CYTOTOXIC AND APOPTOTIC ACTIVITIES OF ISOLATED FRACTIONS FROM MORINGA OLEIFERA LEAF EXTRACT TOWARD HUMAN HCT116 COLON CANCER CELLS

JINTANA TRAGULPAKSEEROJN

2016

GRADUATE SCHOOL

PHARMACEUTICAL SCIENCES

CHIBA UNIVERSITY

JAPAN

CYTOTOXIC AND APOPTOTIC ACTIVITIES OF ISOLATED FRACTIONS FROM MORINGA OLEIFERA LEAF EXTRACT TOWARD HUMAN HCT116 COLON CANCER CELLS

JINTANA TRAGULPAKSEEROJN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

(FRONTIER PHARMACEUTICAL SCIENCES)

DEPARTMENT OF MOLECULAR CELL BIOLOGY

GRADUATE SCHOOL OF PHARMACEUTICAL SCIENCES

CHIBA UNIVERSITY

JAPAN

FEBRUARY, 2016 (tentative)

CONTENTS

INTRODUCTION

Cancer or malignant tumor originates from abnormal growth of cells in the body. The proliferation of cells is uncontrolled and becomes to abnormal large size or tumors (except the leukemia). The cancer cells usually destroy normal cells or other healthy tissues in the process of invasion and metastasis and lead to death (1). Cancer is the third of leading causes of death in the world after cardiovascular and infectious diseases. The cancer incidence is varied in different areas of the world and its trend increases every year. The highest incidence rates are reported in North America, Australia, New Zealand, Europe, and Japan. Furthermore, the cancer incidence in male patient is higher than that in female patient (2). The colorectal cancer is the top five common worldwide of cancer incidence and most common form of malignancy in both Thai's men and women (3-5).

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, lymphomas, leukemia and small cell lung cancer (6). In chemotherapy, drugs are designed to arrest the cell cycle of cancerous cells. However, their mode of action involves targeting rapidly dividing cells, thus they are known to cause severe side effects to rapidly dividing normal cells in the human body (1). Radiation is a conventional cancer treatment. Radiation 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 (7). Since there is less toxicity and undesirable events of natural compounds constituents

presenting in herbal plants, the study of those on cancer has been augmented (8).

Table 1 Major families of drugs used in cancer chemotherapy and novel therapy (4, 9)

Chemotherapy drugs			Novel anticancer drugs
Alkylating agents	Anti-metabolites	Mitosis inhibitors	
DNA crosslinking agent Nitrogen mustard Chlorambucil Melphalan Cisplatin	*Pyrimidine analog* Cytarabine Fluorouracil Gemcitabine *Purine analog* Thioguanine Mercaptopurine *Folic acid antagonist* Methotrexate	Vinblastine Vincristine Paclitaxel (Taxol) Etoposide Topotecan Irinotecan	*Monoclonal antibody* Herceptin *Tyrosine kinase inhibitors* Gefitinib Gleevec *Antisense oligonucleotide* *Matrix metalloproteinase inhibitor*

There are many reasons for the increased use of natural products. Since natural compounds are obtained from diverse sources. Plants are another natural source of anticancer drugs. Several anti-cancer drugs are discovered for current drugs, for example vincristine, vinblastine, ellipticine and taxol (paclitaxel). Phytomedicines are believed to have health benefits and still remain interested. Recently, the edible plant and

medicinal plant continue being a popular study which possesses a wide range of *in vitro* activities (10). *M. oleifera* has common name as drumstick. This plant is a member of Moringaceae family. It is a natural nutrition found in the tropical regions of the world, such as Indonesia, Philippines, Hawaii, Africa, and also Thailand (11, 12).

