ความเป็นพิษต่อเซลล์มะเร็งของพืชสมุนไพรไทยในตำรับยาตรีผลา เบญจกุลและเบญจโลกวิเชียร

นายวรินทร์พิภพ ชยทัตภูมิรัตน์

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CYTOTOXICITY AGAINST CANCER CELLS OF THAI MEDICINAL RECIPES: TRIPHALA, BENJAKUL AND BENJALOKWICHIAN

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วรินทร์พิภพ ชยทัตภูมิรัตน์ : ความเป็นพิษต่อเซลล์มะเร็งของพืชสมุนไพรไทยในตำรับยาตรีผลา เบญจกุลและเบญจโลกวิเชียร. (CYTOTOXICITY AGAINST CANCER CELLS OF THAI MEDICINAL RECIPES: TRIPHALA, BENJAKUL AND BENJALOKWICHIAN) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. จรรยา ชัยเจริญพงศ์, 105 หน้า

จากการทดสอบความเป็นพิษต่อเซลล์มะเร็งของพืชสมุนไพรในตำรับยาไทยที่ออกฤทธิ์ต้านเซลล์มะเร็ง 3 ตำรับยาคือ ตำรับยาเบญจโลกวิเชียร ประกอบด้วยพืช 5 ชนิด ได้แก่ ชิงชี่ ย่านาง คนทา เท้ายายม่อม และมะเดื่อชุมพร ตำรับยาเบญจกุลประกอบด้วยพืช 5 ชนิด ได้แก่ ดีปลี ช้าพลู สะค้าน เจตมูลเพลิงแดง และขิง ตำรับยาตรีผลาประกอบด้วยพืช 3 ชนิด คือ สมอไทย สมอพิเภก และมะขามป้อม ทดสอบความเป็นพิษต่อเซลล์มะเร็งของสารสกัดน้ำและเมทานอลของพืชสมุนไพรทั้งสามตำรับยาด้วยวิธี 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) assay เซลล์มะเร็งที่ใช้ในงานวิจัยมี 5 ชนิด คือ เซลล์มะเร็งลำไส้ใหญ่ (SW620) เซลล์มะเร็งปอด (Chago) เซลล์มะเร็งเต้านม (BT474) เซลล์มะเร็งกระเพาะอาหาร (KATO-III) และเซลล์มะเร็งตับ (Hep-G2) ผลจากการคัดกรองพบว่าสารสกัดเมทานอลของรากเจตมูลเพลิงแดงมีความเป็นพิษต่อเซลล์มะเร็งลำไส้ใหญ่ และเซลล์มะเร็งตับมากที่สุด โดยมีค่าเปอร์เซ็นต์การรอดชีวิตของเซลล์มะเร็งเท่ากับ 11.67 □ 0.01 และ 15.74 □ 0.03 เปอร์เซ็นต์ ตามลำดับ สารสกัดเมทานอลของเหง้าขิงมีความเป็นพิษต่อเซลล์มะเร็งปอด และเซลล์มะเร็งกระเพาะอาหารมากที่สุด โดยมีค่าเปอร์เซ็นต์การรอดชีวิตของเซลล์มะเร็งเท่ากับ 6.67 □ 0.02 และ 14.23 □ 0.01 เปอร์เซ็นต์ ตามลำดับ สารสกัดเมทานอลของเหง้าขิงและรากเจตมูลเพลิงแดงมีความเป็นพิษต่อเซลล์มะเร็งเต้านมมากที่สุดโดยมีค่าเปอร์เซ็นต์การรอดชีวิตของเซลล์มะเร็งเท่ากัน คือ 27.30 □ 0.00 เปอร์เซ็นต์ สารกัดหยาบเมทานอลจากรากเจตมูลเพลิงแดงให้ผลการยับยั้งการเจริญของเซลล์มะเร็งทั้ง 5 ชนิดดีที่สุดจึงถูกเลือกนำมาแยกและหาสารบริสุทธิ์เพื่อทดสอบความสามารถในการยับยั้งการเจริญเติบโตของเซลล์มะเร็ง พบสาร 5 ชนิด คือ 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin), 17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7, 8, 9, 11, 12,14,15,16,17-dodecahydro-1H-cyclopenta[a] phenan thren-3-ol (beta sitosterol), 2,8-dihydroxy-3-methyl-1,4-naphthoquinone (droserone), (3R ,4R)-4,8-dihydroxy-3-methyl-3,4-dihydro-2H-naphthalen-1-one (isoshinanolone) และunknown 5 สารทั้ง 5 ชนิดที่แยกได้สามารถยับยั้งการเจริญเติบโตของเซลล์มะเร็งทั้ง 5 ชนิดได้ดี โดย unknown 5 สามารถยับยั้งการเจริญของเซลล์มะเร็งเต้านม เซลล์มะเร็งปอด เซลล์มะเร็งลำไส้ใหญ่ และเซลล์มะเร็งตับได้ดีที่สุดจากการทดสอบด้วยค่า IC50 เท่ากับ 0.13 □ 0.07, 0.10 □ 0.02, 0.11 □ 0.09 and 0.10 □ 0.02 µg/ml ตามลำดับ สำหรับผลการดสอบกับเซลล์มะเร็งกระเพาะอาหาร สาร 1 ให้ค่า IC50 เท่ากับ 0.0016 □ 0.001 µg/ml ซึ่งน้อยกว่าค่า IC50 ของ doxorubicin 475 เท่า ซึ่งแสดงให้เห็นว่าอาจนำมาพัฒนาเป็นยาในการรักษาโรคมะเร็งกระเพาะอาหารในอนาคตได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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VARINPIPHOB CHAYATHATPHOMMIRAT: CYTOTOXICITY AGAINST CANCER CELLS OF THAI MEDICINAL RECIPES: TRIPHALA, BENJAKUL AND BENJALOKWICHIAN. ADVISOR: CHANYA CHAICHAROENPONG, Ph.D., 117 pp.

Cytotoxic activity of three Thai medicinal plant recipes, Benjalokwichien, Benjakul and Triphala, Benjalokwichien recipe consists of Capparis micracantha, Tiliacora triandra, Harrisonia perforata, Tacca leontopetaloides and Ficus racemosa. Benjakul recipe consists of Piper retrofractum, Piper interruptum, Zingiber officinale, Plumbago indica and Piper sarmentosum. Triphala consists of Terminalia chebula, Terminalia bellerica and Phyllanthus emblica. Cytotoxicity effects of water and methanol extracts from all plants were studied with 5 types of cancer cells; colon cancer (SW620), lung cancer (Chago), breast cancer (BT474), gastric cancer (KATO-III) and liver cancer (Hep-G2). Methanol extract of Plumbago indica root was toxic to colon cancer cells and liver cancer cells with the lowest percent survival of cancer cells at 11.67 □ 0.01 and 15.74 □ 0.03 percent, respectively. Methanol extract of Zingiber officinale rhizome was toxic to lung cancer cells and gastric cancer cells with the lowest percent survival of cancer cells at 6.67 □ 0.02 and 14.23 □ 0.01 percent, respectively. Methanol extracts of Zingiber officinale rhizome and Plumbago indica root were toxic to breast cancer cells with the lowest percent survival of cancer cells at 27.30 □ 0.00 percent. Methanol extract of Plumbago indica root was toxic to five cancer cells with the lowest percent cell survival. Therefore, it was very interesting to isolated compounds from methanol extracts of P. indica L. for anti-cancer activity test. It was separated to obtain 5 compounds; 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin), 17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7, 8, 9, 11, 12,14,15,16,17-dodecahydro-1H-cyclopenta[a] phenan thren-3-ol (beta sitosterol), 2,8-dihydroxy-3-methyl-1,4-naphthoquinone (droserone), (3R ,4R)-4,8-dihydroxy-3-methyl-3,4-dihydro-2H-naphthalen-1-one (isoshinanolone) and unknown 5. All of them showed strong cytotoxicity against cancer cell lines. unknown 5 showed strongest activity against BT474, Chago, SW620 and Hep-G2 cancer cell lines with IC50 0.13 □ 0.07, 0.10 □ 0.02, 0.11 □ 0.09 and 0.10 □ 0.02 µg/ml, respectively. Plumbagin showed strongest activity against KATO-III cancer cell line with IC50 0.0016 □ 0.001 µg/ml less than IC50 of doxorubicin 475 times. The results indicated that compound extracted from roots of Plumbago indica L. may be developed to be human gastric carcinoma drug in the future.

Field of Study: Biotechnology Student's Signature .....

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ABBREVIATIONS

δ	=	Chemical shift
^{o}C	=	Degree Celsius
µg	=	Microgram
µM	=	Micromolar
µl	=	Microliter
µm	=	Micrometer
22Rv1	=	Human prostate carcinoma epithelial cell line
A375.S2	=	Human melanoma cells
A-549	=	Adenocarcinomic human alveolar basal epithelial cells
A.D.	=	Anno domini
ADP	=	Adenosine diphosphate
AIDS	=	Acquired immunodeficiency syndrome
ANOVA	=	Analysis of variance
aq	=	Aqueous solution
ASTM	=	American society for testing and materials
ATCC	=	Global nonprofit bioresource center
ATP	=	Adenosine triphosphate
barcl-95	=	Transplantable mouse thymic lymphoma
BAX	=	Protein inhibitor
BC-8	=	Rat histiocytoma
Bcl-xL	=	B-cell lymphoma-extra large
BGC-823/Doc	=	Human gastric cancer cells
BHA	=	Butylated hydroxyanisole
BHT	=	Butylated hydroxytoluene
BKF	=	Forest herbarium
br s	=	Broad singlet (for NMR spectra)
BT474	=	Human breast cancer cells
CAS	=	Chemical abstracts service
CC	=	Column chromatography
CCD-986Sk	=	Human normal skin fibroblasts

CCl_4	=	Carbon tetrachloride
Cdc25B	=	Regulatory proteins
$CDCl_3$	=	Deuterated chloroform
Chago	=	Human lung cancer cells
$CHCl_3$	=	Chloroform
cm	=	Centimeter
^{13}C-NMR	=	Carbon-13 nuclear magnetic resonance
CO_2	=	Carbon dioxide
COSY	=	Correlated spectroscopy
COX-2	=	Cyclooxygenase
CXCR4	=	Chemokine receptor
d	=	Doublet (for NMR spectra)
dd	=	Doublet of doublet (for NMR spectra)
ddd	=	Doublet of doublet of doublet (for NMR spectra)
D-GalN	=	D-galactosamine
DLA	=	Dalton's lymphoma ascites
DMBA	=	7,12-Dimethylbenz(α)anthracene
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
DU-145	=	Human prostate cancer cells
EC_{50}	=	Half maximal effective concentration
EtOAc	=	Ethyl acetate
EtOH	=	Ethanol
ft	=	Foot
g	=	Gram
h	=	Hour
H460	=	Human lung cancer cells
HCT-15	=	Human colon cancer cell line
HGF	=	Hepatocyte growth factor
Hep-G2	=	Human liver cancer
HIV	=	Human immunodeficiency virus

HK-63	=	Human leukemia
^{1}H-NMR	=	Proton nuclear magnetic resonance
HMBC	=	Heteromolecular Multiple Bond Correlation
HMQC	=	Heteromolecular Multiple Quantum Correlation
HOS-1	=	Human osteosarcoma cell line
HSC-2	=	Human carcinoma
H_2SO_4	=	Sulfuric acid
HT-29	=	Human colon carcinoma cell line
Hz	=	Hertz
IAPs	=	Inhibitors of apoptosis
IC_{50}	=	Half maximal inhibitory concentration
IL-1b	=	Interleukin-1 beta
IL-2	=	Interleukin 2
IL-6	=	Interleukin 6
IMR32	=	Human neuroblastoma
in	=	Inch
iNOS	=	Inducible nitric oxide synthase
J	=	Coupling constant
J774	=	Macrophages cell line
JNK	=	Jun N-terminal kinase
KATO-III	=	Human gastric carcinoma cells
kg	=	Kilogram
L	=	Litre
L929	=	Mouse fibroblasts
LPS	=	Lipopolysaccharide
m	=	Metre
M	=	Molar
MBC	=	Minimum bactericidal concentration
MCF7	=	Human breast adenocarcinoma cell line
MDA-MB-231	=	Human breast cancer cells
MeOH	=	Methanol

mg	=	Milligram
MHz	=	Megahertz
MIC	=	Minimum inhibitory concentration
ml	=	Millilitre
mm	=	Millimetre
mm^2	=	Square millimeter
mm^3	=	Cubic millimeter
m.p.	=	Melting point
mRNA	=	Messenger ribonucleic acid
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	=	Transcription factor
nm	=	Nanometer
NMR	=	Nuclear magnetic resonance
No.	=	Number
$p21^{CIP1/WAF-1}$	=	Cyclin-dependent kinase inhibitor 1
p38	=	Mitogen-activated protein kinases
P388D1	=	Macrophage cell line
p53	=	Tumor suppressor genes
PARP	=	Poly (ADP-ribose) polymerase
PC-3	=	Human prostate cancer cell line
PCC-4	=	Embryonal carcinoma cell line
PI5K-1B	=	1,4-Phopshatidylinositol 5-kinase
PLC	=	Phospholipase C
PP2A	=	Tumor cell
ppm	=	Part per million
PS	=	Phosphatidylserine
q	=	Quartet (for NMR spectra)
R_f	=	Retention factor
ROS	=	Reactive oxygen species
RWEP-1	=	Normal human prostate cell line
s	=	Singlet (for NMR spectra)

SK-MEL-2	=	Human melanoma cell lines
SKOV-3	=	Human ovarian carcinoma cell line
SW620	=	Human colon cancer cells
t	=	Triplet (for NMR spectra)
TLC	=	Thin layer chromatography
TNF-α	=	Tumor necrosis factor alpha
UV	=	Ultraviolet
U937	=	Human leukemic monocyte lymphoma cell line
WHO	=	World health organization
Wi-38	=	Human normal lung fibroblast
wt	=	Weight
XF-389	=	Human tumor cell line
z-DEVD-fmk	=	Caspase 3 inhibitor

CHAPTER I

INTRODUCTION

Cancer is named after the organ or tissue it started and became a disease in which the body's cells become abnormal and divide without control. Cancer cell growth is different from normal cell growth. They can invade nearby tissues and can spread through the bloodstream and lymphatic system to other parts of the body to form lumps or masses of tissue called tumors. Tumors can grow and intervene with body's systems and they can release hormones that alter body function. Tumors that stay in one spot and demonstrate limited growth are generally considered to be benign. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected (Crosta, 2008).

Cancer is becoming a significant health problem in Thailand. It is the first common leading cause of death in Thailand, with an increase in the death rate every year. In 2012, there were 1,309,211 new cancer patients according to 21 groups of causes from health service units, ministry of public health and rates of cancer patients were 22.34 per 1,000 populations (Vatanasapt *et al*., 2002). Nowadays, cancer is the most feared disease in the Asian countries because it is known to be hard to cure. Drug therapy is now a most for cancer treatment. Drugs currently used to treat cancer are mostly directed against all rapidly proliferating cells and also normal cells. The research for new active compounds that do not affect normal cells is required for the cancer treatment.

Medicinal plants have long term been traditionally used for treatment of various diseases including infection, immunological disorders and cancer. Present, according to the World Health Organization (WHO), as many as 80% of the world's people depend on traditional medicine for their primary health care needs. Plants have a long history of use in the treatment of cancer and it is significant that over 60% of currently used anti-cancer agents are derived from natural sources (Cragg and Newman, 2005a). Plants have the efficacy to synthesize widely chemical compounds that are used to perform important biological functions, and to defend against raid from predators such as insects, fungi and herbivorous mammals. In Asian countries, herbal formulations from a mixture of plants are often used by traditional medical

practitioners for the treatment of cancer. Thailand is located in a tropical area with abundance of diverse medicinal plants. Traditional medicines have long been used by Thai people. During the Sukothai period, medicinal herbs were used for the treatment of illnesses. Later in Ayutthaya period, Indian Ayurveda and Chinese traditional medicine principles were used aiming at establishing a balanced condition of four major vital elements. The role of the practitioner is to identify the person's mind and body type and recommend a course of treatment unique to the individual. Many Thai medicinal plant recipes which have anti-cancer activities have been popularly used by Thai people. These recipes combine from many ingredients to ensure effective actions on different targets simultaneously.

Triphala recipe is a combination of three fruits (tri means three and phala means fruit). Triphala consists of fruits of *Terminalia chebula* Retz. Var. Chebula, *Terminalia bellerica* Roxb. and *Phyllanthus emblica* Linn. Triphala is an efficacious cardiotonic mixture which is also prescribed for symptoms of inflammation, heat, infection, obesity, anaemia, fatigue, candida, poor digestion, assimilation, tuberculosis, pneumonia, AIDS and anti-HIV (El-Mekkawy *et al.*, 1995). Triphala is considered as the greatest and more versatile of all the herbal formulations. Currently, Triphala is interesting because many studies have confirmed its activities against cancer cells. Two Thai anticancer medicinal recipes in National List of Essential Medicines are Benjakul and Benjalokwichian recipe. Benjakul is composed of five herbs: fruits of *Piper chaba*, roots *of Piper sarmentosum*, stems of *Peper interruptum*, roots of *Plumbago indica* and rhizomes of *Zingiber officinale*. It is used to adjust a balance of body in theory of Thai traditional medicine. Piperine, plumbagin and 6-gingerol were separated from ethanol extracts of Benjakul recipe that inhibited cell lung cancer (Itharat and Sakpakdeejaroen, 2010), but plumbagin can degrade at 25 degrees celsius. The extracts should be stored at low temperature or store in the refrigerator. Benjalokwichian recipe consists of roots of *Capparis micracantha*, *Tiliacora triandra*, *Harrisonia perforata*, *Tacca leontopetaloides* and *Ficus racemosa*. According to Thai Pharmacopoeia, the ethanol extract of Benjalokwichian showed anti-inflammatory activity (IC_{50} = 40.36 µg/ml) and moderate antioxidant activity (EC_{50} = 40.93 µg/ml). This results supported using Benjalokwichian recipe to treat the inflammatory

conditions which are cause of fever and allergy in Thai traditional medicine (Juckmeta and Itharat, 2012).

Objective of this study

1. To screen Thai medicinal plants from three recipes; Triphala, Benjakul and Benjalokwichian for *in vitro* cytotoxicity against human cancer cell lines.
2. To extract, isolate and determine chemical structure of chemical constituents of the most active plant from three recipes; Triphala, Benjakul and Benjalokwichian.

CHAPTER II

LITERATURE REVIEWS

1. Overview of Cancer

Cancer is a term applied to serious disease characterized by rapid and uncontrolled abnormal cells formation which may mass together to form a growth or proliferate throughout the body, and it may progress until it causes death. Among various diseases attributed to mortality in humans all over the world, cancer is a leading cause and it is one of the most dreaded diseases of the 20th century and spreading further with continuance and increasing incidence in 21st century. Between 2000 and 2020, the total number of cases of cancer is predicted to increase by 73% in the developing world and by 29% in the developed world (Parkin 2001). It was estimated that there were 10.9 million new cases, 6.7 million deaths, and 24.6 million persons living with cancer around the world in 2002 (Parkin *et al*., 2005).

Cancer is the second leading cause of death in the United States (Jemal *et al*. 2004), where one in four deaths is due to cancer. Malignant neoplasm was the leading cause of death in Hong Kong during 1996 to 2001 (Foda and Zucker, 2001). In Thailand the rate of people dying from cancer is still increasing every year and it is the first leading cause of death (Sriplung *et al*., 2006).

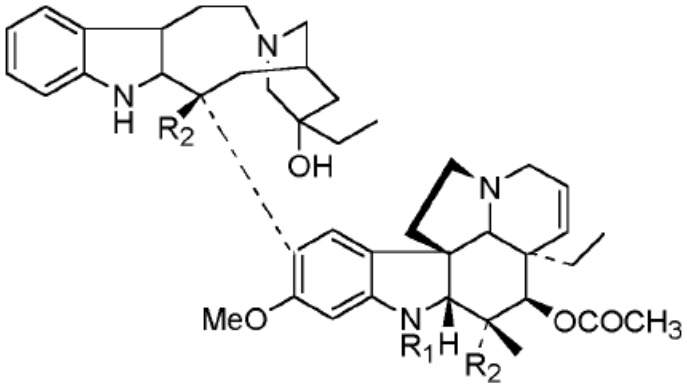
Cancer chemotherapy now plays a significant role in the treatment of many malignancies, either curative (by itself or as an adjuvant to surgery and or radiation) or palliative care, depending upon the specific tumor situation (Ling, 1997). The objective of cancer chemotherapy is to kill cancer cells with as little damage as possible to normal cells (Halliwell *et al*., 1988). Therefore, any discovery of anticancer agents must be related to novel molecular targets; i.e. they should be effective against specific types of cancer cells but less toxic to normal cells, or have a unique mechanism of action for specific types of cancer (Pezzuto, 1997).

2. Discoveries of Novel Anticancer Agents from Plants

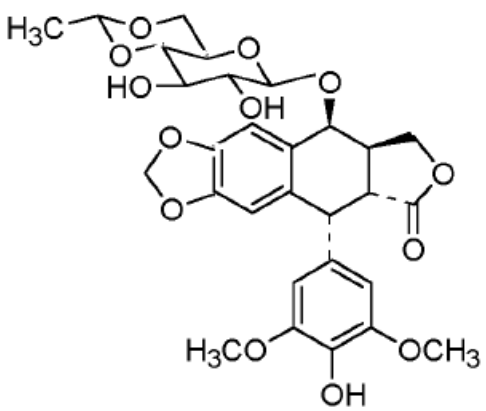
Nature has long been an important source of medicinal agents. The numbers of modern drugs have been isolated from natural sources, based on their use in traditional medicine (Cragg and Newman, 2001). Plants have formed a basis for traditional medicine systems that have been used for thousands of years in countries with ancient civilizations such as China (Chang and But, 1986), India (Kapoor, 1990) and Thailand (Subchareon, 1998). The use of plants in traditional medicine systems of many other cultures has been extensively documented (Schultes and Raffauf, 1990). Plants with antioxidant activities have been reported to possess free radical scavenging activity (Das and Pereira, 1990). Free radicals are known as major contributors to several clinical disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases as a result of deficient natural antioxidant defense mechanism (Parr and Bolwell, 2000). Plant-based systems continue to play an essential role in healthcare and it has been estimated by the WHO that approximately 80% of the world's inhabitants rely mainly on traditional medicine for their primary healthcare (Farnsworth *et al*., 1985). Plant products also play an important role in the healthcare systems of the remaining 20% of the population who reside mainly in developed countries. Analysis of data on prescriptions dispensed from community pharmacies in the United States from 1959 to 1980 indicates that about 25% contained plant extracts or active principles derived from higher plants. Furthermore, at least 119 chemical substances derived from 90 plant species can be considered as important drugs currently in use in one or more countries (Farnsworth *et al*., 1985). About 74% of these 119 drugs were discovered as a result of chemical studies directed at isolation of the active substances from plants used in traditional medicine (Cragg and Newman, 1999). Ethno pharmacological or traditional use of plants often results in the discovery of new biologically active molecules (Houghton, 1995). However, it is important that the investigators understand the principles of folk medicine or mode of action of folk herbs (Nakanishi, 1999).

Plants have a long history of use in the treatment of cancer (Hartwell, 1982). Plant-derived anticancer drugs in clinical use, the best known are the so-called vinca alkaloids, which include vinblastine (**1**) and vincristine (**2**) (Figure 1). These alkaloids were isolated from the Madagascar periwinkle, *Catharanthus roseus* (Linn.) G. Don, Apocynaceae, known in Thailand as Phaeng phuai farang (Itharat and Ooraikul, 2007). Vinblastine and vincristine were first discovered during an investigation of the plant for potential oral hypoglycemic agents (Cragg and Newman, 2005b). Thus, these anticancer agents may be indirectly attributed to the information of an unrelated medicinal use of the source plant. The two clinically-active agents, etoposide (**3**) and teniposide (**4**), which are semi-synthetic derivatives of the natural product epipodophyllotoxin, may be considered to be more closely linked to a plant originally used for the treatment of cancer. Epipodophyllotoxin is an isomer of podophyllotoxin (**5**), which was isolated as the active anti-tumor agent from the roots of various species of the genus *Podophyllum* (Berberidaceae) (Cragg and Newman, 2005b). From the time of Galen (about A.D. 180), the juice expressed from woody nightsha (*Solanum dulcamara* L. Family Solanaceae) has been used to treat cancers, tumors and warts (Itharat and Ooraikul, 2007). The active tumor-inhibitory principle has been identified as the steroidal alkaloid glycoside β-solamarine. Various lichens (e.g. species of *Cladonia*, *Cetraria* and *Usnea*) also have a history of use in folk medicine against cancer since about A.D. 970 (Itharat and Ooraikul, 2007). These are all rich sources of usnic acid, a compound recognized for many years as an antibacterial and antifungal agent but only more recently as an anti-tumor compound. Similarly, many centuries ago, the Druids claimed that mistletoe (*Viscum album*) could be used to cure cancer. Protein fractions with marked anti-tumor activity have been isolated from mistletoe extract. The benzophenanthridine derivatives were found in *Chelidonium majus* (Papaveraceae), a plant with substantial folklore history of use in the treatment of cancers (Dewick, 2002).

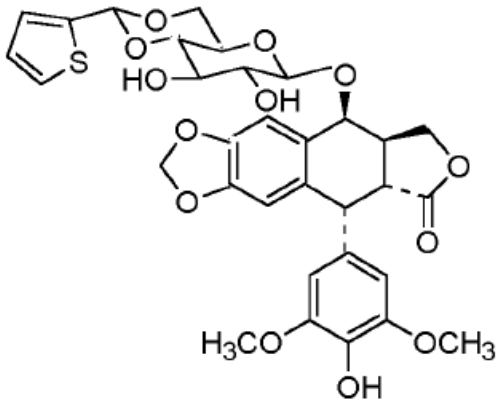
More recent additions to the armamentarium of the naturally derived chemotherapeutic agents are the taxanes and camptothecins. Paclitaxel (**6**) (Taxol®) was initially isolated from the bark of the Pacific or American yew tree, *Taxus brevifolia* Nutt. (Taxaceae), collected in Washington State as part of a random collection program by the U.S. Department of Agriculture for the National Cancer Institute (NCI) (Cragg *et al*., 1993). The use of various parts of *T. brevifolia* Nutt. and other *Taxus* species (e.g., *canadensis, baccata*) by several Native American tribes for the treatment of some noncancerous conditions has been reported (Cragg *et al.,* 1999). The leaves of *T. baccata* are used in the traditional Asiatic Indian (Ayurvedic) medicine system (Kapoor, 1990) that used in the treatment of cancer (Hartwell, 1982). Paclitaxel, along with several key precursors (the baccatins), occurs in the leaves of various *Taxus* species, and the ready semi-synthetic conversion of the relatively abundant baccatins to paclitaxel, as well as active paclitaxel analogs, such as docetaxel (**7**) has provided a major renewable natural source of this important class of drugs (Cortes and Pazdur 1995). Likewise, the clinically active agents, topotecan (hycamptamine) (**8**), irinotecan (**9**) (CPT-11), 9-amino and 9-nitro camptothecin (**10**, **11**) are semi-synthetically derived from camptothecin (**12**), isolated from the Chinese ornamental tree, *Camptotheca acuminate* Decne (Family Cornacea) (Potmeisel and Pinedo, 1995). Camptothecin (as its sodium salt) was advanced to clinical trials by the NCI in the 1970s, but was dropped because of severe bladder toxicity (Cragg and Newman, 2001).



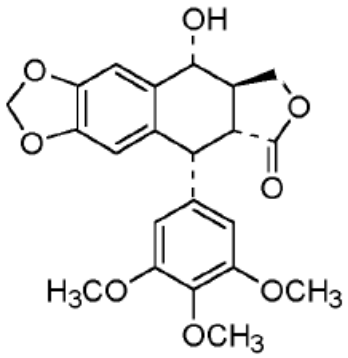
Vinblastine (**1**), R1=CH_3; R2=CO_2CH_3
Vincristine (**2**), R_1=CHO;R_2=CO_2CH_3



Etoposide (**3**)

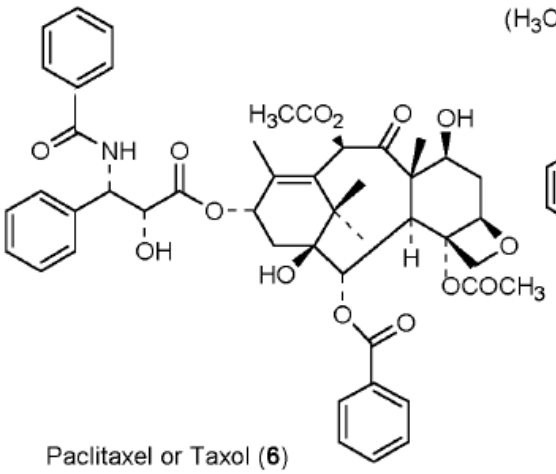


Teniposide (**4**)

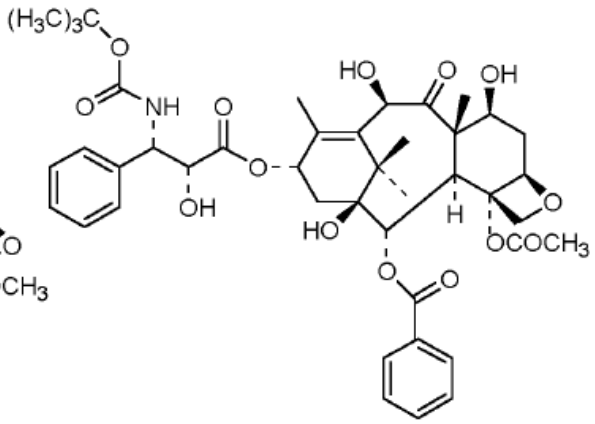


Podophyllotoxin (**5**)

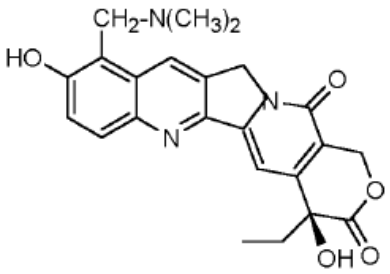
Figure 1 Structures of some anticancer compounds from plants



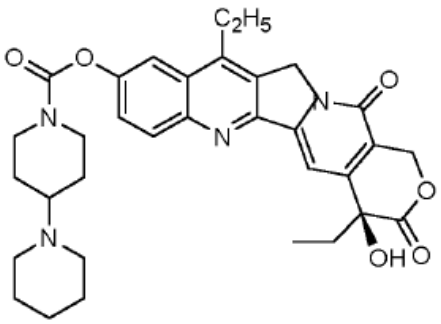
Paclitaxel or Taxol (**6**)



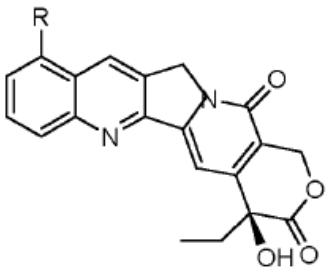
Docetaxel or Taxotere (**7**)



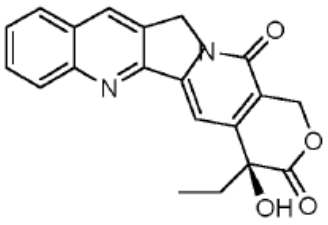
Topotecan or hycamptamin (**8**)



Irinotecan (**9**)



9-Aminocamptothecin (**10**); R=NH_3
9-Nitrocamptothecin (**11**);R=NO_2



Camptothecin (**12**)

Figure 1 (cont.) Structures of some anticancer compounds from plants

3. Thai Medicinal Plant Recipes for Cancer Treatment

There is a long historical use of medicinal plants in Thailand. Some plants have been proven to be useful as pharmaceuticals. Thai medicinal plant recipes are cultural heritage and indigenous wisdom, which have helped take care of the health of Thai people for over a thousand years. Several herbal recipes prepared from the mixture of plants have been traditionally used for the treatment of cancer.

