EFFECT OF MORINGA OLEIFERA LEAVES EXTRACT ON MOLECULAR SIGNALING IN COLON CANCER CELLS

By

Miss Jintana Tragulpakseerojn

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy Program in Pharmaceutical Technology

Graduate School, Silpakorn University

Academic Year 2016

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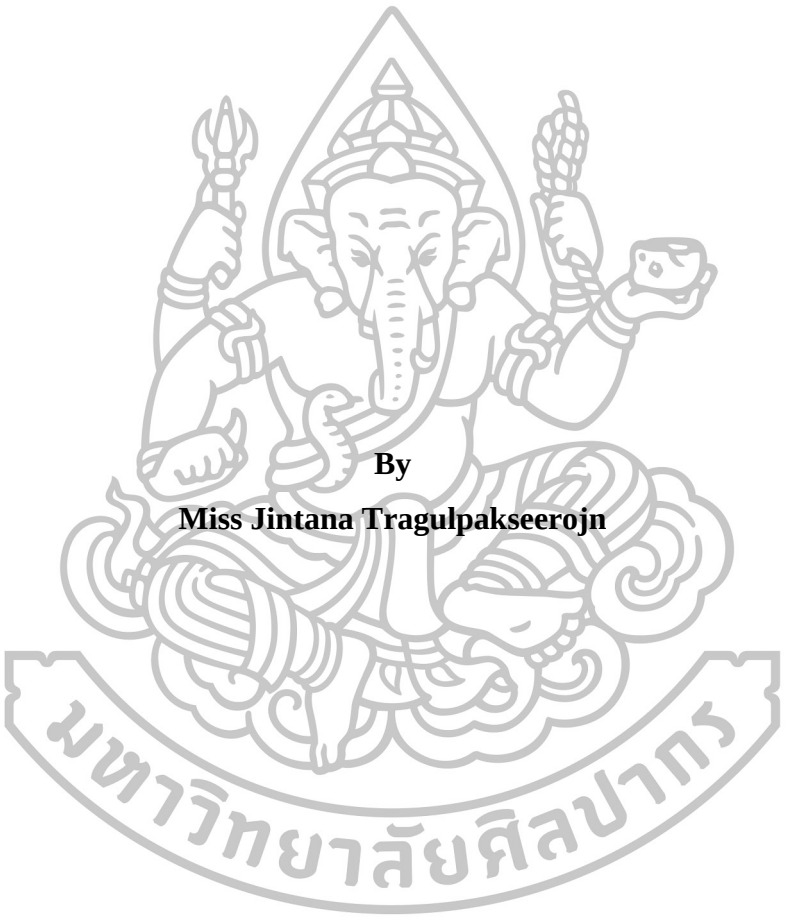

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ผลของสารสกัดใบมะรุมต่อ molecular signaling ในเซลล์มะเร็งลำไส้ใหญ่

โดย

นางสาวจินตนา ตระกูลปักษีโรจน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรดุษฎีบัณฑิต

สาขาวิชาเภสัชศาสตร์ชีวภาพ

บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

ปีการศึกษา 2559

The Graduate School, Silpakorn University has approved and accredited the thesis title of “Effect of *Moringa oleifera* leaves extract on molecular signaling in colon cancer cells” submitted by Miss Jintana Tragulpakseerojn as a partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biopharmaceutical Sciences.

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Moringa oleifera Lam. is an edible plant and used for traditional medicine, with a wide distribution in Thailand. Many studies have examined the nutritional and medicinal properties, especially anti-cancer properties. It has been reported that the crude extract of *M. oleifera* leaves represents the effects of subG1 phase inhibition, apoptotic induction and some molecular signaling involvement in different cancer cell lines. However, the effects of *M. oleifera* leaves on molecular mechanism of cancer in human colon cancer cells have not been studied. Therefore, it is of interest to determine whether *M. oleifera* leaves can affect on colon cancer cells at a molecular level. First, the cytotoxicity effect on colon cancer cells was screened using fractionated *M. Oleifera* leaves extractc. *M. oleifera* leaves extract was fractionated by Sephadex LH-20 column chromatography and then all fractions were analyzed with UV spectrophotometry to yield four pooled fractions (MOL1-MOL4) according to their absorbance profile pattern at 260 nm. The obtained four pooled fraction were evaluated the toxicity on colon HCT116 cancer cells in a comparison to commercial flavonols and flavonol glycosides (kaempferol, astragalin and isoquercetin) which have been found in *M. oleifera* leaves. The four pooled fractions (MOL1-MOL4) displayed a significant anti-proliferative activity against HCT116 cells. Comparatively, the proliferation of MOL2, MOL3 or MOL4 treated cells were more inhibited than that of MOL1 treated cells at 24 and 48 hr. In the other words, MOL2, MOL3 and MOL4 of *M. oleifera* leaves extracts were high toxic on colon cancer cells while MOL1 was less toxic.

Among four pooled fractions of *M. oleifera* leaves, MOL1 and MOL2 were found to decrease pERK1/2 activation of HCT116 cells in a dose-dependent manner. For MOL3 and MOL4, they decreased pERK1/2 activation more than MOL1 and MOL2 which were concomitant with their higher antiproliferative activity. The findings indicated that the *M. oleifera* leaves extracts may inhibit the growth of HCT116 cells through the reduction of pERK1/2 signaling pathway.

Program of Biopharmaceutical Sciences Graduate School, Silpakorn University
Student's signature..... Academic Year 2016
Thesis Advisors' signature 1.....2.....3.....

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คำสำคัญ : มะเร็งลำไส้ใหญ่ / มะรุม / สัญญาณโมเลกุล

จินตนา ตระกูลปักษีโรจน์ : ผลของสารสกัดใบมะรุมต่อ molecular signaling ในเซลล์มะเร็งลำไส้ใหญ่. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ภญ.รศ.ดร.อวยพร อภิรักษ์อร่ามวง, ภก. ผศ.ดร. พีรยศ ภมรศิลปธรรม และ ภญ.รศ.ดร.เพ็ญพรรณ เวชวิทยาขลัง . 87 หน้า.

มะรุมเป็นพืชที่รับประทานได้และใช้เป็นยาพื้นบ้านโดยทั่วไปในประเทศไทย มีการศึกษาวิจัยมากมายเกี่ยวกับคุณค่าสารอาหาร และคุณสมบัติทางยาของมะรุม โดยเฉพาะอย่างยิ่งคุณสมบัติในการต้านมะเร็ง เคยมีรายงานระบุว่า สารสกัดหยาบของใบมะรุมมีฤทธิ์ยับยั้งการแบ่งเซลล์ระยะ subG1, เหนี่ยวนำกระบวนการตายของเซลล์มะเร็ง และส่งผลต่อโมเลกุลสัญญาณบางอย่างในเซลล์มะเร็งหลายชนิด อย่างไรก็ตามยังไม่เคยมีรายงานเกี่ยวกับผลของใบมะรุมต่อกลไกระดับโมเลกุลในเซลล์มะเร็งลำไส้ใหญ่ ดังนั้นการวิจัยเพื่อให้ทราบว่าใบมะรุมมีผลต่อเซลล์มะเร็งลำไส้ใหญ่ในระดับโมเลกุลหรือไม่ จึงเป็นสิ่งที่น่าสนใจ โดยขั้นแรก ทำการตรวจสอบความเป็นพิษต่อเซลล์มะเร็งลำไส้ใหญ่เบื้องต้น ด้วยการใช้สารสกัดจากใบมะรุมที่แยกเป็นส่วนย่อย ๆ สารสกัดจากใบมะรุมถูกนำมาแยกเป็นส่วนย่อย ๆ ด้วยเทคนิคโครมาโตกราฟี ชนิดคอลัมภ์ ที่ใช้ Sephadex LH20 เป็นตัวกลาง หลังจากนั้น fraction ทั้งหมดถูกนำมาวิเคราะห์ด้วย UV spectrophotometry แล้วจึงนำมารวมกันเป็น pool fraction ตามลักษณะค่าการดูดกลืนแสงที่ 260 นาโนเมตร ได้เป็นสารสกัด 4 ส่วน (MOL1-MOL4) ซึ่งสารสกัด 4 ส่วนนี้ใช้ทดสอบความเป็นพิษต่อเซลล์มะเร็งลำไส้ใหญ่ (HCT116) โดยเปรียบเทียบกับสารฟลาโวนอล และฟลาโวนอลไกลโคไซด์บริสุทธิ์ (kaempferol , astragalin และ isoquercetin) ที่มีรายงานว่าพบในใบมะรุม สารสกัด 4 ส่วน (MOL1- MOL4) ยับยั้งการเจริญของเซลล์ HCT116 อย่างมีนัยสำคัญ เมื่อเปรียบเทียบกัน พบว่า MOL2, MOL3 และ MOL4 ยับยั้งการเจริญของเซลล์มากกว่า MOL1 เมื่อบ่มเป็นเวลา 24 และ 48 ชั่วโมง หรือกล่าวได้ว่า MOL2, MOL3 และ MOL4 มีความเป็นพิษสูงต่อเซลล์ลำไส้ใหญ่ ในขณะที่ MOL1 มีความเป็นพิษต่ำกว่า

ในสารสกัด 4 ส่วนจากใบมะรุม MOL1 และ MOL2 ลดการกระตุ้นของ pERK1/2 ในเซลล์ HCT116 ในลักษณะแปรผันตามปริมาณของสารสกัดที่ใช้ สำหรับ MOL3 และ MOL4, สารสกัดทั้งสองส่วนลดการกระตุ้น pERK1/2 ได้มากกว่า MOL1 และ MOL2 ซึ่งสอดคล้องกับฤทธิ์การยับยั้งการเจริญของเซลล์ที่สูงกว่า จากผลการศึกษาชี้ให้เห็นว่าสารสกัดจากใบมะรุมอาจยับยั้งการเจริญของเซลล์มะเร็งลำไส้ใหญ่ HCT116 ผ่านการลดวิถีสัญญาณ pERK1/2

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

®	Registered trademark
% w/v	Percent weight by volume
% v/v	Percent volume by volume
^{o}C	Degree celsius
ϕ	Diameter
µg	Microgram
µL	Microliter
µm	Micrometer
Abs	Absorbance
Akt	Protein kinase B
Avg	Average
BSA	Bovine serum albumin
CC	Column chromatography
cm	Centimetre
cm^2	Square centimetre
DMSO	Dimethyl sulfoxide
e.g.	exempli gratia (Latin); for example
Eq	Equation
ERK1/2	Extracellular signal-regulated kinases 1 and 2
et al.	And others
etc.	et cetera (Latin); and other things/ and so forth
EtOH	Ethanol
FBS	Fetal bovine serum
g	Gram
h	Hour
H-NMR	Proton nuclear magnetic resonance
HCl	Hydrochloride
H_2SO_4	Sulfuric acid

IC_{40}	Inhibition concentration at 40% cell viability
i.e.	id est (Latin); that is
i.d.	Diameter
kV	Kilovolt
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MeOH	Methanol
mg	Milligram
min	Minute
mL	Milliliter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
nm	Nanometer
MOL	Pooled fractions of *M. oleifera* leaves
OH	Hydroxide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
pH	Potentia hydrogenii (latin); power of hydrogen
p-values	Probability values
PVDF	Polyvinylidene difluoride
p38 MAPK	p38 Mitogen-activated protein kinases
rpm	Round per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
STD	Standard
TBS-T	Tris buffer saline with 0.1% tween20
TLC	Thin layer chromatography
UV-Vis	Ultraviolet and Visible Spectrophotometry
WB	Western Blot

CHAPTER 1
INTRODUCTION

1.1 Statement and significance of the research problem

Cancer is a leading cause of death, with 8.2 million deaths in 2012 [1-2]. In fact, the phytochemical constituents present in plant-based foods and medicinal plants are mainly responsible for their anti-cancer effects [3]. Since natural compounds are obtained from diverse sources, many anti-cancer drugs are discovered as a successful story of current drugs, such as ellipticine, paclitaxel, vincristine, and vinblastine [4-5].

Moringa oleifera Lam. (*M. oleifera*), a short, slender and perennial plant, belongs to the Moringaceae family and is widely cultivated in different locations of Southeast Asia, including India, Sri Lanka, Malaysia, and Philippines, and also in Africa, tropical America, and Mexico [6]. *M. oleifera* is an edible plant and used for traditional medicine formulation. Over the past two decades, many studies have examined the nutritional and medicinal properties of *M. oleifera* [7-8]. For example, *M. oleifera* leaves showed anti-bacterial effects [9], anti-fungal activities [10], and other medicinal activities. The extracts from roots and leaves of *M. oleifera* showed inhibition of proliferative of epithelial ovarian cancer cells [11] and human epidermal carcinoma KB cells [12]. Much attention has been paid to phytochemicals that are contained in *M. oleifera* leaves. Some bioactive phytochemicals found in *M. oleifera* leaves are identified as quercetin, chlorogenic acid, astragalin, and kaempferol. Their contents are shown to vary with geography, seasons, and also the methods for collection of leaves and extraction [13-15, 17].

From our preliminary studies, crude methanol extracts from *M. oleifera* leaves showed anti-proliferative activities against human HCT116 colon cancer cells. In this study, *M. oleifera* leaves extracts were fractionated by gel filtration chromatography on Sephadex LH-20 and the fractionated extracts were examined for cytotoxicity activity in HCT116 cells. Furthermore, the effect on cell viability of fractionated extracts was conducted in a comparison to flavonoids; kaempferol, astragalin,

isoquercetin. Kaempferol is a major flavonoid found in *M. oleifera* leaves and it induces apoptosis in HCT116 cells [20]. While astragalin and isoquercetin are flavonol glycosides also found in *M. oleifera* leaves and have been reported for anti-proliferative activity [15, 16]. The result showed that the treatment of HCT116 cells with each pooled fraction (MOL1, MOL2, MOL3 or MOL4) as well as kaempferol and isoquercetin inhibit cell proliferation in a dose-dependent manner whereas astragalin did not inhibit cell proliferation. Recently, it was found that *M. oleifera* leaves extract has a strong relation to Mitogen-activated protein kinase (MAPK) gene that regulates many cellular functions including cell proliferation, differentiation, migration, and apoptosis [18-19]. Therefore, it is of interest to evaluate especially Extracellular signal-related kinase (ERK), a member of MAPK family, pathway for anti-cancer activity of *M. oleifera* leaves extract comparing with the antiproliferative flavonol glycoside "isoquercetin" found in our *M. oleifera* leaves extracts.

1.2 Objective of this research

1. To prepare the fractionated of *M. oleifera* leaves extracts through column chromatography method on Sephadex LH-20.
2. To examine the effects of the fractionated *M. oleifera* leaves extracts on colon cancer cells viability.
3. To study underlying mechanisms of anti-colon cancer effect of active fractionated *M. oleifera* leaves extracts.

1.3 The research of hypothesis

1. Drumstick (*M. oleifera*) leaves extract have phytochemicals with colon tumor suppressing ability.
2. Affecting of some molecular signalings of active fractionated extract on colon cancer should be clarified.

CHAPTER 2
LITERATURE REVIEWS

2.1 *Moringa oleifera* Lam.

2.1.1 General informations

2.1.2 Pharmacological effects

2.1.3 Phytochemical components from leaves

2.2 Cancer

2.2.1 Colorectal cancer

2.2.1.1 Intracellular mechanism

2.2.1.2 Human colorectal carcinoma cells (HCT116 cells)

2.2.2 Plant-derived drug

2.2.3 Phytochemicals from plants

2.1 *Moringa oleifera* Lam.

2.1.1 General informations

M. oleifera is a member of Moringaceae that grows 10-15 meters high. It is a small native tree of the sub-Himalayan regions of North West India and now widely cultivated in tropical and subtropical regions. There are different common names of this tree i.e. Ma-rum (Thailand), Drumstick (India, Nepal, Sri Lanka), Horseradish tree (USA, Indonesia, Malaysia) [21]. It can grow rapidly. The plant has tripinnate leaves, white petals's flowers, pods 15-30 cm length and seeds. *M. oleifera* is an important food commodity which has caught enormous attention as the natural nutrition of the tropical countries such as India, Indonesia, Pakistan, Philippines, Hawaii, Africa, Bangladesh, Afghanistan and Thailand [6, 22]. In nutritional and medicinal view, almost every part of the plant has value for food such as leaves, fruits, flower and immature pods.

(a) (b) (c)

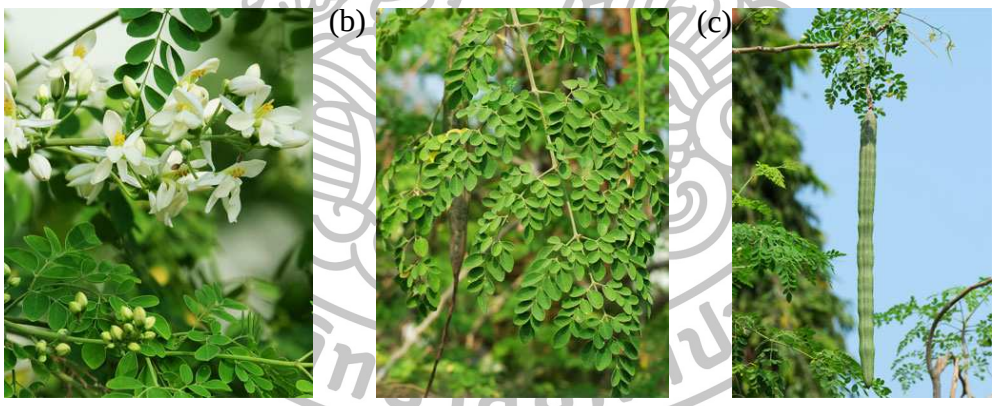


Figure 2.1 *Moringa oleifera* Lam., a typical Moringaceae. The *M. oleifera* tree parts; (a) flowers, (b) leaves and (c) pods.

2.1.2 Pharmacological effects

M. oleifera is used as a traditional medicinal drug in many countries (Table 2.1). Almost all parts of this plant are considered to possess medicinal properties including roots, roots bark, bark, gum, twigs, leaves, flowers, pods and seeds [21].

Table 2.1 Common traditional medicinal uses from various parts of *M. oleifera*

Part of plant	Route	Traditional uses	Country
1. Root	Orally	Abortifacient	East Africa, Nepal
		Amenorrhea	Malaysia
		Relieve fever	Nepal
		Analgesic, hypotensive, sedative, arthritis	Nigeria
		Cardiotonic, antipyretic, stimulant for fainting	Thailand
2. Root bark	Orally	Diuretic, Menstrual promoter	East and West Indies
3. Bark	Orally	Menstrual promoter	Malaysia
		Abortifacient	East Africa, Nepal
		Purgative, vermifuge, antispasmodic	Mauritius
4.Twigs	Orally	Malaria	Togo
5. Gum	Orally	Abortifacient	New Caledonia
6. Flowers	Orally	Aphrodisiac	Nepal
		Cough remedy	West Indies
7. Pod	Orally	Diabetes, ascites, edema, spleen enlargement, inflammatory swelling, abdominal tumors, colic, dyspepsia, fever, ulcers, paralysis, lumbago, skin diseases	Saudi Arabia
8. Seeds	Orally	Treat fevers, tonic	Guam
		Cough remedy	West Indies

Table 2.1 Common traditional medicinal uses from various parts of *M. oleifera* (cont.)

Part of plant	Route	Traditional uses	Country
9. Leaves	Externally	Headach	Fiji
	Ointment	Sore eyes	Fiji
	Rub over the breast	Reduce milk flow	New Caledonia
	Orally	Induce vomiting (useful in poisoning)	Fiji
		Nervous shock	Haiti
		Galactogogue	Philippines
		Malaria	Togo
		Diuretic	USA
		Syphilitic ulcers	West Indies

Source: Ross, I.A. (2003). *"Moringa pterygosperma* Gaertn." **Midicinal Plants of The World**, volume1, 2nd Edition: 368–370.

Nowadays, many studies have shown the biological acitvities of each part of this plant including;

Anti-cancer. Pod exerts suppressive effects in a colitis-related colon carcinogenesis model induced by azosymethan/dextran sodium sulfate in male mice [23]. Isolated compound of seeds, niazimicin, possess to be a potent chemo-preventive agent. This study shows that niazimicin exhibited 50% delay in the promotion of tumors and decreased the incidence of papilloma bearing mice [24]. It has been reported that the leaf extract had potent antiproliferative activity and apoptosis inducing capacity on human epidermal carcinoma (KB) cells and epithelial ovarian cancer cells [11-12], and it also increased the cytotoxicity of chemotherapy on pancreatic cancer cells (Panc-1) [46]. In cancerous human lung cells (A549), Tiloke et al. (2013) reported that leaf extract induced apoptosis and increased oxidative stress [47]. In another study by Purwal et al. (2010), tumorous mice were treated with methanol extract of leaves at concentration of 1 g/kg body weight of mice. The result showed that

methanol extract of leaves were effective in delaying the tumor growth and increased the survival time of mice [48].

Anti-diabetic and anti-hyperglycemic. The ethanolic leaves extract were administered orally to deabetic rats for two weeks. At doses of 250 and 500 mg/kg of extract significantly lowered the fasting blood glucose levels and improved insulin sensitivity and beta-cell function in diabetic rats [28]. The study by Tende et al. (2011) reported that the hypoglycemic and anti-hyperglycemic activity of the leaves may be probably due to the presence of terpenoids, which appeared to be involved in the stimulation of the β-cells and the subsequent secretion of prefored insulin [29]. Soliman (2013) also found that the ethanolic leaves extract was capable of reducing hyperglycemia in streptozocin diabetic male rats [30].

Anti-inflammation. Isolated compounds of pods were investigated with the lipopolysaccharide (LPS)-induced murine macrophage RAW 264.7 cells. In this finding, 4-[(2'-*O*-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate possessed potent NO-inhibitory activity at IC50 of 1.67 μM [31].

Anti-microbial. *In vitro* study, leaves were reported to possess antimicrobial potential both as bacterial [9] and fungal [10]. The seed flour extracts showed the antibacterial activity against four bacteria, *B. cereus*, *S. aureus*, *E. coli* and *Y. enterocolitica*, in the study of Govardhan Singh et al [34].

Anti-oxidant. The flowers and leaves hydroethanolic extracts showed a significant reduction in the severity of the liver damage by oxidative stress in rat [32]. Moreover, the extracts of *M. oleifera* both mature and tender leaves have a potent antioxidant activity against free radicals, prevent oxidative damage to major biomolecules and afford significant protection against oxidative damage [49]. The seed flour extracts showed the presence of polyphenols, gallic acid, vanilin, catechin, caffeic acid, epicatechin, p-coumaric acid, ferulic acid, protocatechuic acid, quercetin, cinnamic acid, and exhibited an effective antioxidant activity [34]

Anti-ulcerogenic. According to Verma et al. (2012), the effect of 50% ethanolic leaf extract of *M. oleifera* on pylorus ligation-induced, ethanol-induced, cold-restraint, stress-induce and aspirin-induced gastric ulcers were investigated. The results of all these assays represented the reduced total ulcerogenic effect, by showing a dose-dependent anti-ulcerogenic activity reduction by the 50% ethanolic leaf extract. The

extract was found to decrease acid-pepsin secretion as well as exhibited ulcer protective properties [33].

2.1.3 Phytochemical components from leaves

Phytomedicines are believed to have benefits over conventional drugs and are regaining interest in current research. Recently, the edible plant and medicinal plant has become a popular study which has been linked to a-wide range of in vitro activities [12]. Several isolated compounds were reported for *M. oleifera* leaves (Table 2.2).

Table 2.2 Some chemical constituents of the isolated molecules from *M. oleifera* leaves.

Chemical compound	Reference
Glucosinolate:	
4-(α-L-rhamno-pyranosyloxy)-benzylglucosinolate	31
4-(α-L-rhamno-pyranosyloxy)-benzylglucosinolate monoacetyl	31
Flavonoids:	
Kaempferol 3-*O*-glucoside (astragalin)	31
Kaempferol 3-*O*-(6"-malonyl-glucoside)	31
3-Methoxy quercetin	21
Quercetin 3-*O*-glucoside (isoquercetin)	31
Quercetin 3-*O*-(6"-malonyl-glucoside)	31
Quercetin 3-*O*-rhamnosylglucoside (Rutin)	31
Phenolic acid:	
Chlorogenic acids (3-caffeoylquinic, 5-caffeoylquinic)	31
Nitriles:	
Niazicin B	21
Niazimicin	23, 24
Niaziminin A and B	21
Niazinin A and B	23, 24
Niazirin	23, 24
Niazirinin	21
Niaziridin	32
Carbamate:	
O-methyl 4-[(2',3',4'-tri-*O*-acetyl-α-L-rhamnosyloxy)benzyl]carbamate	23
O-ethyl 4-[(2',3',4'-tri-*O*-acetyl-α-L-rhamnosyloxy)benzyl]carbamate	23
O-methyl 4-[(4'-tri-*O*-acetyl-α-L-rhamnosyloxy)benzyl]carbamate	23

Table 2.2 Some chemical constituents of the isolated molecules from *M. oleifera* leaves (continued).