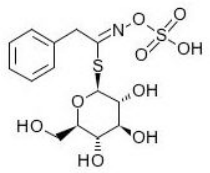
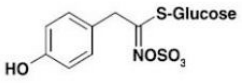
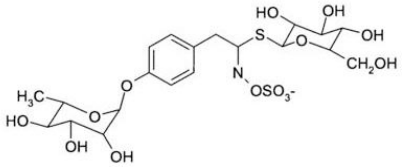
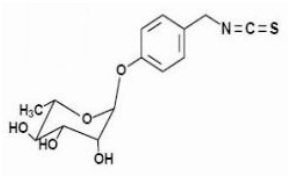
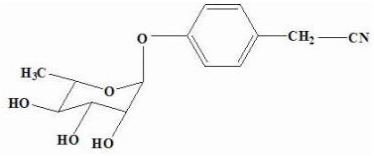
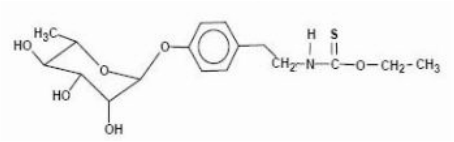
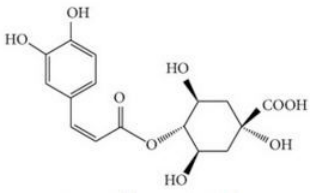
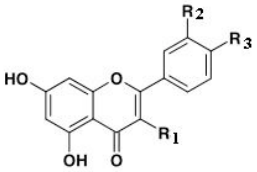
In nutritional view, almost every part of plant has value for being food, for instance, leaves, fruits, flower and immature pods (13). In 2010, Amaglo et al. determined the types and contents of components in the different parts of *M. oleifera*. Nutrients, the high content of crude proteins was found in the extract of leaves, flowers, pods and seeds and the highest amount was found in mature seeds extract. Moreover, it has been reported of highest crude fat in flowers and pods (14). On the other hand, Makkar and Becker studied that the extract of leaves, flowers, pods and seeds showed high crude fat values and the highest crude fat values detected from mature seeds (15). This variation possibly owing to various cultivation factors results in its components content (16). Additionally, *M. oleifera* has been documented retaining a high level of minerals (Ca, K and Mg) especially in leaves while mature pods and seeds have the high potassium and magnesium, respectively. Overall, the leaves of *M. oleifera* have value for dietary nutrients (14).

In phytochemicals view, the amount of glucosinolates in each parts of *M. oleifera* was revealed. Glucosinolates were found in the leaves and seeds very high. The most of them is glucomoringin. *M. oleifera* consist of the complex of flavonoids (quercetin > kaempferol > isorhamnetin) including flavonoid glycosides (glucosides, rutinosides and malonylglucosides). Caffeoylquinic acid (chlorogenic acid), a phenolic acid was found in stem, leaves, flowers and leafstalk (14). Nevertheless, the sample processing affects to lose some compounds and lose the content of compounds (14, 17). For example, the endogenous enzyme

during either tissue damage or rehydration process can cause losing of isothiocyanate and nitriles component. Previously, *M. oleifera* leaves have been reported that the content of flavonoids, multiflorin-B, apigenin-8-*C*-glucoside, quercetin-3-*O*-*β*-D-glucoside, quercetin-3-*O*-acetyl glucoside, kaempferol-3-*O*-glucoside and kaempferol-3-*O*-acetyl glucoside, were lost in the 50% and 70% EtOH extracts of *M. oleifera* leaves, compared to the 90% EtOH extract (17).

The leaves of *M. oleifera* are a potential source of vitamin C, calcium, β-carotene, fatty acid, mineral, potassium as well as essential amino acids (17). Thus, it was used as an alternative for growth stimulators in dairy cows (18-19). Moreover, it contains several antioxidant compounds, such as flavonoids, ascorbic acid, carotenoids and phenolics. *M. oleifera* leaves are abundant nutrients and antioxidants in which the components content is altered by some factors, such as harvest region, leaf age, preparation method and harvest season (20-26). The store of *M. oleifera* leaves (fresh, cooked or dried powder) for a long time without infrigidation or chilling, do not break their nutritional value. Additionally, the leaves are boiled three times that represent more systemic available iron than the uncooked leaves (27). These results are also observed in the dried powder of leaves. Both mature and tender leaves exhibit strong antioxidant activity against free radicals (27).