3.1 Triphala Recipe

Triphala recipe has been used in an Ayurvedic and traditional Thai medicines. It is the combination of ripe, healthy and dried fruits of three plants, *Phyllanthus emblica* Linn., *Terminalia chebula* Retz. and *Terminalia bellerica* Roxb. Triphala becomes one of the highly potential herbal medicines in cancer treatment and prevention because all three compositions of Triphala have been found to possessnotable anticancer properties (Sandhya *et al*., 2006).

Table 1 Compositions of Triphala recipe

Elements	Ratio of the three fruits		
	P. emblica	*T. chebula*	*T. bellerica*
Pitta or bile (fire + water)	4	8	12
Vata or wind (air + space)	8	12	4
Kapha or mucous (water + earth)	12	4	8
Malas or waste product (feces)	8	8	8

From Table 1, different proportions of fruits are based on elements of the human body. Triphala has been described as an important health tonic for detoxification, rejuvenation and balance, especially in the summer season (Wongnoppavich *et al*., 2009). This recipe, rich in antioxidants, is a frequently used Ayurvedic medicine to treat many diseases such as anemia, jaundice, constipation, asthma, fever and chronic ulcers. Many people practicing Ayurvedic medicine consume Triphala recipe as a ‘health tonic’. Recent studies have reported

anticlastogenic (Vani *et al*., 1997), anti-tumor properties of *E. officinalis* (Jose *et al.*, 2001) and anti-proliferative effect of *T. chebula* (Saleem *et al*., 2002). Furthermore, Triphala is a therapeutic agent for treatment of a variety of conditions such as headache, dyspepsia, constipation, liver conditions, fatigue, infections and assimilation. It is also reported to possess many biological activities including antidiabetic (Sabu and Kuttan, 2002), antimutagenic (Kaur *et al*., 2002), antimicrobial (Mehta *et al*., 1993), radioprotective (Jagetia *et al*., 2002), hypocholesterolemic (Jagetia *et al*., 2002), antiviral (El-Mekkawy *et al*, 1995), immunomodulatory (Srikumar *et al*., 2005) and anticancer (Kaur *et al*., 2005).

The anticancer effects of Triphala have been investigated by a few studies. The aqueous extract of Triphala was toxic on human breast cancer cell line (MCF7) and a transplantable mousethymic lymphoma (barcl-95) (Sandhya *et al*., 2006). Triphala at the low concentration (5-10 µg/ml) induced a 3-5 times higher toxicity in the cancer cells as compared to the normal cells. The morphology of tumor cells showed distinct alterations similar to apoptotic cells. The apoptotic cell death induced by Triphala was further confirmed by annexin-V staining for phosphatidyl serine (PS) externalization. Furthermore, Triphala induced the pattern of DNA fragmentation, which is a characteristic of apoptosis in tumor cells. Oral administration of Triphala in mice 7 days after tumor transplantation caused significant reduction in tumor volume. The mechanism of *in vitro* cytotoxicity and tumor growth reduction *in vivo* induced by Triphala seems to involve apoptosis induction. In addition, the components of Triphala may exert synergistic cytotoxic action on tumor reduction (Shi *et al*., 2008).

Gallic acid is one of the major components of Triphala and capable of inhibiting cancer cell proliferation suggesting the key factor responsible for antimutagenic and cytotoxic effects of Triphala (Kaur *et al*., 2005). Gallic acid has been reported cytotoxic activity against human leukemia (HK-63) cell line (Ishihara and Sakagami, 2002). Gallic acid also has been reported cytotoxic effect to HOS-1 cell line (Saleem *et al.*, 2002). Similarly, gallic acid showed higher cytotoxicity against HSC-2 by producing DNA fragmentations compared to normal HGF cells (Mimaki *et al*., 2001).

3.1.1 *Phyllanthus emblica* Linn.

Phyllanthus emblica is commonly known as emblicmyrobalan, Indian gooseberry, amla, amalaka, and ma-kham-pom in Thai. *P. emblica* (syn. Embica officinalis Gaertn.) belongs to Family Euphorbiaceae and a deciduous tree of the family Phyllanthaceae. The plant is small to medium in size, reaching 8 to 18 m in height, with a crooked trunk and spreading branches. The branchlets are glabrous or finely pubescent, 10 to 20 cm long, usually deciduous; the leaves are simple, subsessile and closely set along branchlets, light green, resembling pinnate leaves. The fruit is spherical (15-33 mm), greenish-yellow, quite smooth and drupaceous with six vertical furrows. The taste of fruit is sour and bitter, and it is quite fibrous. The main constituents of *P. emblica* include a number of tannins, flavonoids, and other phenolic compounds. The fruits contain low molecular weight tannoids, mainly emblicanins A and B, punigluconin, pedunculagin and gallic acid (Zhang *et al*., 2001). Moreover, organic acid gallates and other hydrolysable tannins including 1-*O*-galloyl-*β*-*D*-glucose, corilagin, chebulagic acid, elaeocarpusin, and puntranijivan have been isolated from the fruit juice of *P. emblica* (Zhang *et al*., 2001). It is commonly used in Ayurvedic and Chinese medicine as well as Thai herbal medicine. The fruits of this plant have been used for treatment of various ailments, such as anemia, liver disease, dyspepsia, hemorrhage, jaundice and diarrhea (Chawla *et al*., 1982). The extracts of *P. emblica* have been shown to possess several biological activities, e.g. analgesic, antipyretic (Perianayagam *et al*., 2004), antimicrobial, anti-inflammatory (Asmawi *et al*., 1993), antioxidant (Bhattacharya *et al*., 1999), antiviral, antimutagenic (Grover and Kaur, 1989), antidiabetic (Sabu and Kuttan, 2002) and anticancer (Jose *et al*., 2001). Furthermore, The extracts of *P. emblica* have been found to have a protective effect upon radiation-induced chromosomal damage and also hypocholesterolemic (Cohen *et al.*, 2005), hypolipidemic (Mathur *et al*., 1996), cardioprotective (Tariq *et al*., 1977) and anti-atherosclerotic in both humans and experimental animals (Thakur and Mandal, 1984). The fruit extracts of plant possess radioprotective effect against gamma irradiation (Kumar *et al.*, 2004) and *in vivo* heptatoprotective activities against CCl_4 (Lee *et al*., 2006), paracetamol (Gulati *et al*., 1995), ethanol (Pramyothin *et al*., 2006) and antituberculosis drugs (Tasduq *et al.*,

2005). Moreover, several *in vivo* studies have shown inhibitory effect of *P. emblica* on clastogenecity of benzopyrene and cyclophosphamide (Sharma *et al*., 2000), as well as cytoprotective activities against heavy metals (Khandelwal *et al*., 2002), oxidative stress in ischemic-reperfusion injury (Rajak *et al*., 2004) and DMBA-induced genotoxicity (Banu *et al*., 2004).

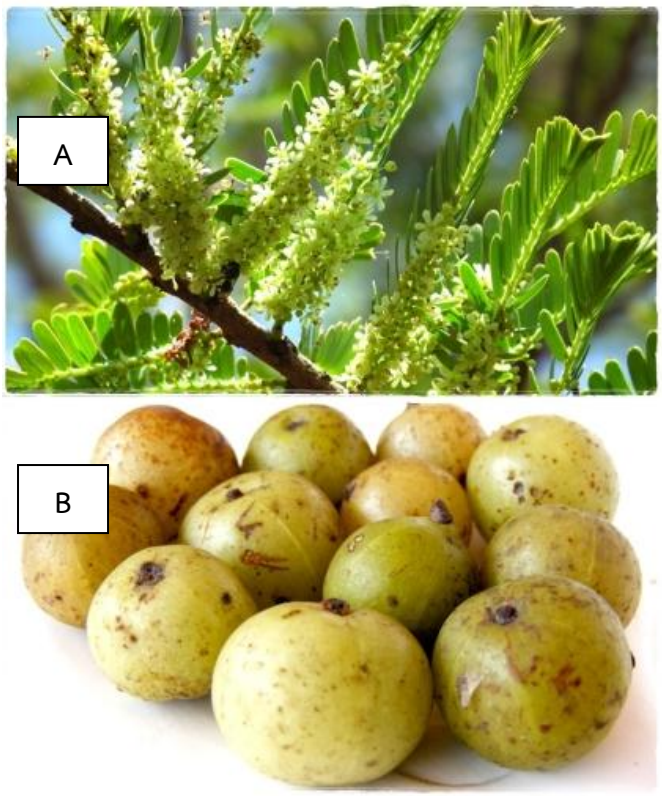


Figure 2 (A) Leaves and (B) fruits of *Phyllanthus emblica* Linn.

source: http://www.natres.psu.ac.th/FNR/vfsouthern/images/pic/img8257/Phyllanthus_emblica_Linn.jpg

The anticancer activity of *P. emblica* has been proved by several reports. Extracts of *P. emblica* fruit inhibited the proliferation of a variety of tumor cell lines *in vitro* (Zhang *et al*., 2004). Many compounds isolated from this plant were absoluted as active components. The inhibition of tumor incidences by fruit extract of this plant has been evaluated on two-stage process of skin carcinogenesis in Swiss albino mice. Chemopreventive potential of *P. emblica* fruit extract on 7,12-dimethylbenz[α]an thracene (DMBA) induced skin tumorigenesis in Swiss albino mice have been found (Sancheti *et al.,* 2004). The aqueous extract of the fruit was cytotoxic to L 929 cells and abled to reduce ascites tumor in mice induced by DLA cells. It also increased life span of tumor bearing mice and reduced tumor volume effectively (Jose *et al*., 2001). The anticarcinogenic activity of the extracts has been reported. The extracts of *P. emblica* inhibited hepatocarcinogenesis induced by *N*-nitrosodiethylaminein animals (Jeena *et al*., 1999). *P. emblica* fruits moderated the immune suppressive effects of chromium on lymphocyte proliferation and restored the production of IL-2 and interferon-γ (Ram *et al*., 2002). In addition, the aqueous fruit extract of *P. emblica* possesses a chemopreventive effect on DMBA-induced skin tumorigenesis in mice (Sancheti *et al*., 2004).

3.1.2 Terminalia bellerica Roxb.

Terminalia bellerica (Gaertn.) Roxb. (syn.: *Myrobalanus bellerica* Gaertn.) is well-known as Bahera, Beleric, astardmyrobalan, Bihara, Bahera in India and samor-phiphek in Thailand belongs to the Family Combretaceae. It is a large deciduous tree common on lower hills in Southeast Asia. The leaves are about 15 cm long, broadly elliptic, clustered towards the end of branches. Flowers are greenish yellow, in solitary, simple, axillary spikes. Shapes of fruits are globular, 1.5 to 2.5 cm in diameter, obscurely 5-angled when dry. The fruit is agloboseor ovoid, 1.3 to 1.9 cm in diameter, covered with wooly hairs with a hard thick walled light yellow putamen and surrounded by a green tissue. Fruits of *T. bellerica* are anti-inflammatory, antihelmintic, expectorant and ophthalmic. Fruits are useful in cough, asthma, bronchitis, dropsy, dyspepsia and cardiac disorders. Ripe fruits used as astringent. Fruits are also useful in eye diseases and scorpion sting. The fruits contain tannins as a major component, both condensed and hydrolysable such as gallic acid, ethyl

gallate and ellagic. Other compositions analysed in the fruit include β-sitosterol, belleric acid, chebulagic acid, glucose, glycosides and various carbohydrates (Mahato *et al*., 1992). The bark of *T. bellerica* is mildly diuretic and is useful in anaemia and leucoderma. Furthermore, *T. bellerica* has been widely used as a laxative as well as an astringent, and also as traditional medicine for several ailments such as fever, cough, diarrhea, oral thrush, inflammation, dyspepsia, skin and liver diseases. Other biological activities of the fruit extract have been reported to possess antimicrobial (Elizabeth, 2005), anti-HIV, antimalarial, antifungal (Valsaraj *et al*., 1997), antidiuretic (Kar *et al*., 2003) and antimutagenic effects (Padam *et al.*, 1996).

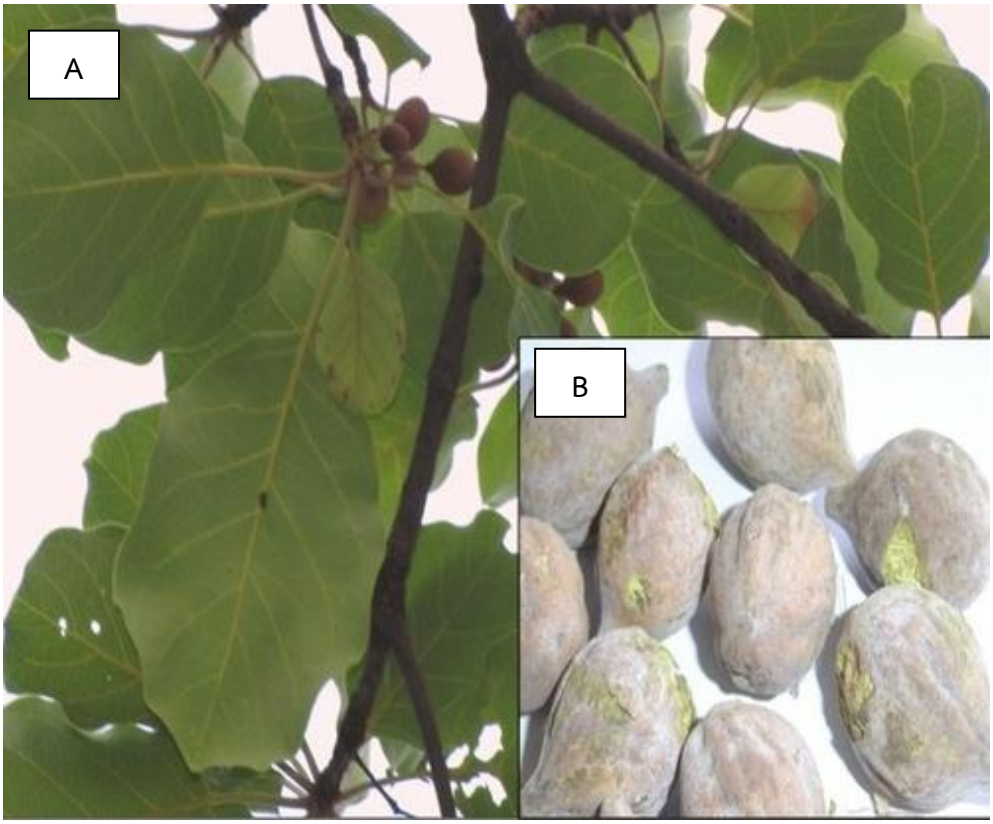


Figure 3 (A) Leaves and (B) dried fruits of *Terminalia bellerica* Roxb.

Source: http://pimg.tradeindia.com/01408611/b/4/Baheda-Medicinal-Seeds-Terminalia-belerica-.jpg

3.1.3 Terminalia chebula Retz.

Terminalia chebula Retz. (syn.: Myrobalanuschebula, Gaertner) belongs to the Family Combretaceae. *T. chebula* is normally known as black myrobalans in English, haradain Hindi, and samorthai in Thai. This plant is widely cultivated in South and Southeast Asia including Thailand. *T. chebula* is tall about 50 to 80 ft in height. It has round crown and spreading branches. The bark is dark brown with some longitudinal yawns. Leaves are ovate and elliptical, with two large glands at the top of the petiole. The flowers are monoecious with a strong unpleasant odour, borne in terminal spikes or short panicles. The fruit of *T. chebula* is about 1-2 in. It has five lines on the outer skin. Fruit is green when unripe and yellowish grey when ripe. The mature fruit is ellipsoid to oval in shape with yellowish orange brown and containing a single seed when dry the fruit becomes five-ridged. The fruits contain high phenolic content, especially hydrolysable tannins. Structures of hydrolysable tannins found in *T. chebula* fruits are gallic acid, chebulic acid, punicalagin, casuarinin, chebulanin, corilagin, neochebulinic acid, terchebulin, ellagic acid, chebulagic acid, chebulinic acid, 1,6-di-*O*-galloyl-*D*-glucose, 3,4,6-tri-*O*-galloyl-*D*-glucose and 1,2,3,4,6-penta-*O*-galloyl-*D*-glucose (Lee *et al*., 1995). *T. chebula* has been used in folk medicines as a laxative, diuretic, cardiotonic, digestive, antiseptic, and carminative (Barthakur and Arnold, 1991). Moreover, it has been reported to exhibit a variety of biological activities including antimutagenic (Kaur *et al*., 1998), antimicrobial (Sato *et al*., 1997), antiviral (Kim *et al*., 2001), antianaphylaxis (Shin *et al*., 2001), anticancer (Saleem *et al*., 2002), antioxidant and free radical scavenging activities (Cheng *et al*., 2003). It also has a potent protective effect against oxidative stress-induced hepatotoxicity (Na *et al*., 2004).

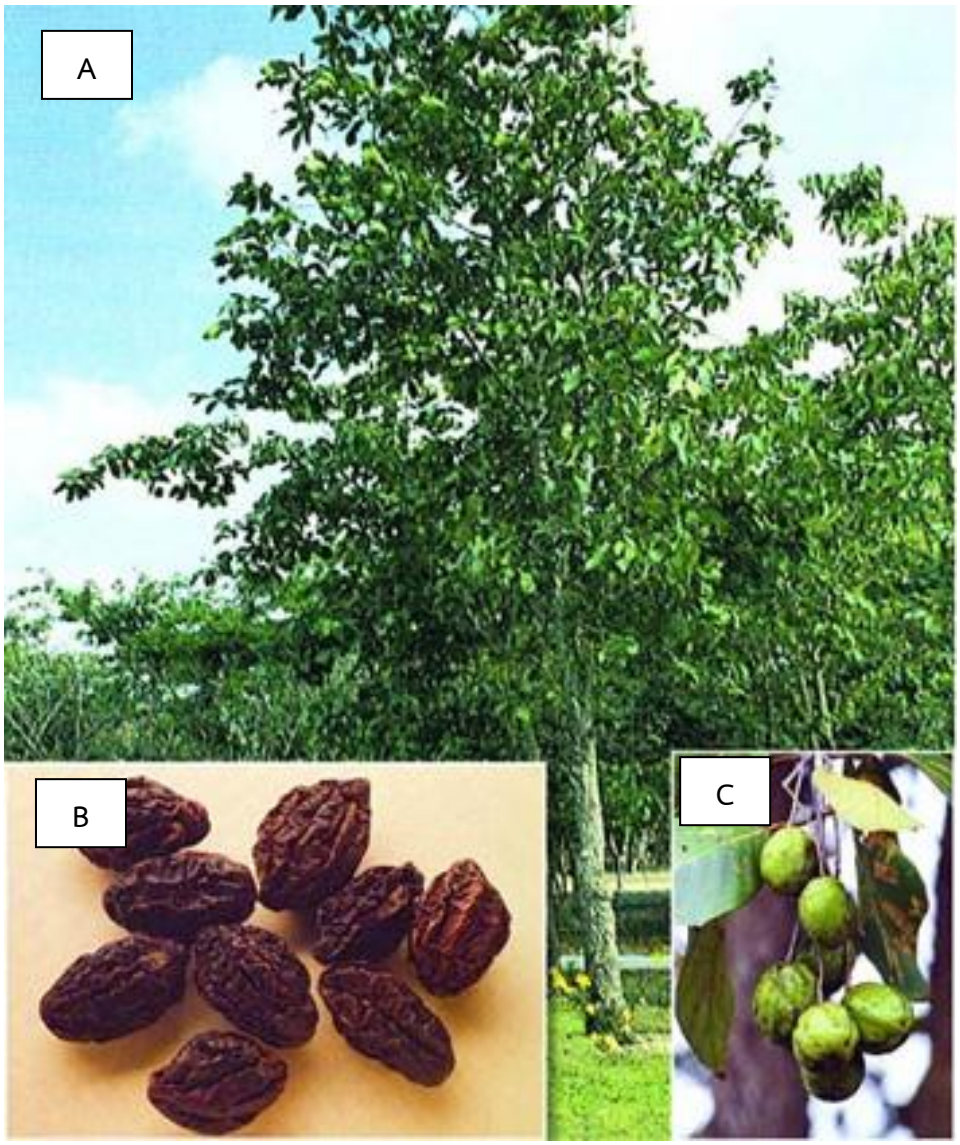


Figure 4 (A) Stem (B) dried fruits and (C) fruits of *Terminalia chebula* Retz.

Source: http://www.ebanglapedia.com/img/H_0219A.JPG

T. chebula has been found to have the cytotoxic effects against human cancer cell lines (Lee *et al*., 1995). The metanolic extract of *T. chebula* containing gallic acid, 1,2,3,4,6-penta-*O*-galloyl-*D*-glucopyranose, chebulagic acid, and chebulinic acid inhibited growth of human cancer cell lines including A-549, SKOV-3, SK-MEL-2, XF-389, and HCT-15. In addition, Cytotoxicity effects of *T. chebula* fruit extract has studied in several human cancer cell lines including breast cancer (MCF7), osteosarcoma (HOS-1) and prostatecancer (PC-3) (Saleem *et al*., 2002). The results

showed the 70% methanol extracts inhibited cell proliferation, and induced cell death in a dose dependent manner. At lower concentration (8.0-40.0 μg/ml), treatment of the metanolic extract of *T. chebula* for 72 h induced apoptotic cell death, whereas at higher concentration (>40 μg/ml) necrotic cell death was observed. The cytotoxic effect of several phenolic compounds and tannic acid in *T. chebula* was also determined by ATP level. The most potent cytotoxic compounds were chibulinic acid (IC_{50} = 53.2 μM) and tannic acid (IC_{50} = 59.0 μg/ml). The ellagic acid (IC_{50} = 78.5 μM) and 2,4-chebulyl-β-*D*-glucopyranose (IC_{50} = 120 μM) showed less cytotoxic activity as comparedto chebulinic acid. These results agree with other studies in which the phenolic compounds, especially hydrolysable tannins exhibit cytotoxic activity and induce apoptotic cell death in various cancer cell lines (Yang *et al.*, 2000). So, these phenolic compounds and their derivatives are possibly responsible for the biological activities of *T. chebula.*

3.2 Benjakul Recipe

Benjakul recipe is one Thai traditional medicine used as complementary medicine in conjunction with other herbal medicines for treatment of cancer. Benjakul recipe is composed of five plants, that is *Piper retrofractum* Vahl., *Piper sarmentosum* Roxb., *Piper interruptum* Opiz., *Plumbago indica* Linn. and *Zingiber officinale* Roscoe. It is a balance body recipe in theory of Thai Traditional Medicine and it is believed to be through balancing "Dhatu" (regulating body chemical and physical function) before cancer chemotherapy (Tappayuthpijarn *et al*., 2007). Bioassay guided fractionation of the ethanolic extract of this herbal formulation revealed active constituents, of which piperine from *P. chaba* Hunter., *P. sarmentosum* Roxb., and *P. interruptum* Opiz. were the major components (78.69%), followed by plumbagin from *P. indica* (17.05%) and 6-gingerol from *Z. officinale* (4.26%) (Itharat and Sakpakdeejaroen, 2010).

This recipe including their active constituents, particularly piperine, has been demonstrated to exhibit a wide range of pharmacological and biological activities. Cytotoxic and anticancer activities against lung, breast and prostate cancers, Benjakul formulation and piperine also possess antioxidant, anti-inflammatory, analgesic, anti-pyretic, central nervous system de-pressant, antiplatelet, antihypertensive, hepatoprotective, antithyroid and immuno-stimulating (promoting natural killer cell activity) activities as well as inhibitory activity on nitric oxide production (Sun *et al*., 2004). Acute and chronic toxicity tests in animals and humans have demonstrated the preparation to be practically non-toxic and well-tolerated (Amorndoljai *et al*., 2012). Nevertheless, there has been no information regarding its pharmacokinetics in humans.

3.2.1 Piper retrofractum Vahl.

Piper retrofractum Vahl. (syn.: *Chavicamaritima*Miquel, *Chavicaofficinarum* Miquel) belongs to the Family Piperaceae. It occurs wild in Indonesia, Malaysia, Philippines, Thailand and Vietnam. It is also found in the Ryuku Islands and Yunnan, and is cultivated in Guangdong, China and Kampuchea. It is a stout climber, with flexuous branches. Leaves are very short petioled, rather coriaceous, 12.5-18.0 cm long, oblong, ovate or lanceolate, acuminate, shining above, base round unequal, cordate. Fruiting spikes 2.5-5.0 cm long, stoutly peduncled, suberect, conico-cylindric, forming a fleshy cone of various fruits. Moreover, this plant was a drug used for tonic earth element and controlled abnormal of earth element such as muscle and tendon painful, stress and dried skin.

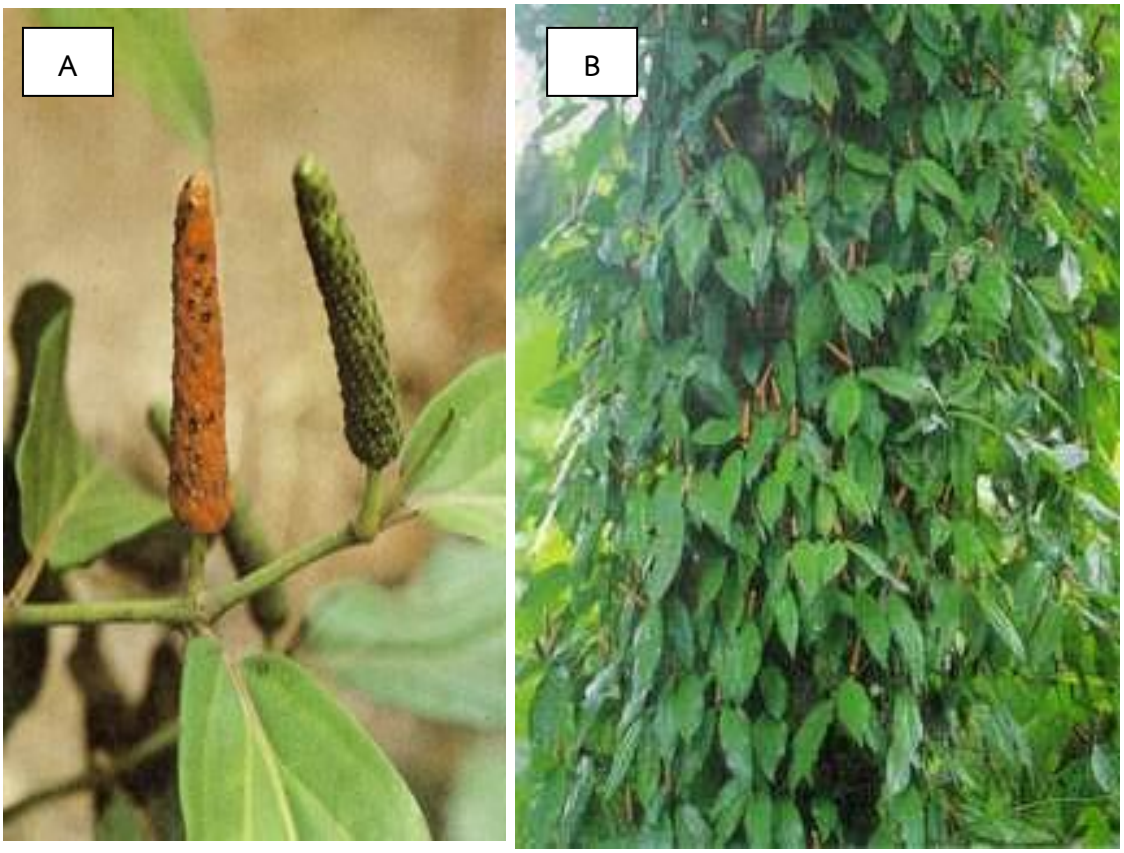


Figure 5 (A) Fruits and (B) leaves of *Piper retrofractum* Vahl.

Source: http://gernot-katzers-spice-pages.com

This plant is used to treat rheumatic pain and body pain after childbirth. Fruits are stimulant, carminative, anthelmintic and expectorant; used in cough, cold, asthma, bronchitis, fever, piles and in hemorrhoidal affections; they improve appetite and taste. The root is alexiteric; useful in asthma, bronchitis and consumption (Fariduddin *et al*., 2009). Stem bark of the plant has been reported to contain lignan and alkaloids, such as piper amine; 2,4-decadienoic acid piperidine; kusunokinin and pellitorine.Two piperidine alkaloids, piperoctadecalidine and pipereicosalidine were isolated from the fruits of *Piper retrofractum* along with known piperidine alkaloids, guineensine, piperine and pipernonaline (Ahn *et al*., 1992). The aerial parts of *P. retrofractum* was found to contain amides such as retrofractamide D, retrofractamide A [*N*-isobutyl-9(3′,4′-methylenedioxyphenyl)2*E*,4*E*,8*E*-nonatrienamide)] and retro fractamide C (Banerji *et al*., 1985). The plant also contained sesamin and 3,4,5-tri

methoxydihydrocinnamic acid as well as two higher homologues of retrofractamide A, viz. pipericide (retrofractamide B) and retrofractamide D. Studies reported seven constituents from *P. retrofractum* fruits: piperic acid, β-sitosterol and phenolic amides, guineensine, pellitorine, piperine, methyl piperate and *N*-siobutyl-2*E*,4*E*,8*Z*-eicosatrienamideguineensine (Nakatani *et* al., 1986). All the phenolic amides compounds were found to possess significant antioxidant activities that were more effective than the naturally occurring antioxidant, α-tocopherol. Naturally occurring antioxidants, therefore, may surpass food preservatives like BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) in their ability to inactivate mutagensin food. Methanolic extract from the fruit was found to have a hepatoprotective effect on *D*-galactosamine (*D*-GalN)/lipopolysaccharide (LPS) induced liver injury in mice (Zhang *et al*., 2008). The fruit also possess gastroprotective activity. From the aqueous acetone extract of the fruit the gastroprotective effects of the principal constituents, piperine, piperanine, pipernonaline, dehydropipernonaline, piperlonguminine, retrofractamide B, guineensine, *N*-isobutyl-(2*E*,4*E*)-octadecadienamide, *N*-isobutyl-(2*E*,4*E*,14*Z*)-eico satrienamide, and methyl piperate. Of these compounds significantly inhibited ethanol-induced gastric lesions at a dose of 25 mg/kg. Aqueous extract of *P. retrofractum* fruits did not produce acute or subchronictoxicity in either female or male rats at doses of 5,000 mg/kg body weight for acute toxicity and 300–1,200 for subchronic toxicity (Jaijoy *et al*., 2010). The results showed no abnormalities in treated groups as compared to the controls. Neither gross abnormalities nor histopathological changes were observed.

P. retrofractum has been found to have the cytotoxic effects against cancer. Studies demonstrated that *E*-piplartine isolated from the roots extract to be a coming candidate touse in combinatorial treatments to resist cancer (Lim, 2012). *E*-piplartine induced a dose-dependent cytotoxicity (2–24 μM) in different tumour cells: rat histiocytoma (BC-8), mouse embryonal carcinoma (PCC4), mouse macrophages (P388D1 and J774), and human neuroblastoma (IMR32) tumour cells. The treatment of piplartine with diferuloylmethane (curcumin), an antiflammatory and anticancer agent, significantly enhanced the piplartine induced cytotoxicity in tumour cells.