Chemical compound	Reference
Thiocarbamate:	
O-methyl 4-[(2',3',4'-tri-*O*-acetyl-α-L-rhamnosyloxy)benzyl]thiocarbamate	23
O-ethyl 4-[(α-L-rhamnosyloxy)benzyl]thiocarbamate	23
Miscellaneous:	
Alpha-tocopherol	21
Amylase	21
Ascobic acid	21
Aurantiamide acetate	35
Beta carotene	21
Choline	21
Gossypitin	21
Moringinine (benzylamine)	35
Nicotinic acid	21
Oxalic acid	21
Protein	21
Quercetagetin	21
Starch	21
Vitamin A, B-1 and B-2	21

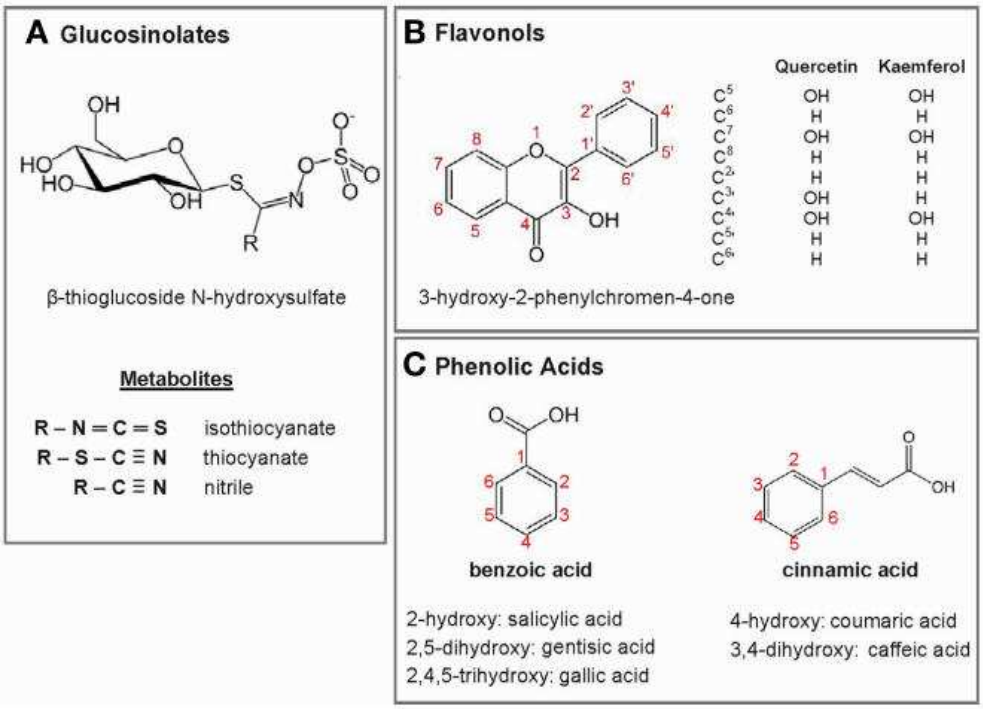


Figure 2.2 Structural of major phytochemicals found *M.oleifera* leaves.

Source: Mbikay, M. (2012). "Therapeutic potential of *Moringa oleifera* leaves in chronic hyperglycemia and dyslipidemia: a review." **Frontiers in Pharmacology** 3, 1-12.

2.2 Cancer

Cancer or malignant tumor originates from abnormal growth of cells in the body. The proliferation of cells is uncontrolable and becomes to abnormal large size (except the leukemia) or tumors. In case of invasion and metastasis of cancer cells, the cancer cells usually destroy normal cells or other healthy tissues and lead to death [21]. Cancers have unique molecular characteristics that make their cells different from normal cells. The molecular characteristics of cancers can be classified into two phenotypes: the overexpression of oncogenes and the down-regulation of tumor suppressor genes [36]. Cancer is one of the leading causes of death worldwide after cardiovascular and infectious diseases. The cancer incidence is varied in different regions of the world and its trend increases every year. The highest incidence rates are reported in North America, Australia, New Zealand, Europe, and Japan. Additionally, the cancer incidence in male patient is higher than that in female patient [22]. Because the human population is continually growing and aging, the incidence of cancer is

becoming even more common. Moreover, environmental factors, which are the major causes of cancer, are likely to contribute to increased cancer mortality in the future because people are becoming more subjected to tobacco, poor diet, obesity, infection, radiation, and environmental pollutants [37].

The treatments of cancer are conventional and novel therapy. Conventional therapies are surgery, radiotherapy and chemotherapy. Novel therapies are the biological therapies and more specific to tumor types or target tumor including: monoclonal antibodies, vaccines, gene therapy and small molecule signaling inhibitors. The kind of surgery varies depending on the type of cancer and the patients' physical fitness. This therapy is not generally an appropriate modality in some cancer, for example the lymphomas, leukaemias and small cell lung cancer [24]. In chemotherapy, drugs are designed to arrest the cell cycle of cancerous cells. However, their mode of action involves targeting rapidly dividing cells, hence they are known to cause severe side effects to rapidly dividing normal cells in the body such as; bone marrow cells, immune cells and hair follicle cells that portray similar characteristices [21]. Radiation as well as conventional cancer treatment, this therapy works by damaging the deoxyribonucleic acid (DNA) of the cancerous cells, but this may also damage the DNA of normal cells leading to adverse side effects [25]. Therefore, due to less toxicity and adverse effects of phytochemicals constituents present in medicinal plants, the research on medicinal plants and cancer has been intensified [26].

2.2.1 Colorectal cancer

Colorectal cancer, which may arise anywhere along the length of the colon or rectum, frequently begin as polyps that are benign outgrowths emerging from the epithelial lining of the colon or rectum. The colorectal cancer is the third most common worldwide cancer incidence and is the top five most common form of malignancy in both Thai’s men and women [1, 4, 23]. The risk of developing this cancer is affected by age, with rates increasing dramatically after 50 years of age. High saturated animal fat and calories are also likely risk factors. And diets low in vegetables or fruits are linked to increased risk, especially smoking and alcohol consumption.

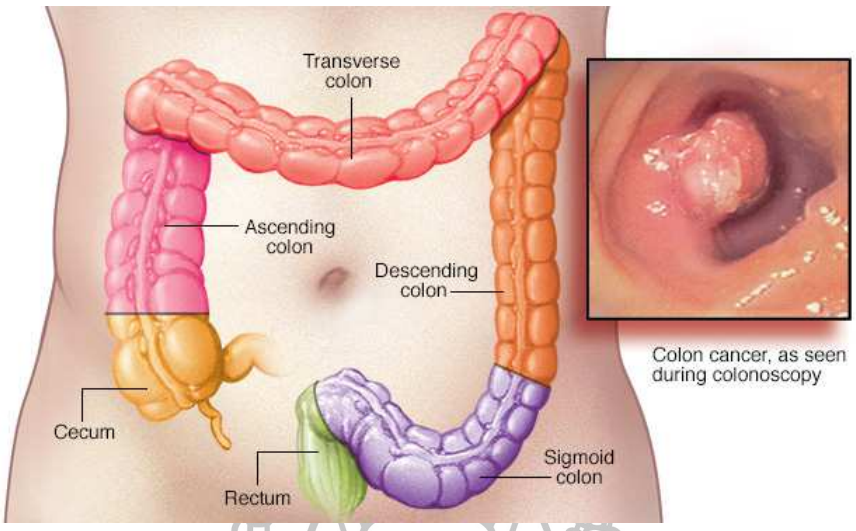


Figure 2.3 Each part of long colon (intestine) and rectal can produce cancer. (Adapted from http://www.mayoclinic.org/diseases-conditions/colon-cancer/home/ovc-20188216)

2.2.1.1 Intracellular mechanism and some molecular targets

All cells in the human body are covered by lipid bilayer membranes. The basic structure of cell membrane consists of lipid bilayer, protein and glycocalyx carbohydrate. Moreover, the membrane structure is composed of the functional domains, called lipid rafts or microdomain. Size of lipid rafts is in the range of 70 to 370 nm [38]. Lipid rafts are evidenced to be essential for many processes such as signal transduction trafficking and adhesion in cells. They contain high content of cholesterol and glycosphingolipid. Because of their tight packing of lipids, lipid rafts are insoluble in nonionic detergents. Many proteins apportion into lipid rafts; for instance, glycosylphosphatidylinositol (GPI)-anchored protein, calveolae, transmembrane proteins and membrane proteins associated with cell signaling [38]. These proteins can change their size and composition in response to intra- or extracellular stimuli. In spite of a small alteration of protein partitioning into lipid rafts, it can cause signaling cascades [39].

The different observations of colorectal cancer lipid rafts can be generally categorized under the following main topics of investigation: cell death-mediated mechanisms, caveolae in cancer cell growth and function, unique structrue-function molecular associations, and intervention studies with bioactive compounds [39].

The Figure 2.4, the lipid bilayer of the cell membrane is depicted in light blue, membrane microdomains or lipid rafts in light purple, and the pear-shaped caveolae associated with these rafts in dark purple. MRP is Multidrug-resistance protein, GlcCer is Glucosyl-ceramide, FADD is Fas-associated protein with death domain, TRADD is Tumor necrosis factor receptor type 1-associated DEATH domain protein, PI3K is Phosphoinositide 3-kinase, Akt is Serine/threonine protein kinase, ERK is Extracellular signal-regulated kinase, MAPK is Mitogen-activated protein kinase, IRS1 is Insulin receptor substrate1, ASK1 is Apoptosis signal-regulating kinase1, SHC is Src homology 2 domain, TNF-α is Tumor necrosis factor-α , IGF-I is Insulin-like growth factor-I, VDR is Vitamin D receptor, Vit D is Vitamin D, RAF is Proto-oncogene serine/threonine-protein kinase, RAS is RAt sarcoma, TfR2 is the second transferrin receptor, Tf is Transferrin, JNKs is c-Jun N terminal kinases, ICAM-I is Intercellular adhesion molecule I, IFN-γ is Interferon-γ, MHC-I is major histocompatibility complex I, FAK is Focal adhesion kinase, ECM is Extracellular matrix, FASE is Fatty acid synthase, SCD-1 is Stearoyl-coenzyme A desaturase 1, ACC1 is Acetyl-CoA carboxylase and Cav is Caveolin.

Most of human colon adenocarcinoma cell lines, lipid rafts divide pro-apototic from anti-apoptotic insulin-like growth factor I (IGF-I) receptor signaling when exposed to tumor necrosis factor-α (TNF-α). In fugure 2.4, the paradoxical pro-apoptotic action of IGF-1 is transported through the PI3K/Akt pathway and that integrity of lipid rafts is important for suitable anti-apoptotic cell signaling. On the other hand, the activation of the ERK1/2 and p38 MAPK pathway that convey the IGF-I anti-apoptotic signaling is independent of lipid rafts [39].

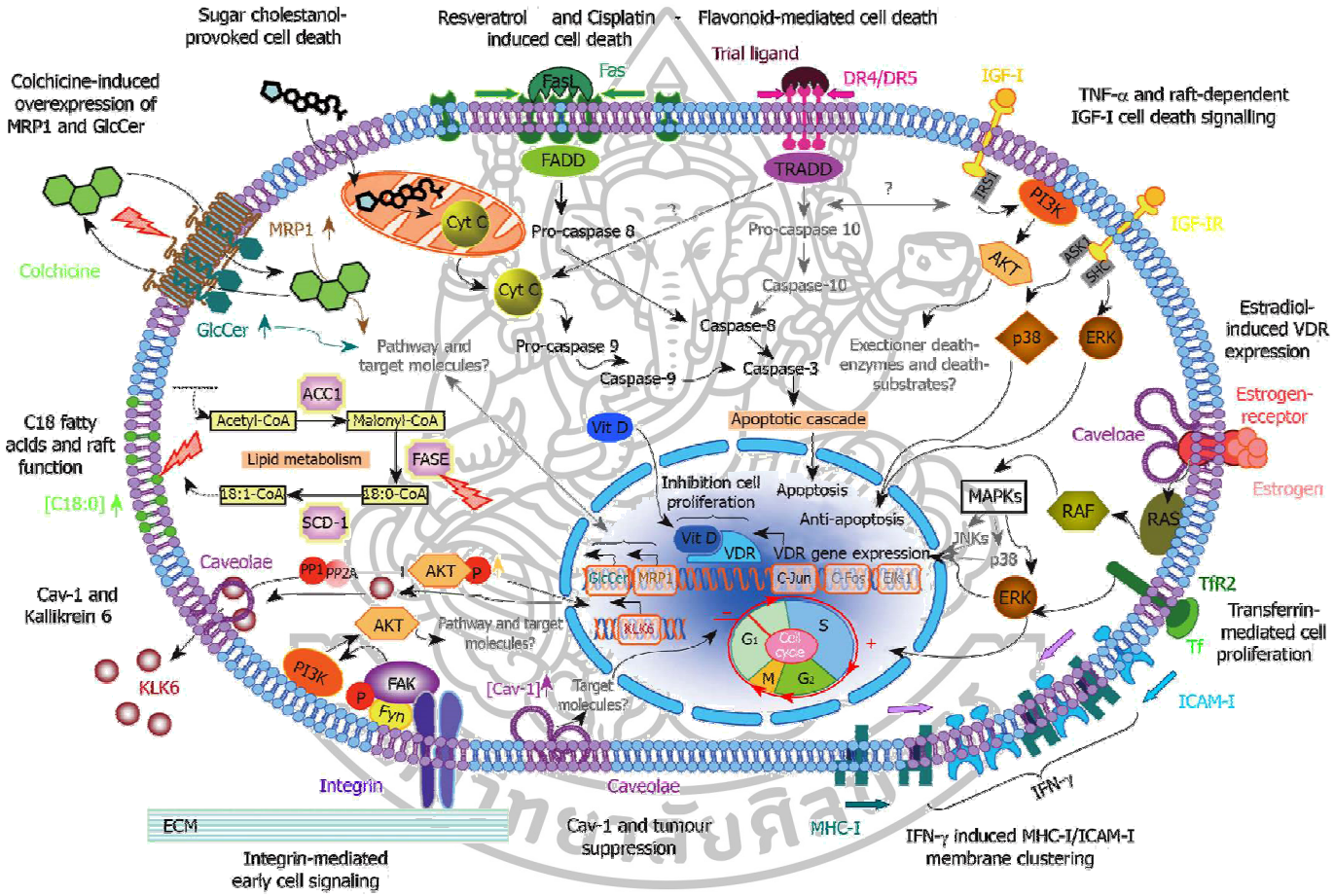


Figure 2.4 Intracellular signaling pathways in colorectal cancer.
Source: Jahn,K. A., Su, Y. and Braet, F. (2011). "Multifaceted nature of membrane microdomains in colorectal cancer." **World Journal of Gastroenterology** 17, 6 (February): 681–690.

The example of bioactive compounds from food and natural product that can induce cell death in colorectal cancer cells are resveratrol and quercetin. Resveratrol belongs to a class of polyphenolic compounds. It was reported to induce apoptosis in SW480 cells via caspase-8/caspase-3-mediated apoptosis cascade. Furthermore, resveratrol reveals induced cell death receptor Fas within lipid rafts on cell surface and caused formation of the death-inducing signaling complex. Quercetin belongs to a class of flavonoid compounds. It was reported to induce apoptosis in SW480 and HT-29 cells. Quercetin exposure enhanced apoptosis caused by TNF-related apoptosis-inducing ligand (TRIAL) via the death receptors (DR) 4 and 5 within lipid rafts on cell membrane [39].

Cisplatin is a strong chemotherapeutic agent and widely used for treatment of various cancers. It belongs to a class of alkylating agent. It induces apoptosis in human colon adenocarcinoma cells through the inhibition of the Na^+/H^+ membrane exchanger-1 and leads to an overall intracellular acidification. It also caused membrane fluidity. Membrane stabilization by cholesterol excess or monosialoganglioside-1 treatment can be counteracted by cisplatin treatment. Additionally, cisplatin, lipid-interfering compound, prevent the aggregation of the Fas receptor on the cell surface of HT-29 cells. Therefore, the action of cisplatin is through the Fas-signaling pathway [39].

The overexpression of cell signaling receptors is one of the common oncogenic alterations in cancer. When the receptors are overexpressed; the downstream signaling pathways are hyperactivated, and tumors are generated with unlimited proliferation potential and an unstable genotype [36].

Extracellular signal-regulated kinase (ERK) is one of members of Mitogen-activated protein kinase (MAPK) family. Extracellular signal-regulated kinases (ERK1 and ERK2) are activated and play a critical role in transmitting signals initiated by EGF, UV, TPA and platelet-derived growth factor (PDGF). The mojority of tumor phenotypes is linked to the deregulation of the ERK pathway [40].

The Figure 2.5, AP-1 is activator protein 1, ATF-1 is Cyclic AMP-dependent transcription factor, EGFR is epidermal growth factor receptor, IκB is inhibitor kappaB, IKK is IκB kinase, MEK is mitogen-activated protein-ERK kinase, MEKK1 is MEK kinase 1, MKK is mitogen-activated protein kinase kinase, MMP is

matrix metallopeptidase, MSK is mitogen- and stress-activated protein kinase, NFAT is Nuclear factor of activated T-cells, NIK is NF-κB-inducing kinase, RSK is ribosomal s6 kinase, S6K is s6 kinase, SFK is Src family kinase, STAT3 is signal transducer and activator of transcription 3 and VEGF is vascular endothelial growth factor.

Generally, cancer cells are initiated by many stimuli outside the cells. When cells are stimulated and EGFR are activated (figure 2.5). The cascades are started. The activated signals lead to stimulate the transcription factors of many genes such as cyclin D1, MMP and VEGF. The expression of those genes results in the imbalance of cell cycle control. Therefore, the abnormal cells can be arise [40].

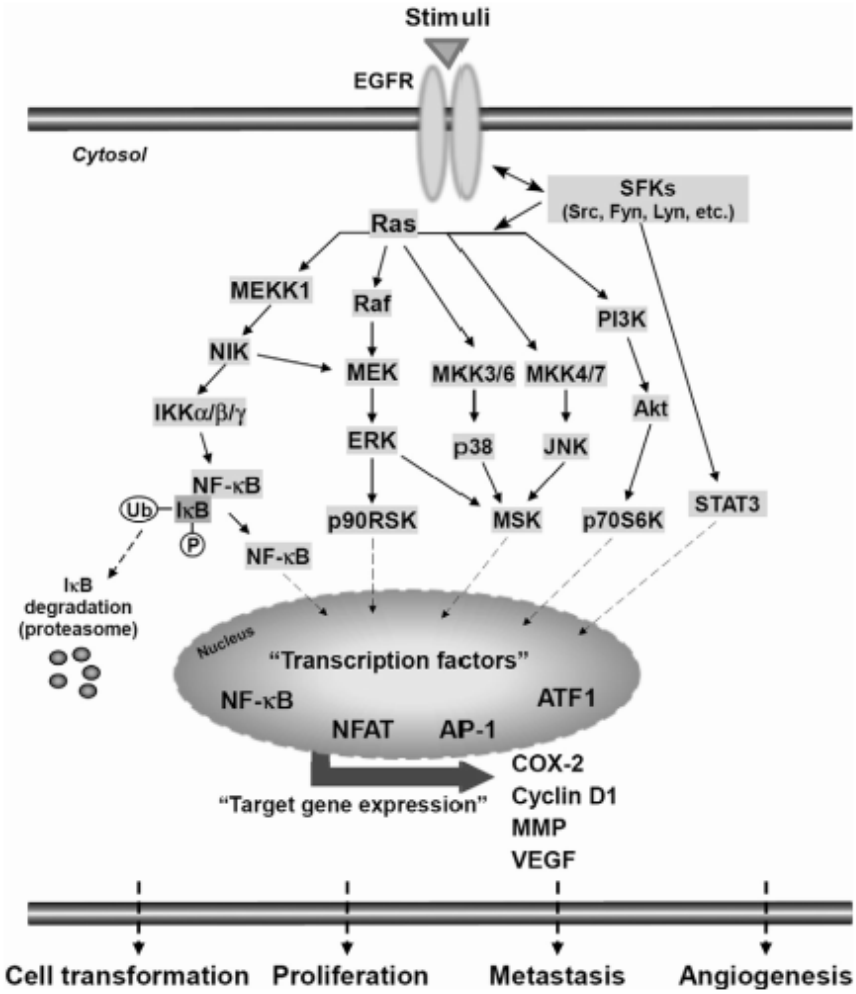


Figure 2.5 General scheme of signaling cascades in cancer cells. The binding of EGF results in the activation and phosphorylation of EGFR on its tyrosine residues and leads to the activation of downstream kinases, such as Ras or STAT3. Once triggered, the signal is amplified and results in the activation of various transcription factors. This event causes a many cellular responses including cell transformation, cell proliferation, metastasis and angiogenesis [40]. Some flavonoid compound targets the Raf1 and MEK1 signaling pathway such as quercetin and myricetin. However, it has not been reported to inhibite the colon cancer cells [41].

Source: Kang, N.J. et al. (2011). "Polyphenols as small molecular inhibitors of signaling cascades in carcinogenesis." **Pharmacology & Therapeutic** 130: 310-324.

2.2.1.2 Human colorectal carcinoma cells (HCT116 cells)

The human colorectal carcinoma cells (HCT116 cells) originated from colon ascendens organ of 48-year old male colorectal carcinoma patient [52]. This cell type is an epithelial cell. HCT116 cells are positive for transforming growth factor β1 and β2 (TGF β1 and β2) expression. This cell line has a mutation in colon 13 of the ras proto-oncogene and can be used as a positive control for PCR assay of mutation in this colon [43]. HCT116 line is a type of colorectal cancer cells because the mutant ras has been identified in colorectal cancer around 50% [44]

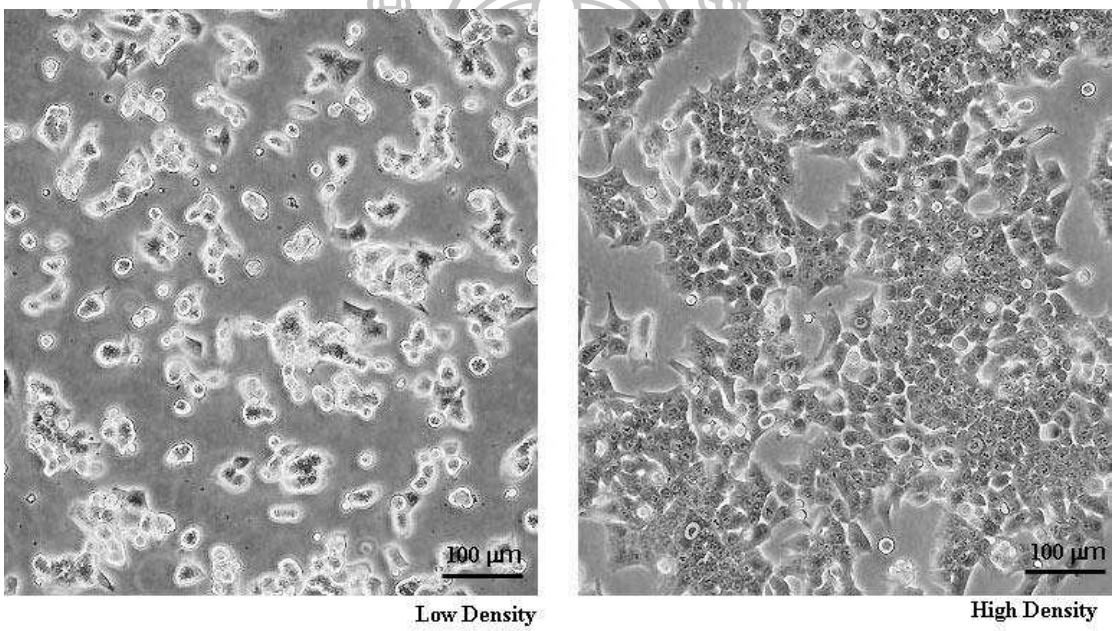


Figure 2.6 Morphology of HCT116 cell line at low and high density. Phase-contrast micrographs depict the individual cell cultures 24 and 72 hr after trypsinization and seeding. Scale bar, 100 µm.

Source: ATCC, American Type Culture Collection: All Products (CCL-247™). <www.atcc.org/Products/All/CCL-247.aspx> (Retrieved 2016-03-03).