Table 2 Structures of previously reported in *M. oleifera*; [a] R1=-Glc, R2=H, R3=OH, [b] R1= -Glc, R2=OH, R3=OH, [c] R1=-GlcMal, R2=H, R3=OH, [d] R1=-GlcMal, R2=OH, R3=OH (14).

Chemical compound	Structure
Benzylgucosinolate (Glucotropaeolin)	
4-Hydroxybenzylglucosinolate (Sinalbin)	
4-*O*-(α-L-Rhamnopyranosyloxy)-benzylglucosinolate (Glucomoringin)	
4-(α-L-Rhamnopyranosyloxy)-benzylisothiocyanate	
Niazirin	
Niazimicin	
5-Caffeoylquinic acid (Chlorogenic acid)	
Kaempferol-3-*O*-Glucoside[a] Quercetin-3-*O*-Glucoside[b] Kaempferol-3-*O*-(6″-Malonylglucoside)[c] Quercetin -3-*O*-(6″-Malonylglucoside)[d]	

In vitro study, leaves are reported to possess antimicrobial potential both bacterial (28) and fungal (29). According to Verma et al. (30), the effect of 50% ethanolic leaf extract of *M. oleifera* on ulcerogenesis were conducted. This study was represented the reduced total ulcerogenic effect in a dose-dependent. The extract decreases acid-pepsin secretion as well as exhibited ulcer protective properties (30). The study by Tende et al. showed that the hypoglycemic and anti-hyperglycemic activity of *M. oleifera* leaves may be resulted from presence of terpenoids, which appeared to be involved in the modulation of the β-cells and the sequential secernment of prefored insulin (31). Saliman also found that the ethanolic leaves extract was capable reducing hyperglycemia in streptozocin diabetic male rats (32).

Furthermore, the *M. oleifera* leaves extract elicits anti-proliferation and apoptosis induction in human epidermal carcinoma cells and epithelial ovarian cancer cells (33, 10), and persuades the toxicity in pancreatic cancer cells (Panc-1) (34). In cancerous human lung cells (A549), Tiloke et al. reported that leaf extract induces apoptosis and increases oxidative stress (35). In another study by Purwal et al., tumorous mice were treated with methanol extract of leaves at concentration of 1 g/kg body weight of mice. The methanol extract of leaves is effective in delaying the tumor growth and increasing viable time of mice (36).

The crude extract of *M. oleifera* leaves represents the inhibitory on subG1 phase, inducing apoptotic and some molecular targeting in different cancer cell lines (34, 35). 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 there are activities of *M. oleifera* leaves on colon cancer cells. First screening, we focused on the apoptosis study.

From our preliminary studies, the crude methanol extracts from seven parts of *M. oleifera* were prepared using Soxhlet Extractor and were evaluated the cytoxicity in HCT116, HT29 and HeLa cell lines. The results showed that the leaves, flowers and pods owned the highest inhibitory effect on all cancer cells. However, the leaves extract was chosen for the further studies because of the limit content from the flowers and pods which could be harvested only in cool season in Thailand.

The objective of this study, we examined the effects of the fractionated *M. oleifera* leaves extract and its active phenolic compound, kaempferol, on cell viability and apoptosis in HCT116 cells. Kaempferol constituent compound in *M. oleifera* leaves is found to induce apoptosis in HCT116 cells (37). Moreover, to clarify the bioactive compound from *M. oleifera* leaves we isolated as well as identified phytochemical compounds from the bioactive fractions that may be responsible for the anti-colon cancer activity. The knowledge gained from this research is to ensure the *M. oleifera* leaves can be ulitized as an alternative or protective anti-colon cancer agent and may expand knowledge of medicinal herb use instead of chemical medicine.