Seven Thai medicinal plants including *P. retrofractum* exhibited promising in-vitro cytotoxic activity against the human cholangiocarcinoma CL-6 cell line with survival of less than 50% at the concentration of 50 mg/ml (Mahavorasirikul *et al*., 2010). *P. retrofractum* had potent cytotoxic activity with mean IC_{50} value of 40.74 mg/ml. All possessed high activity against human laryngeal (Hep-2) cell with mean IC_{50} ranging from 18.93 to 32.40 mg/ml. The extract from *Piper chaba* (IC_{50} = 18.63 mg/ml, SI (selective index) = 9.8) and Pra-Sa-Prao-Yhai recipe (IC_{50} = 20.99mg/ml, SI = 12.5) exhibited the most promising and most selective cytotoxic activity against Hep-2 cell line (Jyothi *et al*., 2009).

3.2.2 Piper sarmentosum Roxb.

Piper sarmentosum Roxb. (syn.: *Piper albispicum*, *Piper pierrei*, *Piper brevicaule*) belongs to the Family Piperaceae, which Thai local name is Chaplu. This plant occurs wild in Southeast Asia, Northeast India, South China and Malaysia. It is tropical, short-lived and mostly climbing shrubs with spikes and swollen nodes. The flower is one-celled ovary with a single ovule and grows to a berry. The kernels have a capacity for abundant endosperm and perisperm. It is an earthly herb, 60 cm high, green trunk and jointed at the nodes. The leave is thin, 7-15 cm long, 5-10 cm wide, dark green color.

P. sarmentosum has been found many phytochemicals such as phenyl propanoids (ascaricin, α-ascarone) (Masuda *et al*., 1991); xanthophylls, tannis, total phenolic compounds (Chanwitheesuk *et al.*, 2005); calcium, iron, vitamin B1, 2, C, E, β-carotene (Subramaniam *et al*., 2003) and β-sitosterol (Masuda *et al*., 1991). The oxalic acid content is high which could combine with calcium in intestine to cause complex formation and block food absorption, and made calculus in bladder (Parmar *et al*., 1997).

According to the herbal medicine, it was used as expectorant, carminative, refreshing throat, enhancing appetite, flatulent and asthma reclieved (Peungvicha *et al*., 1998), muscle pain decreasing property (Ridtitid *et al*., 1998), anti-amoebiasis (Rahman *et al.*, 1999), used to reduce fever in influenza and AIDS in digestion (Rukachaisirikul *et al*., 2004), treatment of diabetes mellitus (Peungvicha *et al.*, 1998). The crude aqueous extract leaves of *P. sarmentosum* reduced blood glucose in

alloxan-induced diabetic rabbits, however, could not reduce blood glucose in normal rabbits (Rukachaisirikul *et al*., 2004).

Figure 6 (A) Leaves and (B) fruits of *Piper sarmentosum* Roxb.

Source: http://img293.imageshack.us/img293/3351/83615015.jpg

P. sarmentosum was able to decrease the intestinal tension and also inhibited the acetycholine-induced intestinal tension in isolated rat ileums (Ridtitid *et al*., 2007). The benzene soluble fraction of the methanolic leaves extract showed antimicrobial activity against *Escherichia coli* and *Bacillus subtilis* (Masuda *et al*., 1991). Ethanol and aqueous extracts of the different parts of this plant were analyzed by HPLC for marker compounds to standardize these extracts. The standardized extracts were verified for antioxidant, anti-TB activity and estimation of total phenolic and amide contents. The ethanol extracts indicated better antioxidant activity as compared to the aqueous extracts. The ethanol extract of leaves were further investigated for dose response relationship and its EC_{50} was found to be 38 μg mL^{-1}. All the extracts have exhibited anti- tuberculosis activity with MIC/MBC 12.5 μg mL^{-1}. The methanolic leaves extract were fractionated and the ethyl acetate fraction exhibited anti- tuberculosis activity with MIC/MBC 3.12 μg mL^{-1} while MIC/MBC of isoniazid (positive control) was found to be 0.5 μg mL^{-1}. This plant possesses promising antioxidant as well as anti-tuberculosis properties (Hussain *et al*., 2009).

3.2.3 Piper interruptum Opiz.

Piper interruptum Opiz. (syn.: *Piper triandrum* F. Muell. or *Piperribesoides* Wall.) belongs to the Family Piperaceae, which Thai local name is Sakan, Sakanlek and Sakanyouak. This plant is commonly found in the North and Northeast in Thailand from 200-1200 m altitude. Malaysia is endowed naturally with a very rich plant life. It widely grown throughout Malaysia and has been locally known as Sirihrimba. These Piper species have been traditionally used in Malaysia for food and medicinal purposes. In Thai herbal medicine, the stem has long been used as carminative, antiflatulent and tonic element (Pichiensunthon and Jeerawongs, 2004).

Description of *P. interruptum* is a climbers dioecious. Petiole 1-2.5 cm, glabrous, sheathed at base only. Leaves with petioles 0.4–3 cm long; lamina ovate to narrowly ovate-elliptic, 5–16 cm long, 2.2–8.5 cm wide, glabrous or with a few short hairs at base and on petiole; base cuneate or lobed, commonly asymmetric; apex acuminate; 1 or 2 pairs of secondary veins radiating from base of midvein, another pair slightly above. Spikes solitary, leaf-opposed, pendulous, 2-4 mm wide; peduncle

0.8–1.8 cm long; bracts adnate to rachis, broadened apically, margins free. Rachis hairy: male 5.2–11.5 cm long; female 7–19 cm long.

Many reports about pharmacological activity of *P. interruptum*, the ethanol extract of stem showed larvicidal effect (Chaithong *et al*., 2006). The methanol extract exhibited inhibitory activity on acetylcholinesterase enzyme and it is reported as good remedy in treatment of diabetes and poultice to reduce swellings (Ingkaninan *et al.*, 2003). *P. interruptum* also commonly used in folk medicine as a carminative (Saralamp *et al*., 1996). Moreover, this plant was a drug used for tonic wind element and controlled abnormal of wind element such as low or high blood pressure, flatulent, headache and dry mouth.

Figure 7 (A) Leaves and (B) nests of *Piper interruptum* Opiz.

Source: http://www.starten.co.th/st2012/images/article/HW_Article/Piper%20interruptum%20Opiz.jpg

3.2.4 Plumbago indica Linn.

Plumbago indica Linn. (syn.: *Plumbago rosea* Linn.) belongs to the Family Plumbaginaceae, which Thai local name are Chettamunphloengdaeng (central), Pitpiudaeng (northern), Faitaidin (peninsular). Its common names in various countries are Rosy-flowered leadwort, Officinal leadwort (English); Mehulatu, Akarbinasa (Indonesia); Setaka, Cherakamerah (Malaysia) and Pampasapit, Laurel (Philippines).

Description of *P. indica* is a shrub up to 1.5 m tall, stems dropping, branched from the base, sometimes rooting of the nodes; leaves oblong, 5-15 cm × 2-8 cm, not corymbose, rachis glabrous, 10-30 cm long; flowers are bright red, 3-5 cm long, forming very long terminal and axillary slender, lax spikes, reaching up to 60 cm. Calyx red, short, cylindric, along the ribs covered with stipitate glands, with a tubular corolla with five petal-like lobes, lobes 2-3 cm in diameter.

In Thai traditional medicine, the roots of this plants are the main source of plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) which is commercially important for its broad range of pharmacological activities. Studies on the pharmacological activities of plumbagin have shown the presence of antitumor (Hazra *et al*., 2002), antimicrobial (Didry *et al*., 1998), anticancer (Kuo *et al*., 2006), antifertility (Satyavati, 1984), antileishmanial (Chan-Bacab and Peña-Rodríguez, 2001) and antiallergic (Tsuboi *et al*., 2004) properties. Therefore, *P. indica* has increased market demand in both domestic and international level. The slow growth rate absence of seeds and lack of fruiting stage of the *P. indica* in traditional agricultural methods necessitate the search for an alternative and effective source to meet with enhanced commercial demand (Gangopadhyay *et al.*, 2011). Hydroponic culture technique can be used to produce medicinal plants in large scale. This plant was a drug used for tonic fire element and controlled abnormal of fire element such as dried cough, lower body temperature, beriberi and anorexia (Gogtay *et al*., 2002).

Figure 8 (A) Flowers and (B) leaves of *Plumbago indica* Linn.

Source: http://www.magnoliathailand.com/webboard/index.php?action=dlattach;topic=1073.0;attach=43256;image

3.2.5 Zingiber officinale Roscoe.

Zingiber officinale Roscoe. (syn.: *Amomum zingiber* L.*, Zingiber missionis* Wall.) belongs to the Family Zingiberaceae. It is common names in various countries are King (Thailand); Luya (Philippines); Jahe, Jae, Lia (Indonesia); Gingembre (Franch); Jahi, Atuja (Malaysia) and Ginger (English). Ginger cultivation began in South Asia and has since spread to East Africa and the Caribbean. In Thai herbal medicine, this plant is used as carminative, expectorant, antiemetic, diaphoretic and antispasmodic. Traditionally, the rhizome is gathered when the stalk withers; it is immediately scalded, or washed and scraped, to kill and prevent sprouting. The fragrant perisperm of Zingiberaceae is used as sweetmeats by Bantu, also as a condiment and sialogogue. Volatile oil from ginger contained borneol, menthol, fenchone, 6-gingerol were increased bile secretion and supported fat digestion (Singh *et al*., 2008). Some studies showed ginger may provide short-term relief of pregnancy-related nausea and vomiting. Studies are inconclusive about effects for other forms of nausea or in treating pain from rheumatoid arthritis, osteoarthritis, or joint and muscle injury. Side effects, mostly associated with powdered ginger, are gas, bloating, heartburn, and nausea (Zick *et al*., 2008)

Botanical illustration of *Z. officinale* (ginger) is a perennial herb with a subterranean, rhizome has a brown corky outer layer (usually removed before use) and a pale yellow centre with a spicy lemon-like scent. The flowering heads, borne on separate shorter stems, are cone-shaped spikes and composed of a series of greenish to yellowish leaf-like bracts. Protruding just beyond the outer edge of the bracts, the flowers are pale yellow in color with a purplish lip that has yellowish dots and striations. Flowering stems are rarely, if ever, produced in cultivated plants. Shoots (pseudostems), up to 1.2 m tall, arise annually from buds on the rhizome. These pseudostems are formed from a series of leaf bases (sheaths) wrapped tightly around one another with the long (up to 7 cm), narrow (up to 1.9 cm wide), mid-green leaf blades arranged alternately.

Rhizome of ginger arises in horizontal, irregularly branching pieces; 3-16 cm long, 3-4 cm wide, up to 2 cm thick; pale yellowish buff or light brown externally, somewhat fibrous; branches known as “finger” occur obliquely from rhizomes, yellowish brown, showing a yellow endodermis separating the narrow cortex from the wide stele, vascular bundles, scattered on the whole surface.

The aromatic rhizome of *Z. officinale* is the source of ginger, a spice used for centuries to add flavour in cooking. In Asia the fresh stem is an essential ingredient of many dishes, whereas the dried, powdered spice is more popular in European cooking. Gingerbread, one of the most popular uses for ginger in Britain, dates to Anglo-Saxon times when preserved ginger (produced by boiling the rhizome in sugar syrup) was used, often medicinally. The characteristic odor and flavor of ginger is caused by a mixture of zingerone, shogaols and gingerols, volatile oils that compose one to three percent of the weight of fresh ginger. In laboratory animals, the gingerols increase the motility of the gastrointestinal tract and have analgesic, sedative, antipyretic and antibacterial properties (Sudarshan *et al.*, 2010).

Ginger has many medicinal used. The fresh or dried rhizome is used in oral or topical preparations to treat a variety of ailments, while the essential oil is applied topically as an analgesic. Evidence suggests ginger is most effective against nausea and vomiting associated with surgery, vertigo, travel sickness and morning sickness. However, safe use of ginger during pregnancy is questionable and pregnant women should exercise caution before taking it. The topical use of ginger may cause allergic reactions. It was classified as a stimulant and carminative and used frequently for dyspepsia, gastroparesis, slow motility symptoms, constipation, and colic (Sudarshan *et al.*, 2010). It was a drug used for tonic air element and controlled abnormal of air element such as blur and tinnitus.

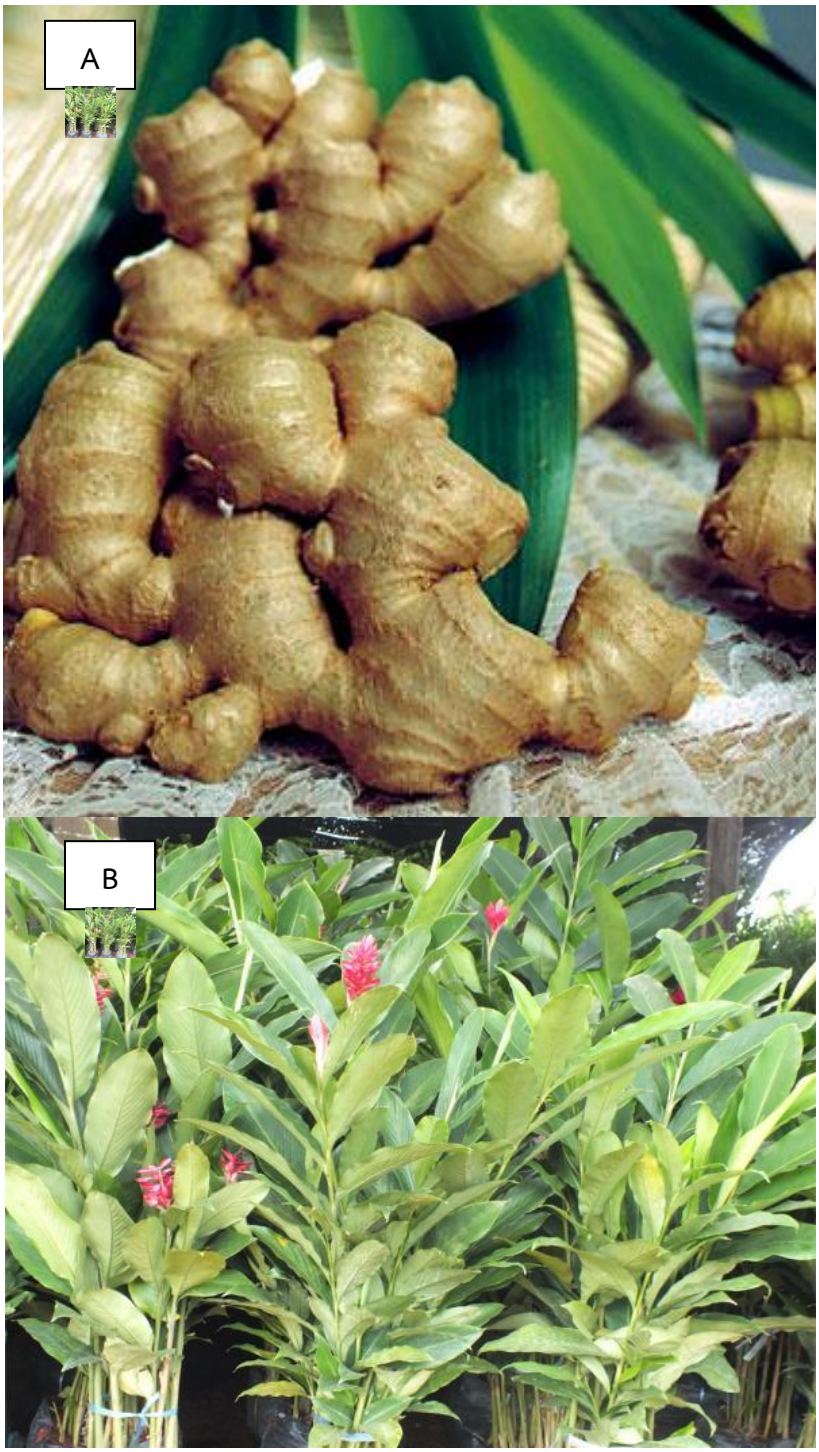


Figure 9 (A) Rhizomes and (B) leaves of *Zingiber officinale* Roscoe.

Source: http://thaiherb.info/wp-content/uploads/2013/08/183119.jpg

3.3 Benjalokwichian Recipe

Benjalokawichian or Ya-Ha-Rak recipe is the drug list in herbal medicinal products A.D. 2011 of Thailand and used as antipyretic drug and treat rash in Thai Traditional Medicine. This recipe consist of five herbal roots, *Ficus racemosa* Linn., *Capparis micracantha* DC., *Clerodendrum petasites* S. Moore, *Harrisonia perforata* Merr. and *Tiliacora triandra.* It is one of Thai traditional medicine used as treat fever, rash and acne. In addition, each plant in this recipe has been found to have anti-inflammatory, anti-allergic and antioxidant (Juckmeta and Itharat, 2012). The extracted material of the medicinal formula Benjalokawichian and each plant component contained phenolic content with good anti-IgE effect. Studies indicated no irritation or allergic reaction to human skin, so this maybe highest safety for drug preparation and other products of external skin treatment (Suwannarat *et al.*, 2013). Its plants extracts were tested for antimicrobial activities by disc diffusion and broth dilution methods. The results showed that four medicinal plants in Benjalokawichian recipe could inhibit the growth of *Propionibacterium acnes*. Among those, *F. racemosa*, *C. petasites*, *H. perforata*, *T. triandra* and Benjalokawichien inhibited the growth of *P. acnes*. *H. perforate* and *T. triandra* exhibited strong inhibitory effects on the acne formation based on the broth dilution method. The MIC values were same, 5 µg/ml for *P. acnes*. *H. perforata* had the greatest inhibitory effect on microbial growth with MIC values (Suwannarat *et al.*, 2013). Ethanolic extract of this recipe contained phenolic content with good anti-IgE effect. The tests showed no irritation nor allergic reaction to human skin, so this maybe highest safety and suitable for drug preparation and other products of external skin treatment (Suwannarat *et al.*, 2013).

3.3.1 Ficus racemosa Linn.

Ficus racemosa Linn. (syn.: *Ficus glomerata* Roxb.) belongs to the Family Moraceae, which Thai local name is Ma duer chumphon. Popularly known as the cluster fig tree, indian fig tree or goolar (gular) fig, this is native to Australia, Malaysia, South-East Asia and the Indian Subcontinent. This plants used in traditional system of medicine for the treatment of several disorders. Various plant parts are used as astringent, carminative, vermifuge and anti-dysentery. The extract of fruit is used in

diabetes, leucoderma and menorrhagia. It is used locally to reduce inflammation of skin wounds, lymphadenitis, in sprains and fibrositis.

Ficus racemosa Linn. can grow over 40 ft tall and 20 to 40 ft wide. Tree is moderate sized deciduous. Leaves are ovate, rich green, 7.5-10 cm long, good shade, glabrous; receptacles little subglobose or piriform, in big clusters from aged nodes of main trunk. Fruits are 2-5 cm in diameter, inclusters, pyriform, arising from trunk or large branches. Fruits look alike the figs and are green when raw, turning orange, and dark crimson on ripening. Fruit of this plant is 3/4 in to 2 in long, circular and grows directly on the stalk. Seeds are small, innumerable and grain-like. Outer surface of the bark consists of simply removable translucent flakes grayish to rusty brown, uniformly hard and non-brittle. Bark is reddish gray or grayish green, soft surface, uneven and cracked, 0.5-1.8 cm thick, inner surface bright brown, fracture fibrous, taste mucilaginous without any characteristic odour. Roots of this plant are long, brownish in colour and inaccurate in shape.

Leaves of this plant contain sterols, triterpenoids (Lanosterol) and alkaloids, tannins and flavonoids. Stem-bark gives gluanol acetate, **β**-sitosterol (Joy *et al*., 2001), cerylbehenate, lupeol acetate and **α**-amyrinacetate (Paarakh, 2009). From bark, lupenol, **β**-sistosteroland stigmasterol were isolated (Chopra *et al*., 1958). Fruit contains gluanol acetate, glucose, tiglic acid (Chopra *et al*., 1958), esters of taraxa sterol, lupeol acetate, friedelin (Babu *et al.*, 2010), higher hydrocarbons and other phytosterols.

The decoction of the bark of *F. racemosa* was found as an antidiuretic and its potential is evaluated in rats using three doses (250, 500 or 1000 mg/kg). It had a rapid onset (within 1 h), peaked at 3 h and lasted throughout the study period (5 h). (Ratnasooriya *et al*., 2003). Methanol extract of bark was tested for it antitussive potential against a cough induced model by sulfur dioxide gas in mice. Studies showed maximum inhibition of 56.9% at a dose of 200 mg/kg 90 min after administration (Bhaskara *et al*., 2003). Ethanol extract of stem bark showed wound healing in excised and incised wound model in rats (Biswas and Mukherjee, 2003). Methanol extract of powered fruits at the dose 1, 2, 3, and 4 g/kg reduced the blood glucose level in normal and alloxan induced diabetic rabbits. In alloxan diabetic

rabbits the treatment with 2, 3 and 4 g/kg body weight of the plant drug produced a significant fall in blood glucose levels. (Akhtar and Qureshi, 1988).

Figure 10 Fruits of *Ficus racemosa* Linn.

Source:http://www.adsthailand.com/bedo/wpcontent/uploads/2013/07/7be7be9ah7eiiched5dck642x335.jpg

3.3.2 Capparis micracantha DC.

Capparis micracantha DC. (syn.: *Capparis micracantha* Caib., *Capparis odorata* Blanco) belongs to the Family Capparaceae, it is a shrub tree. This plant is known as Chingchee in Thai. A book of Thai Herbs mentioned that the flowers of *C. micracantha* can inhibit cancer (Khantikaew and Sakulkhaemaruethai, 2007). This plant is a half-erect shrub or small tree with drooping branches. It is measure 1-6 m tall and rarely with a vine of measuring 2-4 m tall. It is a smooth with drooping branches or a vine growing to 2 to 4 meters, with short, sharp and nearly straight stipular thorns. Leaves are oblong to oblong-elliptic, 8 to 17 cm long, leathery, shiny, with a blunt apex and rounded base. Flowers are in vertical line aloing the branches above the leaf axils, shortly pedicelled, 2 to 6 in a series, the uppermost one of each series opening first. Sepals are pale green. Petals are oblong or elliptic, 1 cm long. Fruit is ovoid or rounded, 4 to 5 cm long, smooth and bright red when ripe. Rind is thin, somewhat stony. Within the rind is a whitish and transparent sweet pulp with a fairly good flavor. Numerous seeds are embedded in the pulp.

Study of hexane extract of the leaves of *C. micracantha* showed lung cancer inhibition. The hexane and dichlormethane extracts of flowers showed anti-tuberculosis activity (Khantikaew and Sakulkhaemaruethai, 2007). Dichloromethane and methanol extract of the leaves were inactive on ant-icancer and anti-tuberculosis assays. The three extracts of the leave were tested cytotoxicity against Vero cells. The result showed that the three extracts were non-cytotoxic (Khantikaew and Sakulkhaemaruethai, 2007).

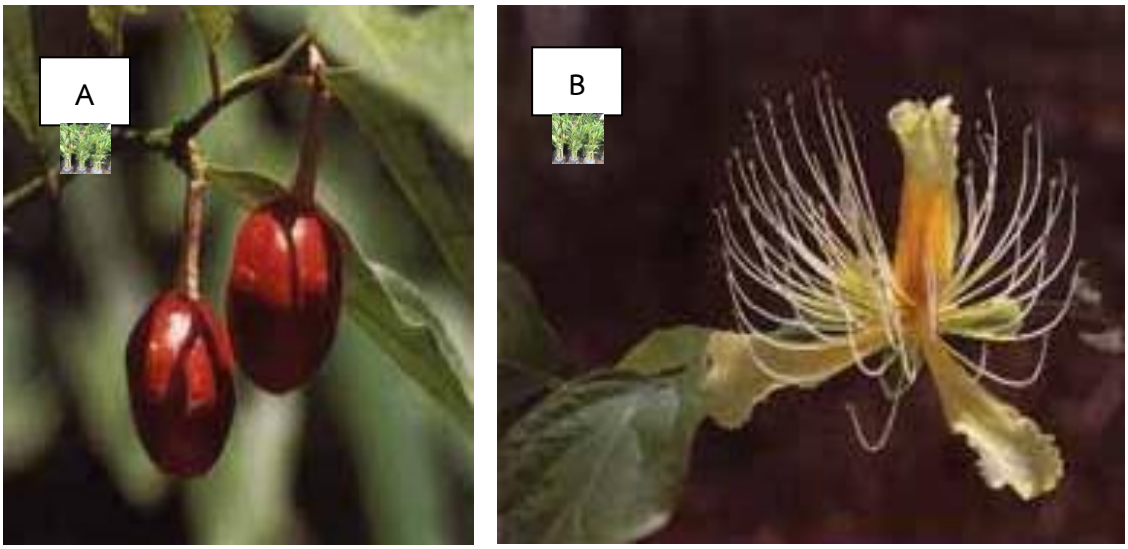


Figure 11 (A) Fruits and (B) flower of *Capparis micracantha* DC.

Source: http://www.ronghosp.org/hosmain/samonpai-thai/herbal/herbimg/g8_14.jpg

3.3.3 Clerodendrum petasites S. Moore.

Clerodendrum petasites S. Moore. belongs to the Family Verbenaceae. This plant is known as Thao Yai Mom in Thai. It is widely grown in India, Malaysia and Thailand. In Thai folklore medicine, its leaf and root are traditionally used for the treatment of fever, inflammation and skin diseases as well as asthma (Tiangburanatham, 1996). In India, fruits of *C. petasites* is used to produce sterility (Lal and Lata, 1980), whereas Chinese use it (part not specified) for the treatment of fever and malaria (Pie, 1985).

Many flavonoid compounds from *C. petasites* which isolated from the stem and root of the plant showed antibacterial activity (Panthong *et al*., 2003). In addition, the active principle responsible for this effect was isolated and identified as the flavonoid hispidulin (Hazekamp *et al*., 2001). The methanol extract from this plant was assessed for anti-inflammatory and antipyretic activities on the experimental animal models. It was found that the extract possessed moderate inhibitory activity on acute phase of inflammation in a dose-related manner as seen in ethyl phenylpropiolate-induced ear edema (ED_{50} = 2.34 mg/ear) as well as carrageenin-induced hind paw edema (ED_{30} = 420.41 mg/kg) in rats (Panthong *et al*., 2003).

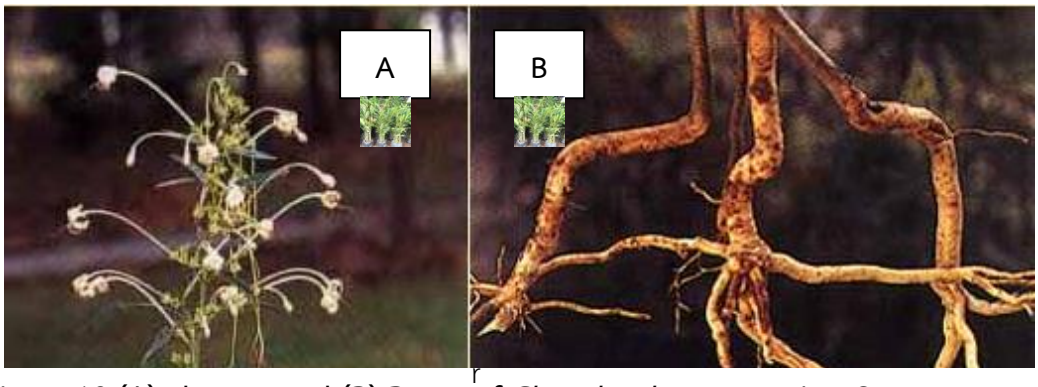


Figure 12 (A) Flowers and (B) Roots of *Clerodendrum petasites* S. Moore.

Source: http://www.biogang.net/upload_img/biodiversity/biodiversity-162783-5.jpg

3.3.4 Harrisonia perforata Merr.

Harrisonia perforata Merr. (syn.: *Harrisonia paucijuga* Olivo. or *Harrisonia bennettii* Benn.) belongs to the Family Simaroubaceae. This plant is known as Khon thaa (central) or Naam chee (Northern) in Thai. It is common throughout Southeast Asia (Vietnam, Philippines and China). It is a climbing to erect prickly shrub up to 4-6 m tall. The stipulate thorns are slightly curved backward or downward, increasing in size to 7 mm. Its leaflets are rhomboid to ovate-lance-shaped, 10-20 mm x 5-15 mm, nearly entire to lobed with narrowly winged rachis. Flowers are with a pedicel, small sepal, triangular lobes, petals are lance-shaped, 6-9 mm x 2-4 mm which are red outside and pale red to white inside. Stamens are (8-)10 with anthers 1.5-4.5 mm long, filaments are 7-10 mm long, at the base with an elongated flattened strap-shaped structure which is densely woolly at the margin, disk is cup-shaped, ovary is slightly lobed, styles 5-8 mm long and pubescent. The fruit is a berry, 4-9 mm x 11-15 mm, exocarp of leathery texture, at least 1 mm thick, endocarp hard, without suture.

A novel compound, 2-hydroxymethyl-3methylalloptaeroxylin and 8 known compounds, heteropeucenin-7methylether, perforatic acid, lupeol, unsubstituted coumarin, 5-hydroxy-6,7-dimethoxycoumarin, saturated long chain aliphatic alcohol, a mixture of steroids (β-sitosterol, campesterol and stigmasterol) and a mixture of steroidal glycosides (β-sitosteryl-3-*O*-glucopyranoside, chloresteryl-3-*O*-glucopyranoside, stigma steryl-3-*O*-glucopyranoside) were isolated from the roots of *Harrisonia perforata* Merr. (Byrne *et al*., 1991). The water extract of *H. perforata* Merr. was studied for acute and subchronic toxicities. The extract at a single dose of 5,000 mg/kg was administered orally to female and male rats. After 14 days, signs and behavioral changes, mortality, gross and histopathological changes of internal organs were examined. The body weight of the male treated rats was significantly decreased when compared to the control group. The extract did not produce signs of toxicity (Sireeratawong *et al*., 2009). Studies indicated that *H. perforata* possessed anti-inflammatory action; the maximum inhibitory effects of *H. perforata* extract (50 µg/ml) were 49.83% and 47.27% for TNF-α and IL-1b respectively. For IL-6, the mamximum inhibitory effect (32.16%) was shown at concentration 12.5 µg/ml. *H.*

perforata extract 50 µg/ml also effectively decreased mRNA expression of important inflammatory enzyme iNOS and COX-2 level up to 88.11% and 93.68% respectively. There was no cytotoxicity at the highest concentration used in this study (Somsil *et al*., 2012).

Figure 13 (A) Flowers and (B) leaves of *Harrisonia perforata* Merr.

Source: http://www.greenerald.com

3.3.5 Tiliacora triandra

Tiliacora triandra (syn.: *Limacia triandra* Miers.) belongs to the Family Menispermaceae. This plant is known as Yanang or Bai yanang in Thai. It is a species of flowering plant native to mainland Southeast Asia and used especially in the meal of Northeast Thailand and Laos. It is a climbing plant with deep green leaves and yellowish flowers, tolerating only very mild frost. In traditional Southeast Asian medicine, it has been used as herbal medicine for fever relief, anti-inflammation, antioxidant, and anti-bacterial. Its leaves contain polyphenols, flavonoids, alkaloids, ***β***-carotene and minerals such as calcium and iron. *T. triandra* juice can be extracted with water by using both hands rubbing on leaves back and forth until all the Chlorophyll in the leaves are out in the water to produce detox drink. It is very refreshing, and has a cool property, believing that could heal so many kinds of illness.

Tiliacora triandra is a climbing plant with deep green leaves and yellowish flowers. It has large roots and green vines and the vines are hairy when young. The leaves are simple, alternate, ovate, rounded at the base and acute at the end. The plant develops clusters of flowers at the nodes near petioles and later small green fruits are developed. The fruits become red and black when ripe. Juice extracted from mature leaves is used as an ingredient of many Thai and Laos recipes including bamboo curry, bamboo soup, cassia curry. Its juice can reduce bitterness of bamboo and cassia. In the Northeast, Yanang juice is also an ingredient for making jackfruit soup, Kaeg Om and araceae curry. Functions of this plant as soluble fiber and helps digestive system. Yanang can reduce heat from fever and eliminate car sick, boat sick, getting drunk and malnutrition symptoms. It also reduces the risk for heart disease and blood sugar (Passakorn, 2008 : online).