2.2.2 Plant-derived drug

There are many reasons for the increased use of natural products. Plants are natural source of anticancer drugs. Several of the drugs obtained from plants act as topoisomerase inhibitors; included in this category are etoposide and teniposide, derived from a substance present in the mayapple plant [42], and topotecan and

irinotecan, derived from a substance present in the bark of the Chinese camptotheca tree. And also the tetracyclic ellipticine comes from *Ochrosia elliptica* plant and acts as an inhibition of DNA topoisomerase II. Another group of plant-derived drugs attack the microtubules that make up the mitotic spindle. This class of drugs includes vinblastine and vincristine, obtained from the *Madagascar periwinkle* and Taxol (Paclitaxel), discovered in the bark of the *Taxus brevifolia* tree. Vinblastine and vincristine block the process of microtubule assembly, whereas Taxol stabilizes microtubules and promotes the formation of abnormal microtubule bundles. In either case, the mitotic spindle is disrupted and cells can not divide [4, 5].

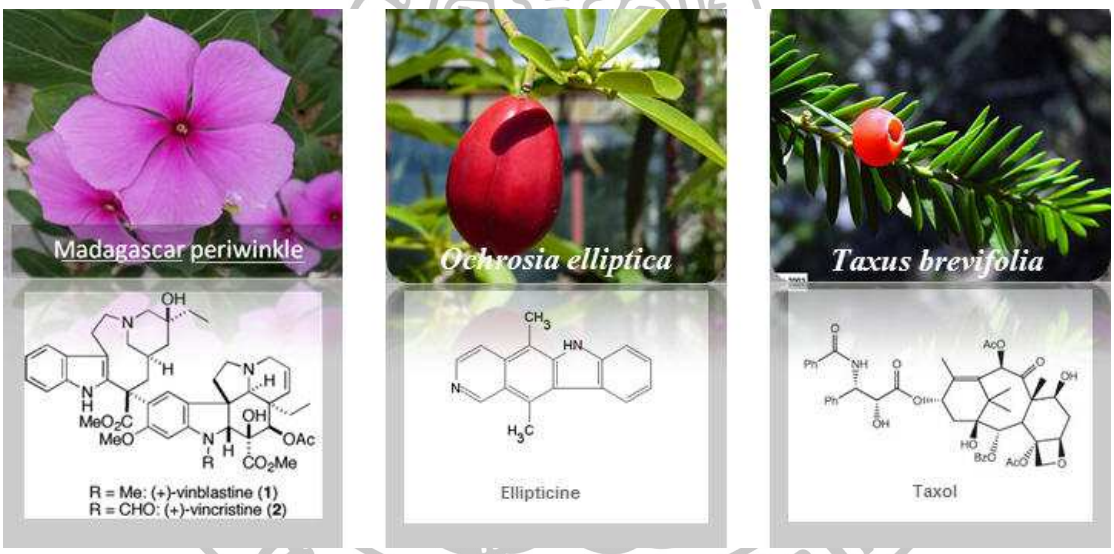


Figure 2.7 Example of an anti-cancer drug that obtained from plants including vinblastine, vincristine, ellipticine and taxol.

2.2.3 Phytochemicals from plants

Phytochemicals are the bioactive nonnutrient plant compounds. Phytochemicals are believed to have health benefits and still remain interested. Recently, the edible plant and medicinal plant continue being a popular study which exhibits a wide range of properties with potential relevance for fighting cancer.

Studies to date have demonstrated that phytochemicals in common fruit and vegetables can have complementary and overlapping mechanisms of action, including scavenging of oxidative agents, hormone metabolism, stimulation of the immune

system and regulation of gene expression in cell proliferation and apoptosis [20, 40, 45-48, 50-51].

Apigenin presents in parsley, celery and other vegetables. It induces apoptosis in human colon cancer cells and increases melanogenesis in B16 cells by activating the p38 MAPK pathway at least partially and suggests that apigenis or its derivatives may potentially be used for treating hypopigmentation disorders [45].

Crocetin comes from flower of saffron. It affects the growth of cancer cells by inhibiting nucleic acid synthesis, enhancing anti-oxidative system, inducing apoptosis and hindering growth factor signaling pathways. [45].

Curcumin, a mojor curcumanoid in the spice turmetic, is a potent inhibitior of NF-κB. It was also demonstrated that curcumin down-modulates Syk activity accompanied by down-regulation of Akt activation [40].

Cyanidin inhibit carcinogenesis by blocking of NF-κB and AP-1 from the MAPK pathway [40]. Cyanidin is able to reduce the proliferation of human colon cancer cells and intestinal tumor development in *apc* mice. Cyanidin initiated a dose-dependent apoptosis in human leukemia Molt 4B cells characterized by DNA fragmentation and this might be due to production of free oxygen, which induces intracellular oxidative stress [46].

Epigallocatechin gallate (EGCG) is a major component of green tea. EGCG can have pro-oxidant effect generating H_2O_2 in a time- and dose-dependent manner when added to cell culture media consequently provoking stressful and/or cytotoxic effects. The apoptosis induced in human oral squamous carcinoma cells by EGCG was attributed to the generation of H_2O_2 in the cell culture medium [47].

Fisetin is found in various sources, for example strawberries and apple. Fisetin pretreatment enhanced the radiosensitivity of p53-mutant HT-29 human colorectal cancer cells. Fisetin treatment also prolonged radiation-induced G_2/M arrest in HT-29 cells [40].

Kaempferol is a flavonoid that can be found in grapefruit and other edible plants. Studies on kaempferol are few but differ as to antiproliferative activity based on concentration. It was reported to induce apoptosis in colon cancer cells [20]. Kaempferol induces autophagy through AMPK and Akt signaling molecules and

causes G2/M arrest via downregulation of CDK1/cyclinB in hepatic cancer cells (SK-HEP-1) [48]. In contrast, it has low toxicity against normal cells [50].

Lycopene is a red pigment and demonstrates antioxidant activity and effect on breast, endometrial, prostate and colon cancer cells. It was also found to suppress insulin-like growth factor-I-stimulated growth [45].

Resveratrol has shown antitumor initiation activities such as inhibition of free radical formation in HL-60 cells treated with 12-O-tetradecanoylphorbol-13-acetate. The anti-initiation activity of resveratrol might be related to its antioxidant and antimutagenic effects. The antitumor effect of resveratrol also correlates with its ability to reduce tumor neovascularization of angiogenesis [46].

Rosmarinic acid is a natural antioxidant found in medicinal herbs such as rosemary. The extracts of rosemary play important roles in anti-inflammation anti-proliferation and anti-tumor. It has been found that rosmarinic acid inhibits migration, adhesion and invasion in Ls174-T human colon cancer cells [45].

Cancer reduction by polyphenolic-rich foods may be mediated by an indirect antioxidant function by 1) inhibiting redox-sensitive transcription factors such as NF-κB and AP-1 2) inhibiting pro-oxidant enzymes such as inducible nitric oxide synthase, lipoxygenases, cyclooxygenases and xanthine oxidase or 3) inducing phase II and antioxidant enzymes such as glutatione S-transferases and superoxide dismutases [51].

Table 2.3 Example of phytochemicals and their main dietary sources.

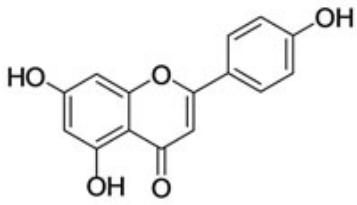
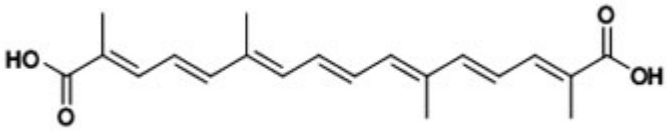
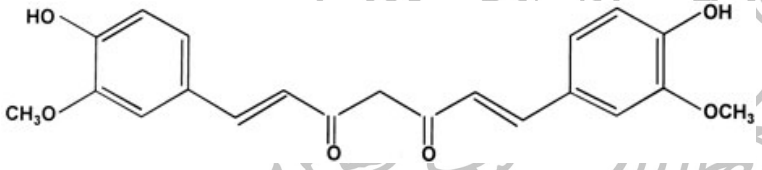
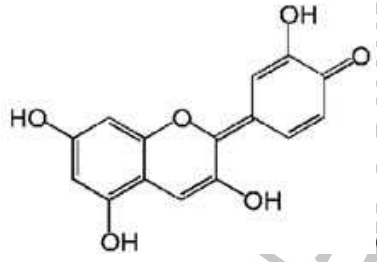
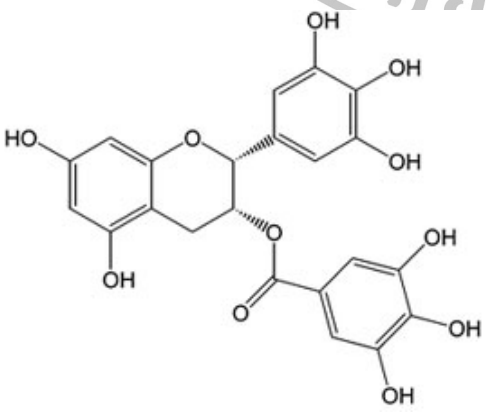
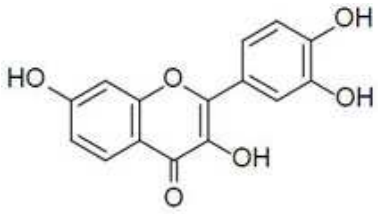
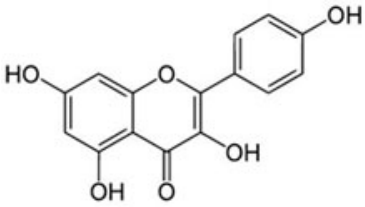
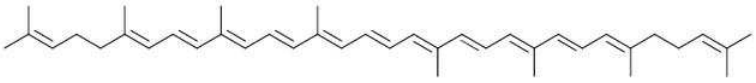
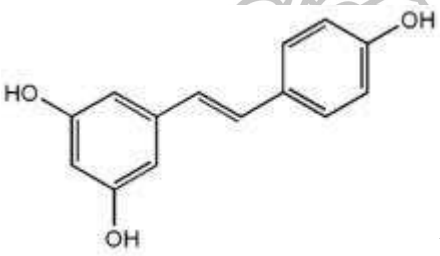
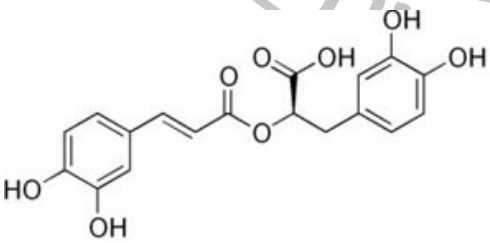
Structure of active compound	Source
 Apigenin	
 Crocetin	
 Curcumin	
 Cyanidin	
 Epigallocatechin-3-gallate	

Table 2.3 Example of phytochemicals and their main dietary sources (continued).

Structure of active compound	Source
 Fisetin	
 Kaempferol	
 Lycopene	
 Resveratrol	
 Rosmarinic acid	

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.2 Equipments

3.3 Methods

3.3.1 Plant material collection and extraction

3.3.2 Fractionation of *M. oleifera* leaves extract

3.3.2.1 Fractionation on Sephadex LH-20 chromatography

3.3.1.2 Detection of fractions using UV-spectrophotometer and TLC

3.3.1.3 TLC procedures

3.3.3 Evaluation of pooled fractions

3.3.3.1 Cytotoxicity assay

3.3.3.2 Intracellular mechanism assay (Western Blot Analysis/ WB)

3.3.4 Statistical analysis

3.1 Materials

Amersham ECL gel 4-12% (GE Healthcare, Waukesha, WI, USA)

Astragalin (Sigma Aldrich®, St. Louis, MO, USA)

Bio-Rad Protein Assay kit ((Bio-Rad Laboratories, USA)

Bovine serum albumin (BSA)

Bradford reagent

Chloroform (VWR Intrnational Ltd. England analytical reagent grade)

Cisplatin (Sigma Aldrich®, St. Louis, MO, USA)

Dimethyl sulphoxide (DMSO) (Fisher Scientific; analytical reagent grade)

Dulbecco's Modified Eagle Medium (D-MEM) (GIBCO™, Grand Island, NY, USA)

Enhanced chemiluminescence (ECL) reagent

Ethanol (Merck, Germany; purity ≥ 99.9%)

Fetal bovine serum (FBS) (GIBCO™, Grand Island, NY, USA)

Glacial acetic acid (Merck, Germany; purity ≥ 99.8%)

Hexane (VWR Intrnational Ltd. England analytical reagent grade)

Horseradish peroxidase–conjugated secondary antibodies

Hydrochloric acid (Scharlau Chemie S.A., Spain; purity ≥ 99.8%)

Isoquercetin (Sigma Aldrich®, St. Louis, MO, USA)

Kaempferol (Sigma Aldrich®, St. Louis, MO, USA)

Leaves of *M. oleifera*

L-glutamine (200 mM) (GIBCO™, Grand Island, NY, USA)

Methanol (Merck, Germany; purity ≥ 99.9%)

Non-essential amino acid (PAA laboratories, Austria)

Penicillin (GIBCO™, Grand Island, NY, USA)

Primary antibodies (p38 MAPK, Akt and pERK1/2 monoclonal antibody)

PVDF membranes (Amersham Hybond™-P, GE Healthcare, Waukesha, WI, USA)

Sephadex LH-20 (GE Healthcare, Waukesha, WI, USA)

Silica gel PSQ 100B (Fuji Sylisia, Kasugai, Japan)

Sodium bicarbonate (Analar® BDH; VWR International Ltd.)

Sterile water for irrigation (General Hospital Products Public Co., Ltd.)

Streptomycin (GIBCO^{TM}, Grand Island, NY, USA)

Sulfuric acid (H_2SO_4) (98%) (Mallinckrodt Baker Inc., USA)

Trypan blue stain 0.4% (GIBCO^{TM}, Grand Island, NY, USA)

Trypsin-EDTA 0.25% (GIBCO^{TM}, Grand Island, NY, USA)

WST-1 reagent

X-ray film

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Dojindo Molecular Technologies, Kumamoto, Japan)

Equipments

Automatic Autoclave (Model: LS-2D, Scientific Promotion Co., Ltd., Bangkok, Thailand)

Cellulose acetate filter 0.2 µm (Sartorius AG. 37070 Goettingen, Germany)

Centrifuge (Hermle Z300K; Labnet®; Lab Focus CO., Ltd.)

CO_2 incubator (HERA Cell 240 Heraeus)

Electrophoresis (horizontal) (GE Healthcare, Waukesha, WI, USA)

Evaporator (BUCHI, Switzerland)

Freeze-dryer (Model: Freezone 2.5, LABCONCO, USA)

Glass syringe 5 mL

Hair dryer

Hot Air Oven (WTB Binder, Germany)

Hot plate (Heidolph®, Germany)

Inverted Microscope (Model: ECLIPSE TE 2000-U, Nikon, Japan)

Laminar air flow (BIO-II-A)

Magnetic stirrer (Framo, Germany) and magnetic bar

Microcentrifuge (Microfuge 16®, Model: A46473, Beckman Coulter Inc., Germany)

Microcentifuge tube (Eppendorf ®, Corning Incorporated, NY, USA)

Micropipette 0.1-2.5 µL, 2–20 µL, 20–200 µL, 100–1000 µL and micropipette tip

Microplate reader (Model No; AOPUS01 and A153601; A Packard bioscience company)

Nylon membrane filter (pore size 0.45 µm, Merck Millipore, Bedford, MA, USA)

Open column chromatography 5 cm diameters, 45 cm length

pH meter (Horiba compact pH meter B-212, Japan)

Reagent spray bottle

Sartorius® filter set (Sartorius BORO 3.3 Goettingen, Germany)

Solvent filtration kit (all glass membrane filter holder, borosilicate glass (47mm) with sintered disc for membrane support, aluminum (duck) clamp, vacuum pump)

Sonicate Bath

Soxhlet Extractor

Spectrofluorometer (RF-1501, Shimadzu, Tokyo, Japan)

Thin-layer chromatography (TLC) developing tank

Tissue culture plate (96-, 6-Well plate) (Corning Incorporated, NY, USA)

UV-Vis spectrophotometer (Agilent model 8453 E, Germany)

Vortex mixer (Model: Labnet, USA)

3.2 Methods

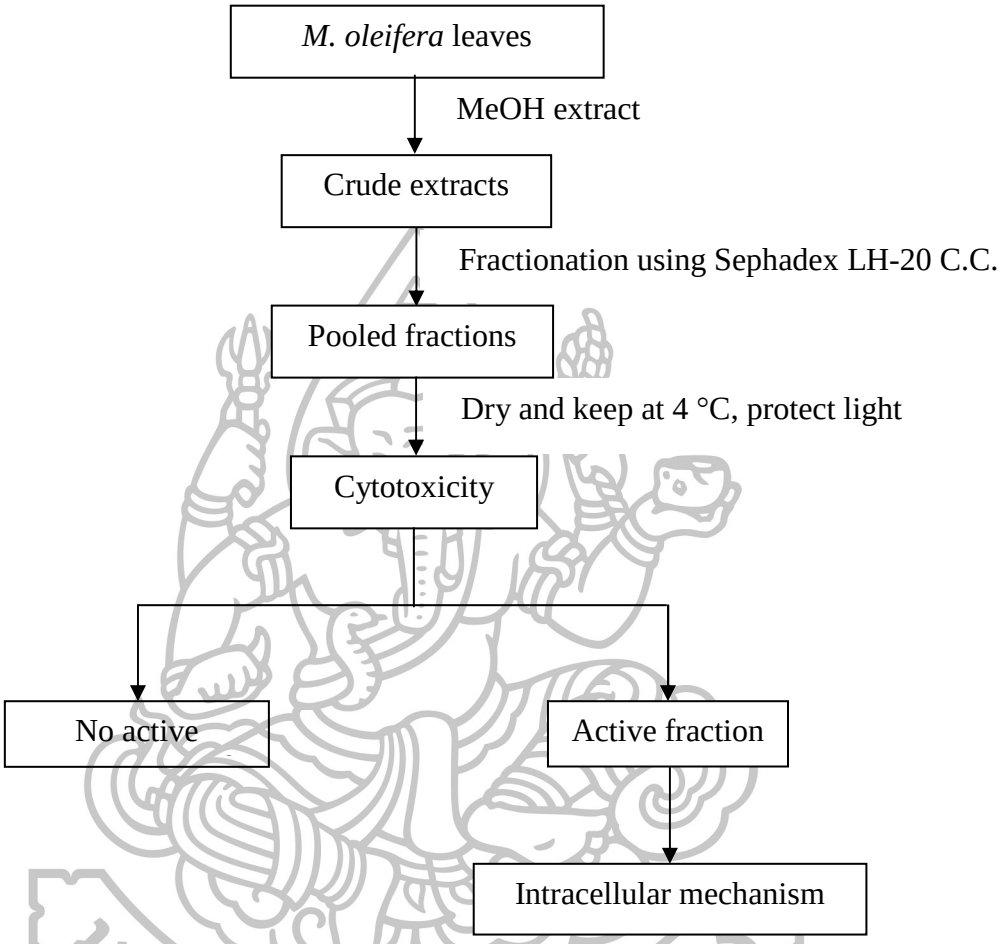


Figure 3.1 Conceptual framework of this research

3.3.1 Plant material collection and extraction

Fresh leaves of *M. oleifera* were collected from January-December 2012-2013 in Nakhon-Pathom province, Thailand. The dried leaves were extracted 100% methanol at 50-60 °C for 3 days using a Soxhlet Extractor and were completely dried using an evaporator. The crude extract was stored at 4 °C with protection from light.

3.3.2 Fractionation of *M. oleifera* leaves extract

3.3.2.1 Fractionation on Sephadex LH-20 chromatography

In this experiment, the crude methanol extract from *M. oleifera* leaves was freshly dissolved in 70% (v/v) aqueous ethanol at 1 g/ 20 ml and filtered

through 0.45-µm pore filter membranes (Merck Millipore, Bedford, MA, USA) just before use. The extract from *M. oleifera* leaves was fractionated using a glass chromatography column (i.d. 5 x 45 cm) packed with swollen Sephadex LH-20 in 70% (v/v) ethanol as the mobile phase. Each fraction was collected every 10 ml until the UV absorbance at 260 nm of each fraction was not detected.

3.3.2.2 Detection of fractions using UV-spectrophotometer and TLC

Each fraction was determined at UV 260 nm using a spectrophotometer and plotted the chromatogram between absorbance at 260 nm and number of fractions. And also each fraction were grouped on the basis of their spectral readings and then it was determined using TLC. Then, the grouped fractions were later grouped again on the basis of their TLC profile. The pooled fractions were concentrated to dryness on a rotary evaporator and freeze-drying and stored at -20 °C in the dark prior to further analysis.

3.3.2.3 TLC procedures

The separation of each grouped fraction on column chromatography was carried out by comparing with standard (STD) compound solutions, isoquercetin, astragalin and kaempferol, prepared in absolute ethanol and applied as a thin line 1 cm from the bottom of the silica plate and dried. The plate was then developed vertically in a closed chamber containing mobile phase (choloform: hexane 7:3) which was previously saturated at room temperature for 15 min. The mobile phase was allowed to migrate for a distance of 8.3 cm from the starting point. Subsequently, the plate was removed from the chamber and air dried. Each sample on plate was directly visualized both under UV irradiation at short (254 nm) and long waveleght (365 nm). The spots of component from pooled fractions were detected by spraying the plate with 50% (w/v) sulfuric acid reagent and heated at 95 ˚C for 2-3 min. The separated components are visualized as coloured bands. The bands containing pure natural product are evaluated the R_f value as equation below;

$$R_f \text{ value} = \frac{\text{distance traveled by substance}}{\text{distance traveled by solvent front}}$$

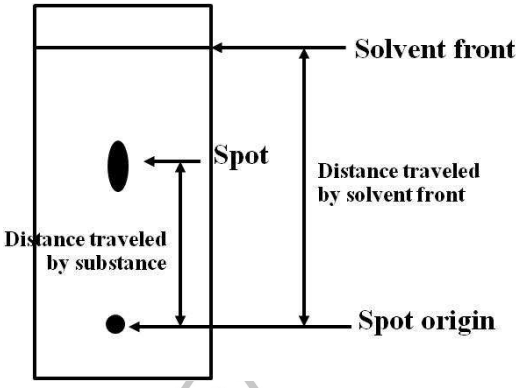


Figure 3.2 TLC plate showing distances traveled by the spot and the solvent after solvent front nearly reached the top of the adsorbent.

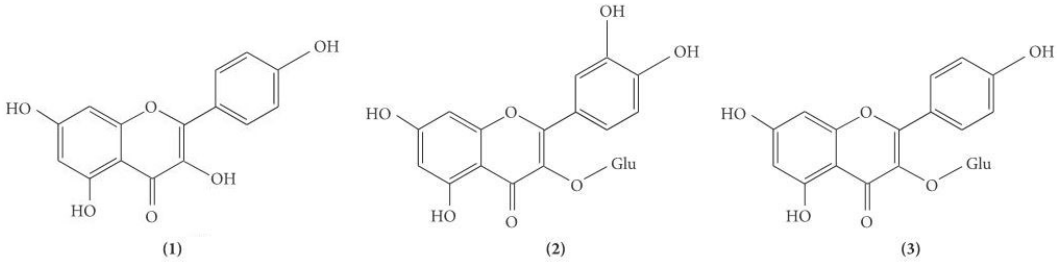


Figure 3.3 Chemical structures of kaempferol (1), isoquercetin (2) and astragalin (3).

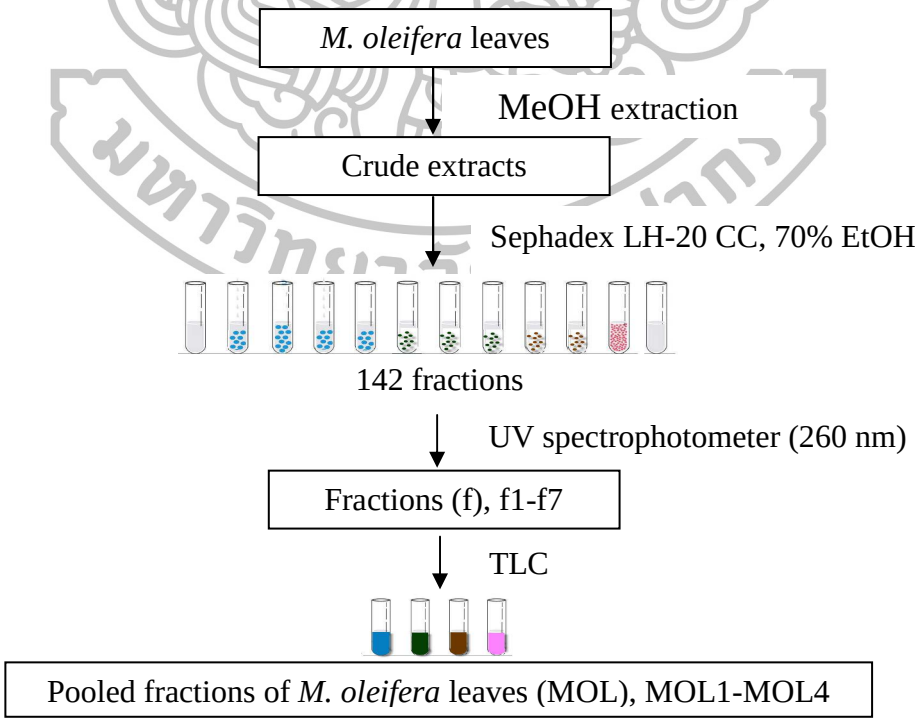


Figure 3.4 Preparation of pooled fractions from *M. oleifera* leaves extract through column chromatography

3.3.3 Evaluation of pooled fractions

Model of experiment studies: colon cancer cell lines HCT116 (from colon ascendens organ of 48-year old male colorectal carcinoma patient) and NHF (from normal human fibroblast) [52].