MATERIALS AND METHODS

Chemicals and antibodies

1. Sephadex LH-20 (GE Healthcare, Waukesha, WI, USA)
2. Silica gel PSQ 100B(Fuji Sylisia, Kasugai, Japan)
3. ODS Chromatorex (Fuji Sylisia, Kasugai, Japan)
4. ODS RP18 F254 TLC plates (Merck, Germany)
5. Dulbecco's Modified Eagle Medium (D-MEM) (Life Technologies, Gaithersburg, MD, USA)
6. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Dojindo Molecular Technologies, Kumamoto, Japan)
7. Adriamycin (ADR) (Sigma, St. Louis, MO, USA)
8. Kaempferol (Sigma, St. Louis, MO, USA)
9. Anti-cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA, USA)
10. Alexa Fluor 647-labeled IgG secondary antibody (Life Technologies)
11. Prolong Antifade^{TM} reagent (Life Technologies)

Methods

1. Plant material collection and extraction

Fresh leaves of *M. oleifera* were harvested from Thailand. The fresh leaves were washed and dried. The dried leaves were blended and extracted in 100% methanol using a Soxhlet Extractor. The crude extracts were dried using an evaporator and stored at 4°C with protection from light.

2. Fractionation on gel filtration chromatography

The crude extract from *M. oleifera* leaves was dissolved in 70% (v/v) EtOH at 0.5 g/ml. Crude extract solution was filtered through 0.45-µm pore filter membranes (Merck Millipore, Bedford, MA, USA). The crude extract solution was then fractionated using a column A (Fig. 1). The eluate was collected at 5 ml per fraction and determined at UV 210 and 260 nm (38-40) using a SmartSpec^{TM} 3000 spectrophotometer (Bio-Rad). The fractions were collected until the reading at 210 and 260 nm was not detected. Finally, the aqueous fractions were dried and stored at -20°C with shielding from light.

3. Biological Assays

3.1 Cell viability

MTT method was used to measure the effect of the extract from *M. oleifera* leaves on cell viability. In brief, human HCT116 colon cells were seeded at a density of 5 x 10^3 cells per well in a 96-well plate and incubated overnight at 37°C before treatment. The cells were maintained D-MEM containing 10% inactivated fetal bovine serum (FBS). The pooled fractions were dissolved in dimethyl sulfoxide (DMSO); its final concentration never exceeded 0.5% (v/v) DMSO in each concentration. After a period of cell incubation, various dilutions of pooled fractions (pf1, pf2, pf3, and pf4) were added to the prepared plate, compared with ADR (0.5 µg/ml) and kaempferol as positive controls and 0.5% DMSO as a negative control. The plates were further incubated for 24 or 48 h in triplicate cultures and were then washed with phosphate-buffered saline (PBS) and replaced with 1 mg/ml MTT solution for 4 h. At last, MTT solution was removed and was added 100 µl

DMSO. The colored products were measured the absorbance at 595 nm. Cell viability (%) of each condition was calculated as previously described (10).

3.2 Double staining for cleaved caspase-3 and propidium iodide

1.0×10^4 cells were seeded in 96-well plates and cultured in D-MEM containing 10% FBS for 24 h. Cells treated with the each sample for 24 h. Cells inducing apoptosis were performed by labeling cells with cleaved caspase-3 antibody and propidium iodide (PI) and analyzed as described Kubota et al (41). Cells were trypsinized, fixed in 4% paraformaldehyde for 30 min, and permeabilized with ice-cold 70% ethanol for 1 h. Cells were washed in PBS containing 0.1% Tween 20 and added with anti-cleaved caspase-3 antibody. The reaction was incubated for 1 h at room temperature. Next, cells were stained with Alexa Fluor 647-conjugated anti-rabbit IgG antibody for 1 h. After that, DNA staining using 200 µg/ml RNase A and 50 µg/ml PI was added at 37°C for 30 min. Representative cells was assessed after treating and was analyzed by flow cytometry. Data were analyzed using Flowing Software version 2.5.1 (Perttu Terho, Centre for Biotechnology, Turku, Finland).