These alkaloids, tiliacorinine, tiliacorine , nortiliacorinine and tiliacorinine 2'-*N*-oxide have been isolated from the roots of *T. triandra* (Wiriyachitra and Phuriyakorn, 1981). A single oral administration of the water extract from *T. triandra* at a dose of 5,000 mg/kg body weight (5 males and 5 females) did not make signs of toxicity, behavioral changes, mortality, changes on gross shape or histopathological changes of internal organs. The subchronic toxicity was determined by oral feeding both male

and female rats daily with the test substance at the doses of 300, 600 and 1,200 mg/kg body weight continuously for 90 days. The examinations of signs, animal behavior and health monitoring showed no abnormalities in the test groups as compared to the controls. The test and control groups (on the 90th day) and the satellite group (on the 118th day) were analyzed by measuring their final body and organ weights, taking necropsy, and examining hematology, blood clinical chemistry and histopathology. The results show that the water extract from the *T. triandra* does not cause acute or subchronic toxicities in either male or female rats (Seewaboon *et al*., 2008). Phytochemical screening of the methanol extract from *T. triandra* showed the presence of alkaloid, flavonoid, tannin and saponins. Flavonoid content was found to be 18.67 ± 0.28 mg quercetin equivalent/g of extract (Rattana *et al*., 2010).

Figure 14 Leaves and fruits of *Tiliacora triandra*

Source: http://www.watbangwaek.com/img_files/Image/AAX%2077.jpg

CHAPTER III

EXPERIMENTAL

1. Plant Materials

Thirteen species of plants from three medicinal plant recipes

Plants in this study	Family	Part used
1. *Phyllanthus emblica*	Phyllanthaceae	fruits
2. *Terminalia chebula*	Combretaceae	fruits
3. *Terminalia bellerica*	Combretaceae	fruits
4. *Piper retrofractum*	Piperaceae	flowers
5. *Piper sarmentosum*	Piperaceae	leaves
6. *Piper interruptum*	Piperaceae	nests
7. *Plumbago indica*	Plumbaginaceae	roots
8. *Zingiber officinale*	Zingiberaceae	rhizomes
9. *Ficus racemosa*	Moraceae	roots
10. *Capparis micracantha*	Capparidaceae	roots
11. *Clerodendrum petasites*	Lamiaceae	roots
12. *Harrisonia perforata*	Simaroubaceae	roots
13. *Tiliacora triandra*	Menispermaceae	nests

All of plant materials were authenticated by botanist and the roots of *P. indica* were authenticated by identification with the voucher specimen no. BKF 185545, deposited in the herbarium of the Royal Forest Department, Ministry of Agriculture and Co-operatives of Thailand.

2. General Techniques

2.1 Analytical Thin Layer Chromatography (TLC)

Technique : One dimension, ascending

Absorbent : Silica gel

Layer thickness : 0.2 mm

Developing distance : 3.5 cm

Temperature : Laboratory room temperature (30-35 ^{o}C)

Detection : 1. Ultraviolet light at wavelength of 365 and 254 nm

: 2. H_2SO_4 in aq. ethanol solution (contains 10% H_2SO_4, 45% ethanol and 45% water) and heat at about 100 ^{o}C for a few minutes.

2.2 Column Chromatography (CC)

2.2.1 Conventional Column Chromatography

Absorbent : 1. Silica gel 60 (CAS number 7631-86-9) (Merck) Particle Size 0.063-0.200 mm (70-230 mesh ASTM)

2. Silica gel 60 (CAS number 7631-86-9) (Merck) Particle Size 5-40 μm

Packing method : Wet packing method

Sample loading : The sample was dissolved in small amount of eluent, then applied gently on top of the column.

Detection : Fractions were examined using TLC technique. In order to detect the compounds, the TLC plate was observed under UV light at wavelength of 365 and 254 nm and then exposed to H_2SO_4 in aq. ethanol solution.

2.3 Spectroscopic Techniques

2.3.1 Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H spectra were recorded at 300 MHz on a Bruker Model AC-F200 spectrometer in $CDCl_3$ with tetramethylsilane (TMS) as internal standard. The ^{13}C spectra were recorded at 100 MHz on JEOL JNM-A500 spectrometer in $CDCl_3$ with TMS as internal standard at the Scientific and Technological Research Equipment, Chulalongkorn University.

2.3.2 Ultraviolet–visible (UV)

UV spectra were recorded on BIO-TEK synergy HT spectrometer in methanol at the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University.

2.4 Physical Property Measurement Apparatus

2.4.1 Melting Points

Melting points were determined on a Stuart® Melting Point SMP11 at the Department of Chemistry, Faculty of Science, Chulalongkorn University.

2.5 Solvents

2.5.1 Chemical reagent for preparation and dissolving of crude extracts

Chemicals and Reagents	CAS No.	Company
n-Heptane 99%, AR	142-82-5	RCI Labscan
n-Hexane 99%, AR	110-54-3	RCI Labscan
Ethyl Acetate, AR	141-78-6	RCI Labscan
Dichloromethane, AR	75-09-2	RCI Labscan
Methanol, AR	67-56-1	RCI Labscan
Ethanol (Absolute), AR	64-17-5	RCI Labscan
Chloroform (1% EtOH), AR	67-66-3	RCI Labscan

For all commercial grades organic solvents were redistilled prior to use.

2.6 Chemical Test for Detection

H_2SO_4 in aq. ethanol solution

The reagent consists of 10 ml of H_2SO_4, 45 ml of ethanol and 45 ml of water. Reagent was sprayed to TLC plates and heat at about 100 ^oC for a few minutes for detection of the spots of compounds.

3. Cytotoxicity Assay

3.1 Materials

3.1.1 Cell Lines

There are 7 cell lines in this experiment. The 5 out of 7 cell lines; Human colon cancer cells (SW620), Human hepatocellular liver carcinoma cells (Hep-G2), Human lung cancer cells (Chago), Human gastric carcinoma cells (KATO-III), Human breast cancer cells (BT474) are cancer cells. The 2 out of 7 cell lines; Human normal lung fibroblast (Wi-38) and Human normal skin fibroblast (CCD-986Sk) are normal cells.

3.1.1.1 Human Colon Cancer Cell Line (SW620)

Human colon cell lines (SW620 ATCC No. CCL-227) were isolated from lymph node of a Caucasian male 51 years old patient with a Dukes' type C of colorectal adenocarcinoma tumor.

3.1.1.2 Human Hepatocellular Liver Carcinoma Cells (Hep-G2)

Human hepatocellular liver carcinoma cells (Hep-G2 ATCC No. HB8065) were derived from the liver tissue of a 15-year-old Caucasian American male with a well-differentiated hepatocellular carcinoma. These cells are epithelial in morphology, have a modal chromosome number of 55, and are not tumorigenic in nude mice.

3.1.1.3 Human lung cancer cells (Chago)

Human lung undifferentiated carcinoma (Chago).

3.1.1.4 Human gastric carcinoma cells (KATO-III)

Human gastric carcinoma cells (KATO-III ATCC No. HTB103) were derived from metastatic pleural effusion, supraclavicular and axillary lymph nodes and Douglas cul-de-sac, of a 55-year-old Asian male.

3.1.1.5 Human breast cancer cells (BT474)

Human breast cancer cells (BT474 ATCC No. HTB20) were derived from the adherent, patchy (The cells form adherent patches of epithelial-like cells The patches are compact multilayered colonies that rarely become confluent) of a 60-year-old Caucasian female. These cells are epithelial in morphology.

3.1.1.6 Human Normal lung fibroblast (Wi-38)

Human normal lung fibroblasts (Wi-38 ATCC No. CCL-75) were derived from the adherent of a 3-month-old Caucasian female. These cells are epithelial in morphology. This cell line was a SV40-tranformed variant of the Wi-38 cell line.

3.1.1.7 Human normal skin fibroblast (CCD-986Sk)

Human normal skin fibroblasts (CCD-986Sk ATCC No.CRL-1947) were derived from skin taken from normal breast tissue removed during breast reduction mammoplasty. The cells have been observed to senesce at about 36 population doublings beyond the biopsy material.

3.1.2 Culture Media

Chemicals and Reagents	**Company**
RPMI 1640 medium	Biochrom
Fetal bovine serum	Biochrom
Trypsinase	Gibco
Sterile water	

3.1.3 Chemical Reagents for Cell Viability Evaluation by MTT Test

Chemicals and Reagents	CAS No.	Company
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT)	298-93-1	Sigma-aldrich
Glycine	4079-10-1	Carlo Erba
Normal saline		GPO, Thailand
Dimethyl sulfoxide (DMSO)	67-68-5	Merck

3.1.4 Chemical reagents for identical chemical structure

Chemicals and Reagents	CAS No.	Company
Chloroform-d 99.8 atom %D	865-49-6	Sigma-aldrich

3.1.5 Equipmenst and Instruments used in this study

Name	Company	Model
Air pump	Emerson	#T55MWCCE-1208
Autoclave	Consolidated	
Biohazard laminar air flow	Faster	90BS
Heamacytometer	Bright-Line	#1110000
Hot-air oven	Contherm	
Microplate reader	Tecan	
Microtiter plate 96 wells	Nunc	#3455
Inverted microscope	Olympus	
Rotary evaporator	Eyela	
Vortex mixer	Scientific Industries	G560
Water bath	Memmert	
Water jacketed CO_2 incubator	Thermo Forma	

3.2 Methods

3.2.1 Cell Count and Dilution

In each experiment, cells were counted on hemocytometer under inverted microscope. To prepare the hemacytometer, the mirror-like polished surface is carefully cleaned with lens paper and ethanol. The coverslips (which is thicker than those for conventional microscopy) should also be cleaned. The coverslip is placed over the counting surface prior to add the cell suspension. The 0.4% trypan blue dye solution and hemacytometer were applied to determine the viable cell number. Trypan blue will only enter across the membranes of non-viable cells. The cell suspensions were diluted with 1:10 culture medium and mixed 20 μl of cells with 20 μl of 0.4% trypan blue suspension, by Pasture pipette and allowed to stand for 10 minutes. Ten microliters of stained cells were placed in a hemocytometer and counted for the number of viable (unstained). The viable cells were counted in the 1 mm middle square and 1 mm four corner squares of the hemacytometer.

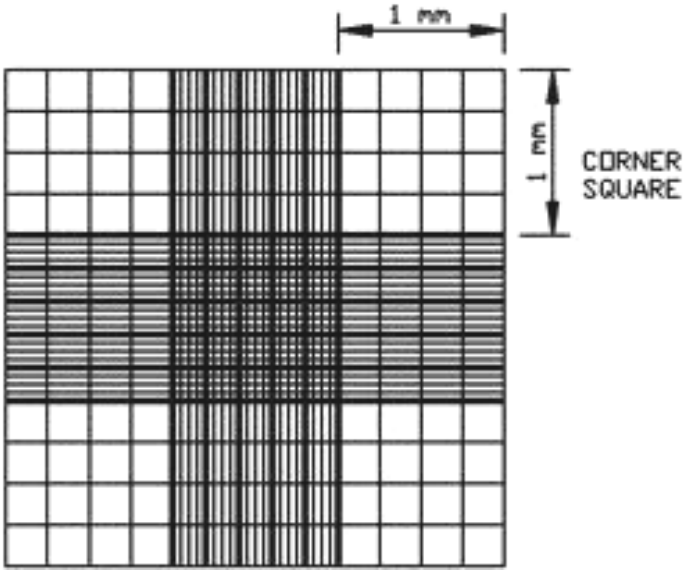


Figure 15 Magnified view of the cell counting chamber grid

From Figure 15, the central 1 mm^2 area is divided into 25 smaller squares, each 1/25 mm^2. There are enclosed by triple rule line and are further subdivided into 16 squares, each 1/400 mm^2.

Cell suspensions should be dilute enough so that the cells do not overlap each other on the grid, and should be uniformly distributed as it is assumed that the total volume in the chamber represents a random sample. This will not be a valid assumption unless the suspension consists of individual well separated cells. Cell clumps will distribute in the same way as single cells and can distort the result. Unless 90% or more of the cells are free from contact with other cells, the count should be repeated with a new sample. Also, the sample will not be representative if the cells are allowed to settle before a sample is taken. Mix the cell suspension thoroughly before taking a sample to count.

The cells in each square of the hemacytometer were equivalent to approximately 1 mm, represent a total volume of 0.1 mm^3 and the subsequent cell density per ml was calculated using the following equation:

Cell density = average cell count per square × dilution factor × 10^4

Cell per ml = (total cell count /5) × (10 × 2) × 10^4

Then, calculate for dilution (desired cell density = 2.5 × 10^4 cells/ml)

Dilution factor (x) = cell per ml/2.5 × 10^4

Diluted cell suspension with culture medium to desirable volume (y)

Media x-1 ml : Cell 1 ml

Media y ml : Cell z ml

(z = cell volume for dilution)

3.2.2 Cytotoxic Assay by MTT Test (Mosmann, 1983)

Cytotoxicity assay was a conventional method to assess cellular damage. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay showed the changing of colorimetric formation for measuring the activity of mitochondrial enzyme in living cells. The intracellular succinate dehydrogenase could reduce the yellow tetrazolium salt MTT to an insoluble purple formazen crystal. The number of formazan product is straight proportional to the number of viable cells (Carmichael *et al*., 1987 and Twentyman, 1985). Therefore, this procedure could be determined the inhibitory dose of plant crude extracts on cancer cells.

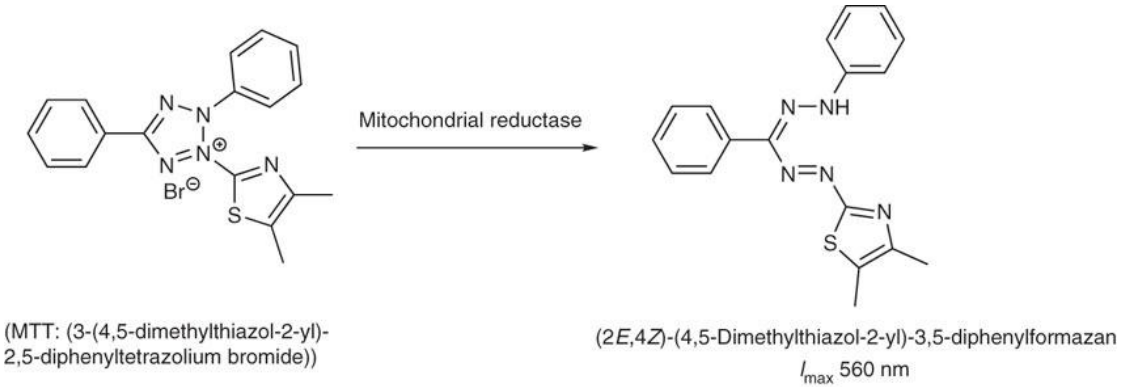


Figure 16 Molecular structure of MTT and its corresponding reaction product

Two hundred microliters of 5×10^3 cells in culture medium were plated into each well of 96-well plate and incubated for 24 h until cell attachment. The cells were treated with the plant crude extracts, 2 μl/well of various concentrations of samples ranging from 0.0001 to 10 μg/ml and incubated at 37 °C, 5% CO_2, for 72 hrs. Plates were added with 10 μl of 5 mg/ml of MTT in normal saline to each well and then incubated under darkness at 37 °C for 4 h. The solutions were discarded and 150 μl of DMSO were added into each well. Plates were then gently shaken to dissolve formazan crystal for complete solubilization. MTT solution was determined by a microplate reader (Tecan, Sunrise) 540 nm. The results were shown in line graph between the percentage of cell survival (Y-axis) and the concentrations of each sample (X-axis) and calculated the concentration of 50% cytotoxicity (IC_{50}).

Calculation of the Percentage of Cell Survival

$$\text{The percent of cell survival} = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of negative control}}\right) \times 100$$

The IC_{50} value could calculate from this curve. It was defined as the 50% reduction of the absorbance or 50% of the percentage of cell survival compared with cells that were treated by DMSO as a negative control in MTT assay.

The results were shown as mean ± standard error mean (S.E.M.) of five replication experiments (n = 5). Statistical analysis was performed using a one-way ANOVA for the analysis of the test results and Duncan analysis of variance at the significance levels of $P < 0.05$ was considered significantly.

4. Extraction and Isolation

4.1 Extraction of Plant Materials for Screening Cytotoxicity Assay

The plants materials were sliced into pieces and dried in hot air oven at 70 $^{\circ}$C. The dried materials were ground into powder. The dried powered plants (10 g) were immersed with methanol (150 ml) for 24 hours for three times. The solutions of plant extraction were filtered through No. 1 filter paper. The total supernatants were evaporated with rotary evaporator under reduced pressure at temperature of approximately 40 $^{\circ}$C. The dried powered plants (10 g) were soaked with water (80 ml) in water bath (60 $^{\circ}$C) for 4 hours for two times and then follow the above method. The plant extracts were subjected to cytotoxicity activity by MTT assay.

4.2 Cytotoxicity Assay of All Plants Extract

Each plant crude extracts were dissolved in 100% DMSO as 10 mg/ml stock solution and stored at 4 $^{\circ}$C. Stock solutions were test with human cancer cell lines and human cell line. All cell lines deaths were observed, at 72 hours intervals, and used for calculation of the percent survival of cells. The most active plant extract was extracted and isolated.

4.3 Extraction of the Most Active Plant Extract from Screening

The dried powered roots from *P. indica* (4 kg) which showed strongest cytotoxicity activity from screening were soaked with methanol (5.50 liters) for 24 hours for four times. The solutions of extraction were filtered through No. 1 filter paper. The total supernatants were evaporated in the vacuum rotary evaporator under reduced pressure at temperature of approximately 40 $^{\circ}$C to give 860.38 g (21.51% w/w) of methanol extract. The obtained extract was separated by solvent extraction with hexane, ethyl acetate and water, respectively.

4.4 Cytotoxicity Assay of Hexane, Ethyl acetate and Water Extracts of the Most Active Plant Extract from Screening

All extracts were subjected to anticancer activity by MTT assay. Each plant crude extract was dissolved in 100% DMSO as 10 mg/ml stock solution and stored at 4 $^{\circ}$C. The cancer cell lines deaths were observed, at 72 hours intervals, and used for calculation of the percent survival of cancer cells.

4.5 Separation of Crude Extracts by Column Chromatography

Hexane and ethyl acetate extracts which showed strong cytotoxicity activity were fractionated using silica gel column chromatography. The polarity of eluent was gradually increased from a low portion to high portion of eluent. Fractions were collected and combined according to their TLC patterns. The fractions were evaporated with rotary evaporator under reduced pressure at temperature of approximately 40 $^{\circ}$C. All fractions were subjected to anticancer activity by MTT assay. The strong cytotoxicity activity fractions were separated until obtained pure compound.

4.6 Cytotoxicity Assay of Pure Compounds

All compounds were tested for cytotoxic activity towards 7 cell lines; SW620, Chago, BT474, KATO-III, Hep-G2, Wi-38 and CCD-986Sk. The *in vitro* activity of all compounds was performed by the MTT test. Stock solution was diluted to test concentration; 0.0001, 0.001, 0.01, 0.1 and 1 μg/ml with 100% DMSO. Cancer cell lines deaths were observed at 72 hours intervals and used for calculation of IC_{50}.

CHAPTER IV
RESULTS AND DISSCUSION

1. Extraction of Plant Materials for Screening Cytotoxicity Assay

Result of plant extracts with different solvent extraction showed different characteristics and quantities of the extracts. The percentage yields and characteristics of the plant extracts are shown in Table 2.

Table 2 The percentage yields and characteristics of the plant extracts

Plants	Part used	Crude extract characteristic		*Yield of extract (%)	
		Methanol	Water	Methanol	Water
Phyllanthus emblica	fruits	brown solid	black solid	7.48	2.50
Terminalia chebula	fruits	black solid	dark brown solid	8.08	4.98
Terminalia bellerica	fruits	brown solid	black solid	5.27	13.13
Piper retrofractum	flowers	brown solid	black oil	2.57	1.30
Piper sarmentosum	leaves	brown gum	brown solid	5.80	0.40
Piper interruptum	nests	brown solid	black gum	2.09	4.48
Plumbago indica	roots	brown gum	black gum	8.76	1.68
Zingiber officinale	rhizomes	black solid	brown oil	4.07	0.40
Ficus racemosa	roots	brown gum	black gum	1.33	2.69
Capparis micracantha	roots	brown gum	black oil	2.11	1.80
Clerodendrum petasites	roots	black oil	brown solid	1.50	0.90
Harrisonia perforata	roots	black gum	brown oil	2.49	2.69
Tiliacora triandra	nests	brown gum	black gum	3.29	3.18

*Percent yield of crude extract (%) = (weight of crude extract / weight of dried plant) × 100

Table 2, 10 grams of dried plant showed yield of methanol extracts range from 1.33 to 8.76 percent and water extracts ranged from 0.40 to 4.98 percent.

2. Cytotoxicity Assay of All Plants Extracts

Cytotoxic assay of methanol and water extracts of thirteen plant species against 5 cancer cell lines: SW620, Chago, BT474, KATO-III and Hep-G2 have been carried out and the results are summarized in Table 3.

Table 3 Cytotoxic assay of methanol and water extracts of 13 plant species against cancer cell lines

Crude extracts	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
C. micracantha (H_2O)	69.64 ± 0.04	60.54 ± 0.11	88.95 ± 0.01	98.02 ± 0.07	39.50 ± 0.01
C. micracantha (MeOH)	25.06 ± 0.01	99.10 ± 0.10	77.89 ± 0.05	81.35 ± 0.07	60.92 ± 0.08
T. triandra (H_2O)	28.92 ± 0.05	48.83 ± 0.04	89.47 ± 0.01	26.98 ± 0.01	84.03 ± 0.02
T. triandra (MeOH)	14.22 ± 0.00	10.81 ± 0.00	41.05 ± 0.03	25.40 ± 0.00	24.79 ± 0.00
T. bellerica (H_2O)	18.55 ± 0.00	32.43 ± 0.07	54.21 ± 0.02	53.57 ± 0.03	46.21 ± 0.01
T. bellerica (MeOH)	20.96 ± 0.00	25.95 ± 0.06	53.16 ± 0.01	45.63 ± 0.03	41.18 ± 0.00
F. racemosa (H_2O)	30.84 ± 0.02	36.58 ± 0.10	102.11 ± 0.02	51.98 ± 0.04	110.08 ± 0.01
F. racemosa (MeOH)	14.22 ± 0.00	14.05 ± 0.01	32.11 ± 0.00	26.59 ± 0.00	26.05 ± 0.00
P. interruptum (H_2O)	58.80 ± 0.03	76.03 ± 0.10	108.42 ± 0.03	91.27 ± 0.09	123.11 ± 0.04
P. interruptum (MeOH)	13.49 ± 0.00	20.90 ± 0.07	31.05 ± 0.00	36.90 ± 0.02	37.82 ± 0.03
H. perforate (H_2O)	30.36 ± 0.10	44.68 ± 0.06	94.74 ± 0.50	69.05 ± 0.05	42.44 ± 0.02
H. perforate (MeOH)	20.96 ± 0.00	18.02 ± 0.01	44.74 ± 0.01	36.90 ± 0.00	38.66 ± 0.01

Table 3 (cont.) Cytotoxic activity of methanol and water extracts of 13 plant species against cancer cell lines.

Crude extracts	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
C. petasites (H_2O)	65.78 ± 0.03	100.72 ± 0.12	78.95 ± 0.01	115.08 ± 0.12	31.09 ± 0.00
C. petasites (MeOH)	35.42 ± 0.04	34.05 ± 0.12	45.79 ± 0.02	33.73 ± 0.01	42.44 ± 0.01
T. chebula (H_2O)	16.63 ± 0.00	51.35 ± 0.07	53.68 ± 0.01	55.95 ± 0.03	37.82 ± 0.01
T. chebula (MeOH)	21.93 ± 0.02	17.66 ± 0.00	54.74 ± 0.02	39.29 ± 0.01	40.34 ± 0.01
P. emblica (H_2O)	35.86 ± 0.10	86.54 ± 0.08	72.70 ± 0.00	32.11 ± 0.06	75.78 ± 0.15
P. emblica (MeOH)	46.41 ± 0.05	70.06 ± 0.10	76.60 ± 0.04	113.80 ± 0.17	91.49 ± 0.52
P. retrofractum (H_2O)	80.17 ± 0.12	116.41 ± 0.21	66.67 ± 0.03	94.93 ± 0.26	88.06 ± 0.14
P. retrofractum (MeOH)	21.24 ± 0.07	13.01 ± 0.12	34.40 ± 0.03	33.24 ± 0.14	31.31 ± 0.09
P. sarmentosum (H_2O)	40.37 ± 0.10	108.59 ± 0.20	99.29 ± 0.09	70.70 ± 0.24	77.16 ± 0.14
P. sarmentosum (MeOH)	12.66 ± 0.01	19.23 ± 0.06	28.37 ± 0.01	38.73 ± 0.06	24.39 ± 0.04
Z. officinale (H_2O)	59.49 ± 0.22	103.14 ± 0.32	97.52 ± 0.02	102.39 ± 0.32	120.07 ± 1.08
Z. officinale (MeOH)	15.33 ± 0.07	6.67 ± 0.02	27.30 ± 0.00	14.23 ± 0.01	18.86 ± 0.01
P. indica (H_2O)	121.52 ± 0.19	105.64 ± 0.24	100.35 ± 0.06	99.86 ± 0.03	87.37 ± 0.21
P. indica (MeOH)	11.67 ± 0.01	26.86 ± 0.13	27.30 ± 0.00	17.32 ± 0.03	15.74 ± 0.03
Positive control (Doxorubicin)	11.78 ± 0.01	9.05 ± 0.02	16.16 ± 0.03	41.28 ± 0.25	33.18 ± 0.01

In this research, terms of the plant extracts that were toxic to cancer cells must have the percent cells survival less than 50 percent. Table 3, almost of methanol extracts inhibited the growth of human cancer cell lines with the percentage growth lower than water extracts.

20 Plant extracts were toxic to colon cancer (SW620); methanol extracts of *C. micracantha*, *T. triandra*, *T. belleria*, *F. racemosa*, *P. interruptum*, *H. perforate*, *C. petasites*, *T. chebula*, *P. emblica*, *P. retrofractum, P. sarmentosum, Z. officinale* and *P. indica* with the percentage growth were 25.06 ± 0.01, 14.22 ± 0.00, 20.96 ± 0.00, 14.22 ± 0.00, 13.49 ± 0.00, 20.96 ± 0.00, 35.42 ± 0.04, 21.93 ± 0.02, 46.41 ± 0.05, 21.24 ± 0.07, 12.66 ± 0.01, 15.33 ± 0.07 and 11.67 ± 0.01 percent, respectively and water extracts of *T. triandra*, *T. belleria*, *F. racemosa*, *H. perforate*, *T. chebula*, *P. emblica* and *P. sarmentosum* with the percentage growth were 28.92 ± 0.05, 18.55 ± 0.00, 30.84 ± 0.02, 30.36 ± 0.10, 16.63 ± 0.00, 35.86 ± 0.10 and 40.37 ± 0.10 percent, respectively.

15 Plant extracts were toxic to lung cancer (Chago); methanol extracts of *T. triandra*, *T. belleria*, *F. racemosa*, *P. interruptum*, *H. perforate*, *C. petasites*, *T. chebula*, *P. retrofractum, P. sarmentosum, Z. officinale* and *P. indica* with the percentage growth were 10.81 ± 0.00, 25.95 ± 0.06, 14.05 ± 0.01, 20.90 ± 0.07, 18.02 ± 0.01, 34.05 ± 0.12, 17.66 ± 0.00, 13.01 ± 0.12, 19.23 ± 0.06, 6.67 ± 0.02 and 26.86 ± 0.13 percent, respectively and water extracts of *T. triandra*, *T. belleria*, *F. racemosa* and *H. perforate* with the percentage growth were 48.83 ± 0.04, 32.43 ± 0.07, 36.58 ± 0.10 and 44.68 ± 0.06 percent, respectively.

9 Plant extracts were toxic to brest cancer (BT474); methanol extracts of *T. triandra*, *F. racemosa*, *P. interruptum*, *H. perforate*, *C. petasites*, *P. retrofractum, P. sarmentosum, Z. officinale* and *P. indica* with the percentage growth were 41.05 ± 0.03, 32.11 ± 0.00, 31.05 ± 0.00, 44.74 ± 0.01, 45.79 ± 0.02, 34.40 ± 0.03, 28.37 ± 0.01, 27.30 ± 0.00 and 27.30 ± 0.00 percent, respectively

13 Plant extracts were toxic to gastric cancer (KATO-III); methanol extracts of *T. triandra*, *T. belleria*, *F. racemosa*, *P. interruptum*, *H. perforate*, *C. petasites*, *T. chebula*, *P. retrofractum, P. sarmentosum, Z. officinale* and *P. indica* with the percentage growth were 25.40 ± 0.00, 45.63 ± 0.03, 26.59 ± 0.00, 36.90 ± 0.02, 36.90

± 0.00, 33.73 ± 0.01, 39.29 ± 0.01, 33.24 ± 0.14, 38.73 ± 0.06, 14.23 ± 0.01 and 17.32 ± 0.03 percent, respectively and water extracts of *T. triandra* and *P. emblica* with the percentage growth were 26.98 ± 0.01 and 32.11 ± 0.06 percent, respectively.

16 Plant extracts were toxic to liver cancer (Hep-G2); methanol extracts of *T. triandra*, *T. belleria*, *F. racemosa*, *P. interruptum*, *H. perforate*, *C. petasites*, *T. chebula*, *P. retrofractum, P. sarmentosum, Z. officinale* and *P. indica* with the percentage growth were 24.79 ± 0.00, 41.18 ± 0.00, 26.05 ± 0.00, 37.82 ± 0.03, 38.66 ± 0.01, 42.44 ± 0.01, 40.34 ± 0.01, 31.31 ± 0.09, 24.39 ± 0.04, 18.86 ± 0.01 and 15.74 ± 0.03 percent, respectively and water extracts of *C. micracantha*, *T. belleria*, *H. perforate*, *C. petasites* and *T. chebula* with the percentage growth were 39.50 ± 0.01, 46.21 ± 0.01, 42.44 ± 0.02, 31.09 ± 0.00 and 37.82 ± 0.01 percent, respectively.

Accordingly, cytotoxic assay of methanol crude extract of thirteen plant species against normal cell line: human normal lung fibroblast (Wi-38) has been carried out and the result was summarized in Table 4.