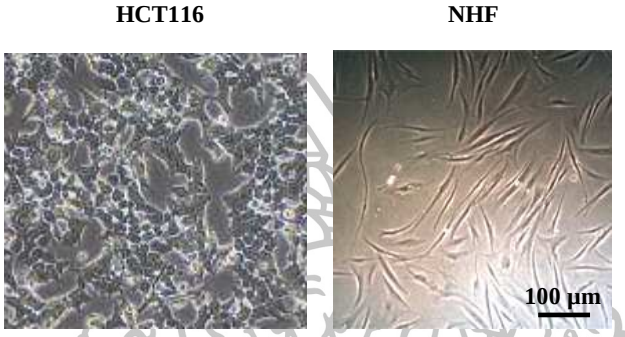


Figure 3.5 Morphology of HCT116 and NHF cell lines. Phase-contrast micrographs depict the individual cell cultures 24 h after trypsinization and seeding. Scale bar, 100 µm.

3.3.3.1 Cytotoxicity assay

HCT116 and NHF cells were maintained in DMEM supplemented with 10% (v/v) heat-inactivated FBS at 37 °C, 5% CO_2. Cells were plated at a density $1x10^4$ cells/well onto 96-well plate. Cells were incubated with varying concentrations of the *M. oleifera* pooled fractions for 24 or 48 h in triplicate cultures, compared with cisplatin as positive controls. Cells incubated with 0.5% DMSO (vehicle) was used as a negative control. After the incubation period, each well was washed with phosphate-buffed saline (PBS) and replaced with 1 mg/ml MTT or 1x WST-1 solution for 4 h incubation. The resulting crystals product from MTT assay was dissolved in 100 µl of 100% DMSO and measured at 550 nm using a microplate reader. The results from WST-1 assay were measured at 550 nm using a microplate reader. The percentage of cell viability was calculated as previously described [12].

3.3.3.2 Intracellular mechanism assay (Western Blot Analysis/ WB)

HCT116 cells were plated at a density $1x10^5$ cells/ mL onto 6-well plate and incubated overnight. Cells were incubated with varying concentrations of

the *M. oleifera* pooled fractions for 24 or 48 h in triplicate cultures, compared with positive and negative controls. After treatment with samples, cells were washed with PBS, pH 7.4 and lysed with lysis buffer (with 1 mM Na_3VO_4 and 1 mM NaF inhibitor) on ice for 15 min. Cell lysates were clarified by centrifugation at 13,000 g for 10 min at 4 °C, and protein concentrations of supernatants were quantified by Bradford assay. Equivalent amounts of total cellular proteins (5-25 μg) were separated by 10% gel SDS-PAGE. Each protein sample was added with sample loading buffer and boiled for 5 min and kept on ice immediately prior to electrophoresis through a 10% gel SDS-PAGE at 110 volts for 90 min. Proteins were then transferred onto PVDF membranes. The process was carried out for 1 h on ice. For immunodetection of the proteins, membranes were blocked in 5% BSA in TBS-T buffer for 1 h. Probing of nitrocellulose or PVDF membranes with primary antibodies at 4 °C overnight and detection of horseradish peroxidase–conjugated secondary antibodies by enhanced chemiluminescence (ECL) was done. For example, the probe used was antibodies against pERK1/2 polyclonal antibody (anti-rabbit ERK1/2). The chemiluminescence reagent was poured into the membrane and incubated for 1-5 min at room temperature and then removed excess chemiluminescence reagent. The membrane was placed and covered with plastic wrap. It must be gently smooth out any air between membrane and plastic wrap. The imaging film was put on top of the membrane for 5-10 min depended on the signal of protein. The film was developed and analyzed using ImageJ software.

3.3.4 Statistical analysis

All experimental measurements were performed in triplicate. The results are expressed as mean ± standard deviation. Statistical analysis of the data was evaluated using one-way analysis of variance (ANOVA) (SPSS software version 16.0). The significance level was set to $p < 0.05$.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Fractionation of *M. oleifera* leaves extract

4.1.1 Detection of fractions using UV-spectrophotometer

4.1.2 Detection of fractions using TLC

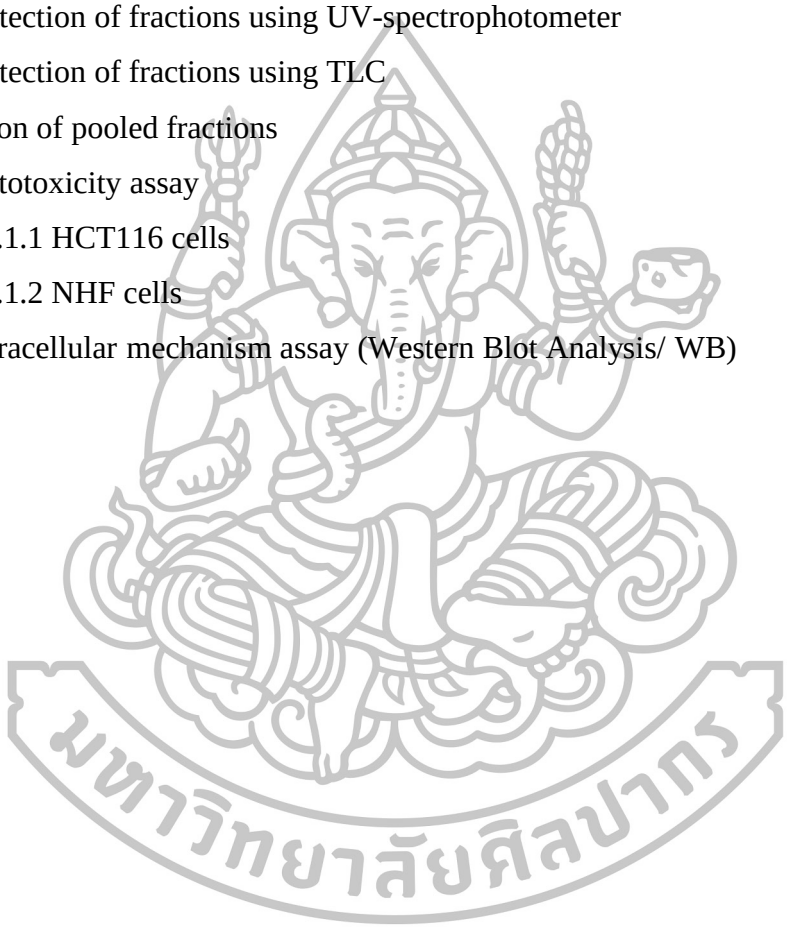
4.2 Evaluation of pooled fractions

4.2.1 Cytotoxicity assay

4.2.1.1 HCT116 cells

4.2.1.2 NHF cells

4.2.2 Intracellular mechanism assay (Western Blot Analysis/ WB)



4.1 Fractionation of *M. oleifera* leaves extract

Detection of fractions using UV-spectrophotometer

In the fractionation process of *M. oliefera* leaves extract, several fractions were collected every 10 ml from Sephadex LH-20 chromatography. The chromatograms of the eluates detected by UV spectrophotometer at 260 nm as shown in Figure 4.1 and Table A.1. The chromatograms showed several inner peaks of fractions from the *M. oleifera* leave extracts. The fractionation of *M. oleifera* leaves was divided into seven groups (f1-f7) according to their absorbance at 260 nm. Because of the absorbance of fraction number 27 to 95 was over 1.000 thus the dilution of these fraction numbers was prepared and then detected the absorbance at 260 nm (Fig. 4.1(b) and Table A.1). According to their absorbance, fraction number 22 to 46 was combined into group 1, f1. Fraction number 47 to 53 was combined into group 2, f2. Fraction number 54 to 76 was combined into group 3, f3. Fraction number 77 to 87 was combined into group 4, f4. Fraction number 88 to 99 was combined into group 5, f5. Fraction number 100 to 131 was combined into group 6, f6. For another fraction from fraction number 131 was combined into last group, f7.

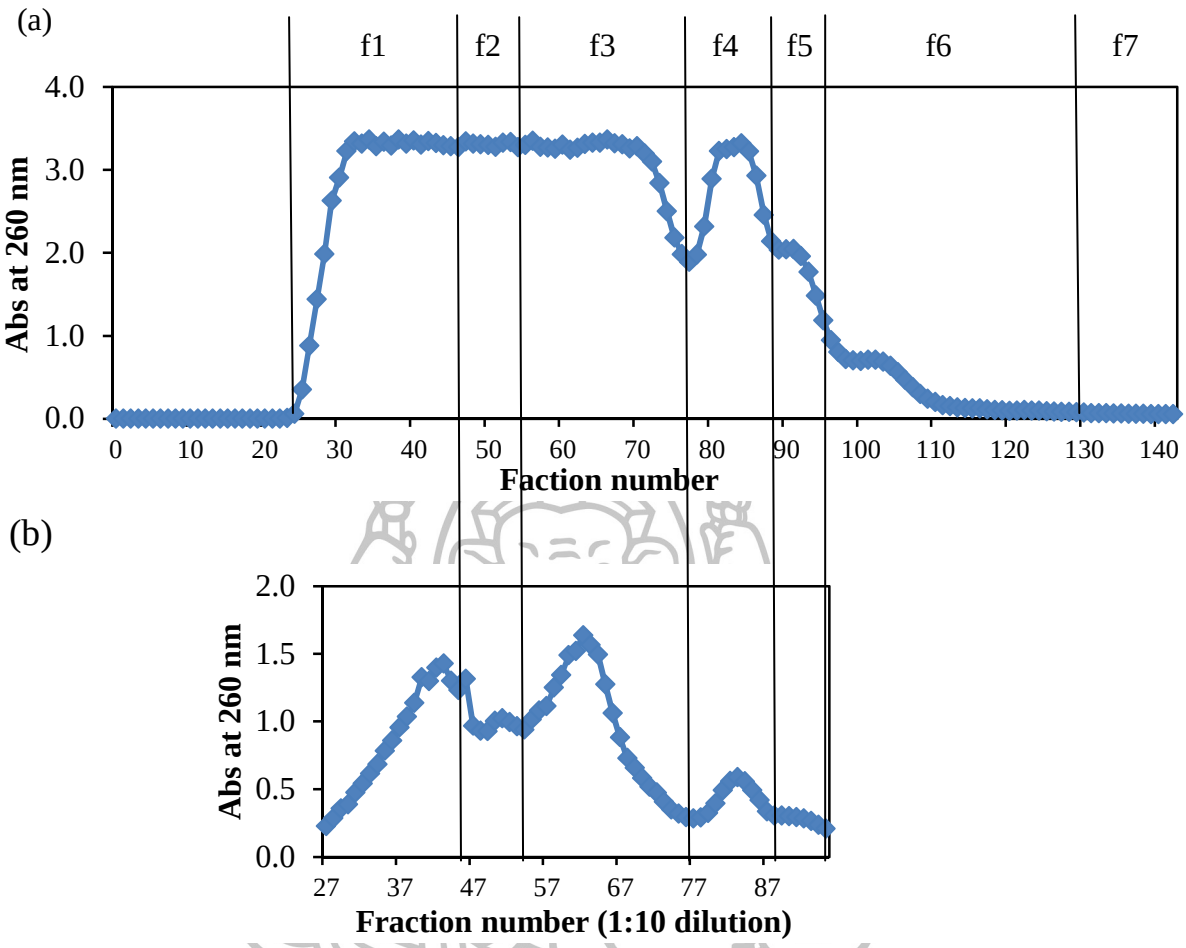


Figure 4.1 Chromatograms of the fractionation from *M. oleifera* leaves extracts. Fractions were collected using 70% EtOH as an eluent. A whole leaf extract at the weight of 1 g was applied onto the column packed with Sephadex LH-20. Collected fractions were measured at OD 260 nm, giving a yield of seven groups, 1-7 (a). Fraction number of 27-95 was diluted and measured at 260 nm to determine more accurately (b)

Detection of fractions using TLC

By TLC analysis, either pooled fractions or STD compounds (astragalin, isoquercetin and kaempferol) were applied on silica plate, using chloroform: hexane (70: 30) as a mobile phase, and sprayed with 50% H_2SO_4 and charred at 95 °C. As show in Figure 4.3, flavonoid astragalin and isoquercetin were found in the fraction 4 and 5. However, some astragalin interfere in the fraction 5. Kaempferol was not found in any fraction. Those STD compounds were revealed the presence of yellow spots. The yellow spot of astragalin, isoquercetin and kaempferol

show the R_f at 0.545, 0.331 and 0.777, respectively (Fig 4.3). The grouped fraction f1, f2 and f3 were combined into MOL1 according to their spots pattern on silica plate. For the grouped fraction f4, f5 and f6, their spot pattern shows an uniqe pattern. Then, the grouped fraction f4, f5 and f6 were renamed to the MOL2, MOL3 and MOL4, respectively. Last grouped fraction f7 did not have any spot. Therefore, seven grouped fractions, f1-f7, were regrouped to four fractions, MOL1 to MOL4.

(a) (b)

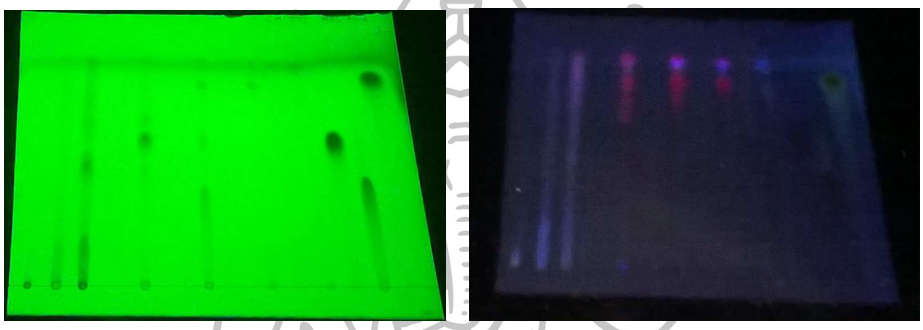


Figure 4.2 The separation of grouped fractions and STD compounds on the silica plates under UV irradiation at (a) 254 nm and (b) 365 nm using chloroform: hexane (70: 30) as a mobile phases. The fraction f1 - f7 represents in the spot 1-7. The STD compounds, astragalin, isoquercetin and kaempferol, represent in the spot 8-10.

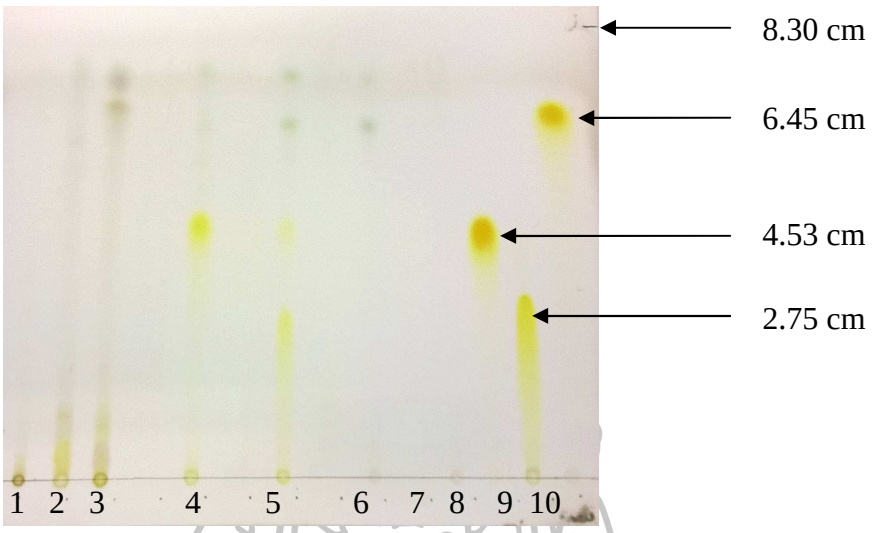


Figure 4.3 The separation of grouped fractions (f1-f7), and STD compounds on the silica plates using chloroform: hexane (70: 30) as a mobile phases after spraying with 50% H_2SO_4 and heating at 95 °C. The fraction f1 - f7 represents in the spot 1-7. The STD compounds, astragalin, isoquercetin and kaempferol, represent in the spot 8-10.

It is not surprising that astragalin and isoquercetin were obtained from *M. oleifera* leaves as they have been reported in previous studies [53]. Astragalin and isoquercetin are a flavonoid glycoside and are obtained from various leaves such as *Diospyros kaki*, mulberry, *Sapium sebiferum* [54-57]. These isolated compounds (astragain, isoquercetin) were also obtained from MOL2 and MOL3 of *M. oleifera* leaves. However, some astragalin in MOL2 was also found in the MOL3 (Fig. 4.3). It should eliminate the interfering astragalin component by removing some fractions from chromatogram (Fig. 4.1) before grouping as the procedure reported by Tragulpakseerojn et al. [16].

Phytochemical	Ether extract	Ethanol extract	Water extract
Gallic tannins	+	+	++
Catechol tennins	+	–	++
Coumarins	–	–	–
Steroids and triterpenoids	+++	++	++
Flavonoids	++	++	++
Saponins	+	+	++
Anthraquinones	+	++	+++
Alkaloids	+	–	++
Reducing sugars	–	++	++

Key -: not detected; +: present in low concentration; ++: present in moderate concentration; +++ present in high concentrations.

Table 4.1 Phytochemicals present in *M. oleifera* leaves using different solvent extract.
Soruce: Kasolo, J.N. et al. (2010) "Phytochemicals and uses of *Moringa oleifera* leaves in Ugandan rural communities." **Journal of Medicinal Plants Research** 4, 9: 753-757.

Selection of the solvent extraction approach is important. For example, Kasolo et al. reported that if *M. oleifera* leaves were extracted using ether or water solvent, the amount of steroids and triterpenoids or anthraquinones were found highest content compared with other compound (Table 4.1) [58]. However, the phytochemicals present in ethanol extract of *M. oleifera* leaves exhibited the steroid and triterpenoids, flavonoids, anthraquinones and reducing sugars in the moderate concentration [58]. Additionally, the previous findings show that among different solvents (absolute EtOH, absolute MeOH, aqueous EtOH (80% v/v), aqueous MeOH (80% v/v)), the extraction made under reflux and shaking techniques using aqueous alcohol (80% v/v of EtOH and MeOH) exhibits highest total phenolics and total flavonoid content [59].

Moreover, they, MOL1 to MOL4, were found to yield of 794.5, 12.3, 9.5 and 14.3 mg per 1 g of dried weight, respectively. In *M. oleifera* leaves, first elution pooled fraction, MOL1, gave the highest yield (79.45%) while subsequent pooled fractions gave the lower yields of 1.23% (w/w), 0.95% (w/w) and 1.43% (w/w), respectively. Each pooled fractions were further evaluated for biological activities.

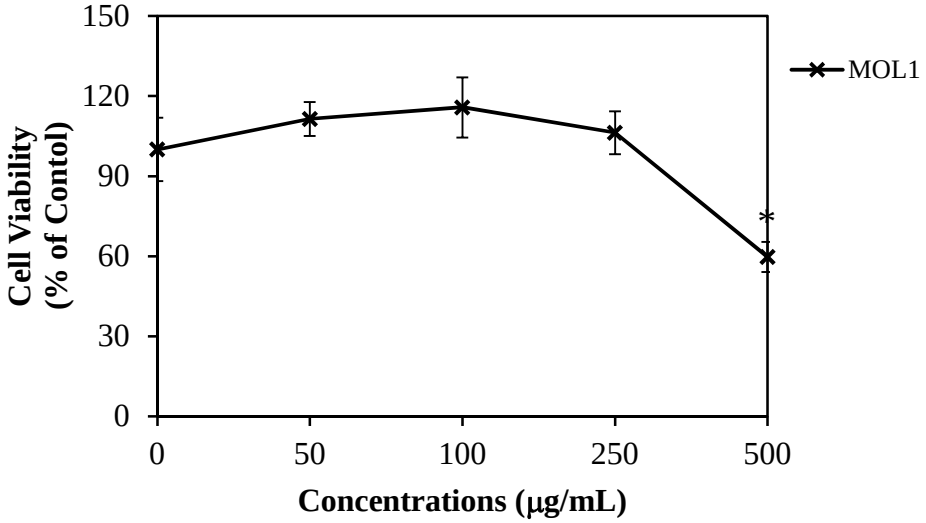
4.2 Evaluation of pooled fractions

4.2.1 Cytotoxicity assay

4.2.1.1 HCT116 cells

A primary screening for antitumor activity was carried out with antiproliferation assay by using the four pooled fractions (MOL1-MOL4). It was found that pooled fractions showed a relatively high antiproliferative activity in HCT116 cells. Firstly, they were examined the antiproliferative activity by WST-1 and MTT reduction assay in colon cancer, HCT116, cells. Studies on cell viability of HCT116 cells with and without the addition of four pooled fractions are illustrated in Figure 4.4 and 4.5. The four pooled fractions, MOL1-MOL4, showed anti-proliferative effects in a dose-dependent manner during 24 and 48 h (Figure 4.4 and 4.5). When cells were incubated for 24 and 48 h, MOL2, MOL3 and MOL4 were significantly more cytotoxic than MOL1. It suggests that the components present in MOL2, MOL3 and MOL4 are more effective than those in MOL1. In addition, slightly decrease of viability in the HCT116 cells was observed in the treatment of kaempferol (Figure 4.6a). HCT116 cells were less affected by kaempferol than that by pooled fractions. As shown in Figure 4.6b, the treatment of astragalin did not effect on HCT116 cell proliferation. When cells were incubated with isoquercetin, a strong decrease of cell viability was observed (Figure 4.6c). It suggests that isoquercetin which could be isolated from *M. oleifera* leaves is more effective than kaempferol and astragalin.

(a)



(b)

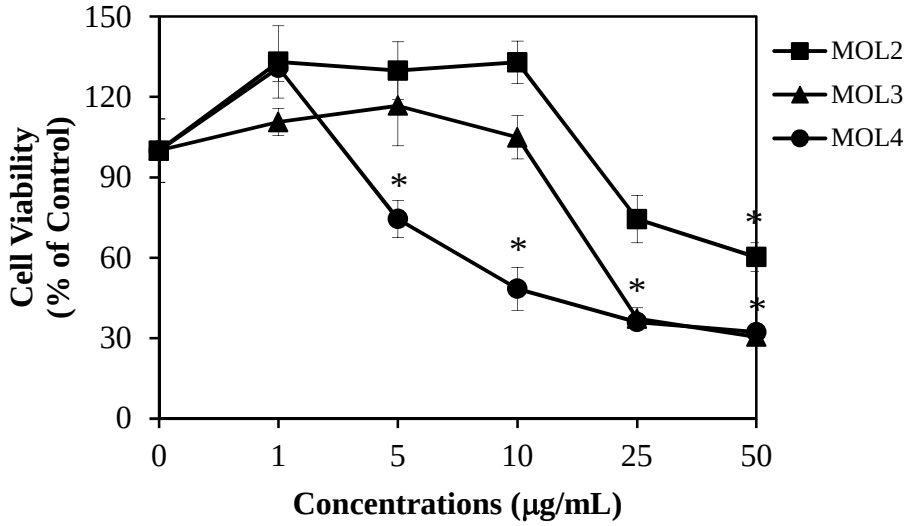
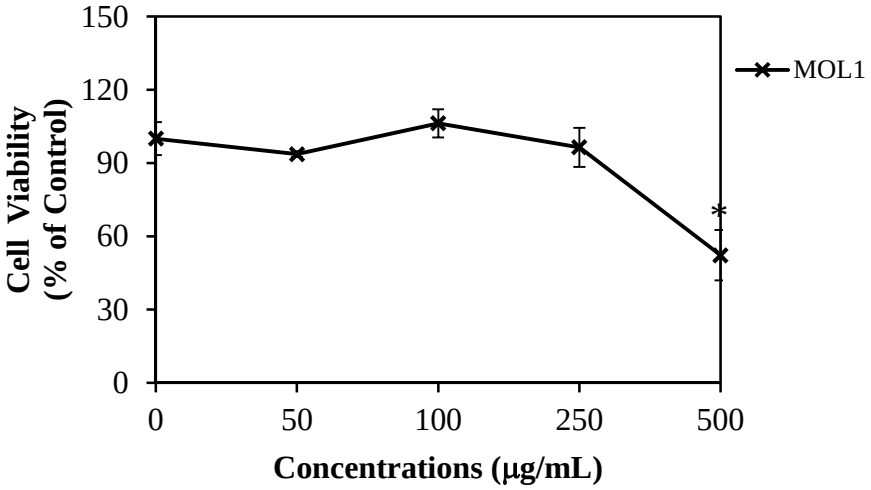


Figure 4.4 Effects of each pooled fraction (MOL1-MOL4) on the growth of HCT116 cells using WST-1 assay. Cells were treated with indicated concentration of each pooled fraction. Cells were continuous exposed to the pooled fractions (a) MOL1 or (b) MOL2-MOL4 at 24 h. Each value is the mean ± SD of triplicate of cultures. **P*<0.05, significantly different from the negative control as treatment with 0.5% of DMSO.