4. Isolation and purification

The bioactive fractions, pf2 and pf3 were resuspended in MeOH and then subjected to ODS (octadecylsilyl) column with different mobile phase. The pf 2 was eluted with a gradient mixture of 30-100% methanol in H_2O using a 2.0 x 21.5 cm column while the pf3 was eluted with a gradient mixture of 30-100% methanol in H_2O using a 1.7 x 21.6 cm column. Note that, the pf2 and pf3 were used for

isolation and purification step that provided from previously study. So the amount of each pooled fraction was not directly from fractionation step.

The dried pf2 (40 mg) was eluted, collected and then analyzed by TLC, yielding 38 fractions which were pooled into sixteen fractions (f1-f16) based on the TLC patterns. The fraction f6, a yellow color spot on TLC, was concentrated using a rotary evaporator, yielding approximately 2.6 mg of dried extract. The f6 (2.6 mg) was further separated by gel filtration, a 2.0 x 35 cm Sephadex LH-20 column, eluted with 80% methanol and then analyzed by thin layer chromatography (TLC). Based on the TLC patterns, the eluted subfractions were combined into seventeen subfractions (sf1- sf17). The sf13 (0.9 mg, compound A) was further subjected to identify using ^{1}H NMR and MS.

The dried pf3 (10 mg) was subjected to ODS column and eluted with similar procedures. The collected eluates were monitored by using Thin-layer chromatography (TLC) to obtain 29 fractions (F1-F29). The F9 (1 mg, compound B) with a yellow color spot on TLC was further subjected to identify using ^{1}H NMR. The isolated pure compounds were identified by comparing their ^{1}H NMR and MS with published data.