Table 4 Cytotoxic activity of methanol extract of 13 plant species against normal cell

Crude extracts	Percent cell survival (%)	Crude extracts	Percent cell survival (%)
	Wi38		Wi38
C. micracantha	51.38 ± 0.03	*T. chebula*	62.92 ± 0.05
T. triandra	54.56 ± 0.04	*P. emblica*	81.07 ± 0.04
T. bellerica	76.97 ± 0.01	*P. retrofractum*	33.15 ± 0.03
F. racemosa	45.01 ± 0.05	*P. sarmentosum*	54.93 ± 0.05
P. interruptum	41.73 ± 0.04	*Z. officinale*	57.81 ± 0.01
H. perforate	51.81 ± 0.05	*P. indica*	54.96 ± 0.06
C. petasites	43.23 ± 0.02	Doxorubicin	100.30 ± 0.02

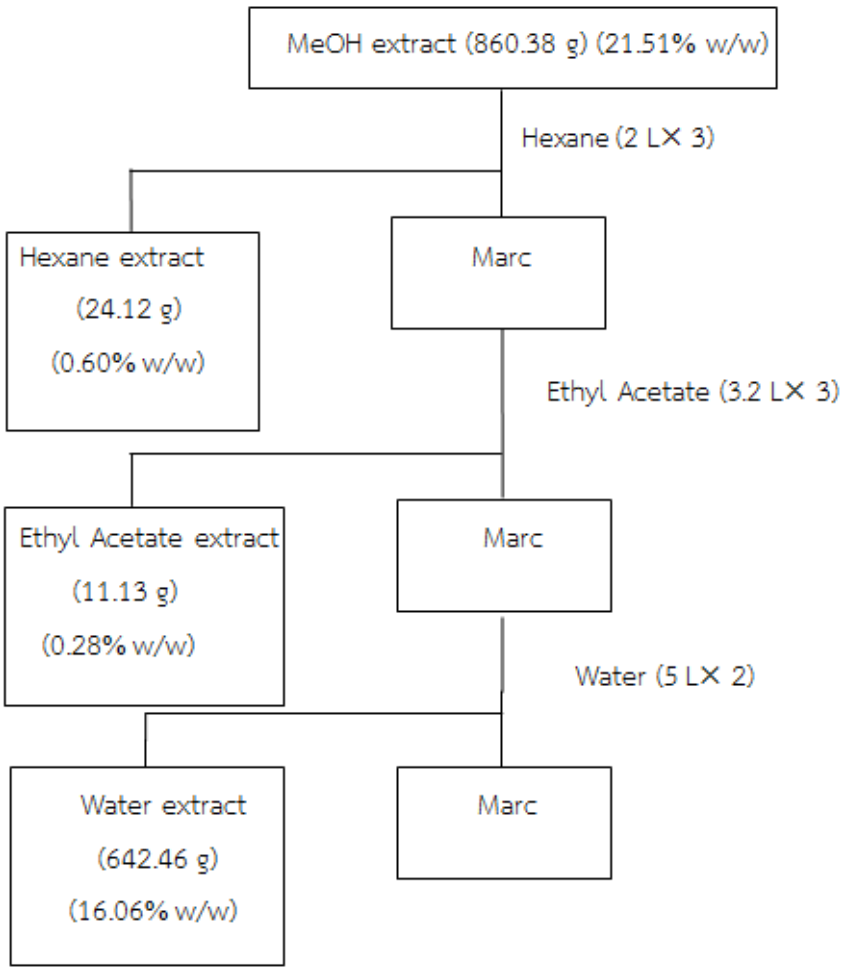
Table 3, it indicated that methanol extract of *P. indica* roots inhibited the growth of SW620 cells with the lowest percentage growth was 11.67 ± 0.01 percent. Methanol extract of *Z. officinale* rhizomes inhibited the growth of Chago cells with the lowest percentage growth was 6.67 ± 0.02 percent. Methanol extracts of *Z. officinale* rhizomes and *P. indica* roots inhibited the growth of BT474 cells with the lowest percentage growth was 27.30 ± 0.00 percent. Methanol extract of *Z. officinale* rhizomes inhibited the growth of KATO-III cells with the lowest percentage growth was 14.23 ± 0.01 percent. Methanol extract of *P. indica* roots inhibited the growth of Hep-G2 cells with the lowest percentage growth was 15.74 ± 0.03 percent. Therefore, it was very interesting to isolate compounds of methanol extracts of *P. indica*.

3. Extraction of Methanol Extracts of Roots of *P. indica*

Methanol extract of roots *P. indica* was 860.38 g. (21.51% w/w). The obtained extract was separated by solvent extraction with hexane, ethyl acetate and water, respectively. Each extract was evaporated under reduced pressure to give 24.12 g of hexane extract (0.60% w/w), 11.13 g of ethyl acetate extract (0.28% w/w) and 642.46 g of water extract (16.06% w/w).

Table 5 Characteristics of the partition extracts of methanol extract

Solvent	Appearance	Weight (g)	% w/w of Crude extract
Hexane	Black-brown oil	24.12	0.60
Ethyl acetate	Red-brown oil	11.13	0.28
Water	Black sticky gum	642.46	16.06



Scheme 1 **Extraction of roots of *P. indica***

4. Cytotoxicity Activity of Crude Partition of Methanol Extract of Roots of *P. indica*

Cytotoxic activity of hexane, ethyl acetate and water extracts from methanol extract against 5 human cancer cell lines: colon cancer (SW620), lung cancer (Chago), breast cancer (BT474), gastric cancer (KATO-III) and liver cancer (Hep-G2) have been carried out and the results are summarized in Table 6.

Table 6 Cytotoxic activity of hexane, ethyl acetate and water extracts against cancer cell lines

Crude extracts	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
Hexane	10.33 ± 0.02	11.95 ± 0.09	11.21 ± 0.02	9.40 ± 0.02	18.76 ± 0.03
Ethyl acetate	18.85 ± 0.02	14.61 ± 0.08	17.02 ± 0.05	11.61 ± 0.05	17.02 ± 0.04
Water	88.56 ± 0.05	80.56 ± 0.05	84.09 ± 0.10	79.12 ± 0.11	82.13 ± 0.13

Table 6 showed that hexane and ethyl acetate extracts inhibited the growth of human cancer cell lines with the low and nearby percentage growth. These promising results prompted us to isolate bioactive constituents from these extracts.

Hexane and ethyl acetate extracts inhibited the growth of cancer cells with the strong cytotoxicity activity (Table 6). This result is in agreement with a previous report (Thitiorul *et al*., 2013) that reported the genotoxicity of an ethanolic extract of *P. indica* roots (25-100 μg/ml) was genotoxic in human lymphocytes and cytotoxic at concentrations of ≥500 μg/ml *in vitro*. These activities of the ethanolic extract of *P. indica* roots could serve its potential therapeutic effects, especially as an anticancer agent.

5. Separation of Crude Partition of Roots of *P. indica* by Column Chromatography

5.1 Separation of Hexane Extract of Roots of *P. indica*

Hexane extract (24.12 g) was fractionated using silica gel column chromatography. The polarity of eluent was gradually increased from hexane to 5% methanol in ethyl acetate. Fractions (50 ml) were collected and combined according to their TLC patterns as shown in Table 7.

Table 7 Combination of fractions from hexane extract by column chromatography

Fraction code	Solvent system	Volume (ml)	Weight (g)	Characteristic of fraction
A	Hexane : Ethyl acetate 100 : 0	50		
	Hexane : Ethyl acetate 90 : 10	450	0.79	Light yellow solid
B	Hexane : Ethyl acetate 85 : 15	1,200	7.35	Yellow solid
C	Hexane : Ethyl acetate 80 : 20	300	0.78	Orange solid
D	Hexane : Ethyl acetate 70 : 30	500	1.25	Light yellow solid
E	Hexane : Ethyl acetate 50 : 50	550	2.70	Orange solid
F	Hexane : Ethyl acetate 50 : 50	200	0.24	Dark orange solid
G	Hexane : Ethyl acetate 20 : 80	200	0.25	Dark brown oil
H	Hexane : Ethyl acetate 20 : 80	200	0.17	Dark brown gum
I	Hexane : Ethyl acetate 0 : 100	200	0.47	Light pink solid
J	Hexane : Ethyl acetate 0 : 100	50	0.08	Dark brown solid
K	Ethyl acetate : Methanol 95 : 5	550	0.27	Dark brown oil

After separation, 11 fractions (A-K) were collected which each fraction has different characteristics (Table 7). Each fraction was subjected to cytotoxicity activity by MTT assay. The cancer cell lines deaths were observed, at 72 hours intervals, and used for calculation of the percent survival of cancer cells. The results of cytotoxicity activity testing are shown in Table 8.

Table 8 Cytotoxic activity of A-K fractions from hexane extract against human cancer cell lines

Fraction code	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
A	13.13 ± 0.02	12.80 ± 0.01	25.22 ± 0.02	42.92 ± 0.01	40.64 ± 0.01
B	15.95 ± 0.03	13.83 ± 0.01	29.81 ± 0.02	47.79 ± 0.00	49.87 ± 0.05
C	17.18 ± 0.03	12.11 ± 0.01	29.45 ± 0.03	46.90 ± 0.01	49.21 ± 0.04
D	14.98 ± 0.02	19.67 ± 0.05	27.87 ± 0.01	43.81 ± 0.02	49.08 ± 0.01
E	14.27 ± 0.03	13.23 ± 0.02	28.22 ± 0.05	41.59 ± 0.01	47.65 ± 0.03
F	18.33 ± 0.03	29.04 ± 0.04	40.04 ± 0.04	44.69 ± 0.04	38.09 ± 0.07
G	50.89 ± 0.07	54.88 ± 0.07	51.23 ± 0.10	56.89 ± 0.12	54.32 ± 0.07
H	67.90 ± 0.09	56.78 ± 0.03	54.55 ± 0.09	67.21 ± 0.04	67.11 ± 0.10
I	66.45 ± 0.04	61.98 ± 0.05	67.23 ± 0.04	60.12 ± 0.08	64.29 ± 0.04
J	58.56 ± 0.03	55.98 ± 0.02	51.28 ± 0.03	54.33 ± 0.04	63.91 ± 0.03
K	87.08 ± 0.04	87.45 ± 0.05	67.54 ± 0.04	82.11 ± 0.05	68.14 ± 0.11

Fractions A, B, C, D, E and F were toxic to human cancer cell lines (Table 8). Fraction A, the percent cell survival was in the range of 12.80-42.92 percent. Fraction B, the percent cell survival was in the range of 13.83-49.87 percent. Fraction C, the percent cell survival was in the range of 12.11-49.21 percent. Fraction D, the percent cell survival was in the range of 14.98-49.08 percent. Fraction E, the percent cell survival was in the range of 13.23-47.65 percent. Fraction F, the percent cell survival was in the range of 18.33-44.69 percent. The promising fractions A-F were toxic to SW620 and Chago with the strong activity. The results prompted us to isolate bioactive constituents from these fractions.

Fractions A-F were fractionated using silica gel column chromatography and by initially eluting it with hexane. The polarity of eluent was gradually increased from a low portion of ethyl acetate in hexane to 80% ethyl acetate in hexane. Fractions (50 ml) were collected and combined according to their TLC patterns to yield of many fractions as shown in Table 9.

Table 9 Separation of fractions from A-F fractions of hexane extract by column chromatography

Fraction code	Solvent system		Volume (ml)	Weight (g)	Characteristic of fraction
AA	Hexane : Ethyl acetate	90 : 10	200	0.44	Yellow crystal
AB	Hexane : Ethyl acetate	50 : 50	100	0.12	Yellow oil
AC	Hexane : Ethyl acetate	20 : 80	100	0.11	Light brown solid
BA	Hexane : Ethyl acetate	90 : 10	1,000	4.30	Yellow crystal
BB	Hexane : Ethyl acetate	80 : 20	400	1.93	Orange crystal
BC	Hexane : Ethyl acetate	50 : 50	100	0.11	Light brown solid
BD	Hexane : Ethyl acetate	20 : 80	100	0.15	Brown solid
CA	Hexane : Ethyl acetate	90 : 10	250	0.63	Yellow solid
CB	Hexane : Ethyl acetate	50 : 50	100	0.11	Dark yellow oil
CC	Hexane : Ethyl acetate	80 : 20	150	0.22	Brown solid
DA	Hexane : Ethyl acetate	90 : 10	200	0.81	Orange crystal
DB	Hexane : Ethyl acetate	50 : 50	200	0.31	Light brown crystal
DC	Hexane : Ethyl acetate	20 : 80	200	0.40	Brown solid
EA	Hexane : Ethyl acetate	90 : 10	200	0.60	Yellow crystal
EB	Hexane : Ethyl acetate	80 : 20	400	1.50	Yellow solid
EC	Hexane : Ethyl acetate	50 : 50	50	0.05	Light yellow solid
ED	Hexane : Ethyl acetate	20 : 80	50	0.04	Light yellow oil
FA	Hexane : Ethyl acetate	80 : 20	50	0.04	Light Yellow oil
FB	Hexane : Ethyl acetate	50 : 50	50	0.05	Yellow oil
FC	Hexane : Ethyl acetate	80 : 20	100	0.13	Orange crystal

Fractions A, C, D and F were separated into 3 fractions. Fractions B and E were separated into 4 fractions. Fraction BA was yellow crystal with the highest weight is 4.30 g. Each fraction (10 mg/ml) was subjected to cytotoxicity activity by MTT assay. Cancer cell lines deaths were observed at 72 hours intervals, and used for calculation of the percent cell survival. The results of cytotoxicity activity testing are shown in Table 10.

Table 10 Cytotoxic activity of separated fractions of A-F fractions against human cancer cell lines

Fraction code	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
AA	12.34 ± 0.02	14.45 ± 0.06	26.78 ± 0.04	37.23 ± 0.05	39.09 ± 0.04
AB	51.23 ± 0.05	59.28 ± 0.02	61.83 ± 0.10	50.83 ± 0.12	91.23 ± 0.12
AC	55.43 ± 0.11	58.13 ± 0.05	75.43 ± 0.02	65.48 ± 0.20	85.63 ± 0.03
BA	13.45 ± 0.12	12.34 ± 0.11	23.56 ± 0.04	31.55 ± 0.03	32.67 ± 0.11
BB	67.89 ± 0.20	68.45 ± 0.12	77.65 ± 0.11	97.19 ± 0.04	87.01 ± 0.09
BC	78.54 ± 0.13	75.09 ± 0.20	88.55 ± 0.20	79.59 ± 0.01	88.53 ± 0.05
BD	56.89 ± 0.12	66.39 ± 0.09	76.11 ± 0.08	85.43 ± 0.00	87.45 ± 0.08
CA	59.87 ± 0.09	63.21 ± 0.06	77.23 ± 0.09	83.17 ± 0.11	89.48 ± 0.01
CB	62.11 ± 0.20	59.45 ± 0.12	69.13 ± 0.12	75.34 ± 0.08	72.47 ± 0.02
CC	67.89 ± 0.07	77.19 ± 0.22	75.23 ± 0.02	87.35 ± 0.06	97.85 ± 0.10
DA	78.98 ± 0.05	58.12 ± 0.07	88.76 ± 0.06	98.38 ± 0.20	78.30 ± 0.03
DB	90.67 ± 0.11	80.56 ± 0.14	94.57 ± 0.08	67.87 ± 0.03	80.27 ± 0.04
DC	67.78 ± 0.09	87.22 ± 0.01	81.28 ± 0.12	97.65 ± 0.02	97.45 ± 0.04
EA	89.45 ± 0.07	67.34 ± 0.02	63.11 ± 0.10	80.23 ± 0.01	67.15 ± 0.10
EB	13.23 ± 0.02	12.78 ± 0.03	22.67 ± 0.02	40.34 ± 0.04	34.12 ± 0.00
EC	56.89 ± 0.10	67.32 ± 0.11	76.39 ± 0.06	57.84 ± 0.12	65.34 ± 0.03
ED	87.56 ± 0.07	64.87 ± 0.10	98.23 ± 0.07	96.34 ± 0.22	78.26 ± 0.11
FA	77.56 ± 0.08	63.34 ± 0.08	70.45 ± 0.13	89.23 ± 0.04	87.98 ± 0.09
FB	50.89 ± 0.10	61.38 ± 0.09	67.83 ± 0.20	89.12 ± 0.01	81.11 ± 0.08
FC	17.54 ± 0.03	25.43 ± 0.02	36.78 ± 0.02	43.23 ± 0.05	32.13 ± 0.04

Fractions AA, BA, EB and FC were toxic to human cancer cells. Fraction AA, the percent cell survival was in the range of 12.34-39.09 percent. Fraction BA, the percent cell survival was in the range of 12.34-32.67 percent. Fraction EB, the percent cell survival was in the range of 12.78-40.34 percent. Fraction FC, the percent cell survival was in the range of 17.54-43.23 percent. These promising results prompted us to isolate bioactive constituents from these fractions.

Fractions AA, BA, EB and FC were fractionated using silica gel column chromatography and by initially eluting it with hexane. The polarity of eluent was gradually increased from a low portion of ethyl acetate in hexane to 80% ethyl acetate in hexane. Fractions (30 ml) were collected and combined according to their TLC patterns.

Table 11 Separation of AA, BA, EB and FC fractions by column chromatography

Fraction code	Solvent system		Volume (ml)	Weight (g)	Characteristic of fraction
AA1	Hexane : Ethyl acetate	90 : 10	150	0.20	Orange crystal
AA2	Hexane : Ethyl acetate	50 : 50	90	0.14	Yellow oil
BA1	Hexane : Ethyl acetate	90 : 10	90	0.11	Yellow oil
BA2	Hexane : Ethyl acetate	80 : 20	900	3.83	Light orange crystal
BA3	Hexane : Ethyl acetate	50 : 50	90	0.01	Light brown solid
BA4	Hexane : Ethyl acetate	20 : 80	90	0.12	Brown oil
EB1	Hexane : Ethyl acetate	90 : 10	210	1.00	White powder
EB2	Hexane : Ethyl acetate	80 : 20	150	0.30	Yellow solid
FC1	Hexane : Ethyl acetate	80 : 20	90	0.04	Orange crystalline solid
FC2	Hexane : Ethyl acetate	50 : 50	90	0.05	Yellow solid

Fractions EB1 and FC1 were pure compounds of separation to give 1.00 g of white powder and 0.04 g of orange crystalline solid, respectively. Each fraction (10 mg/ml) was subjected to cytotoxicity activity by MTT assay. Calculation of the percent cell survival after treated human cancer cells with fractions. Cell lines deaths were observed, at 72 hours intervals. The results of cytotoxicity activity testing are shown in Table 12.

Table 12 Cytotoxic activity of separation of AA, BA, EB and FC fractions against human cancer cell lines

Fraction code	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
AA1	12.73 ± 0.10	11.05 ± 0.05	20.37 ± 0.02	31.12 ± 0.10	34.24 ± 0.00
AA2	55.12 ± 0.03	66.81 ± 0.01	69.41 ± 0.02	57.79 ± 0.10	69.45 ± 0.05
BA1	57.45 ± 0.04	66.04 ± 0.02	75.41 ± 0.03	67.81 ± 0.05	89.01 ± 0.10
BA2	11.98 ± 0.01	20.11 ± 0.04	23.67 ± 0.01	33.12 ± 0.04	39.34 ± 0.05
BA3	64.23 ± 0.07	89.13 ± 0.02	86.22 ± 0.05	81.90 ± 0.01	80.15 ± 0.03
BA4	58.09 ± 0.03	69.11 ± 0.10	60.87 ± 0.04	74.61 ± 0.09	68.01 ± 0.07
EB1	20.89 ± 0.05	40.33 ± 0.09	30.23 ± 0.10	20.85 ± 0.10	14.31 ± 0.05
EB2	77.20 ± 0.10	76.58 ± 0.03	54.55 ± 0.09	77.21 ± 0.03	87.34 ± 0.10
FC1	40.45 ± 0.10	49.22 ± 0.05	47.03 ± 0.04	35.12 ± 0.08	44.09 ± 0.14
FC2	53.56 ± 0.03	55.12 ± 0.02	59.28 ± 0.03	64.53 ± 0.04	69.21 ± 0.03

Fractions AA1, BA2, EB1 and FC1 were toxic to human cancer cells. Fraction AA1, the percent cell survival was in the range of 11.05-34.24 percent. Fraction BA2, the percent cell survival was in the range of 11.98-39.34 percent. Fraction EB1, the percent cell survival was in the range of 14.31-40.33 percent. Fraction FC1, the percent cell survival was in the range of 35.12-47.03 percent. These promising results prompted us to isolate bioactive constituents from AA1 and BA2 fractions. In addition, fractions AA1 and BA2 were toxic to human cancer cells with the strong activity.

Fractions AA1, BA2, EB1 and FC1 were further fractionated using silica gel column chromatography and by initially eluting it with hexane. The polarity of eluent was gradually increased from a low portion of ethyl acetate in hexane to 50% ethyl acetate in hexane. Fractions (20 ml) were collected and combined according to their TLC patterns.

Table 13 Separation of fractions from AA1 and BA2 fractions of hexane extract

Fraction code	Solvent system		Volume (ml)	Weight (g)	Characteristic of fraction
AA1a	Hexane : Ethyl acetate	90 : 10	80	0.15	Yellow crystal
AA1b	Hexane : Ethyl acetate	50 : 50	20	0.03	Yellow solid
BA2a	Hexane : Ethyl acetate	95 : 5	600	3.00	Orange crystal
BA2b	Hexane : Ethyl acetate	90 : 10	100	0.50	Light orange solid

Fraction AA1a was pure compound of separation to give 0.15 g of yellow crystal. Each fraction was subjected to cytotoxicity activity by MTT assay. Human cancer cell lines deaths were observed at 72 hours intervals, and used for calculation of the percent survival of cancer cells. The results of cytotoxicity activity testing are shown in Table 14.

Table 14 Cytotoxic activity of separation of AA1 and BA2 fractions against human cancer cell lines

Fraction code	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
AA1a	2.53 ± 0.02	4.09 ± 0.01	6.21 ± 0.05	3.08 ± 0.02	5.02 ± 0.03
AA1b	53.12 ± 0.11	56.22 ± 0.08	62.22 ± 0.05	67.65 ± 0.11	68.35 ± 0.05
BA2a	17.22 ± 0.10	16.23 ± 0.03	25.34 ± 0.03	37.51 ± 0.05	39.67 ± 0.10
BA2b	66.34 ± 0.01	90.45 ± 0.14	68.33 ± 0.01	63.10 ± 0.04	79.14 ± 0.05

Fractions AA1a and BA2a were toxic to human cancer cells. Fraction AA1a, the percent cell survival was in the range of 2.53-6.21 percent. Fraction BA2a, the percent cell survival was in the range of 16.23-39.67 percent. These promising results prompted us to isolate bioactive constituents from BA2a fraction.

Fractions BA2a was further fractionated using silica gel column chromatography and by initially eluting it with hexane. The polarity of eluent was gradually increased from a low portion of ethyl acetate in hexane to 10% ethyl acetate in hexane. Fractions (10 ml) were collected and combined according to their TLC patterns.

Table 15 Separation of fractions from BA2a fraction of hexane extract by column chromatography

Fraction code	Solvent system	Volume (ml)	Weight (g)	Characteristic of fraction
BA2a1	Hexane : Ethyl acetate 95 : 5	200	2.00	Yellow crystal
BA2a2	Hexane : Ethyl acetate 90 : 10	80	0.30	Brown oil

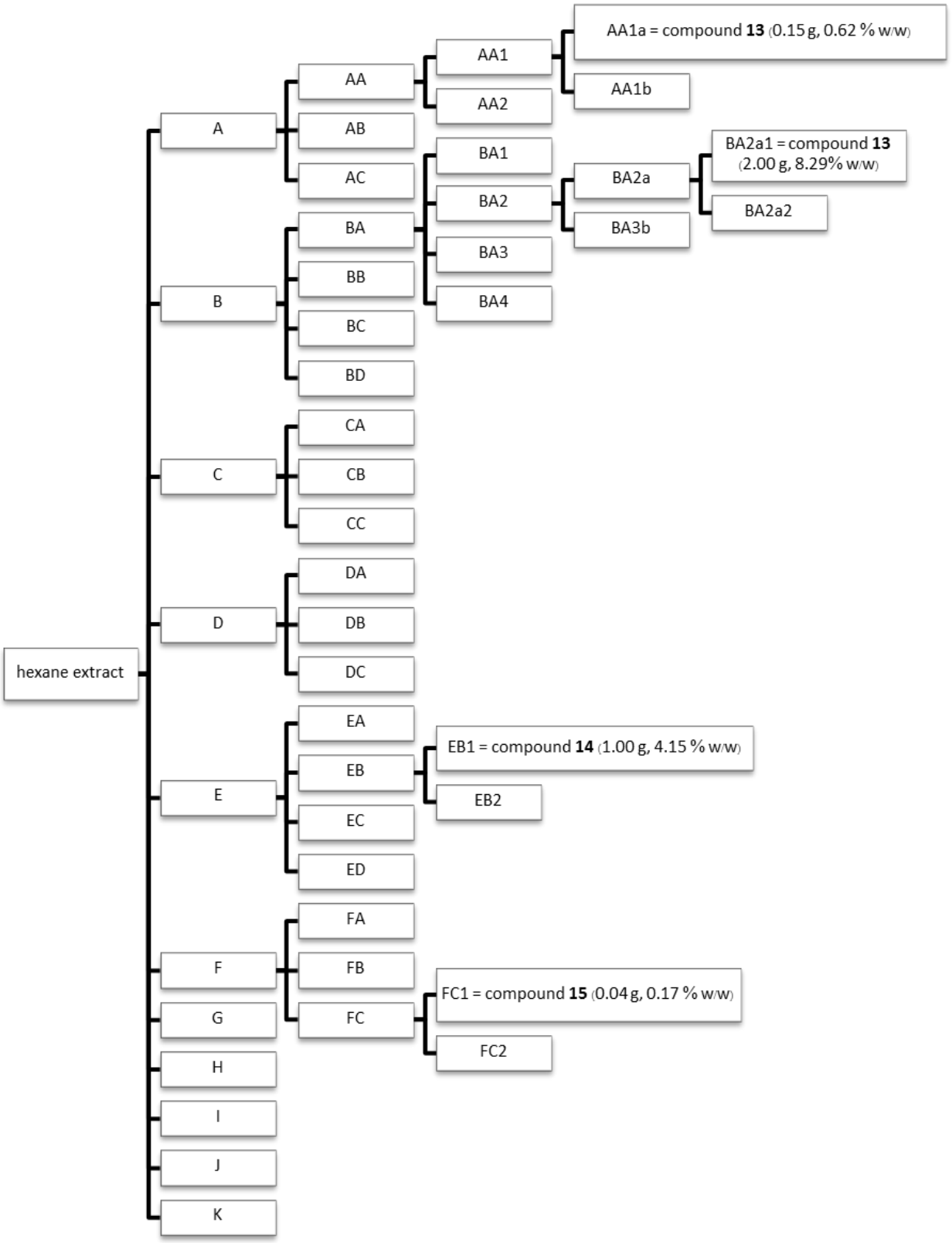
Fraction BA2a1 was pure compound of separation to give 2.00 g of yellow crystal. Each fraction was subjected to cytotoxicity activity by MTT assay. The results of cytotoxicity activity testing are shown in Table 16.

Table 16 Cytotoxic activity of separation of BA2a fraction against human cancer cell lines.

Fraction code	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
BA2a1	2.45 ± 0.01	4.12 ± 0.05	6.01 ± 0.03	3.11 ± 0.00	5.19 ± 0.05
BA2a2	54.03 ± 0.09	53.34 ± 0.02	59.07 ± 0.11	60.32 ± 0.01	67.14 ± 0.10

Fraction BA2a1 was strongly toxic to human cancer cells with the low percent cell survival**.** The percent cell survival was in the range of 2.45-6.01 percent.

Isolated 3 compounds from hexane extracts, fractions AA1a and BA2a1 were compound **13**, fraction EB1 was compound **14** and fraction FC1 was compound **15**. Isolation of hexane extract of roots of *P. indica* is briefly summarized in Scheme 2.



Scheme 2 Isolation of hexane extract of roots of *P. indica*

Scheme 2, fraction AA1a was compound **13** which separated from hexane extract to give 0.15 g (0.62 % w/w of hexane extract). Fraction BA2a1 was compound **13** which separated from hexane extract to give 2.00 g (8.29 % w/w of hexane extract). Fraction EB1 was compound **14** which separated from hexane extract to give 1.00 g (4.15 % w/w of hexane extract). Fraction FC1 was compound **15** which separated from hexane extract to give 0.04 g (0.17 % w/w of hexane extract).

5.2 Separation of Ethyl Acetate Extract of Roots of *P. indica*

Ethyl acetate extract (11.13 g) was fractionated using silica gel column chromatography and by initially eluting it with hexane. The polarity of eluent was gradually increased from a low portion of ethyl acetate in hexane to 100% ethyl acetate. Fractions (50 ml) were collected and combined according to their TLC patterns to yield of 3 fractions (A-C) as shown in Table 17.

Table 17 Combination of fractions from ethyl acetate extract by column chromatography

Fraction code	Solvent system		Volume (ml)	Weight (g)	Characteristic of fraction
A	Hexane : Ethyl acetate	80 : 20	550	0.23	Light brown crystal
B	Hexane : Ethyl acetate	50 : 50	500	0.24	Brown oil
C	Hexane : Ethyl acetate	0 : 100	1,000	1.19	Dark brown oil

Ethyl acetate extract was separated into 3 fractions, fraction A was light crystal brown to give 0.23 g (2.07% w/w of ethyl acetate extract), fraction B was brown oil to give 0.24 g (2.16% w/w of ethyl acetate extract) and fraction c was dark brown oil to give 1.19 g (10.69% w/w of ethyl acetate extract). Each fraction (10 mg/ml) was subjected to cytotoxicity activity by MTT assay. Human cancer cell lines deaths were observed, at 72 hours intervals, and used for calculation of the percent cell survival. The results of cytotoxicity activity testing are shown in Table 18.

Table 18 Cytotoxic activity of A-C fractions from ethyl acetate extract against cancer cell lines

Fraction code	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
A	12.25 ± 0.01	14.18 ± 0.02	28.92 ± 0.03	52.21 ± 0.05	59.19 ± 0.03
B	13.92 ± 0.01	15.81 ± 0.03	33.16 ± 0.02	53.32 ± 0.04	57.14 ± 0.05
C	16.01 ± 0.01	18.30 ± 0.02	31.81 ± 0.02	61.07 ± 0.04	64.23 ± 0.04

Fractions A, B and C inhibited the growth of human cancer cell lines with the nearby percentage. These promising results prompted us to isolate bioactive constituents from these fractions.

Fractions A-C were fractionated using silica gel column chromatography and by initially eluting it with hexane. The polarity of eluent was gradually increased from a low portion of ethyl acetate in hexane to 100% ethyl acetate. Fractions (30 ml) were collected and combined according to their TLC patterns to yield of many fractions as shown in Table 19.

Table 19 Separation of fractions of A-C fractions of ethyl acetate extract

Fraction code	Solvent system		Volume (ml)	Weight (g)	Characteristic of fraction
AA	Hexane : Ethyl acetate	80 : 20	540	0.20	Dark orange crystal
AB	Hexane : Ethyl acetate	20 : 80	60	0.01	Brown oil
BA	Hexane : Ethyl acetate	80 : 20	210	0.17	Orange solid
BB	Hexane : Ethyl acetate	50 : 50	70	0.01	Light pink solid
BC	Hexane : Ethyl acetate	20 : 80	90	0.03	Brown oil
BD	Hexane : Ethyl acetate	0 : 100	60	0.01	Brown solid
CA	Hexane : Ethyl acetate	90 : 10	210	0.16	Orange crystal
CB	Hexane : Ethyl acetate	50 : 50	210	0.15	Red brown oil
CC	Hexane : Ethyl acetate	20 : 80	600	0.35	Brown oil

Fractions A and C were separated into 3 fractions. Fraction B was separated into 4 fractions. Each fraction was subjected to cytotoxicity activity by MTT assay. The results of cytotoxicity activity testing are shown in Table 20.