(a)



(b)

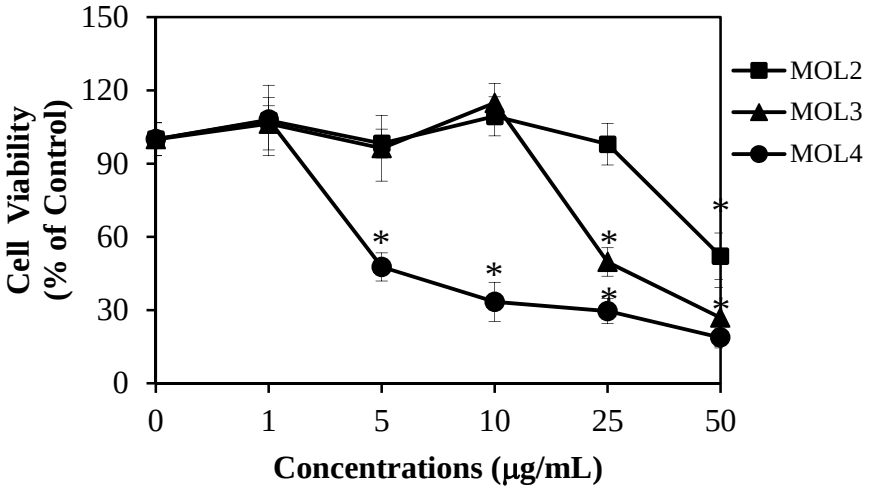


Figure 4.5 Effects of each pooled fraction (MOL1-MOL4) on the growth of HCT116 cells using WST-1 assay. Cells were treated with indicated concentration of each pooled fraction. Cells were continuous exposed to the pooled fractions (a) MOL1 or (b) MOL2-MOL4 at 48 h. Each value is the mean ± SD of triplicate of cultures. *P<0.05, significantly different from the negative control as treatment with 0.5% of DMSO.

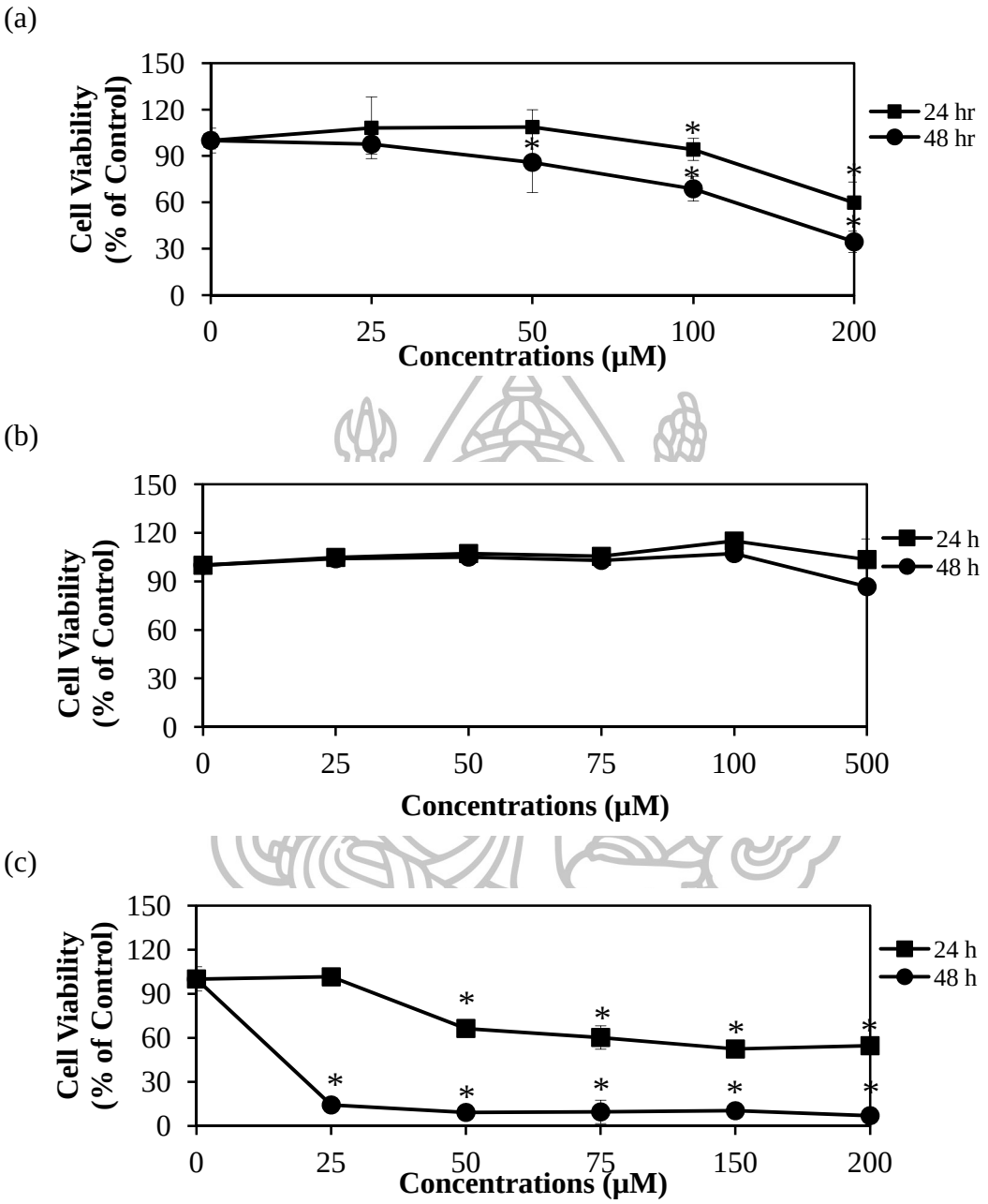


Figure 4.6 Effects of STD compounds on the growth of HCT116 cells using MTT assay. Cells were exposed to the STD compounds, kaempferol (a), astragalin (b) and isoquercetin (c) at 24 or 48 h. Each value is the mean ± SD of triplicate of cultures. **P*<0.05, significantly different from the negative control as treatment with 0.5% of DMSO.

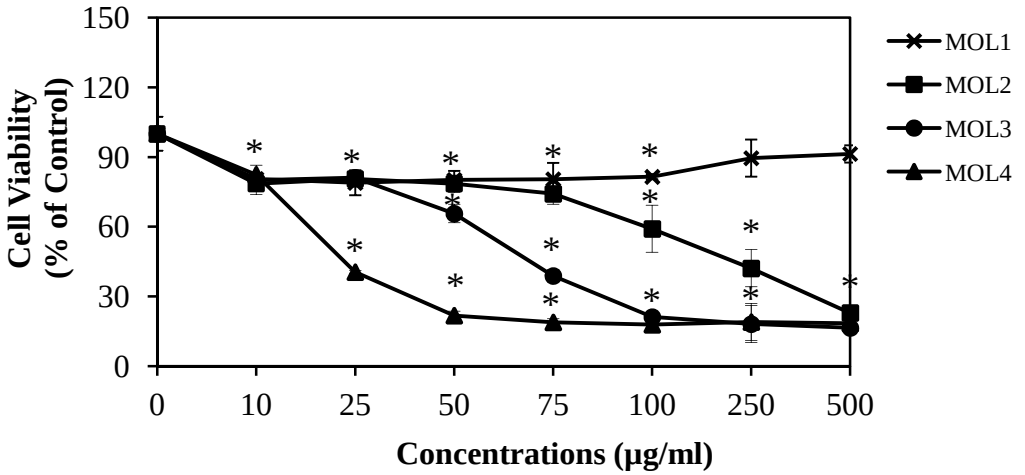
Since cisplatin (cis-diamminedichloroplatinum II) is an anti-cancer drug using for chemotherapy of many cancers including colon cancer [60], it was used as a positive control in this study. When HCT116 cells were treated with 100 μg/ml cisplatin for 24 and 48 h, it showed low toxicity to the cells (Table A.2 and A.6). This concentration of cisplatin may be not enough to reduce HCT116 cell proliferation. Sergent et al. reported that cisplatin at high dose (200 μg/ml) exhibits apoptosis induction on colon cancer HCT116 cells. Additionally, the efficiency of cisplatin is low in colorectal cancer (CRC), with fewer than 20% clinical responses when used alone [62]. Moreover, dysregulation of apoptosis pathways is generally assumed to be important for resistance to cisplatin [61]. It suggests that the HCT116 cells are quite tolerant to to cisplatin treatment.

4.2.1.2 NHF cells

To evaluate whether the effect of four pooled fractions (MOL1-MOL4) on colon cancer (HCT116) cells differed from that on human normal fibroblast (NHF) cells, the antiproliferative assay was carried out.

It was found that pooled fractions showed antiproliferative activity effect on NHF cells in a dose-dependent manner during 24 and 48 h. (Figure 4.7). When cells were incubated for 24 and 48 h, MOL2, MOL3 and MOL4 were more cytotoxic than MOL1. Its results were concomitant to the results from HCT116 cells. It suggests that the components present in MOL2, MOL3 and MOL4 are more effective than those in MOL1. Moreover, it was noticed that cisplatin was not cytotoxic at concentration of 100 μg/ml on NHF cells (Table A.10 and A.14). Generally, the efficiency of chemotherapeutic drugs, such as cisplatin, is low in non-cancer cells because normal cells do not have a rapid proliferation therefore NHF cells show a decrease sensitivity to cisplatin [61].

(a)



(b)

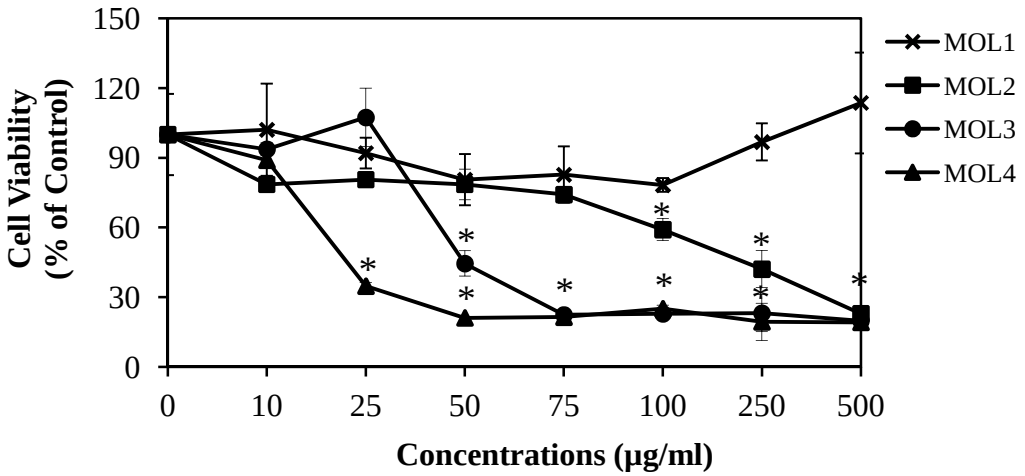


Figure 4.7 Effects of each pooled fraction (MOL1-MOL4) on the growth of NHF cells using WST-1 assay. Cells were treated with indicated concentration of each pooled fraction. Cells were continuous exposed to the pooled fractions MOL1-MOL4 at 24 h (a) or 48 h (b). Each value is the mean ± SD of triplicate of cultures. *P<0.05, significantly different from the negative control as treatment with 0.5% of DMSO.

The toxicity of each pooled fractions in both cells was also done at 24 and 48 h, determining the effect of different cell line. The results showed that the cytotoxicity of each pooled fractions was dose-dependent. The cytotoxicities of

MOL4 at both inclubation times were extremely higher toxic in colon cancer (HCT116) cells than that in human normal fibroblast (NHF) cells (Table 4.1). Moreover, the cytotoxicities of all pooled fractions, except the MOL1 were higher in HCT116 cells than that in NHF cells at 24 and 48 h. The results also suggested that the cytotoxic effect of almost pooled fractions from *M. oleifera* leaves in HCT116 cells was higher than that in NHF cells.

From the effect of STD compounds on HCT116 cell proliferation (Tabel 4.2) by MTT reduction assay, the results show that isoquercetin was strongest effective in cell proliferation at both 24 and 48 h. The results of kaempferol revealed that it decrease the viability of HCT116 cells in an inclubation time-dependent manner. However, the results of astragalin indicated that it was ineffective on HCT116 cells at 24 and 48 h.

Table 4.2 Toxicity of each pooled fractions in HCT116 and NHF cells at 24 and 48 h.

Samples	IC_{40} (approximately) (μg/mL)			
	24 h		48 h	
	HCT116	NHF	HCT116	NHF
MOL1	517.540	> 500	462.600	>500
MOL2	43.799	106.190	46.290	51.520
MOL3	21.145	52.498	24.869	39.197
MOL4	8.936	17.041	4.031	17.697

Table 4.3 Toxicity of STD compounds in HCT116 cells at 24 and 48 h.

STD compound	IC_{40} (approximately) (μM)	
	24 h	48 h
Astragalin	> 500.000	> 500.000
Kaempferol	205.896	126.648
Isoquercetin	68.518	5.412

Note that, the cytotoxicity test between each MOL and STD compound should be tested in the same method. In this study, the WST-1 stock solution was limited therefore, the similar principle assay, MTT method, was selected to use in STD compounds cytotoxicity assay.

The difference in colon cancer cell proliferation inhibition between MOL1, MOL2, MOL3 and MOL4 was probably due to the presence of different components and/or different amounts of active components in different pooled fraction of *M. oleifera* leaves extract. Since isoquercetin is one of components obtained from MOL3, the strong inhibitory effect of MOL3 on colon cancer cell growth from cell growth inhibition activity of isoquercetin may be partly.

It is worth to note that cancer cells, compared to normal cells, are more susceptible to be killed by anticancer drugs and polyphenols as well. This is probably because cancer cells are rapidly dividing cells [61]. In fact, by using the same concentration, each MOL decreases cell proliferation in cancer cell line, but having a little effect in normal cells.

The dose-dependent effect of MOL on cell proliferation inhibition was demonstrated in colon HCT116 cells, i.e., MOL3 and MOL4 at a low concentration (20-50 ug/ml) decreased HCT116 cell proliferation, while MOL1 at higher concentration (more than 500 ug/ml) could caused the antiproliferative activity.

4.2.2 Intracellular mechanism assay (Western Blot Analysis/ WB)

To this session, the investigation whether each fractionated fraction (MOL) induced growth arrest in the HCT116 cell was associated with the activation of ERK, cell lysate from MOL-treated cells at different times (24 and 48 h) and concentrations (2 times of IC_{40}, IC_{40} and half times of IC_{40} value) were subjected to western blot analysis using an anti-phospho-ERK antibody to detect phosphorylated ERK. However, the maximum concentration of MOL1 is 1.5 times of IC_{40} value because of the limited of the % of DMSO. Normally, the % of DMSO must be lower than 1% v/v of DMSO. The same blots were subsequently reblotted with an antibody that recognized total tubulin to verify equal amounts of the protein in various samples. As shown in Figure 4.8, treatment of HCT116 cell with isoquercetin, MOL1, MOL2,

MOL3 and MOL4 possess different effect on ERK signaling. Treatment of HCT116 cells with 11, 5.5, 2.7 μM of isoquercetin showed a slight effect on pERK signaling at 24 hr. Although, it mediated up-regulation of pERK at 48 hr, thereby further reduced proliferation of HCT116 cells might be due to another signaling pathway.

As shown in Figure 4.8 (b), treatment of HCT116 cell with MOL1 and MOL2 led to a dose-dependent reduction of pERK. MOL1 and MOL2 reduced the cellular levels of antiproliferative protein pERK1/2. It suggested that the blockage of the serine/ threonine kinase ERK activity by MOL1 and MOL2 is important for inhibition of colon cancer cell proliferation because active phosphorylated ERK enhances the proliferative of cells [44].

In addition, a MOL3 and MOL4 possing strong antiproliferative activity showed stronger effect on phosphorylation of ERKs reduction (Figure 4.8 (c)). Moreover, the strong antiproliferation activity of isoquercetin may involve other mechanisms. It has been reported that isoquercetin inhibit colon, HCT116, DLD-1 and SW480, cancer growth through Wnt/β-catenin signaling pathway [63]. Therefore, it might be worthfully to make a further experiment for MOL3 with Wnt/β-catenin signaling pathway.

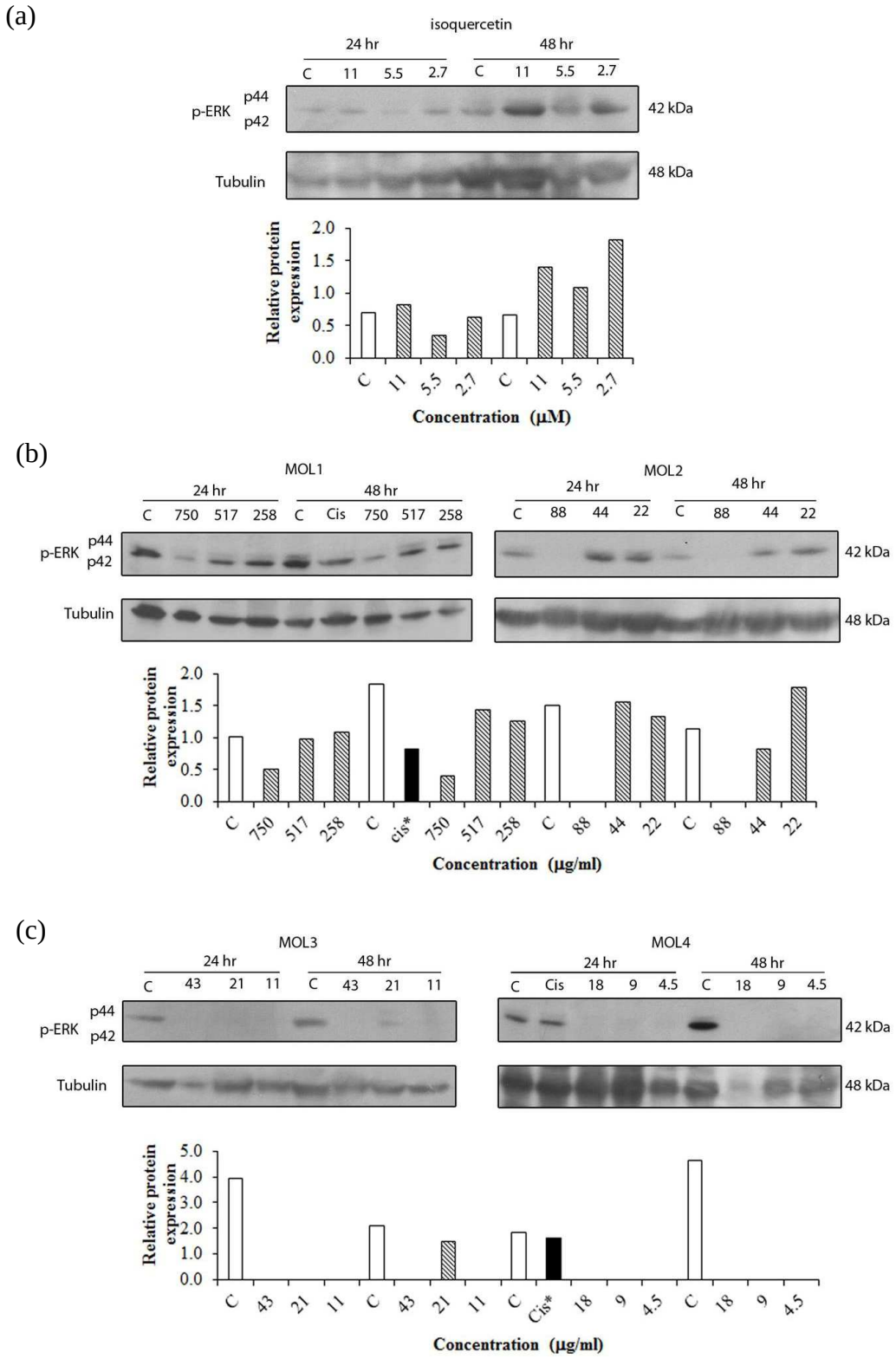
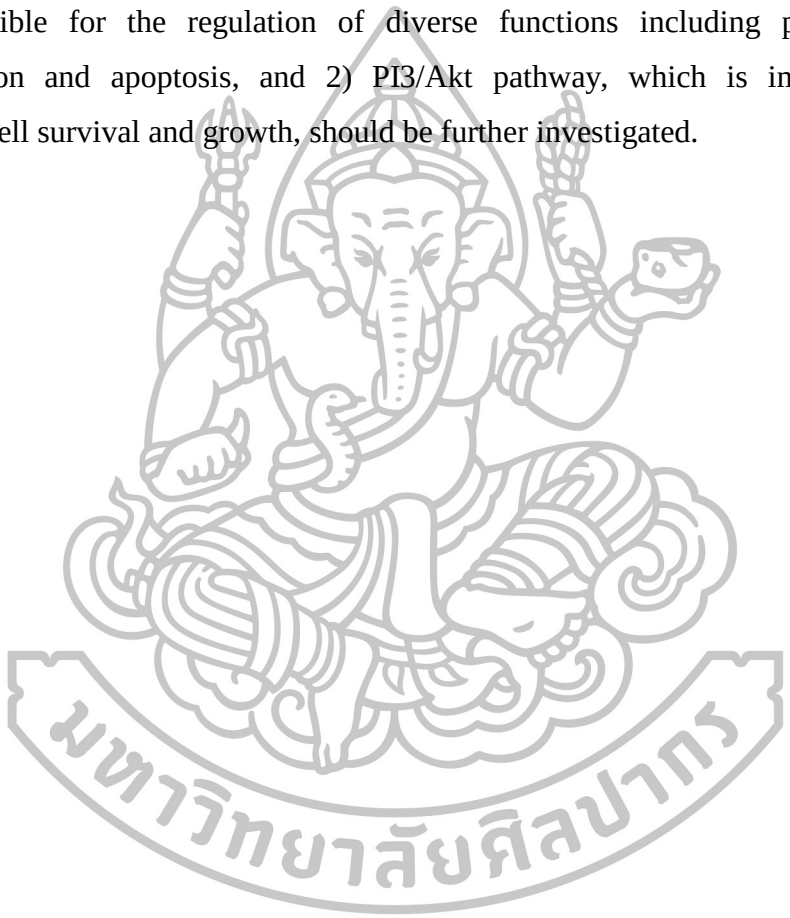


Figure 4.8 Effect of treatment with each pooled fraction ,(a) isoquercetin, (b) MOL1, MOL2, (c) MOL3 and MOL4 for 24 and 48 h on phospho-ERK expression in HCT116 cells, using western bolt. Tubulin was used as loading control. *Cisplatin 100 ug/ml was used as control.

The results MOL1, MOL2, MOL3 and MOL4 indicated that the inhibition of HCT116 cell growth was related to the reduction of pERK1/2 signaling pathway.

However, the pERK1/2 signaling data alone is insufficient to conclude that bioactivities of each pooled fraction from *M. oleifera* leaves promote cytotoxicity by dimimishing pERKs signaling. Other intracellular signaling of cancer i.e. 1) some member of the MAPK family, p38 kinase or c-Jun N-terminal kinase (JNKs) which are responsible for the regulation of diverse functions including proliferation, differentiation and apoptosis, and 2) PI3/Akt pathway, which is important for promoting cell survival and growth, should be further investigated.



