCI-H226] with IC_{50} values of 24 - 64 µg/ml respectively, but they had been reported the results of anti-inflamatory and anti-allergic activities which was the most potent with IC_{50} values 5.38±0.17 and 6.16±0.73 µg/ml respectively, has been presented by Champasuri [2015].

Reversed phase high performance liquid chromatography [HPLC] method to control quality of APSL remedy was developed. In this method, piperine and eugenol as the compounds having the most potent inhibition of cytotoxic activity were investigated by Thongmee [2015] that used to be markers. The method of HPLC was developed and showed good sensitivity, precision, and accuracy.The result showed that piperine in the ethanolic extract of APSL was present in the highest content [104.64±7.77 mg/g of extract] following by Eugenol contents with 98.83±6.09, mg/g of extract. The results of cytotoxic activity of these two compounds against human lung cell lines [A549, COR-L23, NCI-H226 and MRC-5] showed IC_{50} values of 4-10 µg/ml respectively. A HPLC method for studying chemical fingerprints of ethanolic extract of APSL and quantifying piperine, has been presented by Rattarom [2010] that piperine showed cytotoxic activity against human lung carcinoma cell lines [COR-L23 and A549] of piperine with IC_{50} values 17.00±0.14 and more than 20 µg/ml respectively. The Cytotoxic activity against human lung cell line [MRC-5] had the IC_{50} values more than 20 µg/ml.

Stability of cytotoxic activity by SRB assay of the 95% ethanolic extracts of APSL remedy showed high cytotoxic activity agianst human lung large cell line [COR-L23] with IC_{50} values in range of 10.16-32.02 µg/ml and testing cytotoxic activity with human lung adenocacinoma cell line [A549] showed IC_{50} values in range of 25.07-45.14 µg/ml These results indicated that under accelerated condition at 40±2^{o}C with 75±5% RH for 6 months [Days 15, 30, 60, 90, 120, 150 and 180] were

stable because IC_{50} of day0 and day180 showed no significant different [*p*-value >0.05]. However, the stability of cytotoxic activity of APSL remedy against human lung squamous carcinoma cell line [NCI-H226] showed IC_{50} values in range of 35.68-88.72 µg/ml that unstable because IC_{50} of day0 and day180 showed significant different [*p*-value <0.05] but the stability was to good activity if it keep in accelerate condition for long time. This result may conclude that the chemical component should be changed in high temperature to be active cytotoxic compound.

The purposes of this study were to investigate the cytotoxic effects of ethanolic extracts and aqueous extracts of APSL and its plant ingredients. The results the ethanolic extract of remedy and some plant showed highly potent cytotoxic activity with lung cancer cell lines [COR-L23]. These results can support that the recommended for used of Thai medicinal remedy for preventing cancer in lung human in the future. The biological activity of this remedy has been studied such as anti-oxidant and anti-inflammatory. Thus, this study can develop to medicine for treat lung cancer in future.

APSL and its plant ingredients had been used for treating COPD that been cause of lung cancer and it was known that can treat lung cancer such as preventing lung cancer, anti-inflammation lung and promote blood circulation in lung. However, the mechanism of action of these components is not yet known. Thus, it should be further tested in animals and human to support the experimental results.

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APPENDICES

APPENDIX A
CHEMICAL REAGENTS

1. Reagents for cell culture

1.1 FBS [inactivated]

Slowly thaw the frozen FBS, heat inactivate at 56 degree Celsius

[Aliquot and stored in -20 degree Celsius]

1.2 PBS

PBS	1 tablet
DI water	100 ml

[Autoclave 121 degree Celsius, 15 min and stored in 4 degree Celsius]

1.3 Penicillin-Streptomycin

Slowly thaw the frozen P/S in water bath at 37 degree Celsius until complete thaw, Aliquot and stored in -20 degree Celsius

1.4 Trypsin-EDTA

Slowly thaw the frozen trypsin in water bath at 37 degree Celsius until complete thaw, Aliquot and stored in -20 degree Celsius