Table 20 Cytotoxic activity of separated fractions of A-C fractions against human cancer cell lines

Fraction code	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
AA	2.37 ± 0.02	4.04 ± 0.08	6.12 ± 0.04	3.21 ± 0.10	5.10 ± 0.03
AB	53.45 ± 0.01	55.81 ± 0.03	63.22 ± 0.02	50.45 ± 0.04	57.13 ± 0.00
BA	15.00 ± 0.08	16.30 ± 0.05	30.34 ± 0.05	35.67 ± 0.03	34.56 ± 0.05
BB	75.70 ± 0.09	77.89 ± 0.10	78.74 ± 0.05	85.67 ± 0.00	84.23 ± 0.07
BC	84.01 ± 0.10	82.30 ± 0.00	90.67 ± 0.05	95.12 ± 0.03	94.09 ± 0.08
BD	84.23 ± 0.05	83.00 ± 0.10	89.17 ± 0.03	93.11 ± 0.05	90.33 ± 0.09
CA	55.23 ± 0.07	56.10 ± 0.07	60.94 ± 0.15	65.07 ± 0.00	74.32 ± 0.04
CB	19.76 ± 0.01	18.90 ± 0.05	32.64 ± 0.05	39.83 ± 0.00	39.75 ± 0.09
CC	10.04 ± 0.08	11.40 ± 0.04	20.98 ± 0.05	25.22 ± 0.03	24.46 ± 0.05

Fractions AA, BA, CB and CC were toxic to human cancer cell lines. Fraction AA, the percent cell survival was in the range of 2.37-5.10 percent. Fraction BA, the percent cell survival was in the range of 15.00-35.67 percent. Fraction CB, the percent cell survival was in the range of 18.90-39.83 percent. Fraction CC, the percent cell survival was in the range of 10.04-25.22 percent. These promising results prompted us to isolate bioactive constituents from BA, CB and CC fractions.

Fraction AA was pure compound of separation to give 0.20 g of dark orange crystal. Fractions BA, CB and CC were further fractionated using silica gel column chromatography and by initially eluting it with hexane. The polarity of eluent was gradually increased from a low portion of ethyl acetate in hexane to 80% ethyl acetate in hexane. Fractions (20 ml) were collected and combined according to their TLC patterns to yield of many fractions as shown in Table 21.

Table 21 Separation of BA, CB and CC fractions

Fraction code	Solvent system		Volume (ml)	Weight (g)	Characteristic of fraction
BA1	Hexane : Ethyl acetate	90 : 10	200	0.10	Dark orange crystal
BA2	Hexane : Ethyl acetate	50 : 50	60	0.02	Brown solid
CB1	Hexane : Ethyl acetate	90 : 10	100	0.07	Yellow crystal
CB2	Hexane : Ethyl acetate	80 : 20	60	0.02	Brown solid
CB3	Hexane : Ethyl acetate	50 : 50	40	0.01	Dark brown solid
CB4	Hexane : Ethyl acetate	20 : 80	40	0.03	Dark brown solid
CC1	Hexane : Ethyl acetate	90 : 10	60	0.20	Yellow crystal
CC2	Hexane : Ethyl acetate	50 : 50	180	0.09	Brown oil
CC3	Hexane : Ethyl acetate	20 : 80	40	0.03	Dark brown oil

Fraction BA was separated into 2 fractions. Fraction CB was separated into 4 fractions. Fraction CC was separated into 3 fractions. Each fraction was subjected to cytotoxicity activity by MTT assay. The results of cytotoxicity activity testing are shown in Table 22.

Table 22 Cytotoxic activity of separation of BA, CB and CC fractions against human cancer cell lines

Fraction code	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
BA1	13.45 ± 0.05	15.08 ± 0.09	27.11 ± 0.10	33.20 ± 0.10	35.45 ± 0.03
BA2	50.98 ± 0.03	54.23 ± 0.05	55.45 ± 0.09	52.32 ± 0.04	51.23 ± 0.05
CB1	17.70 ± 0.12	18.59 ± 0.07	31.54 ± 0.04	35.23 ± 0.03	32.21 ± 0.07
CB2	65.12 ± 0.09	67.32 ± 0.09	71.11 ± 0.08	82.33 ± 0.05	80.89 ± 0.09
CB3	74.67 ± 0.10	80.87 ± 0.05	89.37 ± 0.15	91.10 ± 0.05	93.23 ± 0.09
CB4	84.03 ± 0.05	88.00 ± 0.12	90.88 ± 0.09	90.17 ± 0.06	90.90 ± 0.10
CC1	10.06 ± 0.05	11.10 ± 0.05	20.04 ± 0.15	25.00 ± 0.07	24.11 ± 0.04
CC2	59.23 ± 0.08	58.90 ± 0.05	52.14 ± 0.09	69.67 ± 0.09	79.05 ± 0.09
CC3	60.04 ± 0.07	61.40 ± 0.03	60.99 ± 0.09	75.20 ± 0.03	74.39 ± 0.05

Fractions BA1, CB1 and CC1 were toxic to cancer cells. Fraction BA1, the percent cell survival was in the range of 13.45-35.45 percent. Fraction CB1, the percent cell survival was in the range of 17.70-35.23 percent. Fraction CC1, the percent cell survival was in the range of 10.06-25.00 percent. These promising results prompted us to isolate bioactive constituents from these fractions.

Fractions BA1, CB1 and CC1 were further fractionated using silica gel column chromatography and by initially eluting it with hexane. The polarity of eluent was gradually increased from a low portion of ethyl acetate in hexane to 50% ethyl acetate in hexane. Fractions (10 ml) were collected and combined according to their TLC patterns to yield of many fractions as shown in Table 23.

Table 23 Separation of BA1, CB1 and CC1 fractions

Fraction code	Solvent system		Volume (ml)	Weight (g)	Characteristic of fraction
BA1a	Hexane : Ethyl acetate	80 : 20	50	0.05	Dark orange crystal
BA1b	Hexane : Ethyl acetate	50 : 50	20	0.02	Light orange solid
CB1a	Hexane : Ethyl acetate	90 : 10	50	0.05	colorless crystalline solid
CB1b	Hexane : Ethyl acetate	80 : 20	10	0.01	Light yellow oil
CC1a	Hexane : Ethyl acetate	90 : 10	15	0.15	Dark orange solid
CC1b	Hexane : Ethyl acetate	50 : 50	30	0.03	Light orange solid

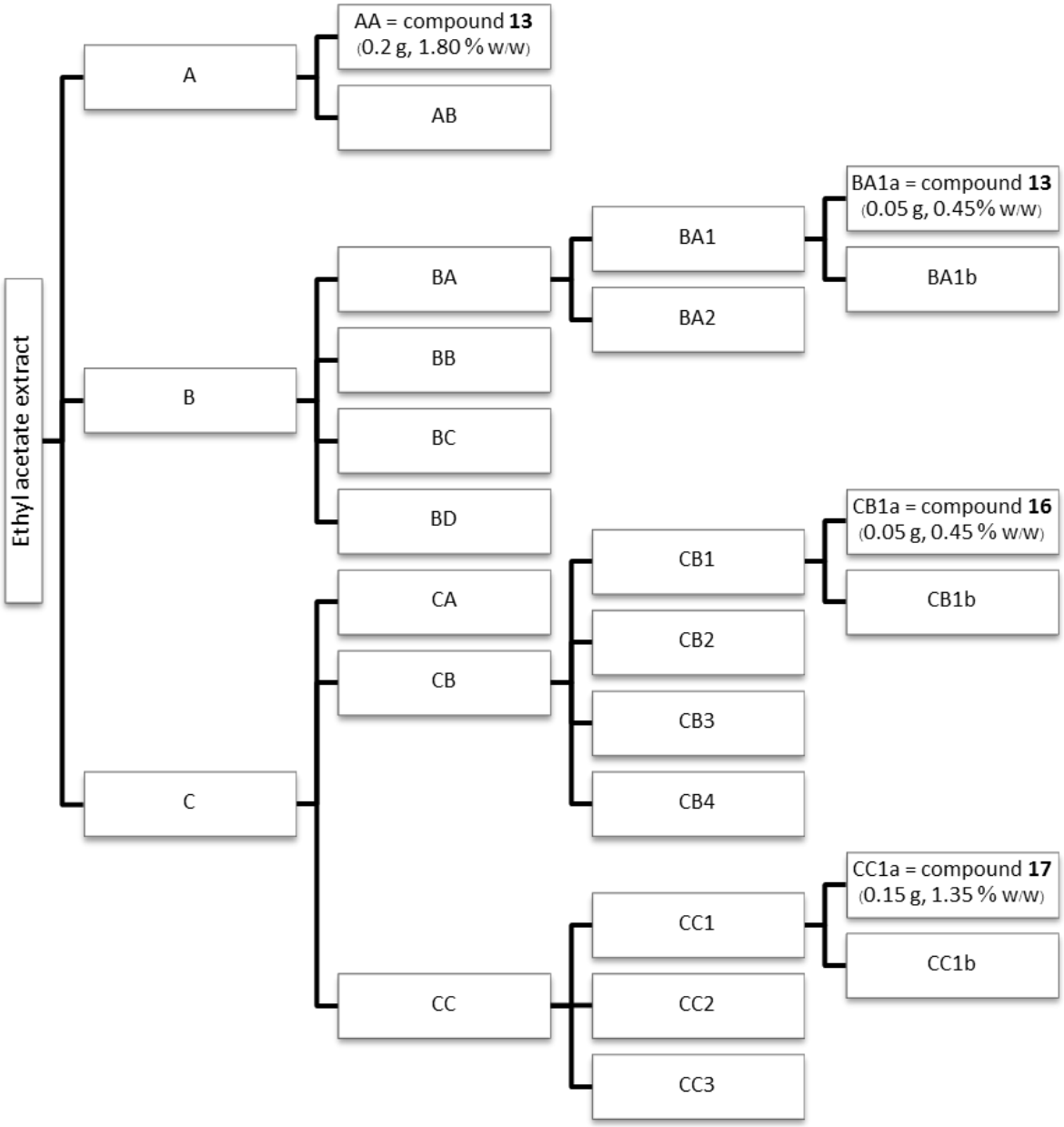
Fractions BA1a, CB1a and CC1a were pure compounds and obtained 0.50 g of dark orange crystal, 0.05 g of colorless crystalline solid, 0.15 g of dark orange solid, respectively. Each fraction was subjected to cytotoxicity activity by MTT assay. The results of cytotoxicity activity testing are shown in Table 24.

Table 24 Cytotoxic activity of separation of BA1, CB1 and CC1 fractions against human cancer cell lines

Fraction code	Percent cell survival (%)				
	SW620	Chago	BT474	KATO-III	Hep-G2
BA1a	2.33 ± 0.05	4.10 ± 0.07	6.05 ± 0.08	3.18 ± 0.10	5.07 ± 0.05
BA1b	51.23 ± 0.03	65.23 ± 0.05	66.15 ± 0.09	66.32 ± 0.05	71.23 ± 0.04
CB1a	39.70 ± 0.10	48.19 ± 0.07	41.64 ± 0.04	32.11 ± 0.04	40.11 ± 0.07
CB1b	75.82 ± 0.10	60.32 ± 0.09	70.90 ± 0.09	81.33 ± 0.05	83.89 ± 0.09
CC1a	2.07 ± 0.01	3.07 ± 0.05	6.07 ± 0.09	11.10 ± 0.07	5.03 ± 0.03
CC1b	84.61 ± 0.05	88.34 ± 0.12	90.48 ± 0.09	91.97 ± 0.06	89.70 ± 0.10

Fractions BA1a, CB1a and CC1a were toxic to human cancer cells. Fraction BA1a, the percent cell survival was in the range of 2.33-5.07 percent. Fraction CB1a, the percent cell survival was in the range of 32.11-48.19 percent. Fraction CC1a, the percent cell survival was in the range of 2.07-11.10 percent.

Isolated 3 compounds from ethyl acetate extracts, fractions AA and BA1a were compound **13**, fraction CB1a was compound **16** and fraction CC1a was compound **17.** Isolation of ethyl acetate extract of roots of *P. indica* was briefly summarized in Scheme 3.



Scheme 3 Isolation of ethyl acetate extract of roots of *P. indica*

Scheme 3, fraction AA was compound **13** which separated from ethyl acetate extract to give 0.20 g (1.80 % w/w of ethyl acetate extract). Fraction BA1a was compound **13** which separated from ethyl acetate extract to give 0.05 g (0.45 % w/w of ethyl acetate extract). Fraction CB1a was compound **16** which separated from ethyl acetate extract to give 0.05 g (0.45 % w/w of ethyl acetate extract). Fraction CC1a was compound **17** which separated from ethyl acetate extract to give 0.15 g (1.35 % w/w of ethyl acetate extract).

6. Results of Isolated Compounds for Cytotoxicity Activity

Isolated compounds were tested for cytotoxic activity towards 7 cell lines; SW620, Chago, BT474, KATO-III, Hep-G2, Wi-38 and CCD-986Sk. The *in vitro* activity of all compounds was performed by the MTT test. Stock solution was diluted to test concentration (0.0001, 0.001, 0.01, 0.1 and 1 μg/ml) with 100% DMSO. Cell lines deaths were observed, at 72 hours intervals, and used for calculation of IC_{50}.

Table 25 Cytotoxicity activity of isolated compounds against cell lines at 72 hours intervals

Isolated compound	IC_{50} (μg/ml)						
	BT474	Chago	KATO-III	SW620	Hep-G2	Wi38	CCD-986Sk
Compound **13**	0.31 ± 0.12	0.56 ± 0.12	0.0016±0.00	0.56 ± 0.23	0.35 ± 0.10	0.94 ± 0.09	0.98 ± 0.12
Compound **14**	0.92 ± 0.24	0.85 ± 0.22	0.42 ± 0.12	0.54 ± 0.21	0.41 ± 0.18	0.88 ± 0.17	0.99 ± 0.24
Compound **15**	0.91 ± 0.10	0.94 ± 0.28	0.51 ± 0.19	0.81 ± 0.22	0.82 ± 0.15	0.99 ± 0.13	0.93 ± 0.22
Compound **16**	0.43 ± 0.25	0.93 ± 0.27	0.28 ± 0.09	0.76 ± 0.31	0.50 ± 0.17	0.67 ± 0.17	0.85 ± 0.20
Compound **17**	0.13 ± 0.07	0.10 ± 0.02	0.14 ± 0.02	0.11 ± 0.09	0.10 ± 0.02	0.90 ± 0.01	0.83 ± 0.19
Doxorubicin	0.57 ± 0.13	0.82 ± 0.30	0.76± 0.22	0.17 ± 0.03	0.48 ± 0.19	1.53 ± 0.13	1.07 ± 0.30

All isolated compounds showed strong cytotoxicity against human cancer cell lines. Compound **13** showed stronger activity than doxorubin against human cancer cell lines except SW620. Compound **14** showed stronger activity than doxorubicin against KATO-III and Hep-G2. Compound **15** showed stronger activity than doxorubicin against KATO-III. Compound **16** showed stronger activity than doxorubicin against BT474 and KATO-III. Compound **17** showed the strongest activity against BT474, Chago, SW620 and Hep-G2 cancer cell lines with IC_{50} 0.13 ± 0.07, 0.10 ± 0.02, 0.11 ± 0.09 and 0.10 ± 0.02 μg/ml, respectively. Compound **13** showed the strongest activity against KATO-III cancer cell line with IC_{50} 0.0016 ± 0.001 μg/ml less than IC_{50}

of doxorubicin 475 times. The results indicated that compounds **17** extracted from roots of *P. indica* may be developed to be human gastric carcinoma drug in the future.

7. Structure Elucidation of Isolated Compounds from Roots of *P. indica*

7.1 Structure Elucidation of Compound 13

Compound **13** was obtained from hexane and ethyl acetate extracts. It was yellow needle shaped crystals and obtained 2.4 g (2.15 g from hexane extract and 0.25 g from ethyl acetate extract, 0.06% w/w of dried weight) with melting point of 70-72 ^{o}C, compound **13** in methanol showed UV absorption maxima at 420 nm.

^{1}H-NMR spectrum of compound **13** ($CDCl_3$, Figure 20) showed signal at δ2.19 ppm (s, CH_3) which was the methyl protons that was located at C-11 (Figure 17). The signal at δ6.81 ppm (s, 1H) indicated the presence of olefinic proton which could be placed at C-3 in association with a C-2-C-3 double bond (Figure 17). The olefinic protons of aromatic ring at δ7.26 ppm (d, 1H), δ7.59 ppm (d, 1H) and δ7.64 ppm (d, 1H) were the signal of protons at C-6, C-8 and C-7, respectively (Figure 17). The signal at δ11.97 ppm indicated the presence of proton of hydroxyl group that attach to C-5 and form hydrogen bond with the carbonyl group at C-4 (Figure 17).

^{13}C-NMR spectrum of compound **13** ($CDCl_3$, Figure 21) showed 11 carbons resonances, eight of which were olefinic carbons at δ115.1, δ119.3, δ124.1, δ132.0, δ135.4, δ136.1, δ149.6 and δ161.1 corresponding with C-10, C-8, C-6, C-9, C-3, C-7, C-2 and C-5, respectively (Figure 17). The chemical shift of CH_3 at δ16.5 ppm and the signal arising from the carbonyl groups appeared at δ184.7 ppm and δ190.2 ppm.

The combination of spectroscopic data lead to compare that compound **13** could be plumbagin or 5-hydroxy-2-methyl-1,4-naphthoquinone ($C_{11}H_8O_3$). Plumbagin was previously isolated from *P. indica* (Vijver, 1972). The ^{1}H and ^{13}C-NMR chemical shifts of compound **13** were compared with that of plumbagin to confirm the structure (Table 26).

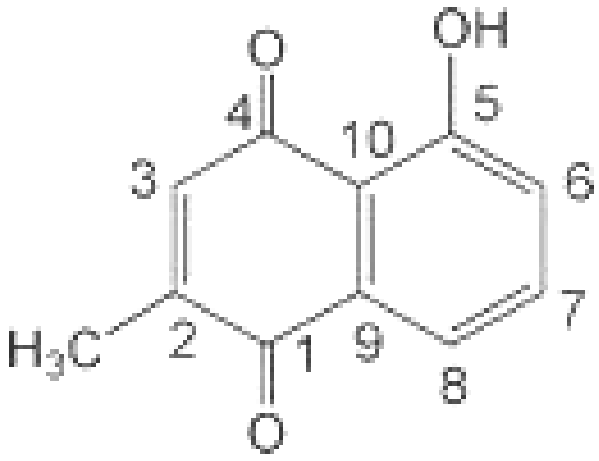


Figure 17 Structure of plumbagin

Table 26 ^{1}H and ^{13}C-NMR chemical shift of compound 13 compared to those of plumbagin (Chen *et al*., 2010)

Position	Chemical shift δ_C (ppm)			
	Compound **13**		plumbagin	
	^{1}H	^{13}C	^{1}H	^{13}C
1		184.7		184.8
2		149.6		149.6
3	6.81 (s)	135.4	6.83 (s)	136.1
4		190.2		190.3
5		161.1		161.2
6	7.26 (d, *J* = 8.2 Hz)	124.1	7.28 (d, *J* = 8.5 Hz)	124.2
7	7.64 (d, *J* = 7.4 Hz)	136.1	7.66 (d, *J* = 7.5 Hz)	135.5
8	7.59 (d, *J* = 6.1 Hz)	119.3	7.60 (d, *J* = 6.2 Hz)	119.3
9		132.0		132.0
10		115.1		115.2
11	2.19 (s)	16.5	2.22 (s)	16.5
OH	11.97 (s)		11.99 (s)	

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) was isolated from Chinese medicinal plants such as the Plumbaginaceae, Droseraceae and Ebenaceae. Plumbagin was reported to have various pharmacological activities including antiinflammatory, antibacterial, antifungal activities *in vitro* and *in vivo*, (Parimala and Sachdanandam, 1993) anti-cancer, (Itoigawa *et al*. 1991) cardiotonic (Bhargava, 1984) and antifertilityaction (Padhye *et al*., 2012). Plumbagin also exhibited antiproliferative effect in various cancer cell lines via intracellular reactive oxygen species (ROS) generation, apoptosis induction and cell cycle arrest but the mechanism was not understood (Tian *et al*., 2012). Moreover, plumbagin induced cell apoptosis via modulation of cellular redox status and generation of ROS (Powolny and Singh, 2008) in human prostate cancer cells, (Qiu *et al*., 2013), tongue carcinoma cells (Xu and Lu, 2010) and lung cancer cells (Shieh *et al*., 2010). Plumbagin inhibited invasion and migration of breast and gastric cancer cells through down-regulation of the expression of chemokine receptor CXCR (Manu *et al*., 2011). According to the mechanism of action of the plumbagin, its up-regulated the expression of p53 and $p21^{CIP1/WAF1}$ causing cell cycle arrest in the G2/M-phase by down-regulating G2/M regulatory proteins (cyclinB1 and Cdc25B) in H460 cells (Gomathinayagam *et al*., 2008). Furthermore, it activated the JNK/p38 signaling, leading to caspase-3 activation resulting in the induction of apoptosis.

In this study, plumbagin showed strong activity against human cancer cell lines (Table 25). This result is in agreement with a previous report (Wang *et al*., 2008) indicated that plumbagin could also induce cell cycle arrest in human melanoma A375.S2 cells.

7.2 Structure Elucidation of Compound 14

Compound **14** was isolated from hexane extract. It was white solid (1.0 g, 0.025% w/w) with melting point of 134-136 ^{o}C, compound **14** in EtOH showed UV absorption maxima at 205 nm.

^{1}H-NMR spectrum of compound **14** ($CDCl_3$, Figure 22) showed signals at δ 0.68-1.30 ppm which were the signals of methyl protons that located at C-28, C-29 and at side chain of the steroidal compounds, C-19, C-24, C-26 and C-27 (Figure 18). The signals at δ 1.50-1.77 ppm indicated the presence of proton which could be placed at C-1, C-2, C-8, C-16 and C-12 (Figure 18). The signal at δ 3.51 ppm (m, 1H) was the signal of proton at C-3 and that at δ 5.39 ppm (m, 1H) could be assigned to H-6 which the olefinic proton of cyclic (Figure 18).

^{13}C-NMR spectrum of compound **14** ($CDCl_3$, Figure 23) showed 29 carbons resonances, two of which were olefinic carbons at δ 140.8 ppm and δ 121.7 ppm corresponding with C-5 and C-6 (Figure 18). The signals appeared at δ 71.8 ppm was signal of carbon attached to heteroatom, C-3. The signals between δ 11.9-56.8 ppm were signals of sp^3 carbons. The signals of compound **14** that were close to signals from **β**-sitosterol which were similar to literature values (Ingkaninan, 1994) as shown in Table 27.

The combination of spectroscopic data lead to compare that compound **14** could be **β**-sitosterol or 17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-cyclopenta[α]phenanthren-3-ol ($C_{29}H_{50}O$). The ^{13}C-NMR chemical shift of compound **14** was compared with that of **β**-sitosterol to confirm the structure (Table 27).

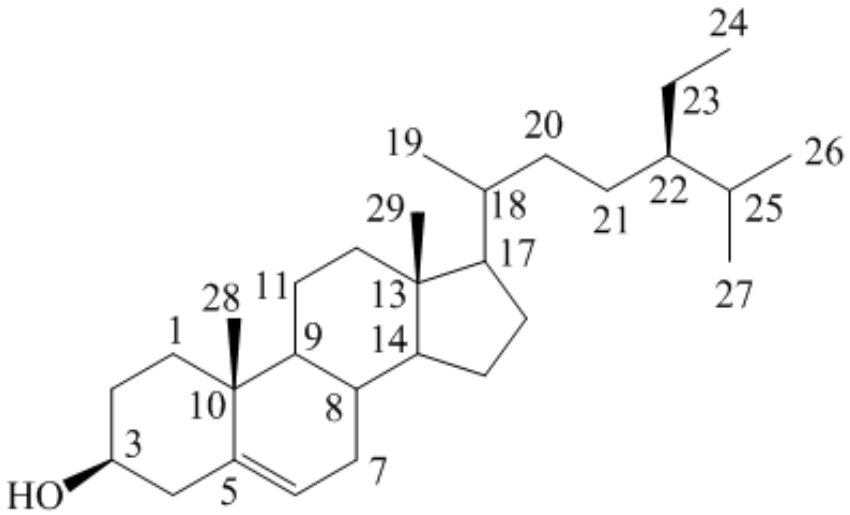


Figure 18 Structure of β-sitosterol

Table 27 ^{13}C-NMR chemical shift of compound 14 compared to those of *β*-sitosterol (Sosinska *et al*., 2013)

Position	Chemical shift δ_C (ppm)	
	Compound **14**	*β*-sitosterol
1	37.3	37.3
2	31.7	31.5
3	71.8	71.7
4	42.3	42.2
5	140.8	140.8
6	121.7	121.7
7	31.9	31.8
8	31.9	31.8
9	50.1	50.1
10	36.5	36.5
11	21.1	21.1
12	39.8	39.8
13	45.8	42.2
14	56.8	56.7
15	24.3	24.3
16	28.2	28.3
17	56.1	56.0
18	36.1	36.1
19	18.8	18.7
20	34.0	33.9
21	26.1	26.0
22	50.1	45.8
23	23.1	23.0
24	11.9	12.0
25	29.2	29.1
26	19.4	19.0
27	19.0	19.8
28	19.8	19.3
29	11.9	11.9

β-Sitosterol, 17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12, 14,15,16,17-dodecahydro-1*H*-cyclopenta[*α*]phenanthren-3-ol, is the main dietary phyto- sterol in plants and has been shown to inhibit proliferation and induce apoptosis in human solid tumors such as colon and breast cancers (Park, 2008). It was to examine the anti-cancer activity on HT-29 cells, a human colon cancer cell line (Awad *et al*., 1996). The study indicated that 8 and 16 μM **β**-sitosterol were effective at cell growth inhibition after supplementation with 16 μM **β**-sitosterol for 9 days, cell growth was only one-third that of cells supplemented with equimolar concentration of cholesterol. The previous study was designed to examine the effect of the two most common dietary phytosterols, **β**-sitosterol and campesterol, as compared to cholesterol, on growth, apoptosis and cytotoxicity of MDA-MB-231 human breast cancer cells in culture (Awad *et al*., 2000). Breast cancer cell growth was found to be inhibited by 66% after 3 days and 80% after 5 days with 16 μM **β**-sitosterol. **β**-Sitosterol supplementation for 3 days at 16 μM resulted in a 6-fold increase in apoptosis in cells when compared to cholesterol treated cells. **β**-Sitosterol treatment was found to have no effect on the level and content of tumor cell PP2A.

In comparison the cytotoxicity activity of **β**-sitosterol which isolated from other plants against human cancer cells, *in vitro* study was to evaluate the inhibitory effect of different cocoa polyphenols extracts, alone or combined with **β**-sitosterol, on two human prostate cancer cell lines (nonmetastatic 22Rv1 cells and metastatic DU145 cells) and a normal human prostate cell line (RWEP-1) (Jourdain *et al*., 2006). Cocoa polyphenols extracts were more active against local cancer cells than against metastatic cells. Moreover, **β**-sitosterol induced low growth inhibition of both cancer cell line. Cocoa polyphenols extracts, however, were significantly more active and showed a strong and fast inhibition of cell growth than **β**-sitosterol alone.

β-Sitosterol induced cytotoxicity and apoptosis in leukemic U937 cells (Park *et al.*, 2007). The increase in apoptosis induced by **β**-sitosterol was associated with down-regulation of Bcl-2, degradation of poly-(ADP-ribose) polymerase (PARP) and phospholipase C (PLC)-gamma1 protein, and activation of caspase-3. **β**-Sitosterol induced apoptosis was not associated with changes in the expression of Bcl-xL, Bax, or inhibitor of apoptosis proteins (IAPs). z-DEVD-fmk, a caspase-3 specific inhibitor, blocked caspase-3 activation and PARP degradation, and significantly attenuated **β**-sitosterol-induced apoptosis. This suggested that caspase-3 activation was partially essential for **β**-sitosterol-induced apoptosis. Bcl-2 overexpression also significantly blocked caspase-3 activation and the decrease in PARP cleavage by **β**-sitosterol, and

effectively attenuated the apoptotic response to **β**-sitosterol. These results showed that **β**-sitosterol potently induced apoptosis in U937 cells and **β**-sitosterol-induced apoptosis was related to the selective activation of caspase-3 and induction of Bax/Bcl-2 ratio.

7.3 Structure Elucidation of Compound 15

Compound **15** was isolated from hexane extract. It was orange crystalline solid (0.04 g, 0.001% w/w) with melting point of 166-167 ^{o}C. Compound **15** in CH_3OH showed absorption maxima at 413 nm**.**

^{1}H-NMR spectrum of compound **15** ($CDCl_3$, Figure 24) showed signals at δ 11.1 ppm (s, 1H) which was the signal of the exchangeable with hydrogen bond chelated the hydroxyl group. The signals at δ 7.6-7.7ppm (dd, 3H) and δ 7.1 ppm (d, 1H) were the olefinic protons of aromatic compound. The signal at δ 2.1 ppm (s, 3H) may be the signal of methyl protons. The signal at δ 0.9 ppm (m, 3H) and δ 2.3 ppm (m, 1H) may be the signal of protons attached to sp^3 carbon atoms.

^{13}C-NMR spectrum of compound **15** ($CDCl_3$, Figure 25) showed signals at δ 184.5 ppm was the signal of carbonyl groups. The signals at δ 161.2, 152.8, 137.5, 132.7, 123.2, 121.8 and 119.7 were the signals of sp^2 carbon of -CH- and -C-. The signals at δ 10.0-42.0 ppm were the chemical shift of sp^3 carbons. From ^{1}H and ^{13}C spectra, the main structure of this compound look like core structure of plumbagin (compound **13**) but the substituted group could not identified. The combination of spectroscopic data could not identify the actual structure of compound **15**. More spectroscopic data and mass spectrum need to elucidate this compound.

7.4 Structure Elucidation of Compound 16

Compound **16** was isolated from ethyl acetate extract. It was colorless crystalline solid (0.05 g, 0.00125% w/w) with melting point of 255-256 ^{o}C. Compound **16** in EtOH showed absorption maxima at 335 nm.

^{1}H-NMR spectrum of compound **16** ($CDCl_3$, Figure 26) showed signals at δ 1.17 ppm (d, 3H) which was the methyl protons that was located at C-11 (Figure 19). The signal at δ 2.44 (m, 1H) which was the proton at C-2 (Figure 19). The signals at δ 6.92**,** δ 6.94 and δ 7.48 indicated the presence of aromatic protons at C-6, C-8 and C-7, respectively (Figure 19). The signal at δ 12.41 ppm (s, 1H) which was the signal of the exchangeable with hydrogen bond chelated hydroxyl group at C-5 (Figure 19).

^{13}C-NMR spectrum of compound **16** ($CDCl_3$, Figure 27) showed the chemical shift of CH_3 at δ 16.2 ppm and the signals at δ 34.4 and 40.7 ppm were the presence of C-2 and C-3 (Figure 19). The signal at δ 114.9, 118.2, 118.6 and 136.9 ppm indicated the presence of olefinic carbon which could be placed at C-5, C-9, C-7 and C-6, respectively (Figure 19). The chemical shift at δ 204.7 ppm was the signal arising from the carbonyl groups at C-4. The signals appeared at δ 71.1 ppm and δ 145.0 ppm were the presence of C-1 and C-8 (Figure 19).

The combination of spectroscopic data lead to compare that compound **16** could be isoshinanolone or (3*R*,4*R*)-4,8-dihydroxy-3-methyl-3,4-dihydro-2*H*-naphtha len-1-one ($C_{11}H_{12}O$) which was isolated from *Dioncophyllum thollonii* (Tezuka *et al*., 1973). The ^{1}H and ^{13}C-NMR chemical shifts of compound **16** were compared with that of isoshinanolone to confirm the structure (Table 28).