CHAPTER 5
CONCLUSIONS

Base on the findings of this study, the following conclusions were made;

5.1 Fractionation of *M. oleifera* leaves extract

Fractionated the extract from *M. oleifera leaves* by gel filtration chromatography on Sephadex LH-20 is used for fractionation of natural products on the basis of molecular size. The fractionation of *M. oleifera* leaves was divided into four groups (MOL1-MOL4) according to their absorbance at 260 nm and TLC profile.

5.2 Evaluation of pooled fractions

5.2.1 Cytotoxicity assay

MOL2, MOL3 and MOL4 were observed to be significantly more cytotoxic on colon HCT116 cancer cells than MOL1. It may be deduced that the components present in MOL2, MOL3 and MOL4 are more effective than those in MOL1. While all pooled fraction was observed to be more specifically effect on colon cancer cells than normal cells.

Among the STD compound in *M. oleifera* leaves, isoquercetin showed strongest effect on HCT116 cells.

5.3 Intracellular mechanism assay (WB)

Molecular target of MOL1, MOL2, MOL3 and MOL4 is pERKs, which cooperates in MEK/ERK activation. This could partially explain the potent anti-proliferative effect it was observed *in vitro*.

REFERENCES

[1] WHO, World Health Organization (2014): World cancer burden 2012. <http://publications.cancerresearchuk.org/cancerstats/statsworldwide/worldfactsheet.html> (Retrieved 2015-05-10).

[2] Brenner, H., Kloor, M. and Pox, C.P. (2014). "Colorectal cancer." **The Lancet** 383, 9927: 1490-1502.

[3] Zhou, J.R., et al. (2003): "Soy phytochemicals and tea bioactive components synergistically inhibit androgen-sensitive human prostate tumors in mice." **Journal of Nutrition** 133, 516-521.

[4] Sultana, S., et al. (2014). "Medicinal plants combating against cancer-a green anticancer approach." **Asian Pacific journal of cancer prevention** 15, 4385-4394.

[5] เกษร นันทจิต และ สมจริง รุ่งแจง (2555). "เคมีของยา: ยาที่เกี่ยวข้องกับการรักษามะเร็ง." พิมพ์ครั้งที่3, 99.

[6] Sharma, J., et al. (2013). "Natural polymers-promising potential in drug delivery." **International Journal of PharmTech Research** 5, 684-699.

[7] Anwar, F. et al. (2007). "Moringa oleifera: a food plant with multiple medicinal uses." **Phytotherapy Research** 21, 17-25.

[8] Mbikay, M. (2012). "Therapeutic potential of Moringa oleifera leaves in chronic hyperglycemia and dyslipidemia: a review". **Frontiers in Pharmacology** 3, 1-12.

[9] Peixoto, J.R.O.et al. (2011). "In vitro antibacterial effect of aqueous and ethanolic Moringa leaf extracts." **Asian Pacific Journal of Tropical Medicine** 4, 201-204.

[10] Chuang, P.et al. (2007). "Anti-fungal activity of crude extracts and essential oil of Moringa oleifera Lam." **Bioresource Technology** 98, 232-236.

[11] Bose, C.K. (2007). "Possible role of Moringa oleifera Lam. root in epithelial ovarian cancer." **Medscape general medicine** 9, 26.

[12] Sreelatha, S. et al. (2011). "Antiproliferation and induction of apoptosis by Moringa oleifera leaf extract on human cancer cells." **Food and Chemical Toxicology** 49, 1270-1275.

[13] Iqbal, S. and Bhanger, M.I. (2006). “Effect of season and production location on antioxidant activity of Moringa oleifera leaves grown in Pakistan.” **Journal of Food Composition and Analysis** 19, 544-551.

[14] Makkar, H.P.S. and Becker, K. (1996). “Nutritional value and antinutritional components of whole and ethanol extracted Moringa oleifera leaves.” **Animal Feed Science and Technology** 63, 211-228.

[15] Khalafalla, M.M. et al. (2010). “Active principle from Moringa oleifera Lam leaves effective against two leukemias and a hepatocarcinoma.” **African Journal of Biotechnology** 9, 8467-8471.

[16] Tragulpakseerojn, J. et al. (2014). "Apoptosis activities of the extract from *Moringa oleifera* leaves on human HCT116 colon cancer cells." **Fundamental Toxicological Sciences** 1, 4: 143-149.

[17] Karthivashan, G. et al. (2013). “Identification of bioactive candidate compounds responsible for oxidative challenge from hydro-ethanolic extract of Moringa oleifera leaves.” **Journal of Food Science** 78, C1368-C1375.

[18] Slattery, M.L. et al. (2012). “MAPKinase genes and colon and rectal cancer.” **Carcinogenesis**, 33(12), 2398-2408.

[19] McCubrey, J.A. et al. (2007). “Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance.” **Biochimica et Biophysica Acta** 1773, 8: 1263-84.

[20] Li, W. et al. (2009). “Kaempferol induces apoptosis in human HCT116 colon cancer cells via the Ataxia-Telangiectasia mutated-p53 pathway with the involvement of p53 upregulated modulator of apoptosis.” **Chemico-Biological Interactions** 177, 121-127.

[21] Ross, Ivan A. (2003). "Moringa pterygosperma" **Medicinal Plants of the World** , 367-376.

[22] Charoensin, S. (2014). “Antioxidant and anticancer activities of *Moringa oleifera* leaves.” **Journal of Medicinal Plants Research** 8, 7: 318-325.

[23] Budda, Sirintip et al. (2011). "Suppressive effects of Moringa oleifera Lam pod against mouse colon carcinogenesis induced by azosymethane and dextran

sodium sulfate." **Asian Pacific Journal of Cancer Prevention** 12, 3221-3228.

[24] Guevara, Amelia P. et al. (1999). "An antitumor promoter from Moringa oleifera Lam." **Mutation Research** 440, 181-188.

[25] Berkovich, L. et al. (2013). "*Moringa oleifera* aqueous leaf extract down-regulates nuclear factor-kappaB and increases cytotoxic effect of chemotherapy in pancreatic cancer cells." **Complementary and Alternative Medicine**13, 212-219.

[26] Tiloke, C. et al. (2013). "The antiproliferative effect of *Moringa oleifera* crude aqueous leaf extract on cancerous human alveolar epithelial cells." **Complementary and Alternative Medicine** 13, 226.

[27] Purwal, L. et al. (2010). "*In vivo* anticancer activity of the leaves and fruits of moringa oleifera on mouse melanoma." **Pharmacologynoline** 1, 655-665.

[28] Chinedu, Anyanwu Anthony et al. (2014). "Effect of the ethanolic leaf extract of *Moringa oleifera* on insulin resistance in streptozotocin induced diabetic rats." **Journal of Plant Sciences** 2, 6-1: 5-12.

[29] Tende, J.A. et al. (2011). "Effect of ethanolic leaves extract of *Moringa oleifera* on blood glucose levels of streptozocin-induced diabetics and normoglycemic wister rats." **British Journal of Pharmacolog and Toxicology** 2, 1: 1-4.

[30] Soliman, G.Z.A. (2013). "Anti-diabetic activity of dried *Moringa oleifera* leaves in normal and streptozotocin (Stz)-induced diabetic male rats." **Biochemistry** 3, 9: 18-23.

[31] Cheenpracha, Sarot et al. (2010). "Potential anti-inflammatory phenolic glycosides from the medicinal plant *Moringa oleifera* fruits." **Bioorganic and Medicinal Chemistry** 13, 6598-6602.

[32] Fakurazi, Sharida et al. (2012). "*Moringa oleifera* hydroethanolic extracts effectively alleviate acetaminophen-induced hepatotoxicity in experimental rats through their antioxidant nature." **Molecules** 17, 8334-8350.

[33] Verma, V.K. et al. (2012). “Anti-ulcer and antioxicant activity of *Moringa oleifera* (Lam) leaves against aspirin and ethanol induced gastric ulcer in rats.” **Research Journal of Pharmaceuticaly** 2, 2: 46-57.

[34] Govardhan Singh, R.S. et al. (2013). "Phenolic composition, antioxidant and antimicrobial activities of free and bound phenolic extracts of *Moringa oleifera* seed flour." **Journal of Functional Foods** 5, 1883-1891.

[35] Mbikay, Majambu. (2012). "Therapeutic potential of *Moringa oleifera* leaves in chronic hyperglycemia and dyslipidemia: a review." **Frontiers in Pharmacology** 3, 1-12.

[36] Mitra, A.K. et al. (2014). “Advanced drug delivery in cancer therapy.” **Advanced drug delivery**, 323-340.

[37] Kleinsmith, L.J. (2006), "Identifying the causes of cancer." **Principles of cancer biology**. 60-79.

[38] Sittiwet, C. (2014), "Rafts, Caveolae and ıntracellular cholesterol trafficking." วารสารวิทยาศาสตร์บูรพา 19(1), 147-159.

[39] Jahn, K. A. et al. (2011). “Multifaceted nature of membrane microdomains in colorectal cancer.” **World Journal of Gastroenterology** 17, 6 (February): 681–690.

[40] Kang, N. J. et al. (2011). "Polyphenols as small molecular inhibitors of signaling cascades in carcinogenesis." **Pharmacology and Therapeutics** 130, 310-324.

[41] Bode, A. M. and Dong, Z. (2013). "Signal transduction and moleular targets of selected flavonoids". Antioxidants and redox signaling. 19(2): 163-179.

[42] Ross, Warren et al. (1984). "Role of Topoisomerase II in mediating epipodophyllotoxin-induced DNA cleavage." **Cancer research** 44, 55857-5860.

[43] ATCC, American Type Culture Collection: All Products (CCL-247^{TM}). <www.atcc.org/Products/All/CCL-247.aspx> (Retrieved 2016-03-03).

[44] Ruddon, R.W. (1995). “Cancer Biology”. Oxford University Press: New York.

[45] Wang, H. et al. (2012). "Plants against cancer: A review on natura phytochemicals in preventing and treating cancers and their druggability." **Anti-Cancer Agents in Medicinal Chemistry** 12, 10: 1281-1305.

[46] Nichenametla, S. N. et al. (2014). "A review of the effects and mechanisms of polyphenolics in cancer" **Critical Reviews in Food Science and Nutrition** 46, 161-183.

[47] Archivio, M. D. et al. (2008). "Modulation effects of polyphenols on apoptosisinduction: relevance for cancer prevention." **International Journal of Molecular Sciences** 9, 213-228.

[48] Huang, Wen-Wen et al. (2013). "Kaempferol induces autophagy through AMPK and Akt signaling molecules and causes G2/M arrest via downregulation of CDK1/cyclin B in SK-HEP-1 human hepatic cancer cells." **International Journal of oncology** 42, 2069-2077.

[49] Sreelatha, S. and Padma, P.R. (2009). "Antioxidant activity and total phenolic content of *Moringa oleifera* leaves in two stages of maturity." **Plant Foods for Human Nutrition** 64, 303-311.

[50] Yoshida, Tatsushi et al. (2008). "Kaempferol sensitizes colon cancer cells to TRAIL-induced apoptosis." **Biochemical and Biophysical Research Communications** 375, 129-133.

[51] Finley, J. W. (2005). "Proposed criteria for assessing the efficacy of cancer reduction by plant foods enriched in carotenoids, glucosinolates, polyphenols and selenocompounds." **Annals of Botany** 95, 1075-1096.

[52] Ahmed, D. et al. (2013). “Epigenetic and genetic features of 24 colon cancer cell lines.” **Oncogenesis** 2, 1-8.

[53] Vongsak, B. et al. (2013). “Simultaneous determination of crypto-chlorogenic acid, isoquercetin, and astragalin contents in *Moringa oleifera* leaf extracts by TLC-densitometric method.” **Evidence-Based Complementary and Alternative Medicine,** 1-7.

[54] Xue, Y., Miyakawa et al. (2011). “Isolation and tyrosinase inhibitory effects of polyphenols from the leaves of Persimmon, Diospyros kaki.” **Journal of Agricultural and Food Chemistry** 59, 6011-6017.

[55] Katsube, T. et al. (2006). “Antioxidant flavonol glycosides in mulberry (Morus alba L.) leaves isolated based on LDL andtioxidant activity.” **Food Chemistry** 97, 25-31.

[56] Fu, R., Zhang, Y.et al. (2015). “Phenolic composition and effects on allergic contact dermatitis of phenolic extracts Sapium sebiferum (L.) Roxb. leaves.” **Journal of Ethnopharmacology** 162, 176-180.

[57] Choi, J.et al.(2013). “Antioxidant effect of astragalin isolated from the leaves of *Morus alba* L. against free radical-induced oxidative hemolysis of human red blood cells.” **Archives of Pharmacal Research** 36, 912-917.

[58] Kasolo, J.N. et al. (2010). "Phytochemicals and uses of *Moringa oleifera* leaves in Ugandan rural communities." **Journal of Medicinal Plants Research** 4, 9: 753-757.

[59] Sultana, B., Anwar, F. and Ashraf, M. (2009). “Effect of Extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts.” **Molecules** 14, 2167-2180.

[60] Cepeda, V., Fuertes, M. A., Castilla, J., Alonso, C., Quevedo, C., Perez, J. M. (2007). “Biochemical mechanisms of cisplatin cytotoxicity.” **Anticancer Agents Med Chem** 7(1), 3-18.

[61] Fayad, W. et al. (2009). "Restriction of cisplatin induction of acute apoptosis to a subpopulation of cells in a three-dimensional carcinoma culture model." **International Journal of Cancer** 125, 2450-2455.

[62] Sergent, C. et al. (2002). "Human colon cancer cells surviving high doses of cisplatin or oxaliplatin in vitro are not defective in DNA mismatch repair proteins." **Cancer Chemother Pharmacol** 49, 445-452.

[63] Amado, N.G. et al. (2014). "Isoquercitrin suppresses colon cancer cell growth in vitro by tarteting the Wnt/ β-catenin signaling pathway." **The Journal of Biological Chemistry** 289, 51: 35456-35467.

APPENDIX

APPENDIX A

1. Fractionation of *M. oleifera* leaves extracts

Table A.1 Detection of each fraction from fractionation of *M. oleifera* leaves extract using UV-spectrophotometer at 260 nm

Fraction no.	n (no dilution)				n (1:10 dilution)			
	1	2	3	Avg	1	2	3	Avg
1	0.000	0.000	0.000	0.000	-	-	-	-
2	0.000	0.000	0.000	0.000	-	-	-	-
3	0.000	0.000	0.000	0.000	-	-	-	-
4	0.000	0.000	0.000	0.000	-	-	-	-
5	0.000	0.000	0.000	0.000	-	-	-	-
6	0.000	0.000	0.000	0.000	-	-	-	-
7	0.000	0.000	0.000	0.000	-	-	-	-
8	0.000	0.000	0.000	0.000	-	-	-	-
9	0.000	0.000	0.000	0.000	-	-	-	-
10	0.000	0.000	0.000	0.000	-	-	-	-
11	0.000	0.000	0.000	0.000	-	-	-	-
12	0.000	0.000	0.000	0.000	-	-	-	-
13	0.000	0.000	0.000	0.000	-	-	-	-
14	0.000	0.000	0.000	0.000	-	-	-	-
15	0.000	0.000	0.000	0.000	-	-	-	-
16	0.000	0.000	0.000	0.000	-	-	-	-
17	0.000	0.000	0.000	0.000	-	-	-	-
18	0.000	0.000	0.000	0.000	-	-	-	-
19	0.000	0.000	0.000	0.000	-	-	-	-
20	0.000	0.000	0.000	0.000	-	-	-	-
21	0.000	0.000	0.000	0.000	-	-	-	-
22	0.001	0.001	0.001	0.001	-	-	-	-
23	0.010	0.011	0.010	0.010	-	-	-	-
24	0.061	0.060	0.060	0.060	-	-	-	-
25	0.353	0.353	0.352	0.353	-	-	-	-
26	0.882	0.882	0.882	0.882	-	-	-	-
27	1.441	1.440	1.439	1.440	0.229	0.228	0.228	0.228
28	1.980	1.988	1.986	1.985	0.288	0.288	0.287	0.288
29	2.621	2.649	2.616	2.629	0.358	0.358	0.357	0.358

Table A.1 Detection of each fraction from fractionation of *M. oleifera* leaves extract using UV-spectrophotometer at 260 nm (continued)

Fraction no.	n (no dilution)				n (1:10 dilution)			
	1	2	3	Avg	1	2	3	Avg
30	2.873	2.919	2.924	2.905	0.389	0.389	0.388	0.389
31	3.199	3.247	3.226	3.224	0.478	0.476	0.476	0.477
32	3.282	3.339	3.399	3.340	0.543	0.543	0.543	0.543
33	3.240	3.330	3.372	3.314	0.617	0.616	0.615	0.616
34	3.350	3.341	3.409	3.367	0.686	0.686	0.686	0.686
35	3.275	3.238	3.336	3.283	0.784	0.782	0.784	0.783
36	3.286	3.336	3.390	3.337	0.860	0.859	0.860	0.860
37	3.300	3.218	3.359	3.292	0.956	0.956	0.956	0.956
38	3.372	3.342	3.377	3.364	1.036	1.035	1.035	1.035
39	3.317	3.305	3.317	3.313	1.138	1.137	1.136	1.137
40	3.445	3.285	3.324	3.351	1.327	1.326	1.326	1.326
41	3.266	3.377	3.266	3.303	1.299	1.297	1.298	1.298
42	3.301	3.395	3.339	3.345	1.356	1.406	1.431	1.398
43	3.353	3.249	3.361	3.321	1.400	1.432	1.452	1.428
44	3.235	3.252	3.400	3.296	1.294	1.302	1.307	1.301
45	3.237	3.346	3.268	3.284	1.233	1.232	1.231	1.232
46	3.236	3.338	3.243	3.272	1.318	1.312	1.315	1.315
47	3.292	3.390	3.339	3.340	0.972	0.969	0.965	0.969
48	3.298	3.344	3.305	3.316	0.932	0.931	0.927	0.930
49	3.315	3.292	3.311	3.306	0.938	0.929	0.922	0.930
50	3.295	3.296	3.310	3.300	1.003	1.004	1.003	1.003
51	3.205	3.328	3.291	3.275	1.024	1.025	1.024	1.024
52	3.252	3.305	3.420	3.326	0.996	0.995	0.996	0.996
53	3.296	3.353	3.354	3.334	0.965	0.965	0.965	0.965
54	3.223	3.293	3.295	3.270	0.939	0.941	0.940	0.940
55	3.342	3.297	3.256	3.298	1.015	1.016	1.015	1.015
56	3.293	3.374	3.376	3.348	1.083	1.087	1.070	1.080
57	3.239	3.306	3.286	3.277	1.114	1.113	1.114	1.114
58	3.265	3.310	3.226	3.267	1.251	1.251	1.251	1.251
59	3.265	3.210	3.281	3.252	1.343	1.344	1.343	1.343
60	3.270	3.258	3.377	3.302	1.488	1.489	1.490	1.489

Table A.1 Detection of each fraction from fractionation of *M. oleifera* leaves extract using UV-spectrophotometer at 260 nm (continued)

Fraction no.	n (no dilution)				n (1:10 dilution)			
	1	2	3	Avg	1	2	3	Avg
61	3.240	3.269	3.219	3.243	1.522	1.521	1.521	1.521
62	3.235	3.269	3.292	3.265	1.638	1.637	1.637	1.637
63	3.279	3.339	3.317	3.312	1.565	1.566	1.565	1.565
64	3.276	3.313	3.388	3.326	1.494	1.497	1.495	1.495
65	3.264	3.361	3.353	3.326	1.280	1.271	1.269	1.273
66	3.280	3.388	3.425	3.364	1.063	1.062	1.062	1.062
67	3.253	3.325	3.373	3.317	0.883	0.884	0.883	0.883
68	3.303	3.296	3.320	3.306	0.734	0.728	0.727	0.730
69	3.253	3.227	3.292	3.257	0.661	0.658	0.656	0.658
70	3.264	3.260	3.322	3.282	0.584	0.583	0.581	0.583
71	3.162	3.206	3.212	3.193	0.521	0.519	0.521	0.520
72	3.082	3.085	3.125	3.097	0.474	0.473	0.474	0.474
73	2.830	2.831	2.856	2.839	0.407	0.407	0.407	0.407
74	2.501	2.506	2.497	2.501	0.350	0.350	0.350	0.350
75	2.177	2.183	2.187	2.182	0.320	0.320	0.320	0.320
76	1.986	1.981	1.981	1.983	0.296	0.295	0.295	0.295
77	1.890	1.889	1.890	1.890	0.284	0.284	0.284	0.284
78	1.981	1.978	1.979	1.979	0.292	0.292	0.292	0.292
79	2.319	2.318	2.320	2.319	0.325	0.324	0.323	0.324
80	2.848	2.904	2.919	2.890	0.396	0.396	0.396	0.396
81	3.185	3.192	3.299	3.225	0.494	0.493	0.493	0.493
82	3.217	3.301	3.226	3.248	0.564	0.559	0.554	0.559
83	3.299	3.292	3.227	3.273	0.587	0.587	0.587	0.587
84	3.304	3.300	3.350	3.318	0.557	0.557	0.557	0.557
85	3.158	3.209	3.295	3.221	0.492	0.492	0.492	0.492
86	2.913	2.937	2.941	2.930	0.420	0.419	0.418	0.419
87	2.444	2.460	2.473	2.459	0.338	0.338	0.338	0.338
88	2.134	2.147	2.144	2.142	0.306	0.306	0.306	0.306
89	2.035	2.036	2.035	2.035	0.305	0.305	0.305	0.305
90	2.043	2.037	2.041	2.040	0.301	0.301	0.300	0.301
91	2.037	2.046	2.044	2.042	0.294	0.294	0.294	0.294

Table A.1 Detection of each fraction from fractionation of *M. oleifera* leaves extract using UV-spectrophotometer at 260 nm (continued)

Fraction no.	n (no dilution)				n (1:10 dilution)			
	1	2	3	Avg	1	2	3	Avg
92	1.956	1.964	1.957	1.959	0.286	0.286	0.286	0.286
93	1.772	1.771	1.773	1.772	0.267	0.267	0.267	0.267
94	1.486	1.486	1.486	1.486	0.238	0.238	0.238	0.238
95	1.187	1.189	1.189	1.188	0.209	0.209	0.209	0.209
96	0.948	0.949	0.949	0.949	-	-	-	-
97	0.803	0.804	0.804	0.804	-	-	-	-
98	0.724	0.722	0.716	0.721	-	-	-	-
99	0.706	0.704	0.699	0.703	-	-	-	-
100	0.699	0.697	0.696	0.697	-	-	-	-
101	0.712	0.711	0.712	0.712	-	-	-	-
102	0.714	0.713	0.714	0.714	-	-	-	-
103	0.690	0.690	0.690	0.690	-	-	-	-
104	0.640	0.641	0.640	0.640	-	-	-	-
105	0.562	0.563	0.563	0.563	-	-	-	-
106	0.470	0.467	0.467	0.468	-	-	-	-
107	0.380	0.379	0.379	0.379	-	-	-	-
108	0.300	0.300	0.300	0.300	-	-	-	-
109	0.242	0.241	0.241	0.241	-	-	-	-
110	0.203	0.203	0.203	0.203	-	-	-	-
111	0.167	0.165	0.165	0.166	-	-	-	-
112	0.151	0.151	0.150	0.151	-	-	-	-
113	0.136	0.135	0.138	0.136	-	-	-	-
114	0.128	0.128	0.128	0.128	-	-	-	-
115	0.126	0.126	0.126	0.126	-	-	-	-
116	0.124	0.124	0.124	0.124	-	-	-	-
117	0.114	0.113	0.114	0.114	-	-	-	-
118	0.106	0.106	0.106	0.106	-	-	-	-
119	0.106	0.101	0.098	0.102	-	-	-	-
120	0.094	0.093	0.093	0.093	-	-	-	-
121	0.097	0.097	0.097	0.097	-	-	-	-
122	0.104	0.104	0.104	0.104	-	-	-	-

Table A.1 Detection of each fraction from fractionation of *M. oleifera* leaves extract using UV-spectrophotometer at 260 nm (continued)

Fraction no.	n (no dilution)				n (1:10 dilution)			
	1	2	3	Avg	1	2	3	Avg
123	0.096	0.097	0.097	0.097	-	-	-	-
124	0.103	0.099	0.096	0.099	-	-	-	-
125	0.092	0.092	0.092	0.092	-	-	-	-
126	0.085	0.085	0.085	0.085	-	-	-	-
127	0.083	0.083	0.082	0.083	-	-	-	-
128	0.082	0.082	0.082	0.082	-	-	-	-
129	0.077	0.077	0.078	0.077	-	-	-	-
130	0.076	0.076	0.076	0.076	-	-	-	-
131	0.068	0.068	0.068	0.068	-	-	-	-
132	0.069	0.069	0.068	0.069	-	-	-	-
133	0.067	0.067	0.067	0.067	-	-	-	-
134	0.063	0.064	0.064	0.064	-	-	-	-
135	0.062	0.063	0.063	0.063	-	-	-	-
136	0.060	0.060	0.060	0.060	-	-	-	-
137	0.061	0.061	0.061	0.061	-	-	-	-
138	0.059	0.059	0.059	0.059	-	-	-	-
139	0.054	0.055	0.054	0.054	-	-	-	-
140	0.057	0.057	0.057	0.057	-	-	-	-
141	0.055	0.055	0.055	0.055	-	-	-	-
142	0.056	0.056	0.056	0.056	-	-	-	-