1.5 RPMI 1640 [incomplete media]

RPMI 1640 1X with L-glutamine	1 pack
$NaHCO_3$	2.0 g

[Adjust volumn with sterile water to 1000 ml pH 7.0-7.2 by 1 N NaOH or 1N HCl and filtered sterile at a pore size of 0.2 microM, and stored in 4 degree Celsius]

RPMI 1640 [complete media]

RPMI 1640 [incomplete media]	1000 ml
10% FBS	100 ml
1% Penicillin-Streptomycin [Stored in 4 degree Celsius]	10 ml

APPENDIX A
CHEMICAL REAGENTS (Continued)

1.6 MEM [incomplete media]

MEM 1X with Earle's salts, L-glutamine	1 pack
$NaHCO_3$	2.2 mg

[Adjust volumn with sterile water to 1000 ml pH 7.0-7.2 by 1 N NaOH or 1N HCl and filtered sterile at a pore size of 0.2 microM, and stored in 4 degree Celsius]

MEM [complete media]

MEM [incomplete media]	1000 ml
10% FBS	100 ml
1% Penicillin-Streptomycin	10 ml

[Stored in 4 degree Celsius]

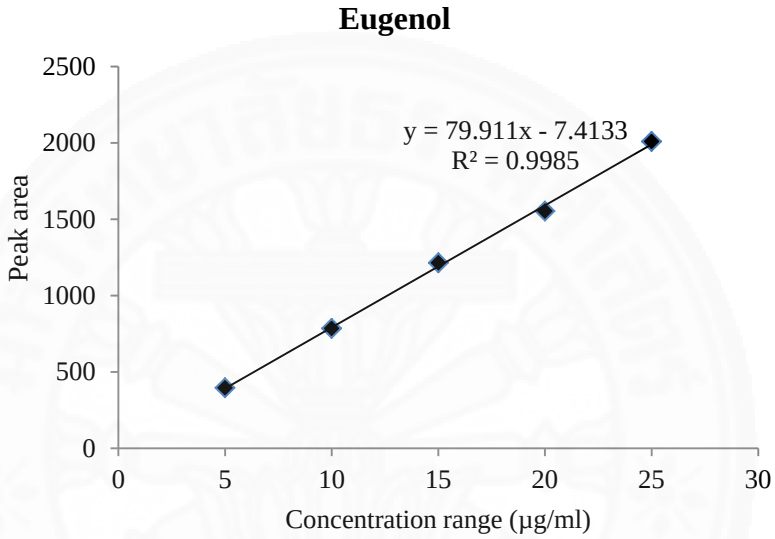
2. Reagent for Thin Layer Chromatography [TLC]

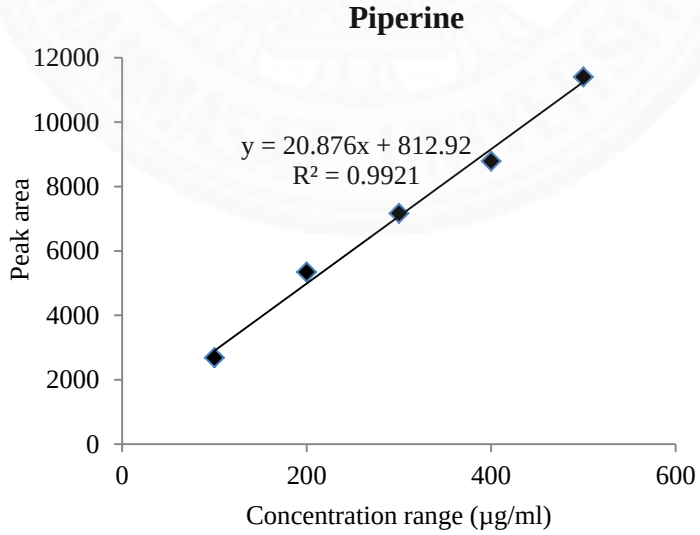
2.1 Anisaldehyde reagent

Anisaldehyde	0.5 ml
MeOH	8.5 ml
Acetic acid	10 ml
H_2SO_4	5 ml

APPENDIX B

STANDARD CURVES OF PURE COMPOUNDS ANALYZED BY HPLC





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