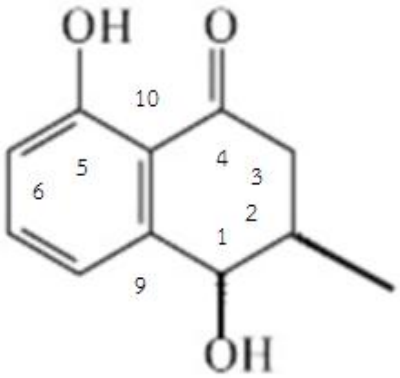


Figure 19 Structure of isoshinanolone

Table 28 ^{1}H **and** ^{13}C**-NMR chemical shift of compound 16 compared to isoshinanolone (Bringmann *et al.*, 1999)**

Position	Chemical shift δ_C (ppm)			
	Compound **16**		Isoshinanolone	
	^{1}H	^{13}C	^{1}H	^{13}C
1	4.74 (d, *J* = 2.7 Hz)	71.1	4.75 (d, *J* = 2.5 Hz)	71.2
2	2.44 (m)	34.4	2.44 (m)	34.4
3	2.57 (ddd, *J* = 17.4, 4.5, 0.9 Hz) 2.86 (dd, *J* = 17.7, 11.1 Hz)	40.7	2.56 (ddd, *J* = 17.7, 4.3, 0.9 Hz) 2.87 (dd, *J* = 17.7, 11.0 Hz)	40.7
4		204.7		204.7
5		114.9		114.9
6	6.92 (d, *J* = 7.2 Hz)	136.9	6.92 (d, *J* = 7.3 Hz)	136.9
7	7.48 (dd, *J* = 8.4, 7.5 Hz)	136.9	7.48 (dd, *J* = 8.3, 7.3 Hz)	136.9
8	6.94 (dd, *J* = 8.5, 1.2 Hz)	145.0	6.94 (dd, *J* = 8.5, 1.2 Hz)	145.0
9		118.2		118.2
10		162.7		162.7
11	1.17 (d, *J* = 6.9 Hz)	16.2	1.19 (d, *J* = 6.7 Hz)	16.1
C_5-OH	12.41 (s)		12.42 (s)	

Isoshinanolone constitutes an acetogenic natural tetralone of wide-spread occurrence in various plant families, like Iridaceae, Plumbaginaceae, Nepenthaceae, Dioncophyllaceae, Ancistrocladaceae and Ebenaceae (Bringmann *et al*., 1998). In Ancistrocladaceae and Dioncophyllaceae, isoshinanolone and its obvious naphtha quinone precursor, plumbagin, may be formed in major amounts under various conditions of chemical, physical, and biotic stress (Bringmann *et al*., 1998) apparently as an alternative to the normally predomnant formation of acetogenic naphthyliso quinoline alkaloids (Bringmann and Pokorny, 1995).

Trans-isoshinanolone and its *cis*-isomer are widely distributed naturally occurring tetralones isolated from a variety of different plant families, biosynthetically formed via the acetate-malonate pathway (Bringmann *et al*., 1998). The isolating, separating and evaluating the antimicrobial properties of compounds from the root extract of *P. zeylanica*, *trans*-isoshinanolone is more active with a MIC of 12.5-25 µg/ml whereas neoisoshinanolone was recorded a MIC of 50-100 µg/ml (Jetty *et al*., 2010). The activities were compared with plumbagin (0.78-3.13 µg/ml) and standards streptomycin for bacteria and nystatin for fungi. Recently study showed that isoshinanolone and plumbagin were tested for their mosquito larvicidal activity against fourth instar larvae of *Aedes aegypti*. Isoshinanolone and plumbagin showed excellent toxicity with LC_{50} values of 1.26 and 5.43 µg/ml (Sreelatha *et al*., 2010).

7.5 Structure Elucidation of Compound 17

Compound **17** was isolated from ethyl acetate extract. It was orange solid (0.15 g, 0.00375% w/w) with melting point of 176-178 ^{o}C. Compound **17** in EtOH showed absorption maxima at 400 nm.

^{1}H-NMR spectrum of compound **17** ($CDCl_3$, Figure 28) showed signals at δ 2.01 ppm (d, *J* = 1.5 Hz, 3H) which were the methyl protons**.** The signal at δ 6.81 ppm (q, *J* = 1.5 Hz, 1H) was the signal of the olefinic protons. The signals at δ7.20 ppm (d, *J* = 8.7 Hz, 1H) and 7.30 ppm (d, *J* = 8.7 Hz, 1H) were the signals of the olefinic protons of aromatic compound. The signal appeared at δ 12.58 ppm (s, 2H) was the signal of the exchangeable with hydrogen bond chelated hydroxyl group.

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^{13}C-NMR spectrum of compound **17** ($CDCl_3$, Figure 29) showed 11 carbons resonances, the signal arising from the signal of sp^2 carbon of –CH- and –C- appeared at δ 115.56, 124.27, 128.38, 134.95, 135.55, 138.02, 150.06 and 161.45 ppm. The chemical shift of CH_3 at δ 16.55 ppm and the signal arising from the carbonyl groups in the compound **17** appeared at δ 185.18 ppm and δ 190.51 ppm. All of the proton-proton spin systems were traced by using data from a COSY experiments (Figure 30). Heteronuclear single quantum correlation experiment, HMBC (Figure 31) and HSQC (Figure 32) allowed unambigouous assignments of all the ^{1}H and ^{13}C resonances in the compound **17**. The COSY data (Figure 30) was showed methyl proton at δ 2.01 ppm correlation with the olefinic protons at δ 6.81 ppm. The HSQC data was indicated that the compound **17** having the signal of methyl proton at δ 2.01 ppm correlation with the chemical shift of carbon at δ 16.55 ppm (Figure 32). The signal of the olefinic protons at δ 6.81 ppm correlated with the olefinic carbon at δ 134.95 ppm (Figure 32). The olefinic protons of aromatic compound at δ 7.20 and 7.30 ppm correlation with the chemical shift of carbon at δ 138.02 and 124.27 ppm, respectively (Figure 32). The HMBC data showed the signal of methyl proton at δ 2.01 ppm correlation with the chemical shift of carbon at δ 134.95, 150.06 and 185.18 ppm (Figure 31). The signal of olefinic protons at δ 6.81 ppm correlated with the signal of carbon at δ 16.55 and 185.18 ppm (Figure 31). The signals of the olefinic protons of aromatic compound at δ 7.20 ppm correlation with the signal of carbon at δ 128.38 and 161.45 ppm (Figure 31). The olefinic protons of aromatic compound at δ 7.30 ppm correlated with the signal of carbon at δ 115.56 and 134.95 ppm (Figure 31). The signals of the exchangeable with hydrogen bond chelated hydroxyl group at δ 12.58 ppm correlation with the signal of carbon at δ 124.27 and 161.45 ppm (Figure 31).

From all data, the main structure of this compound looks like core structure of plumbagin (compound **13**). The combination of spectroscopic data could identify the compound **17** has two carbonyl groups, methyl group and two hydroxyl groups. More spectroscopic data and mass spectrum need to elucidate this compound. In this study, compound **17** showed strongest activity against BT474, Chago, SW620 and Hep-G2 cancer cell lines with IC_{50} 0.13 ± 0.07, 0.10 ± 0.02, 0.11 ± 0.09 and 0.10 ± 0.02 µg/ml, respectively.

CHAPTER V
CONCLUSION

Cytotoxicity effects of methanol and water extracts from plants of Triphala, Benjakul and Benjalokwichian recipes were evaluated cytotoxicity activity on 5 types of cancer cells; colon cancer (SW620), lung cancer (Chago), breast cancer (BT474), gastric cancer (KATO-III) and liver cancer (Hep-G2). Almost of methanol extracts of tested plants inhibited the growth of human cancer cell lines with the percentage growth lower than water extracts. Twenty plant extracts were toxic to colon cancer cell line (SW620); methanol extracts of *C. micracantha*, *T. triandra*, *T. belleria*, *F. racemosa*, *P. interruptum*, *H. perforate*, *C. petasites*, *T. chebula*, *P. emblica*, *P. retrofractum, P. sarmentosum, Z. officinale* and *P. indica* with the percentage growth were 25.06 ± 0.01, 14.22 ± 0.00, 20.96 ± 0.00, 14.22 ± 0.00, 13.49 ± 0.00, 20.96 ± 0.00, 35.42 ± 0.04, 21.93 ± 0.02, 46.41 ± 0.05, 21.24 ± 0.07, 12.66 ± 0.01, 15.33 ± 0.07 and 11.67 ± 0.01 percent, respectively and water extracts of *T. triandra*, *T. belleria*, *F. racemosa*, *H. perforate*, *T. chebula*, *P. emblica* and *P. sarmentosum* with the percentage growth were 28.92 ± 0.05, 18.55 ± 0.00, 30.84 ± 0.02, 30.36 ± 0.10, 16.63 ± 0.00, 35.86 ± 0.10 and 40.37 ± 0.10 percent, respectively. Fifteen plant extracts were toxic to lung cancer cell line (Chago); methanol extracts of *T. triandra*, *T. belleria*, *F. racemosa*, *P. interruptum*, *H. perforate*, *C. petasites*, *T. chebula*, *P. retrofractum, P. sarmentosum, Z. officinale* and *P. indica* with the percentage growth were 10.81 ± 0.00, 25.95 ± 0.06, 14.05 ± 0.01, 20.90 ± 0.07, 18.02 ± 0.01, 34.05 ± 0.12, 17.66 ± 0.00, 13.01 ± 0.12, 19.23 ± 0.06, 6.67 ± 0.02 and 26.86 ± 0.13 percent, respectively and water extracts of *T. triandra*, *T. belleria*, *F. racemosa* and *H. perforate* with the percentage growth were 48.83 ± 0.04, 32.43 ± 0.07, 36.58 ± 0.10 and 44.68 ± 0.06 percent, respectively. Nine plant extracts were toxic to brest cancer cell line (BT474); methanol extracts of *T. triandra*, *F. racemosa*, *P. interruptum*, *H. perforate*, *C. petasites*, *P. retrofractum, P. sarmentosum, Z. officinale* and *P. indica* with the percentage growth were 41.05 ± 0.03, 32.11 ± 0.00, 31.05 ± 0.00, 44.74 ± 0.01, 45.79 ± 0.02, 34.40 ± 0.03, 28.37 ± 0.01, 27.30 ± 0.00 and 27.30 ± 0.00 percent, respectively. Thirteen plant extracts were toxic to gastric cancer cell line (KATO-III); methanol extracts of *T. triandra*, *T. belleria*, *F. racemosa*, *P. interruptum*, *H.*

perforate, *C. petasites*, *T. chebula*, *P. retrofractum, P. sarmentosum, Z. officinale* and *P. indica* with the percentage growth were 25.40 ± 0.00, 45.63 ± 0.03, 26.59 ± 0.00, 36.90 ± 0.02, 36.90 ± 0.00, 33.73 ± 0.01, 39.29 ± 0.01, 33.24 ± 0.14, 38.73 ± 0.06, 14.23 ± 0.01 and 17.32 ± 0.03 percent, respectively and water extracts of *T. triandra* and *P. emblica* with the percentage growth were 26.98 ± 0.01 and 32.11 ± 0.06 percent, respectively. Sixteen plant extracts were toxic to liver cancer cell line (Hep-G2); methanol extracts of *T. triandra*, *T. belleria*, *F. racemosa*, *P. interruptum*, *H. perforate*, *C. petasites*, *T. chebula*, *P. retrofractum, P. sarmentosum, Z. officinale* and *P. indica* with the percentage growth were 24.79 ± 0.00, 41.18 ± 0.00, 26.05 ± 0.00, 37.82 ± 0.03, 38.66 ± 0.01, 42.44 ± 0.01, 40.34 ± 0.01, 31.31 ± 0.09, 24.39 ± 0.04, 18.86 ± 0.01 and 15.74 ± 0.03 percent, respectively and water extracts of *C. micracantha*, *T. belleria*, *H. perforate*, *C. petasites* and *T. chebula* with the percentage growth were 39.50 ± 0.01, 46.21 ± 0.01, 42.44 ± 0.02, 31.09 ± 0.00 and 37.82 ± 0.01 percent, respectively. Methanol extract of *P. indica* root was toxic to colon cancer cell line and liver cancer cell line with the strongest cytotoxicity activity of cancer cells at 11.67 ± 0.01 and 15.74 ± 0.03 percent, respectively. Methanol extract of *Z. officinale* rhizome was toxic to lung cancer cell line and gastric cancer cell line with the strongest cytotoxicity activity of cancer cells at 6.67 ± 0.02 and 14.23 ± 0.01 percent, respectively. Methanol extracts of *Z. officinale* rhizome and *P. indica* root were toxic to breast cancer cell line with the strongest cytotoxicity activity of cancer cells at 27.30 ± 0.00 percent. Therefore, methanol extract of root of *P. indica* was further separated and purified for anti-cancer active compounds.

P. indica roots were extracted with methanol for cytotoxicity activity test; the obtained extract was separated by solvent extraction with hexane, ethyl acetate and water, respectively. Hexane and ethyl acetate extracts inhibited the growth of cancer cells with the strong cytotoxicity activity. These promising results prompted us to isolate bioactive constituents from these extracts. The chemical constituents found in the roots of *P. indica* from hexane and ethyl acetate extracts, were 5 compounds as 5-hydroxy-2-methyl-naphthalene-1,4-dione (plumbagin), 17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-cyclopenta[α]phenanthren-3-ol (β-sitosterol), unknown **15**, (3*R*,4*R*)-4,8-dihydroxy-3-methyl-3,4-

dihydro-2*H*-naphthalen-1-one (isoshinanolone) and unknown **17**. All of them showed strong cytotoxicity against cancer cell lines. Unknown **17** showed strongest activity against BT474, Chago, SW620 and Hep-G2 cancer cell lines with IC_{50} 0.13 ± 0.07, 0.10 ± 0.02, 0.11 ± 0.09 and 0.10 ± 0.02 µg/ml, respectively. Plumbagin showed stronger activity than doxorubin against human cancer cell lines except SW620. *β*-Sitosterol showed stronger activity than doxorubicin against KATO-III and Hep-G2. Unknown **15** showed stronger activity than doxorubicin against KATO-III. Isoshinanolone showed stronger activity than doxorubicin against BT474 and KATO-III. Plumbagin showed strongest activity against KATO-III cancer cell line with IC_{50} 0.0016 $\pm$ 0.001 µg/ml less than IC_{50} of doxorubicin 475 times. The results indicated that plumbagin extracted from roots of *P. indica* may be developed to be human gastric carcinoma drug in the future.