2. Determination of pooled fractions yield from fractionation

$$\% \text{ Yield of pooled fraction} = \frac{W_1}{W_0} \times 100\%$$

W_1 = the weight of pooled fraction after dryness

W_0 = the weight of the initial dried *M. oleifera* leaves extract (1 g)

3. Cytotoxicity evaluation using WST-1 assay

Table A.2 The percentage of cell viability of MOL1 on HCT116 cells at 24 h.

n	Concentration (μg/ml)						
	control	DMSO*	Cisplatin*	50	100	250	500
1	95.25	111.16	89.78	104.20	102.96	100.72	53.97
2	113.40	102.46	109.75	115.56	120.20	106.52	59.93
3	91.35	100.39	89.86	114.56	124.10	111.58	65.24
Avg	100.00	104.67	96.46	111.44	115.75	106.27	59.71
SD	11.77	5.72	11.51	6.29	11.25	5.43	5.64

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control

Table A.3 The percentage of cell viability of MOL2 on HCT116 cells at 24 h.

n	Concentration (μg/ml)							
	control	DMSO*	Cisplatin*	1	5	10	25	50
1	95.25	111.16	89.78	126.42	135.04	135.78	73.94	64.41
2	113.4	102.46	109.75	148.63	117.38	123.10	65.82	62.17
3	91.35	100.39	89.86	124.18	137.03	139.76	83.56	54.13
Avg	100	104.67	96.46	133.08	129.81	132.88	74.44	60.24
SD	11.77	5.72	11.51	13.52	10.81	8.70	8.88	5.41

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control

Table A.4 The percentage of cell viability of MOL3 on HCT116 cells at 24 h.

n	Concentration (μg/ml)							
	control	DMSO*	Cisplatin*	1	5	10	25	50
1	95.25	111.16	89.78	110.91	126.91	107.27	41.86	33.49
2	113.4	102.46	109.75	105.61	99.72	107.85	34.32	30.92
3	91.35	100.39	89.86	115.56	123.43	99.64	35.56	27.02
Avg	100	104.67	96.46	110.69	116.69	104.92	37.25	30.48
SD	11.77	5.72	11.51	4.98	14.80	4.58	4.04	3.26

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control

Table A.5 The percentage of cell viability of MOL4 on HCT116 cells at 24 h.

n	Concentration (μg/ml)							
	control	DMSO*	Cisplatin*	1	5	10	25	50
1	95.25	111.16	89.78	125.01	66.48	46.50	34.73	34.90
2	113.4	102.46	109.75	133.63	78.25	52.89	35.89	31.00
3	91.35	100.39	89.86	134.04	78.75	45.84	37.39	30.67
Avg	100	104.67	96.46	130.89	74.50	48.41	36.00	32.19
SD	11.77	5.72	11.51	5.10	6.94	3.89	1.33	2.35

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control

Table A.6 The percentage of cell viability of MOL1 on HCT116 cells at 48 h.

n	Concentration (μg/ml)						
	control	DMSO*	Cisplatin*	50	100	250	500
1	95.91	95.12	96.13	94.95	109.97	91.41	40.49
2	96.30	95.34	103.12	92.81	99.67	99.62	60.18
3	107.79	98.31	78.83	93.03	109.10	98.31	56.08
Avg	100.00	96.26	92.69	93.59	106.25	96.45	52.25
SD	6.75	1.78	12.50	1.18	5.72	4.41	10.39

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control

Table A.7 The percentage of cell viability of MOL2 on HCT116 cells at 48 h.

n	Concentration (μg/ml)							
	control	DMSO*	Cisplatin*	1	5	10	25	50
1	95.91	95.12	96.13	105.87	103.55	108.49	93.38	57.52
2	96.30	95.34	103.12	122.86	99.27	104.95	92.68	57.61
3	107.79	98.31	78.83	94.29	91.93	114.69	107.83	41.10
Avg	100.00	96.26	92.69	107.67	98.25	109.38	97.96	52.07
SD	6.75	1.78	12.50	14.37	5.88	4.93	8.56	9.51

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control

Table A.8 The percentage of cell viability of MOL3 on HCT116 cells at 48 h.

n	Concentration (μg/ml)							
	control	DMSO*	Cisplatin*	1	5	10	25	50
1	95.91	95.12	96.13	117.31	111.41	110.85	44.46	25.68
2	96.30	95.34	103.12	95.87	92.11	123.99	48.65	15.16
3	107.79	98.31	78.83	105.87	85.43	109.80	55.99	39.87
Avg	100.00	96.26	92.69	106.35	96.32	114.88	49.70	26.90
SD	6.75	1.78	12.50	10.73	13.49	7.91	5.84	12.41

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.9 The percentage of cell viability of MOL4 on HCT116 cells at 48 h.

n	Concentration (μg/ml)							
	control	DMSO*	Cisplatin*	1	5	10	25	50
1	95.91	95.12	96.13	114.03	52.89	43.11	25.37	16.25
2	96.30	95.34	103.12	102.72	48.65	38.13	35.25	22.97
3	107.79	98.31	78.83	107.18	41.45	18.87	28.00	17.08
Avg	100.00	96.26	92.69	107.98	47.66	33.37	29.54	18.77
SD	6.75	1.78	12.50	5.70	5.79	12.80	5.11	3.67

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.10 The percentage of cell viability of MOL1 on NHF cells at 24 h.

n	Concentration (μg/ml)									
	control	DMSO*	Cisplatin*	10	25	50	75	100	250	500
1	103.82	90.78	98.00	79.51	77.53	81.45	75.95	81.22	92.81	94.93
2	104.63	89.70	97.23	79.42	74.46	75.99	76.94	80.59	87.31	87.58
3	91.55	91.37	95.48	82.89	84.88	83.16	88.44	82.76	88.53	91.46
Avg	100.00	90.62	96.90	80.61	78.95	80.20	80.44	81.52	89.55	91.33
SD	7.33	0.85	1.29	1.98	5.35	3.74	6.94	1.11	2.89	3.68

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.11 The percentage of cell viability of MOL2 on NHF cells at 24 h.

n	Concentration (μg/ml)									
	control	DMSO*	Cisplatin*	10	25	50	75	100	250	500
1	103.82	90.78	98.00	80.77	81.45	75.63	69.59	47.94	41.22	22.46
2	104.63	89.70	97.23	81.72	78.70	82.76	78.52	67.65	42.62	22.73
3	91.55	91.37	95.48	73.11	81.67	77.07	74.46	61.83	42.53	23.63
Avg	100.00	90.62	96.90	78.53	80.61	78.49	74.19	59.14	42.12	22.94
SD	7.33	0.85	1.29	4.72	1.66	3.77	4.47	10.13	0.78	0.61

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.12 The percentage of cell viability of MOL3 on NHF cells at 24 h.

n	Concentration (μg/ml)									
	control	DMSO*	Cisplatin*	10	25	50	75	100	250	500
1	103.82	90.78	98.00	77.03	79.96	63.27	38.06	19.75	18.36	16.51
2	104.63	89.70	97.23	81.63	84.70	63.45	36.76	20.79	17.63	15.74
3	91.55	91.37	95.48	81.45	78.83	70.26	41.85	23.05	18.49	17.32
Avg	100.00	90.62	96.90	80.04	81.16	65.66	38.89	21.20	18.16	16.52
SD	7.33	0.85	1.29	2.61	3.11	3.98	2.65	1.68	0.46	0.79

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.13 The percentage of cell viability of MOL4 on NHF cells at 24 h.

n	Concentration (μg/ml)									
	control	DMSO*	Cisplatin*	10	25	50	75	100	250	500
1	103.82	90.78	98.00	79.65	40.50	20.11	17.99	18.17	19.03	17.90
2	104.63	89.70	97.23	80.55	39.87	21.65	18.08	17.27	19.17	17.41
3	91.55	91.37	95.48	87.09	41.13	23.72	20.66	18.27	18.90	20.11
Avg	100.00	90.62	96.90	82.43	40.50	21.83	18.91	17.90	19.03	18.48
SD	7.33	0.85	1.29	4.06	0.63	1.81	1.51	0.55	0.14	1.44

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.14 The percentage of cell viability of MOL1 on NHF cells at 48 h.

n	Concentration (μg/ml)									
	control	DMSO*	Cisplatin*	10	25	50	75	100	250	500
1	119.65	109.18	87.09	122.93	86.26	74.18	70.78	81.63	108.22	124.79
2	94.16	102.76	118.75	99.81	99.23	93.32	94.99	76.17	89.53	88.57
3	86.19	87.80	89.79	83.49	90.56	74.31	82.47	77.07	92.68	127.36
Avg	100.00	99.91	98.54	102.08	92.01	80.60	82.74	78.29	96.81	113.57
SD	17.48	10.97	17.55	19.82	6.61	11.01	12.11	2.93	10.01	21.69

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.15 The percentage of cell viability of MOL2 on NHF cells at 48 h.

n	Concentration (μg/ml)									
	control	DMSO*	Cisplatin*	10	25	50	75	100	250	500
1	119.65	109.18	87.09	79.58	76.17	56.65	52.47	29.80	24.28	27.75
2	94.16	102.76	118.75	77.26	72.90	69.69	46.95	37.32	26.27	25.05
3	86.19	87.80	89.79	83.24	76.49	61.01	48.23	28.39	22.29	22.22
Avg	100.00	99.91	98.54	80.03	75.19	62.45	49.22	31.83	24.28	25.01
SD	17.48	10.97	17.55	3.01	1.99	6.64	2.89	4.80	1.99	2.76

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.16 The percentage of cell viability of MOL3 on NHF cells at 48 h.

n	Concentration (μg/ml)									
	control	DMSO*	Cisplatin*	10	25	50	75	100	250	500
1	119.65	109.18	87.09	95.50	121.71	42.58	22.03	22.67	23.76	19.40
2	94.16	102.76	118.75	94.93	101.80	40.33	21.00	25.11	22.41	19.78
3	86.19	87.80	89.79	90.69	98.59	50.67	24.34	20.87	23.31	20.55
Avg	100.00	99.91	98.54	93.71	107.36	44.53	22.46	22.89	23.16	19.91
SD	17.48	10.97	17.55	2.63	12.53	5.44	1.71	2.13	0.69	0.59

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.17 The percentage of cell viability of MOL4 on NHF cells at 48 h.

n	Concentration (μg/ml)									
	control	DMSO*	Cisplatin*	10	25	50	75	100	250	500
1	119.65	109.18	87.09	103.85	33.20	21.84	21.32	26.72	19.33	19.72
2	94.16	102.76	118.75	83.88	35.13	20.68	22.41	24.98	19.91	19.27
3	86.19	87.80	89.79	79.25	36.16	20.94	20.68	23.70	19.08	18.69
Avg	100.00	99.91	98.54	89.00	34.83	21.15	21.47	25.13	19.44	19.23
SD	17.48	10.97	17.55	13.07	1.50	0.61	0.88	1.51	0.43	0.52

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

4. Cytotoxicity evaluation using MTT assay

Table A.18 The percentage of cell viability of astragalin on HCT116 cells at 24 h.

n	Concentration (μM)							
	control	DMSO*	Adriamycin*	25	50	75	100	500
1	102.165	105.848	46.042	99.548	103.328	106.042	116.995	101.002
2	99.160	101.874	39.160	109.435	110.307	108.078	109.725	117.383
3	98.675	96.543	33.538	105.460	108.271	102.359	118.158	92.181
Avg	100.000	101.422	39.580	104.814	107.302	105.493	114.960	103.522
SD	1.890	4.669	6.263	4.975	3.589	2.899	4.570	12.789

*0.5% of DMSO as a negative control and 0.5 μg/ml of adriamycin as a positive control.

Table A.19 The percentage of cell viability of kaempferol on HCT116 cells at 24 h.

n	Concentration (μM)							
	control	DMSO*	Adriamycin*	25	50	100	200	500
1	101.124	87.828	79.963	70.225	98.127	83.333	46.816	32.772
2	107.303	75.094	86.704	101.685	88.202	75.281	60.674	45.880
3	91.573	82.022	54.869	93.071	79.775	72.097	39.139	76.217
Avg	100.000	81.648	73.845	88.327	88.702	76.904	48.876	51.623
SD	7.925	6.375	16.776	16.258	9.186	5.791	10.915	22.285

*0.5% of DMSO as a negative control and 0.5 μg/ml of adriamycin as a positive control.

Table A.20 The percentage of cell viability of isoquercetin on HCT116 cells at 24 h.

n	Concentration (μM)							
	control	DMSO*	Cisplatin*	25	50	75	150	200
1	99.830	96.657	91.446	96.617	63.981	66.331	57.046	58.104
2	91.798	101.084	79.104	102.063	63.589	57.634	48.387	51.091
3	108.371	97.754	89.604	105.786	71.542	56.732	51.796	62.374
Avg	100.000	98.498	86.718	101.489	66.371	60.232	52.410	54.597
SD	8.288	2.306	6.658	4.611	4.483	5.301	4.362	4.959

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.21 The percentage of cell viability of astragalin on HCT116 cells at 48 h.

n	Concentration (μM)							
	control	DMSO*	Cisplatin*	25	50	75	100	500
1	97.757	99.595	85.596	106.736	100.992	100.992	108.056	85.234
2	96.697	100.992	83.836	101.768	109.557	103.502	108.056	85.596
3	105.546	99.595	94.394	103.502	104.485	104.485	105.546	89.426
Avg	100.000	100.060	87.942	104.002	105.011	102.993	107.219	86.752
SD	4.832	0.807	5.656	2.522	4.307	1.801	1.449	2.323

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.22 The percentage of cell viability of kaempferol on HCT116 cells at 48 h.

n	Concentration (μM)							
	control	DMSO*	Cisplatin*	25	50	100	200	500
1	101.648	101.019	36.848	99.041	79.898	61.654	32.355	6.201
2	104.434	102.367	42.061	103.355	107.669	77.561	42.241	6.561
3	93.919	95.986	31.995	90.503	70.012	67.136	28.760	5.752
Avg	100.000	99.790	36.968	97.633	85.860	68.784	34.452	6.171
SD	5.448	3.363	5.034	6.541	19.524	8.081	6.981	0.405

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

Table A.23 The percentage of cell viability of isoquercetin on HCT116 cells at 48 h.

n	Concentration (μM)							
	control	DMSO*	Cisplatin*	25	50	75	150	200
1	99.331	102.340	73.438	14.605	7.380	9.617	10.183	7.174
2	104.217	98.714	77.064	12.754	8.974	7.945	10.003	6.917
3	96.452	93.006	73.181	15.582	11.365	11.211	11.031	6.223
Avg	100.000	98.020	74.561	14.314	9.240	9.591	10.405	7.046
SD	3.926	4.706	2.171	1.437	2.006	1.633	0.549	0.182

*0.5% of DMSO as a negative control and 100 μg/ml of cisplatin as a positive control.

4. Calculation of toxicity value of pooled fractions

The toxicity values were expresses as IC_{60} (60% Inhibitory Concentration). The IC_{60} were calculated by using the following example: For Table 4.1, the following calculations were performed.

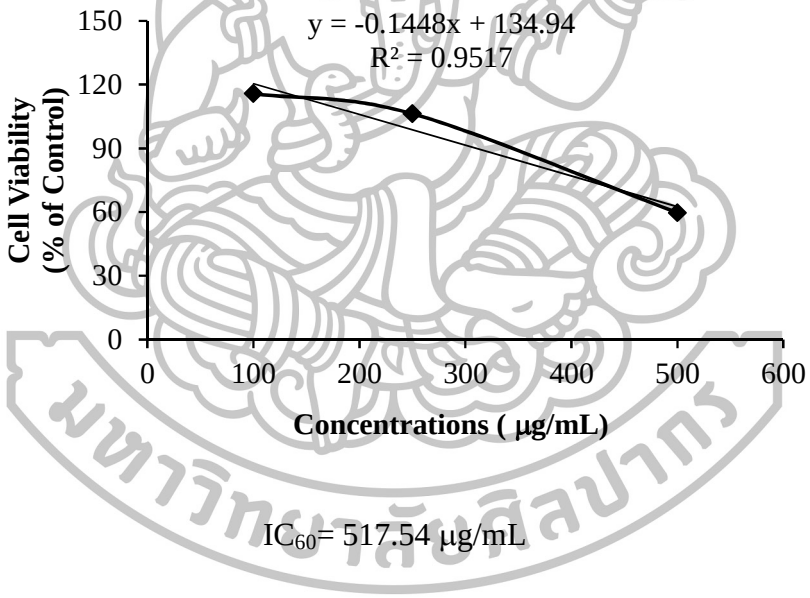
$$y = -30.57\ln(x) + 126.95 \qquad (y = 60)$$

$$\ln(x) = (60-126.95)/-30.57$$

$$x = e^{(60-126.95)/-30.57)}$$

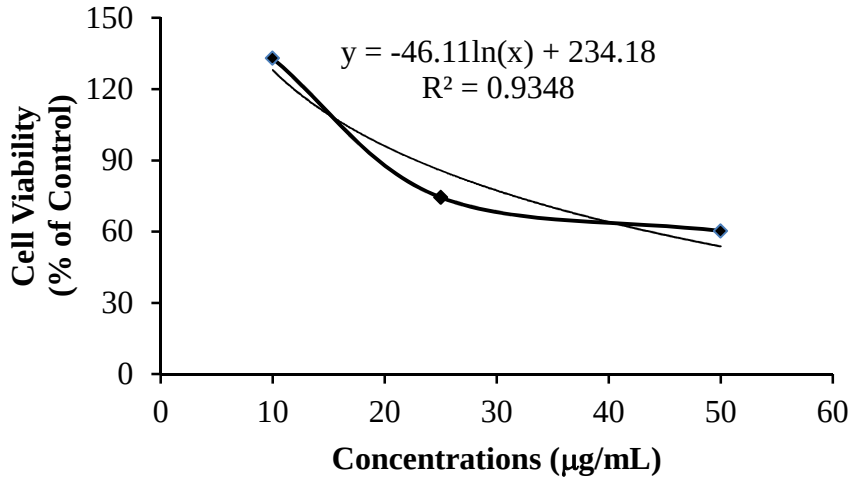
$$x = 8.936\ \mu g/mL$$

$\therefore$ IC_{60} of MOL4 in HCT116 at 24 h = 8.936 μg/mL



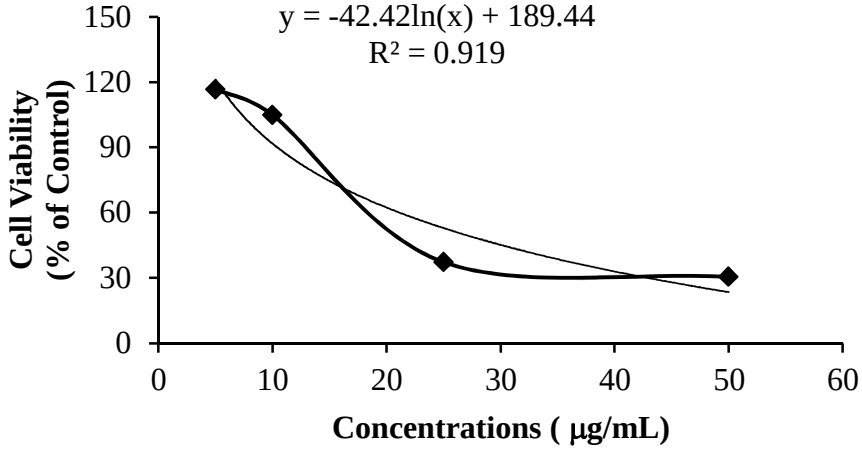
IC_{60}= 517.54 μg/mL

Figure A.1 IC_{60} data of MOL1 in HCT116 cells at 24 h.



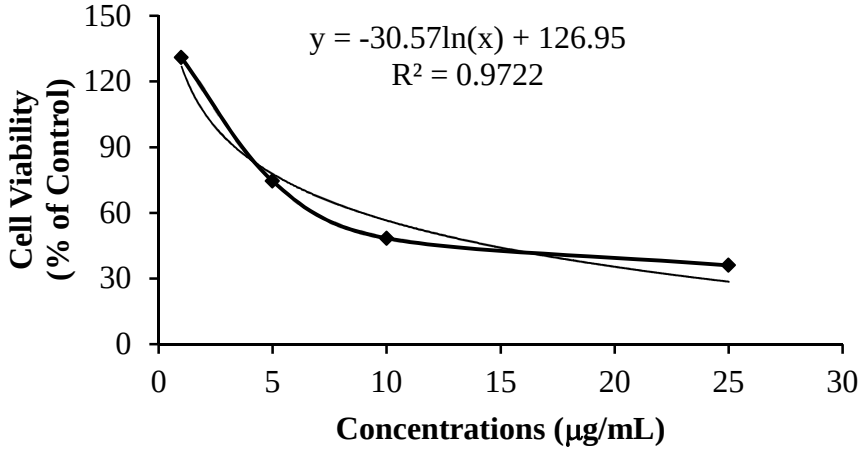
IC_{60} = 43.799 µg/mL

Figure A.2 IC_{60} data of MOL2 in HCT116 cells at 24 h.



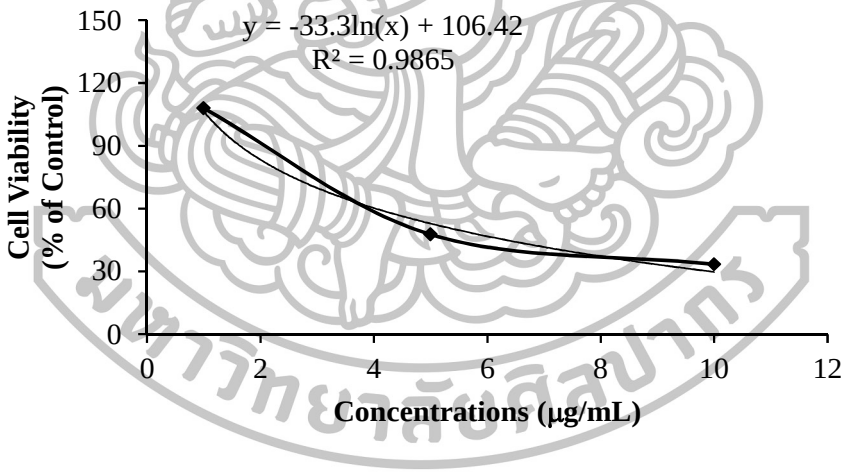
IC_{60} = 21.145 µg/mL

Figure A.3 IC_{60} data of MOL3 in HCT116 cells at 24 h.



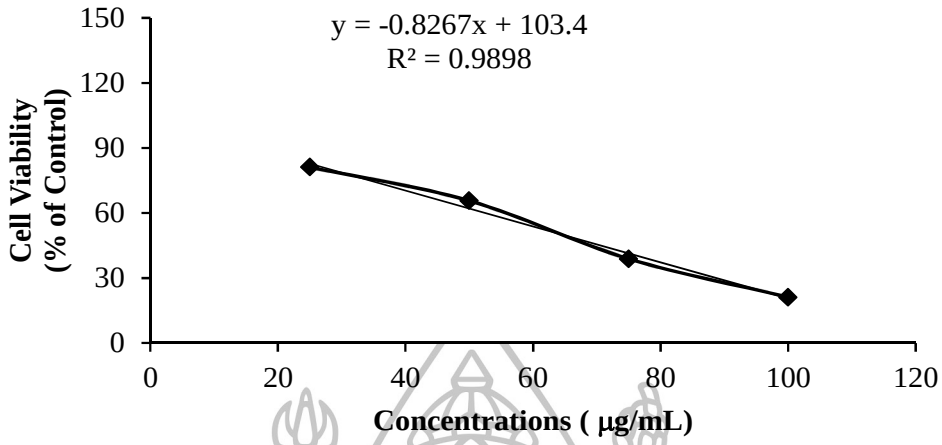
IC_{60} = 8.936 μg/mL

Figure A.4 IC_{60} data of MOL4 in HCT116 cells at 24 h.