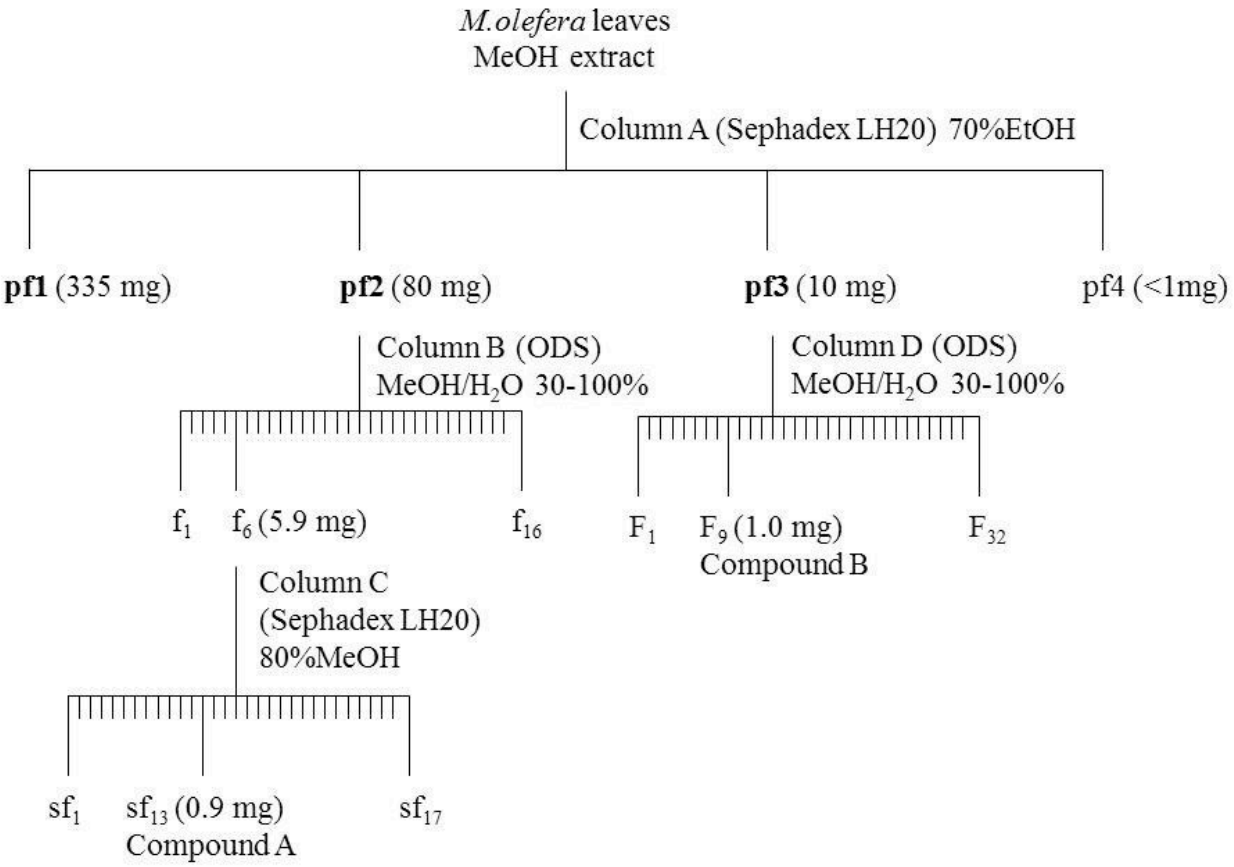


Fig. 1. Separation scheme (the active fractions are bolded).

5. General experimental procedure

5.1 Analytical thin-layer chromatography (TLC)

TLC was done with glass pre-coated ODS RP18 F_{254} plates, developing with gradient methanol-H_2O (40:60-80:20). Spots were visualized both under UV irradiation at 254 and 365 nm and by spraying with 50% H_2SO_4, then, the plate was heated at 90–100 °C at least 2-3 min.

5.2 Open column chromatography

Column A

Column size: 2.5 cm diameters, 37.5 cm length

Adsorbent: Sephadex LH20

Solvent: 70% ethanol

Column B

Column size: 2.0 cm diameters, 21.5 cm length

Adsorbent: ODS Chromatorex

Solvent: Gradient mixture of 30-100% methanol in H_2O and 100% methanol with 0.1%TFA

Column C

Column size: 2.0 cm diameters, 35.0 cm length

Adsorbent: Sephadex LH20

Solvent: 80% ethanol

Column D

Column size: 1.7 cm diameters, 21.6 cm length

Adsorbent: ODS Chromatorex

Solvent: Gradient mixture of 30-100% methanol in H_2O and 100% methanol with 0.1%TFA

5.3 Instruments for determination of compounds

Mass Spectra (MS) was measured on a JEOL AccuTOF-T100LP spectrometer (JEOL, Tokyo, Japan). ^1H Nuclear Magnetic Resonance (NMR) spectra were recorded on an ECA 600 spectrometer (JEOL, Tokyo, Japan) with deuterated solvent.

6. Statistical analysis

The results are presented as means ± SD (standard deviation) of three experiments. The statistical significance of the results was evaluated by two-way T-test. The *p*-values (probability values) less than 0.05 or 0.005 was considered to be significant.

RESULTS

Fractionation on gel filtration chromatography

The chromatograms of the eluates detection by UV spectrophotometer and a recorder were shown in Fig. 2. The chromatograms showed several fractions and several inner peaks from the *M. oleifera* leave extracts. The fractionation of *M. oleifera* leaves was divided into four groups (pf1-pf4) according to their absorbance at 210 and 260 nm. As it was indicated as the number 1 to 4, it meant to those four groups of fraction, pf1 to pf4, respectively. Moreover, they, pf1 to pf4, were found to contain a high, intermediate, small and smallest amount of yield, respectively. Each pooled fractions were further evaluated for biological activities.