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APPENDICES

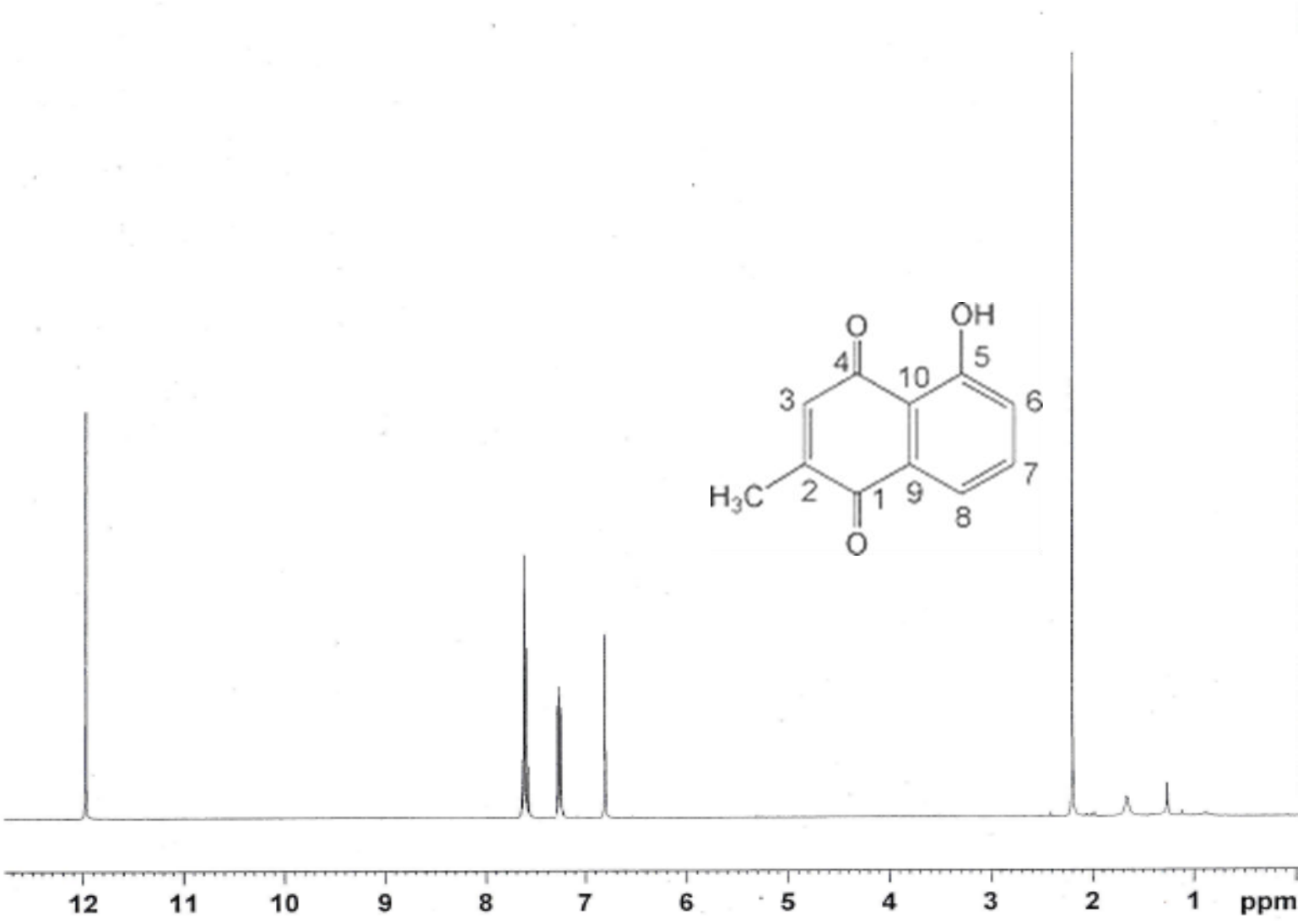

Figure 20 The ^{1}H-NMR spectrum of Compound 13

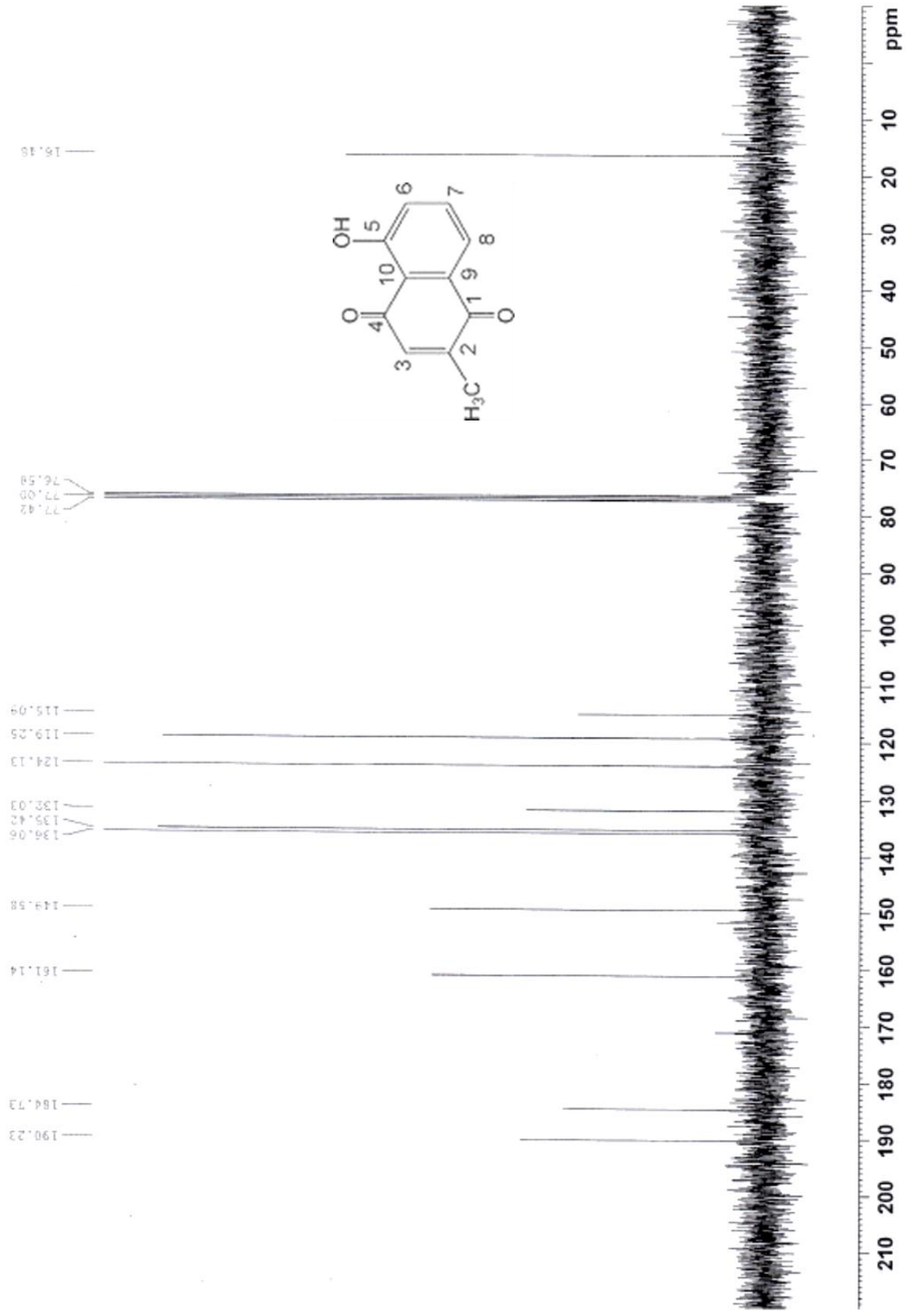

Figure 21 The ^{13}C-NMR spectrum of Compound 13

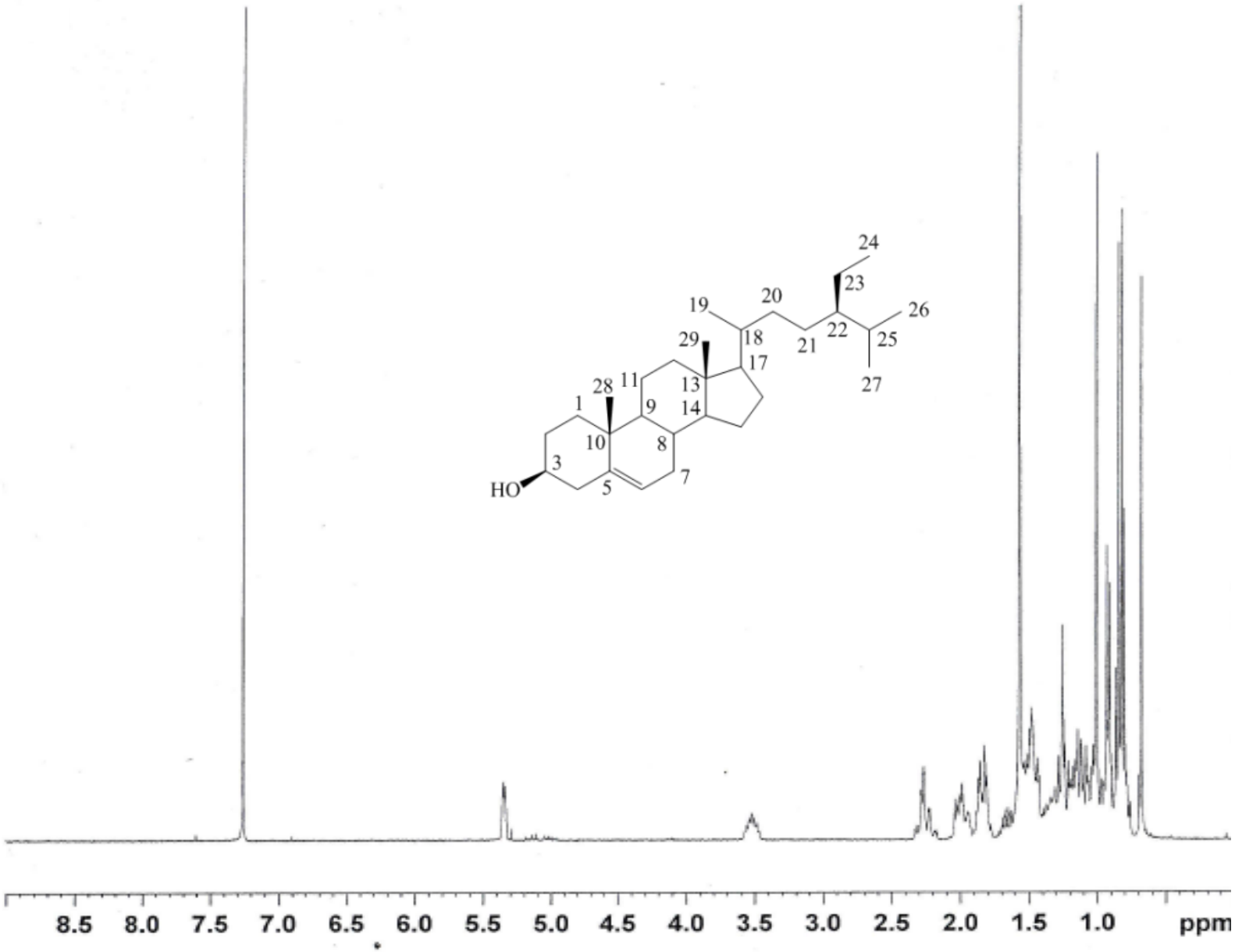

Figure 22 The ^{1}H-NMR spectrum of Compound 14

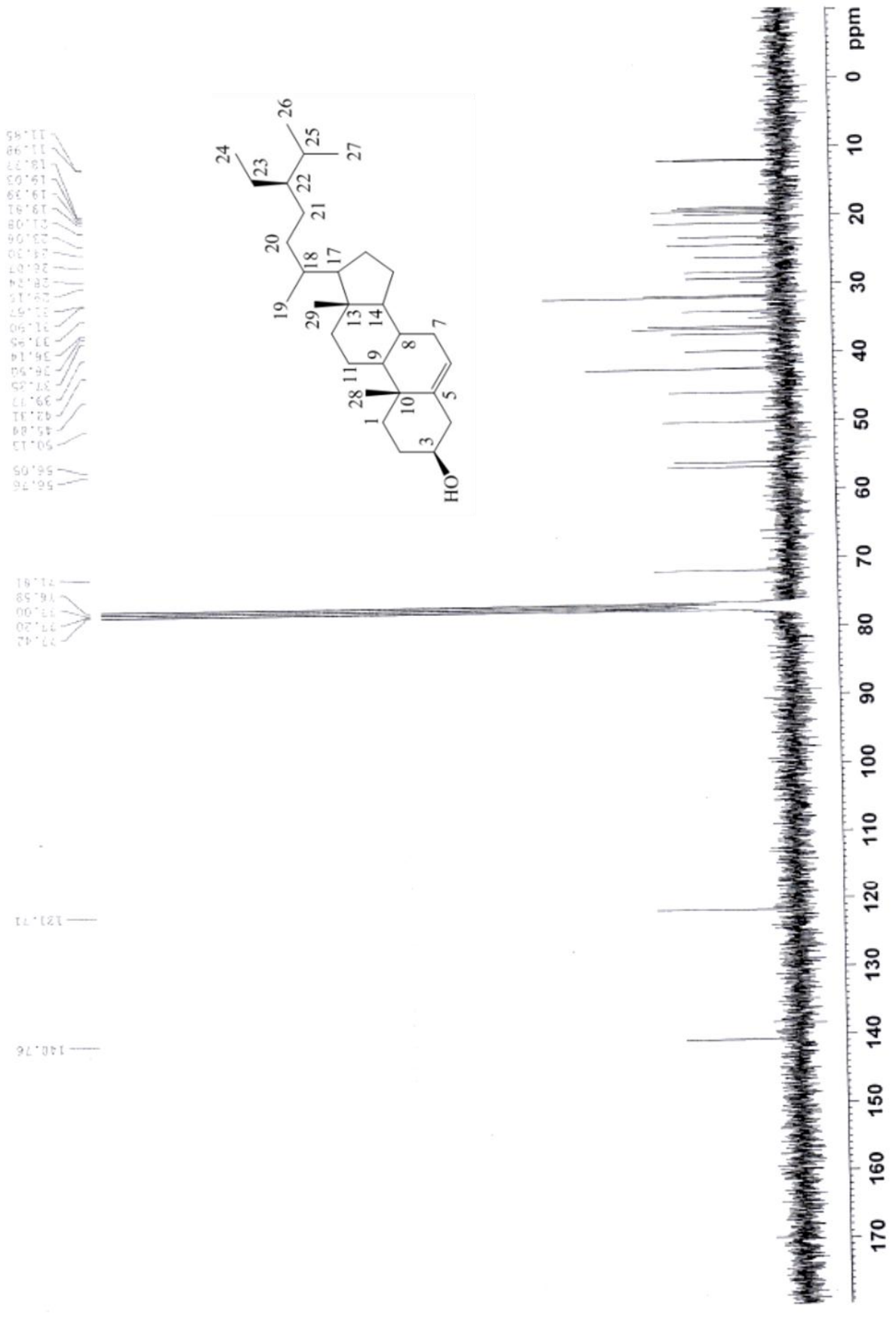

Figure 23 The ^{13}C-NMR spectrum of Compound 14

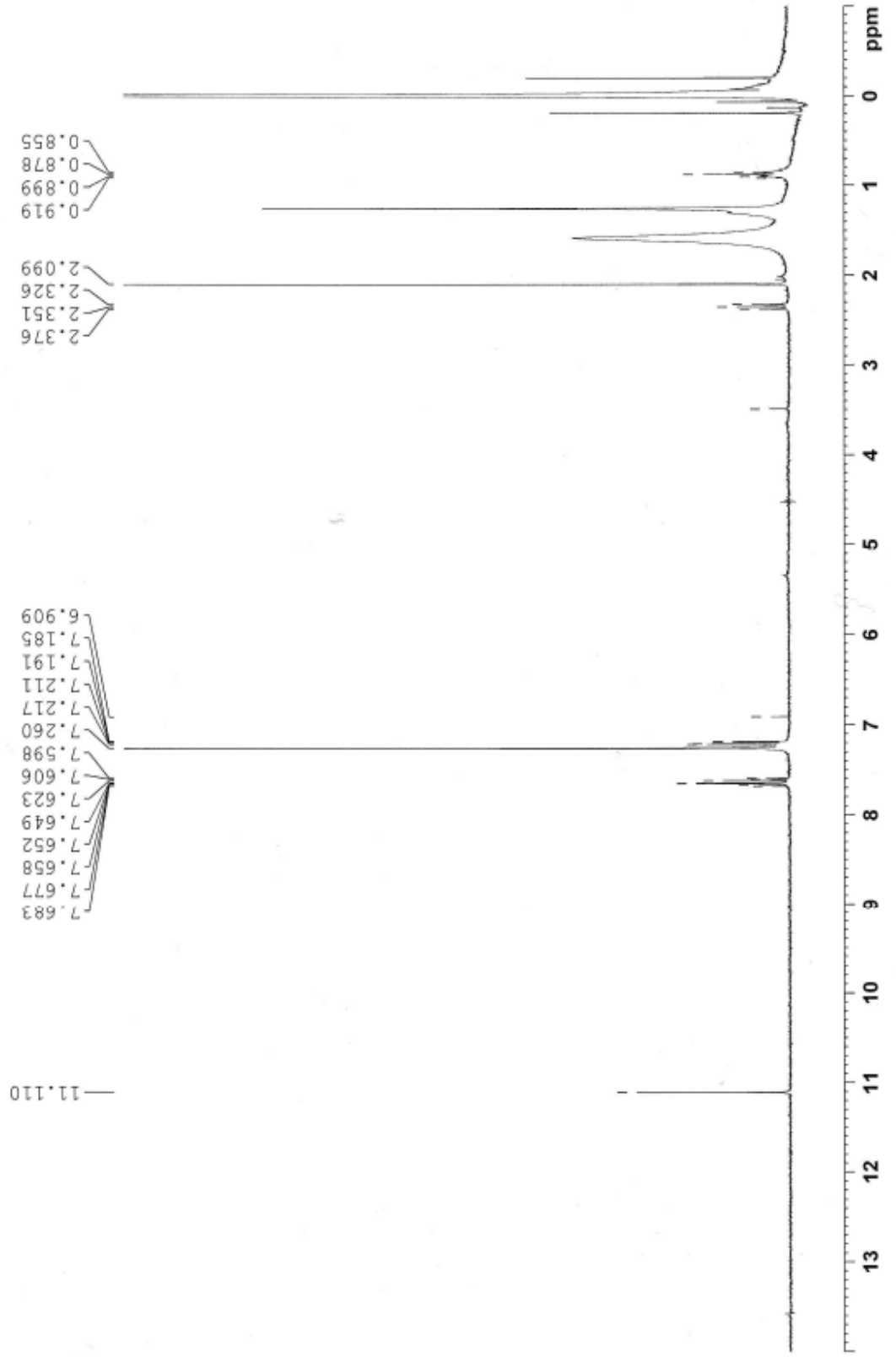

Figure 24 The ^{1}H-NMR spectrum of Compound 15

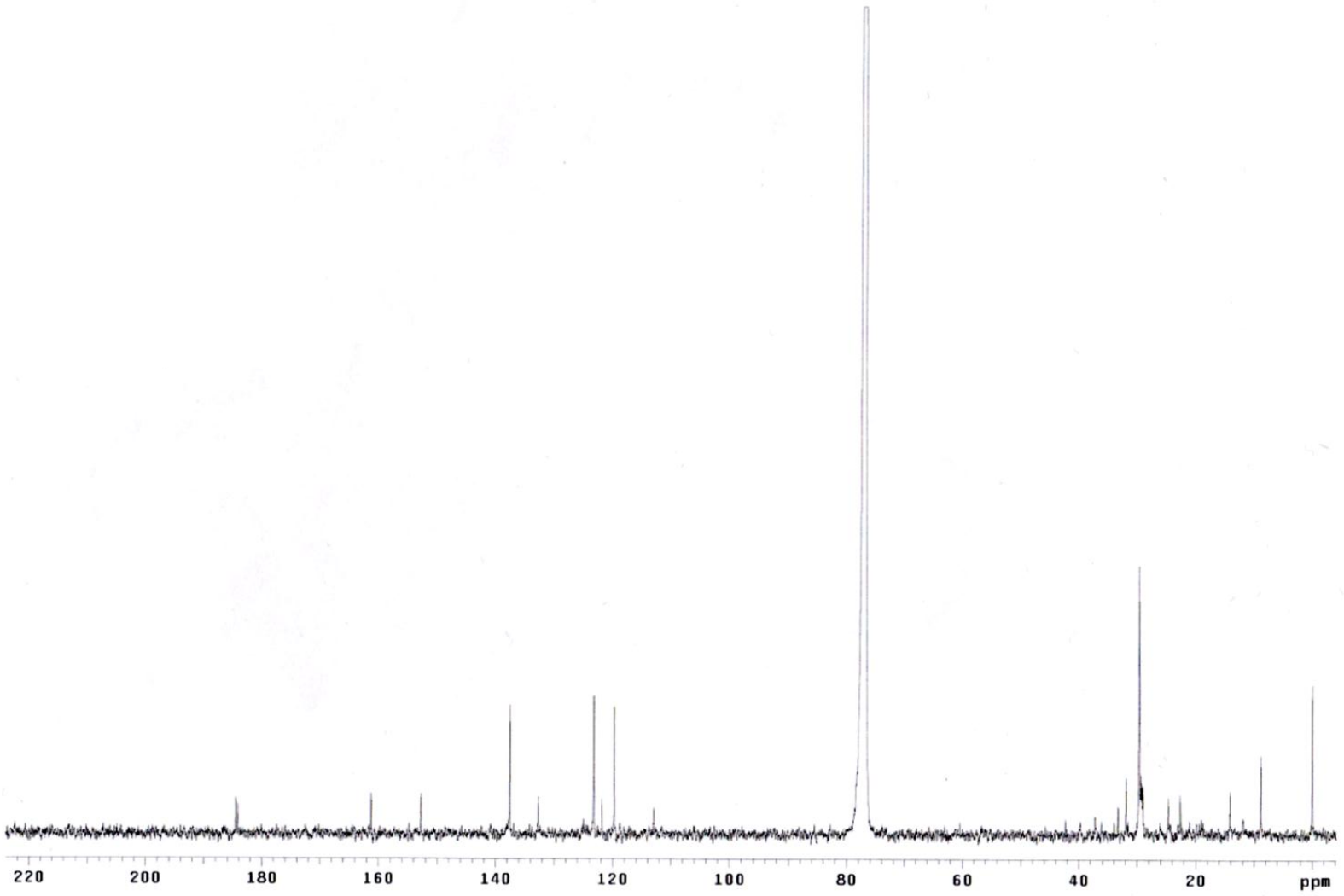

Figure 25 The ^{13}C-NMR spectrum of Compound 15

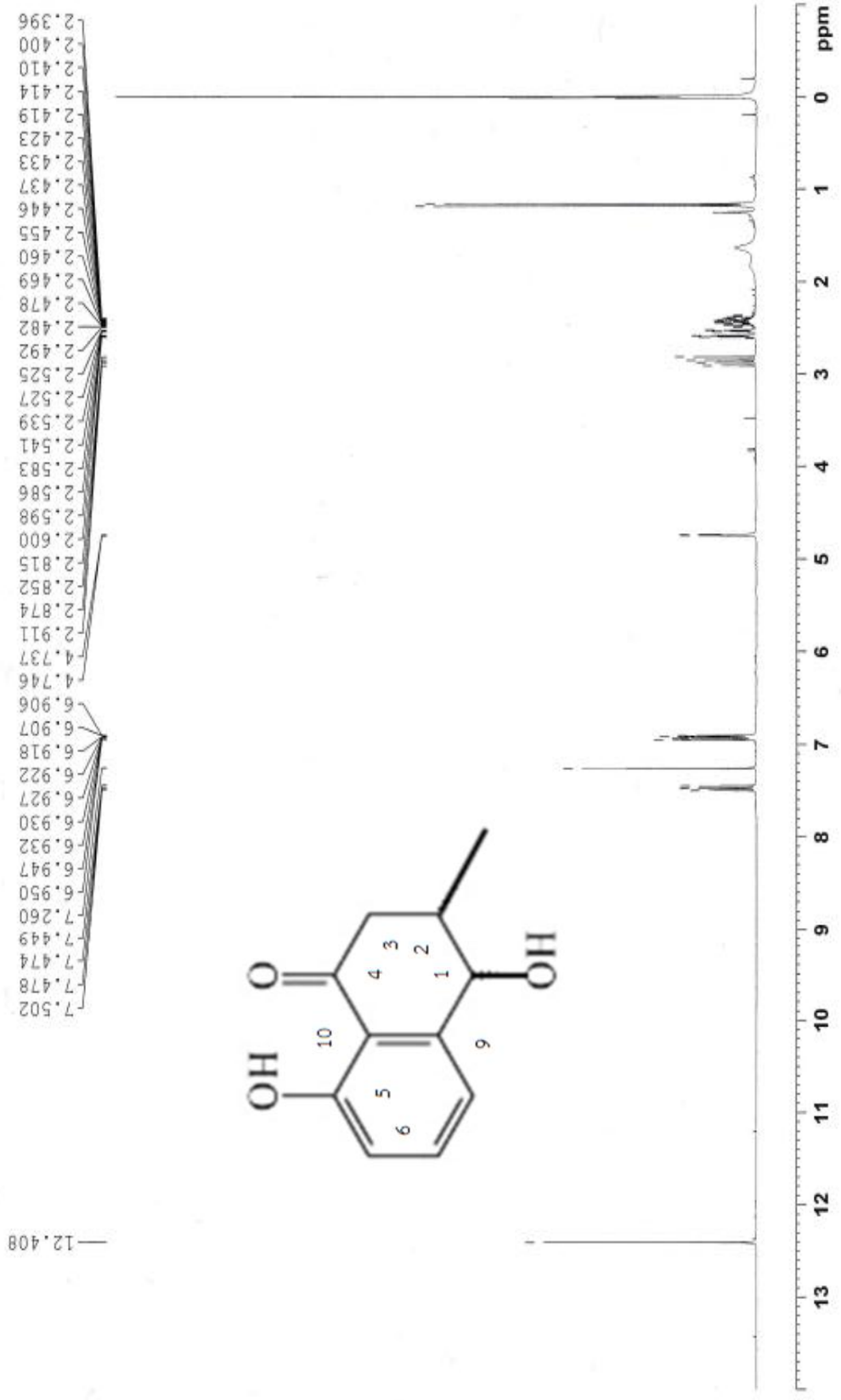

Figure 26 The ^{1}H-NMR spectrum of Compound 16

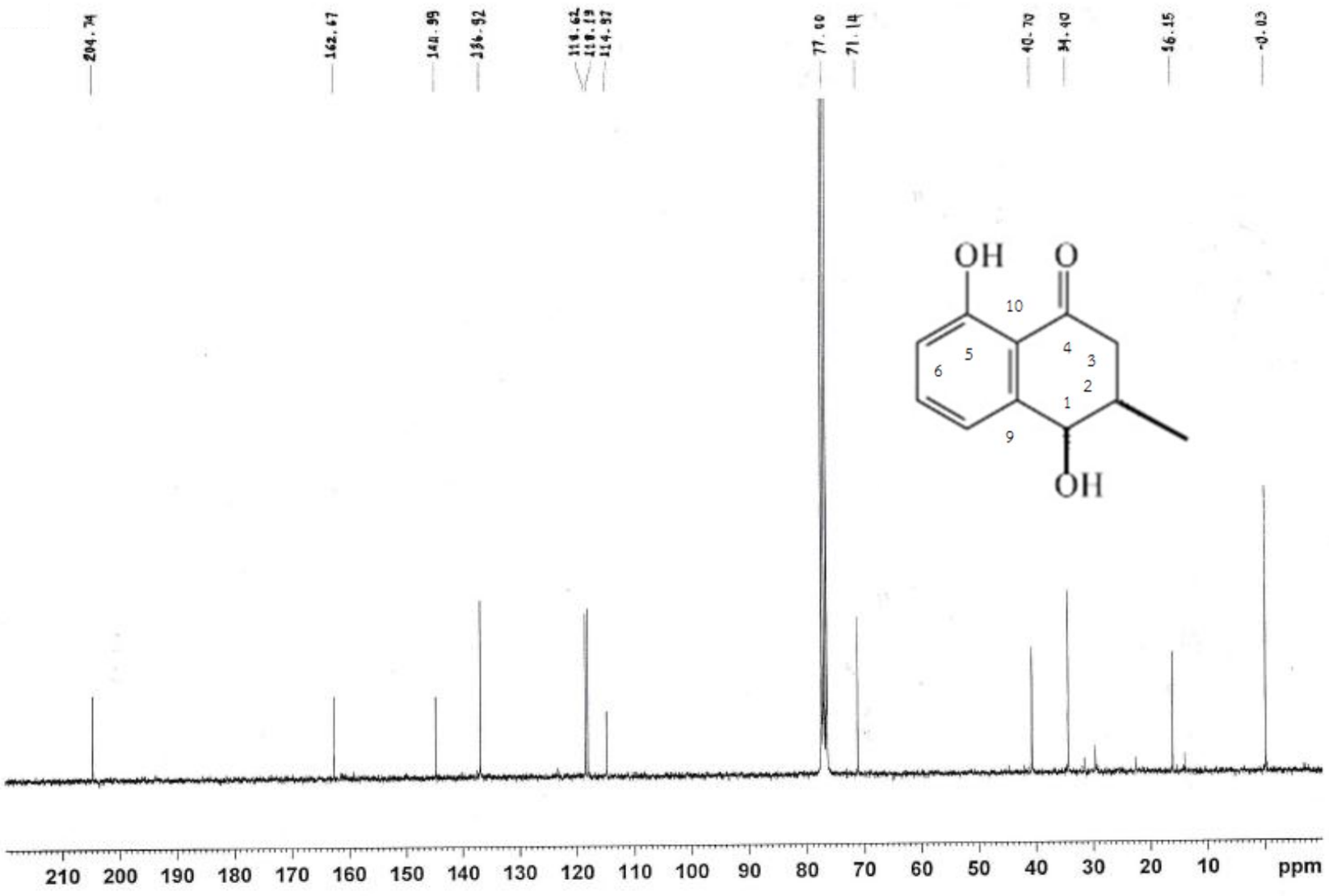

Figure 27 The ^{13}C-NMR spectrum of Compound 16

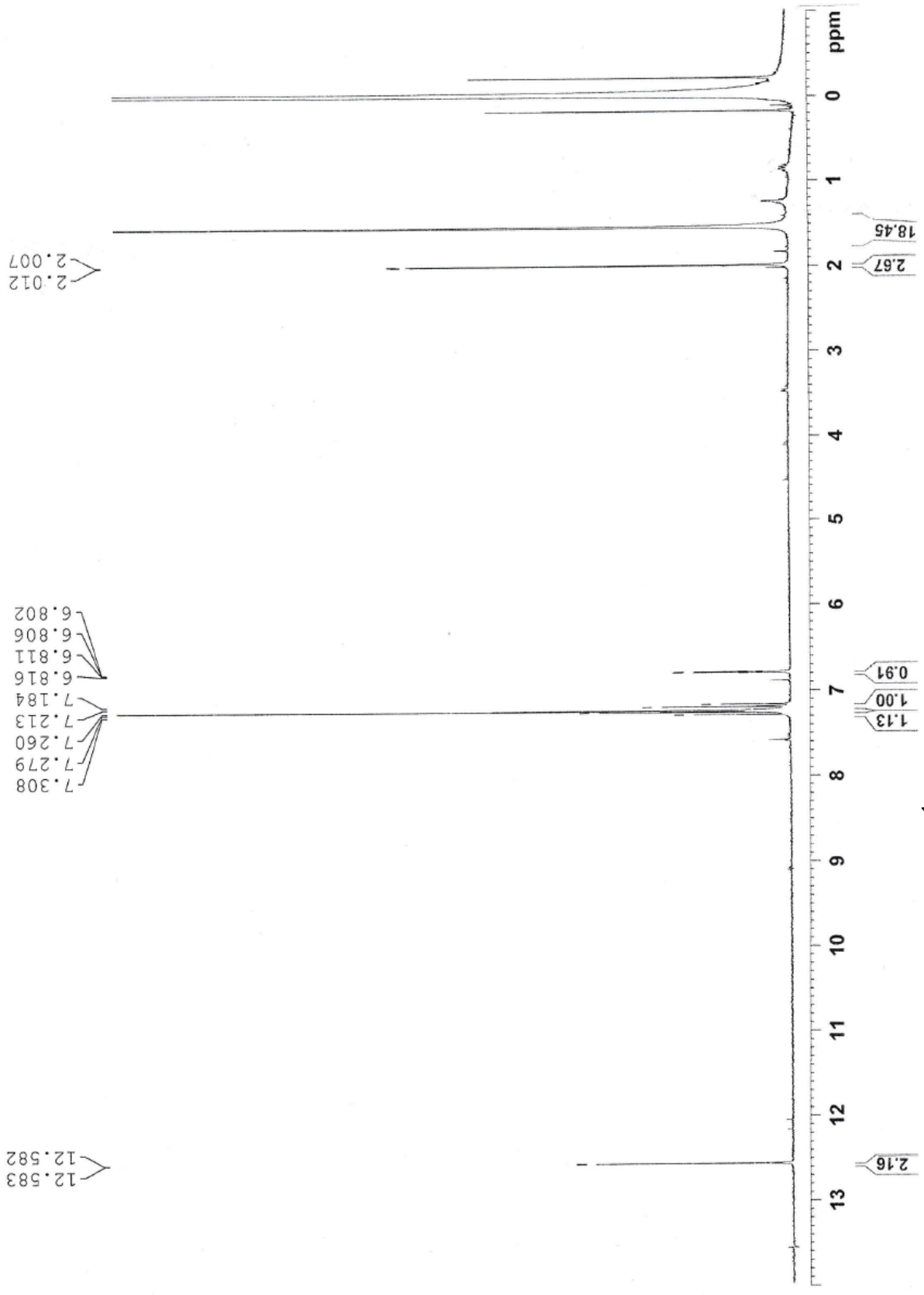

Figure 28 The ^{1}H-NMR spectrum of Compound 17

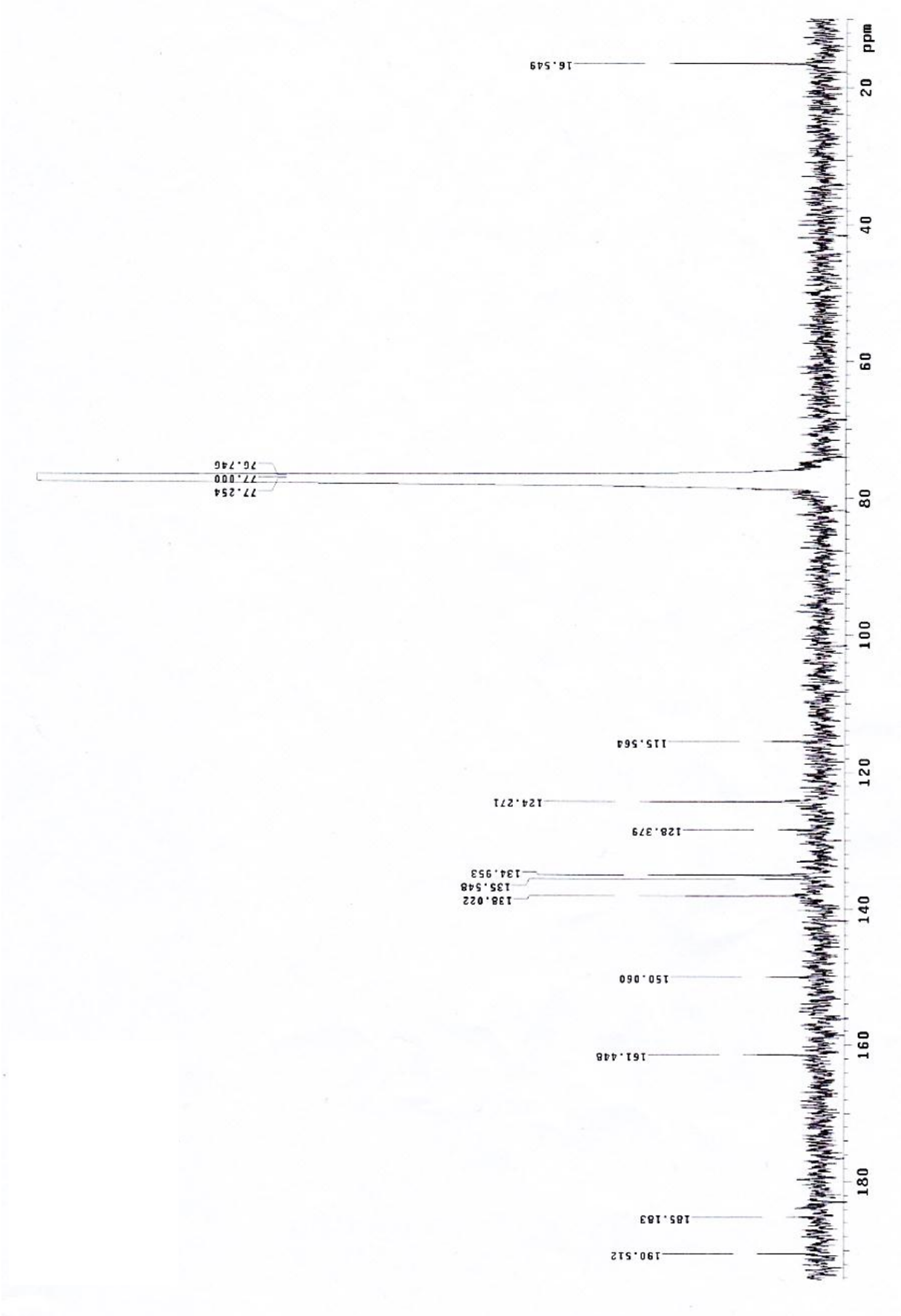

Figure 29 The ^{13}C-NMR spectrum of Compound 17

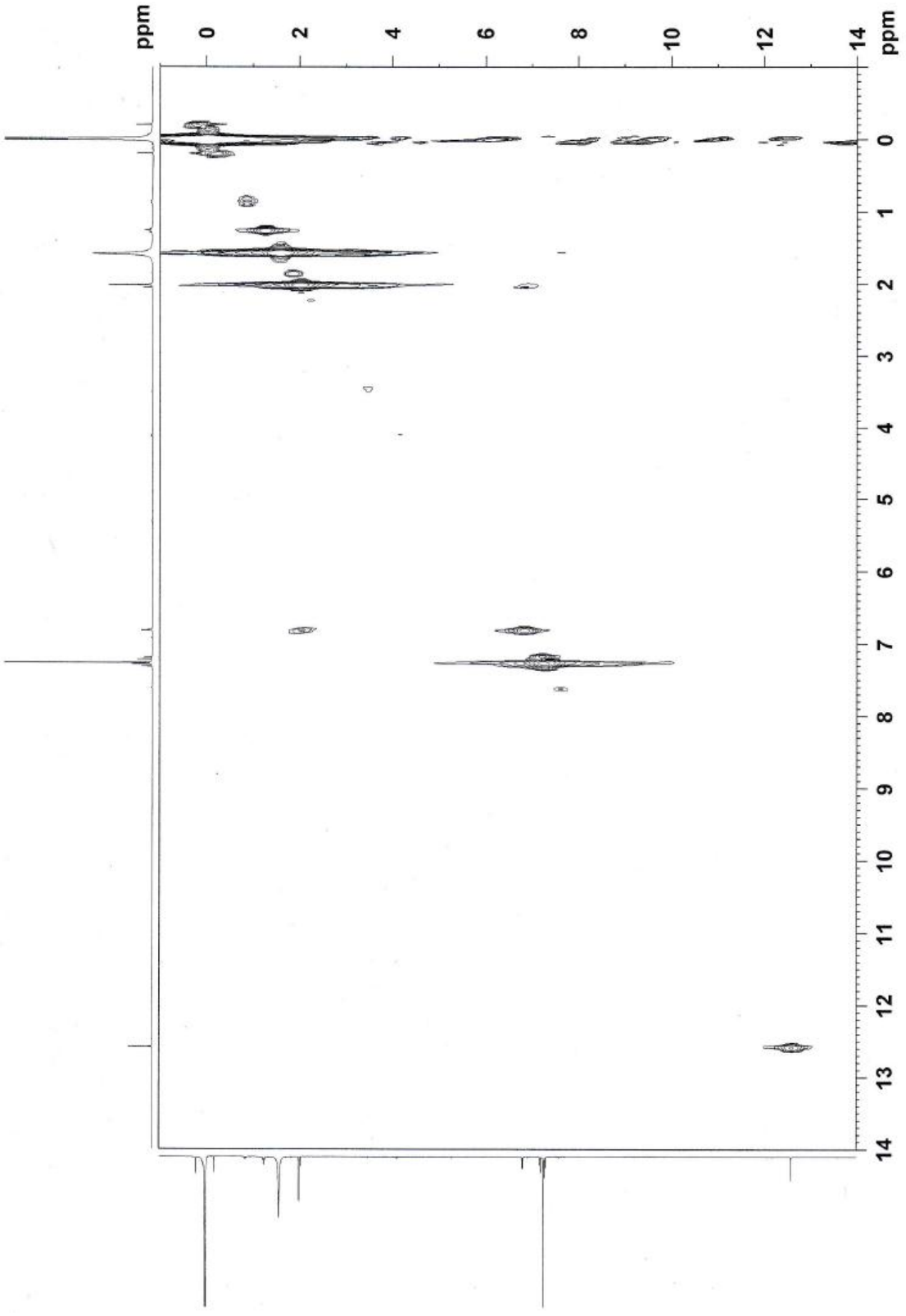

Figure 30 The COSY spectrum of Compound 17

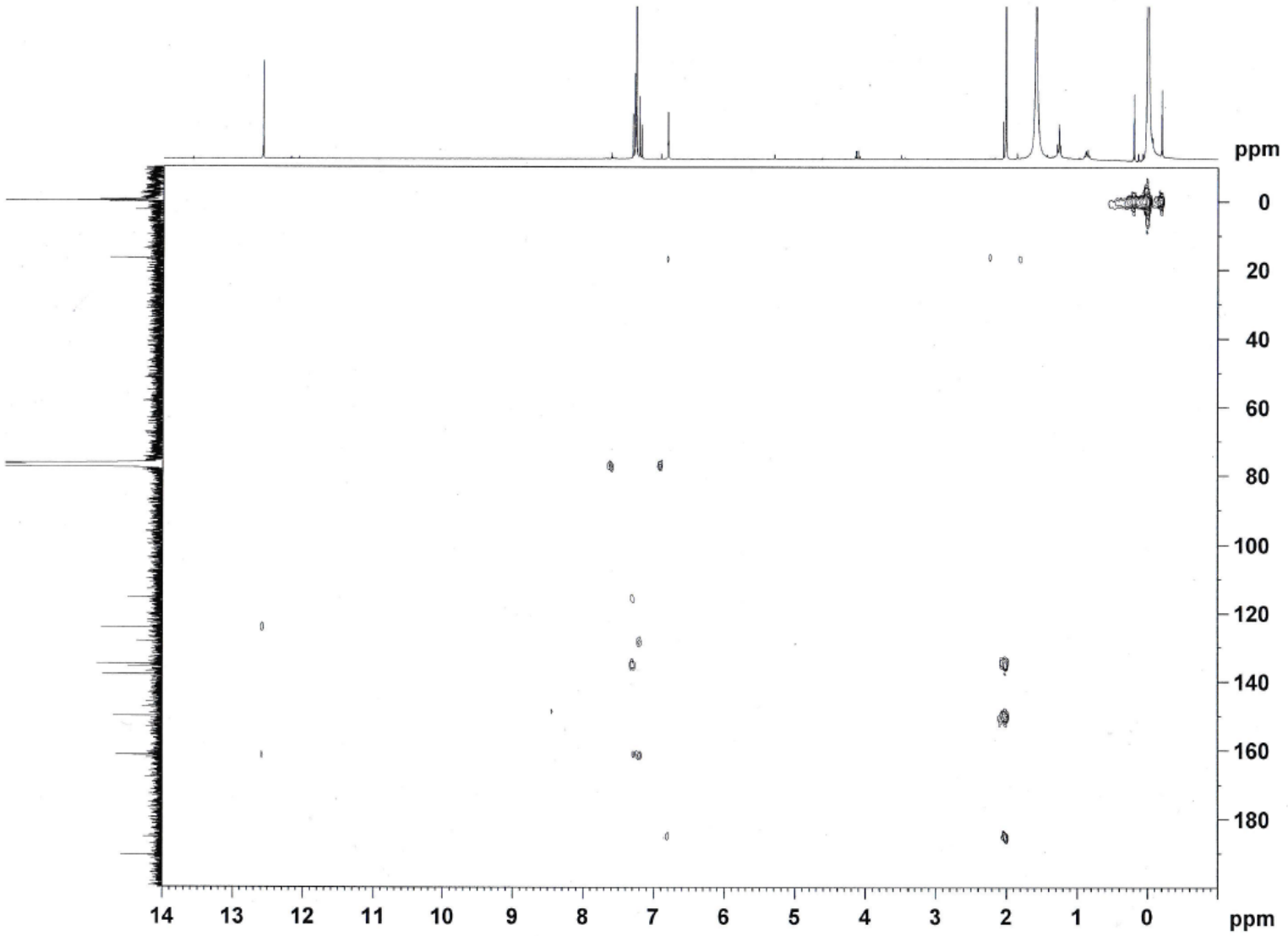

Figure 31 The HMBC spectrum of Compound 17

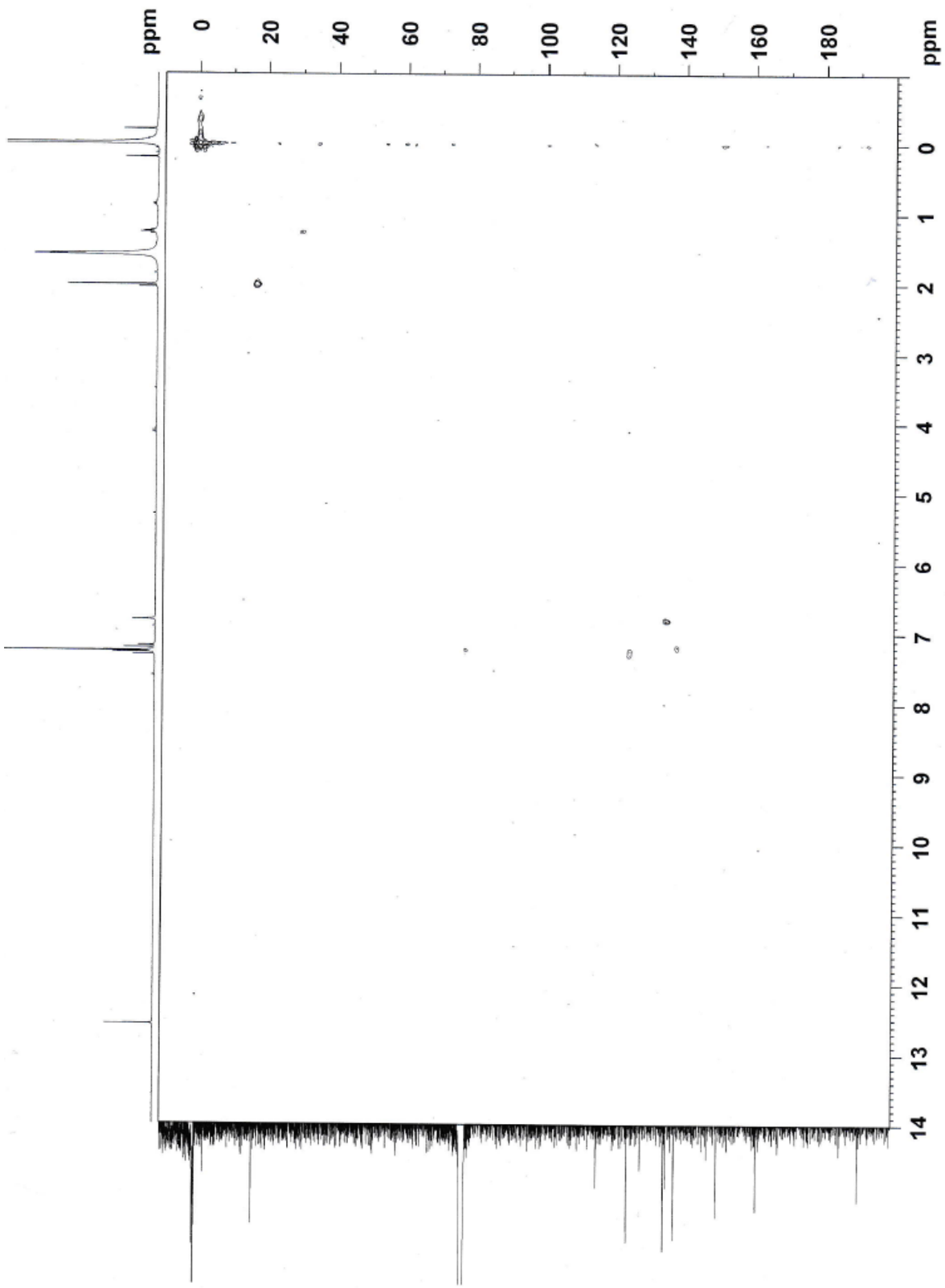

Figure 32 The HSQC spectrum of Compound 17

VITA

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