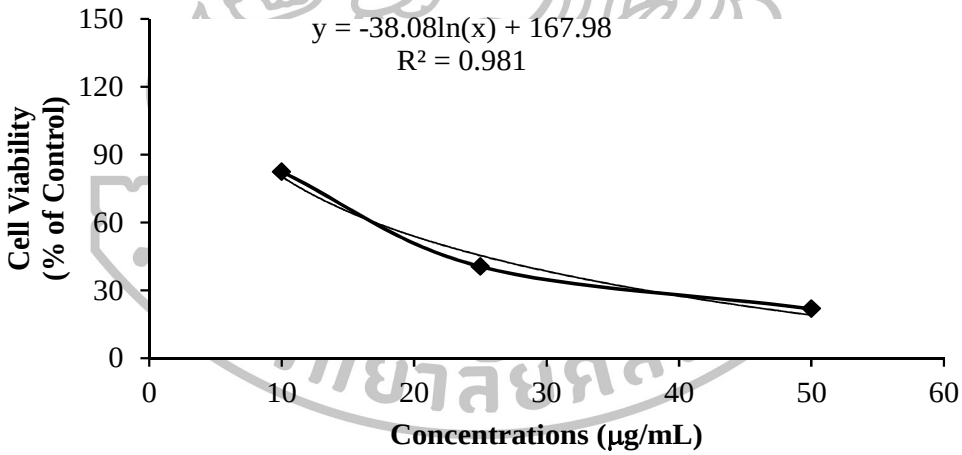
IC_{60} = 106.19 μg/mL

Figure A.5 IC_{60} data of MOL2 in NHF cells at 24 h.



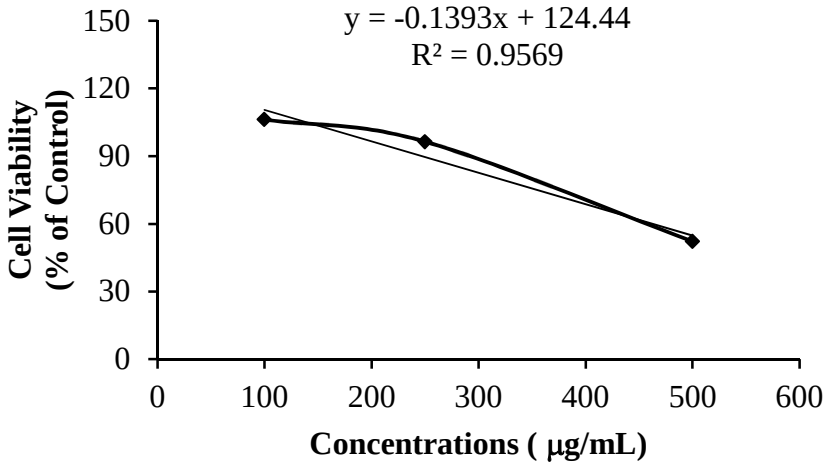
IC_{60}= 52.498 μg/mL

Figure A.6 IC_{60} data of MOL3 in NHF cells at 24 h.



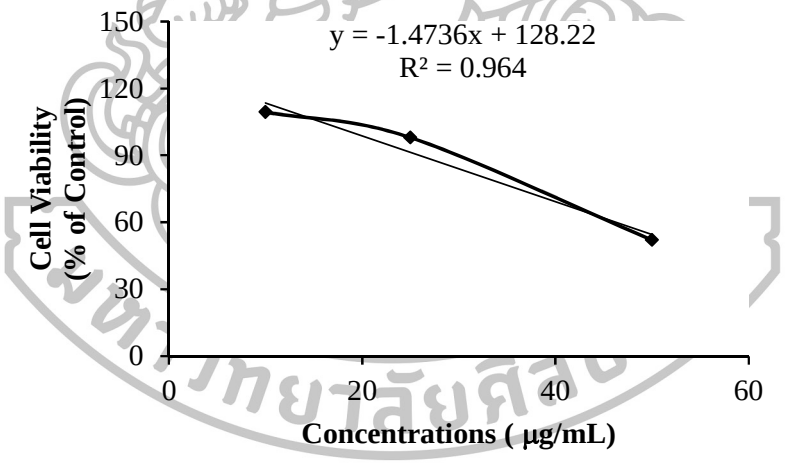
IC_{60} = 17.041 μg/mL

Figure A.7 IC_{60} data of MOL4 in NHF cells at 24 h.



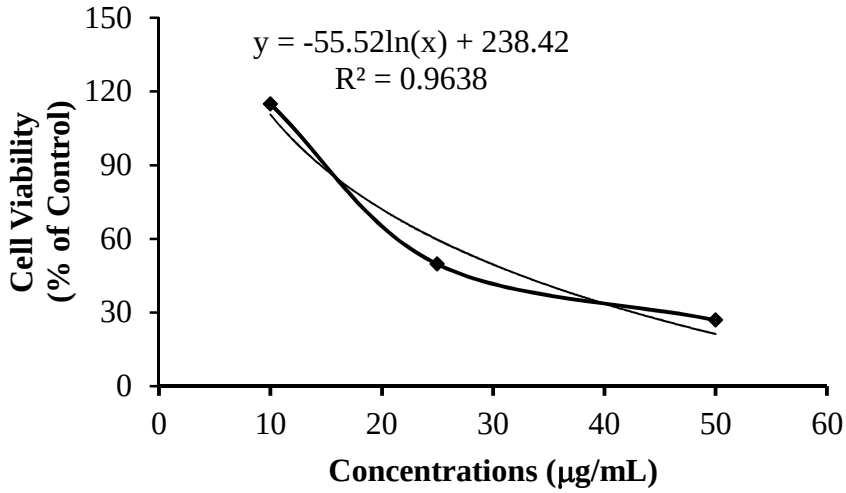
IC_{60} = 462.600 µg/mL

Figure A.8 IC_{60} data of MOL1 in HCT116 cells at 48 h.



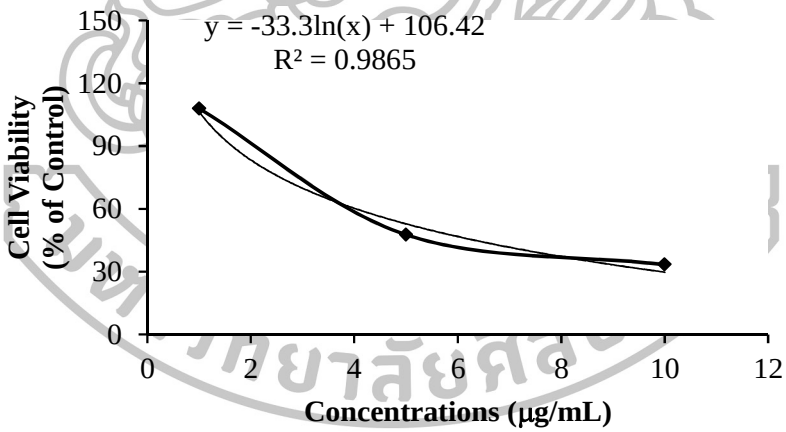
IC_{60} = 46.290 µg/mL

Figure A.9 IC_{60} data of MOL2 in HCT116 cells at 48 h.



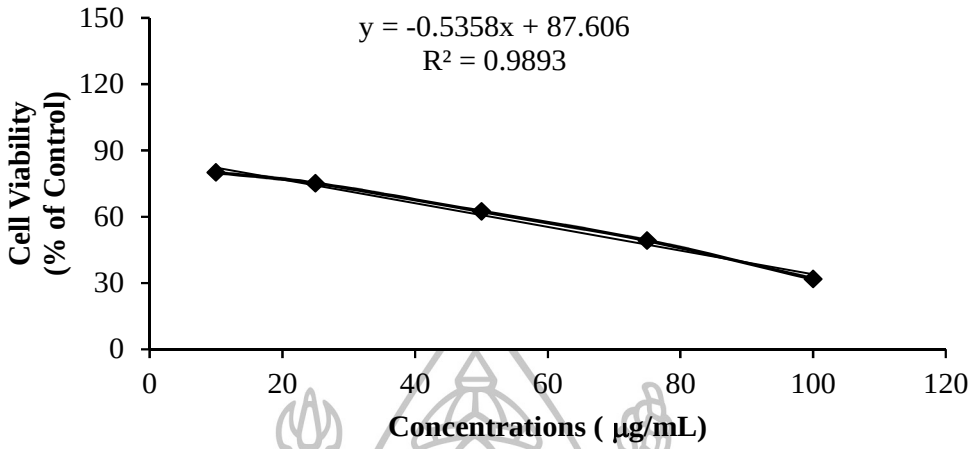
IC_{60} = 24.869 μg/mL

Figure A.10 IC_{60} data of MOL3 in HCT116 cells at 48 h.



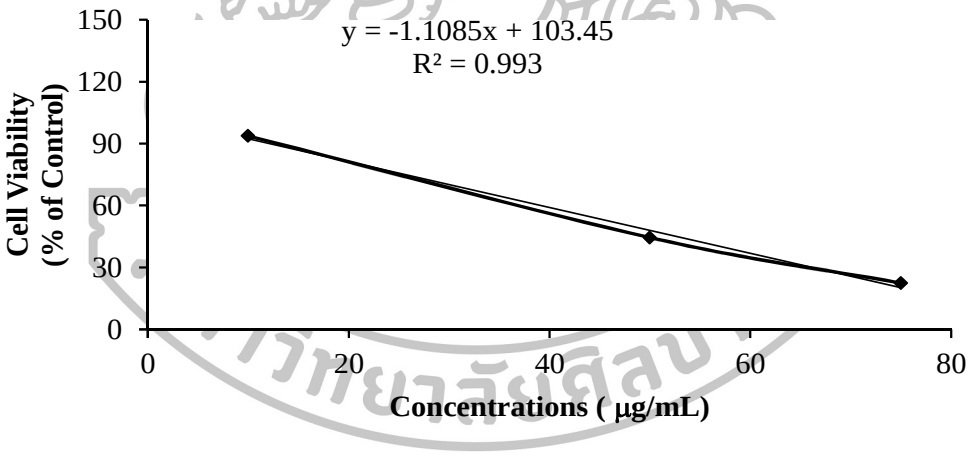
IC_{60} = 4.031 μg/mL

Figure A.11 IC_{60} data of MOL4 in HCT116 cells at 48 h.



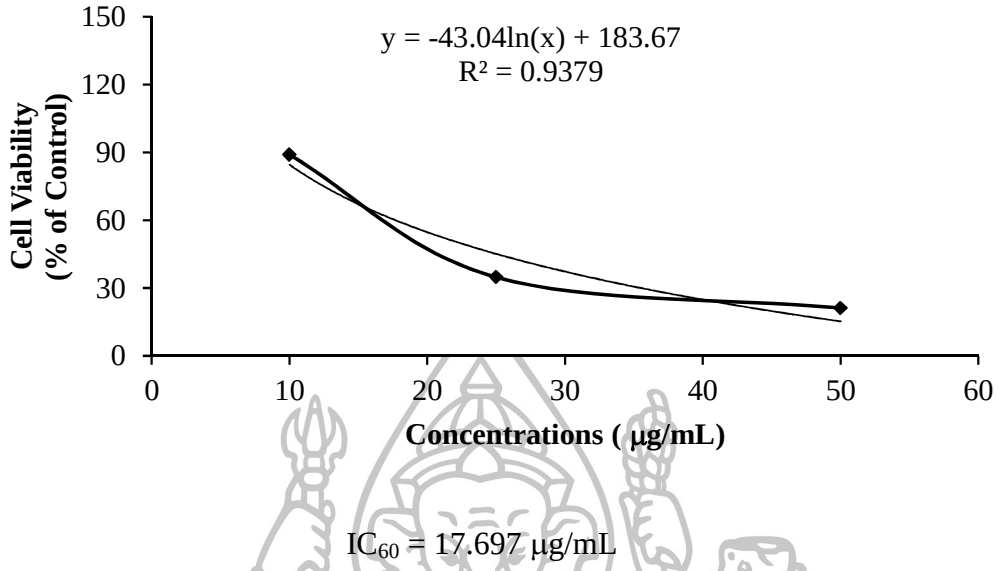
IC_{60} = 51.52 μg/mL

Figure A.12 IC_{60} data of MOL2 in NHF cells at 48 h.



IC_{60} = 39.197 μg/mL

Figure A.13 IC_{60} data of MOL3 in NHF cells at 48 h.



IC_{60} = 17.697 µg/mL

Figure A.14 IC_{60} data of MOL4 in NHF cells at 48 h.

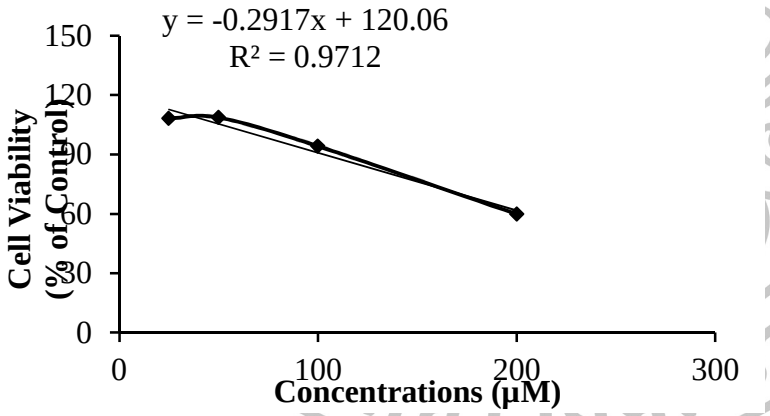


Figure A.15 IC_{60} data of kaempferol in HCT116 cells at 24 h.

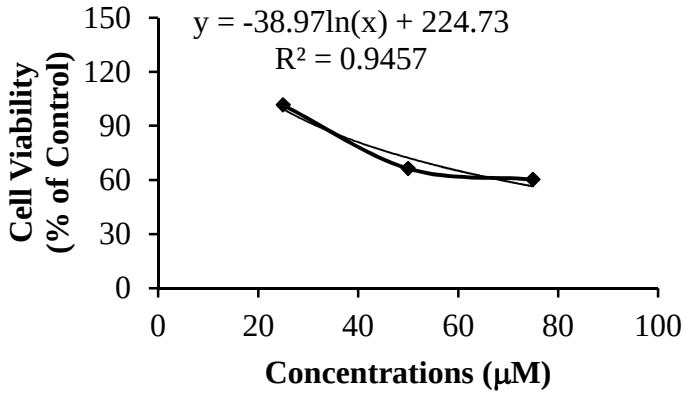


Figure A.16 IC_{60} data of isoquercetin in HCT116 cells at 24 h.

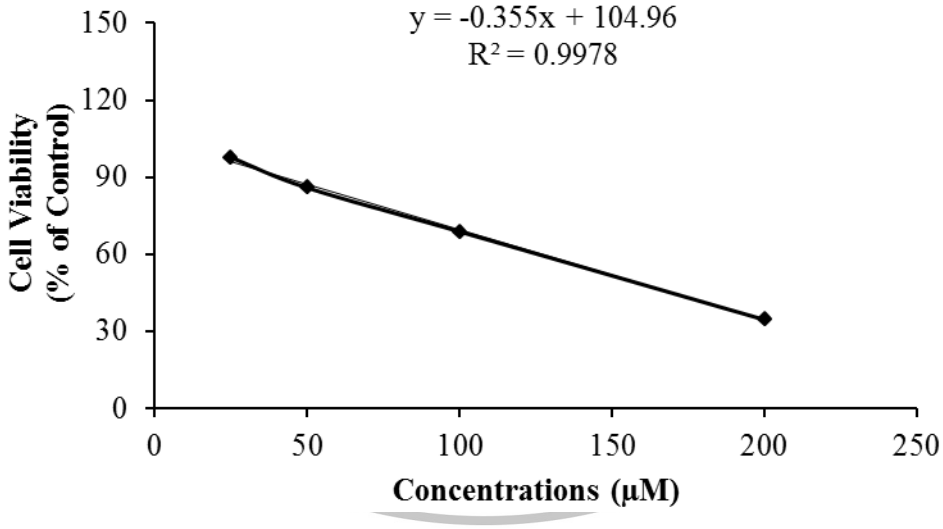


Figure A.17 IC_{60} data of kaempferol in HCT116 cells at 48 h.

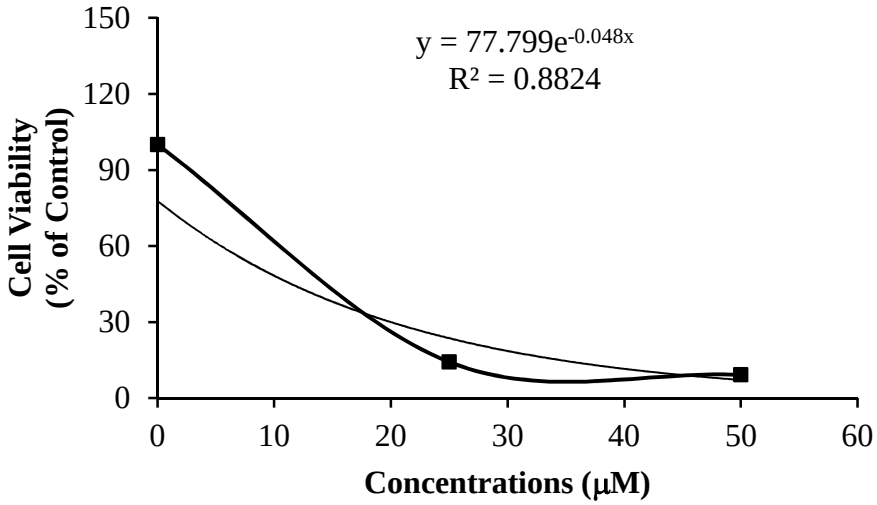


Figure A.18 IC_{60} data of isoquercetin in HCT116 cells at 48 h.

5. Calculation of protein amount of each treatment

Plot a calibration graph using absorbance values at 550 nm and concentration of protein standard or BSA. Note that the zero protein (dye only) value should be included as a data point.

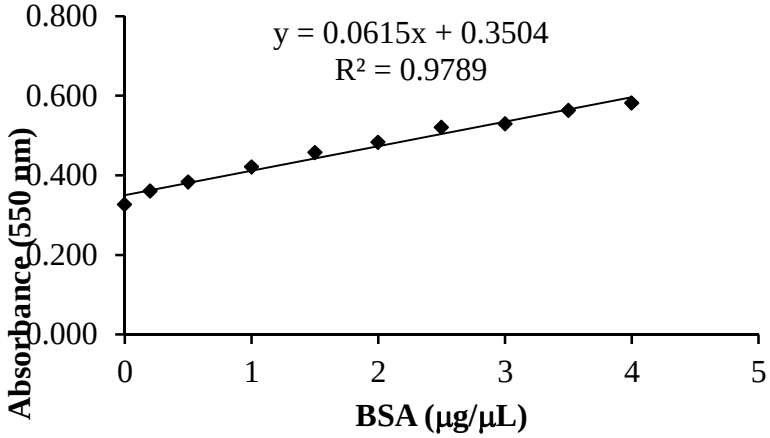


Figure A.19 Standard curve of BSA

Calculate the concentration of the unknown sample base on the linear equation of the calibration curve (Fig. A 19).

The amount of protein was calculated by using the following example:

y = 0.0615x + 0.3504 (y = 0.607 or absorbance of unknown protein)

0.607 = 0.0615x + 0.3504

x = (0.607 – 0.3504)/0.0615

x = 4.172 μg/μL

Amount of unknow protein = 4.172 μg/μL

6. Calculation of relative protein expression values of each treatment

The relative protein expressions values were analyzed using ImageJ software and calculated follow this equation;

$$\text{Relative protein expression} = \frac{\text{pERK protein level}}{\text{Total tubulin protein level}}$$

Table A 24 The relative protein expression values (pERK1/2) from Image J analysis.

Treatment	pERk1/2 level		Tubulin level		Relative protein expression	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
Control	15.099	18.399	14.825	10.022	1.018	1.836
Cisplatin	-	11.801	-	14.384	-	0.820
MOL1-750	5.112	4.762	10.302	11.733	0.496	0.406
MOL1-517	10.703	12.12	10.969	8.366	0.976	1.449
MOL1-258	15.289	6.714	14.115	5.283	1.083	1.271
Control	14.882	10.307	9.939	9.034	1.497	1.141
MOL2-88	0	0	10.805	14.139	0.000	0.000
MOL2-44	26.296	11.431	16.892	14.098	1.557	0.811
MOL2-22	22.411	14.673	16.868	8.226	1.329	1.784
Control	42.54	41.579	10.863	19.655	3.916	2.115
MOL3-43	0	0	4.768	13.119	0.000	0.000
MOL3-21	0	15.881	18.274	10.613	0.000	1.496
MOL3-11	0	0	13.461	9.247	0.000	0.000
Control	24.558	49.881	13.44	10.714	1.827	4.656
Cisplatin	25.561	-	15.622	-	1.636	-
MOL2-18	0	0	14.814	2.665	0.000	0.000
MOL2-9	0	0	15.746	7.616	0.000	0.000
MOL2-4.5	0	0	9.808	9.576	0.000	0.000
Control	4.56	12.833	6.442	19.318	0.708	0.664
Isoq.-11	5.27	32.665	6.475	23.09	0.814	1.415
Isoq.-5.5	3.289	14.19	9.4	12.899	0.350	1.100
Isoq.-2.7	6.959	20.234	11.269	11.108	0.618	1.822

APPENDIX B

BUFFER FORMULAR

Table 10x Phosphate buffer saline (PBS)

Component	Amount
NaCl	80 g
KCl	2 g
$Na_2HPO_4.7H_2O$	21.7 g
KH_2PO_4	2 g
dH_2O q.s to	1,000 mL

Store at room temperature.

Table 10x Sample loading buffer

Component	Amount
β-mercaptomethanol	5 mL
Glycerol	5 mL
Bromophenol blue	0.02%

Store at -20 °C protected from light.

Table 10x SDS PAGE Running buffer

Component	Amount
Tris base	30.3 g
SDS	10 g
Glycine	144.1 g
dH_2O q.s to	1,000 mL

Store at room temperature.

Table 10x Transfer buffer (pH 7.0)

Component	Amount
Glycine	288 g
Tris	60.4 g
dH_2O q.s to	1,000 mL

Store at 4 °C.

Table 1x Transfer buffer

Component	Amount
10x Transfer buffer	100 mL
EtOH	200 mL
dH_2O q.s to	1,000 mL

Store at 4 °C.

Table 5x Tris buffer saline (TBS) (pH 7)

Component	Amount
Tris	6.057 g
NaCl	87.66 g
dH_2O q.s to	1,000 mL

Store at room temperature.

Table 1x Tris buffer saline-tween (TBS-T)

Component	Amount
1x TBS	40 mL
Tween-20	0.2 mL
dH_2O q.s to	200 mL

Store at room temperature.

Table 5% BSA/Tris buffer saline-tween (BSA/TBS-T)

Component	Amount
BSA	2.5 g
1x TBS-T q.s to	50 mL

Store at 4 °C.

BIOGRAPHY

Name Jintana Tragulpakseerojn, Miss

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Education

2012-2016 Doctor of Philosophy, Ph.D. in Biopharmaceutical Sciences, Silpakorn University, Thailand

2008-2009 Master of Science in Pharmaceutical Sciences of Pharmacy, Silpakorn University, Thailand

2001-2005 Bachelor of Science in Microbiology, Silpakorn University, Thailand

Work experience

2006-2007 Research assistance at Pharmacy, Silpakorn University, Thailand

2010-2011 Research assistance at Pharmacy, Silpakorn University, Thailand

Presentation

Poster

1. **Jintana Tragulpakseerojn,** Perayot Pamonsinlapatham, Chavalit Sithisombut, and Auayporn Apirakaramwong**.** "Effect of *Moringa oleifera* L. extracts on *in vitro* colony formation of human tumor cells." The 4th International Conference on Natural Products for Health and Beauty (NATPRO4), 28th – 30th Nov 2012, Chiang Mai, Thailand.
2. **Jintana Tragulpakseerojn,** Perayot Pamonsinlapatham, Penpun Wetwitayaklung, Nopparat Nuntharatanapong, and Auayporn Apirakaramwong**.** "The effect of *Moringa oleifera* ethanolic leaf extract and its 2 major active components on colon cancer cell viability." The 6th International Conference on Natural Products for Health and Beauty (NATPRO6), 21st – 23rd Jan 2016, Khon Kaen, Thailand.

Scientific Publication:

1. **Jintana Tragulpakseerojn,** Perayot Pamonsinlapatham, Penpun Wetwitayaklung, Tatsuro Yoneyama, Naoki Ishikawa, Masami Ishibashi, Naoto Yamaguchi and Auayporn Apirakaramwong. Anti-proliferative effect of *Moringa oleifera* leaf extract on human colon cancer HCT116 cell line. Tropical Journal of Pharmaceutical Research. 2017; 16(2).