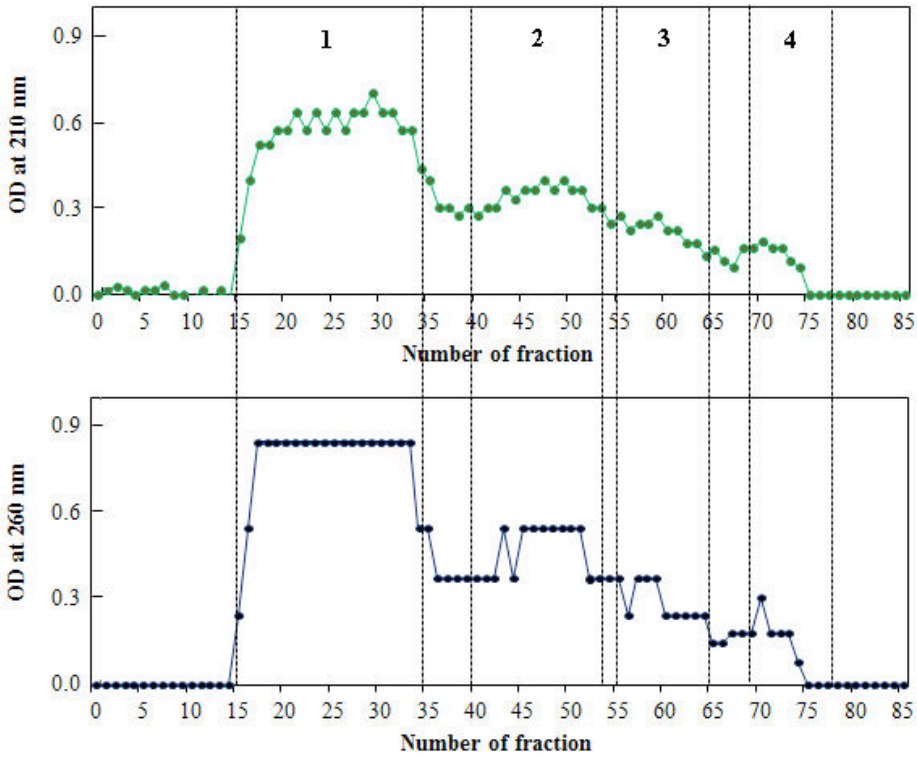


Fig. 2. Chromatograms of the extracts from *M. oleifera* leaves. Fractions were collected using 425 ml of 70% EtOH as an eluent. A whole leaf extract at the weight of 0.5 g was applied onto the column packed with Sephadex LH-20. Collected fractions were measured at OD 210 and 260 nm, giving a yield of four pooled fractions, 1-4.

Biological assays

1. Cell viability

A primary screening for antitumor activity was carried out with MTT method by using the four pooled fractions (pf1-pf4). Due to limited quantities of the fractions, only three pooled fractions (pf1-pf3) from *M. oleifera* leaves extract were investigated for their biological activities. It was found that pooled fractions showed a relatively high antiproliferative activity in HCT116 cells. Firstly, they were examined the antiproliferative activity by MTT reduction assay in colon cancer, HCT116, cells. Studies on cell viability of HCT116 cells with and without the addition of three pooled fractions are illustrated in Fig. 3, using ADR as a positive control and 0.5% (v/v) DMSO as a negative control. The pf1, pf2 and pf3 showed anti-proliferative effects in a dose-dependent manner during 24 and 48 h (Fig. 3A and 4B). When cells were incubated for 24 h, pf2 and pf3 were observed to be significantly more cytotoxic than pf1. It suggests that the components present in pf2 and pf3 are more effective than those in pf1. In addition, slightly decrease of viability in the HCT116 cells was observed in the treatment of kaempferol (Fig. 4). HCT116 cells were least affected by kaempferol, compared to the pooled fractions. From all of the above data, the pf2 and pf3 were very potent in reducing growth of HCT116 cells.

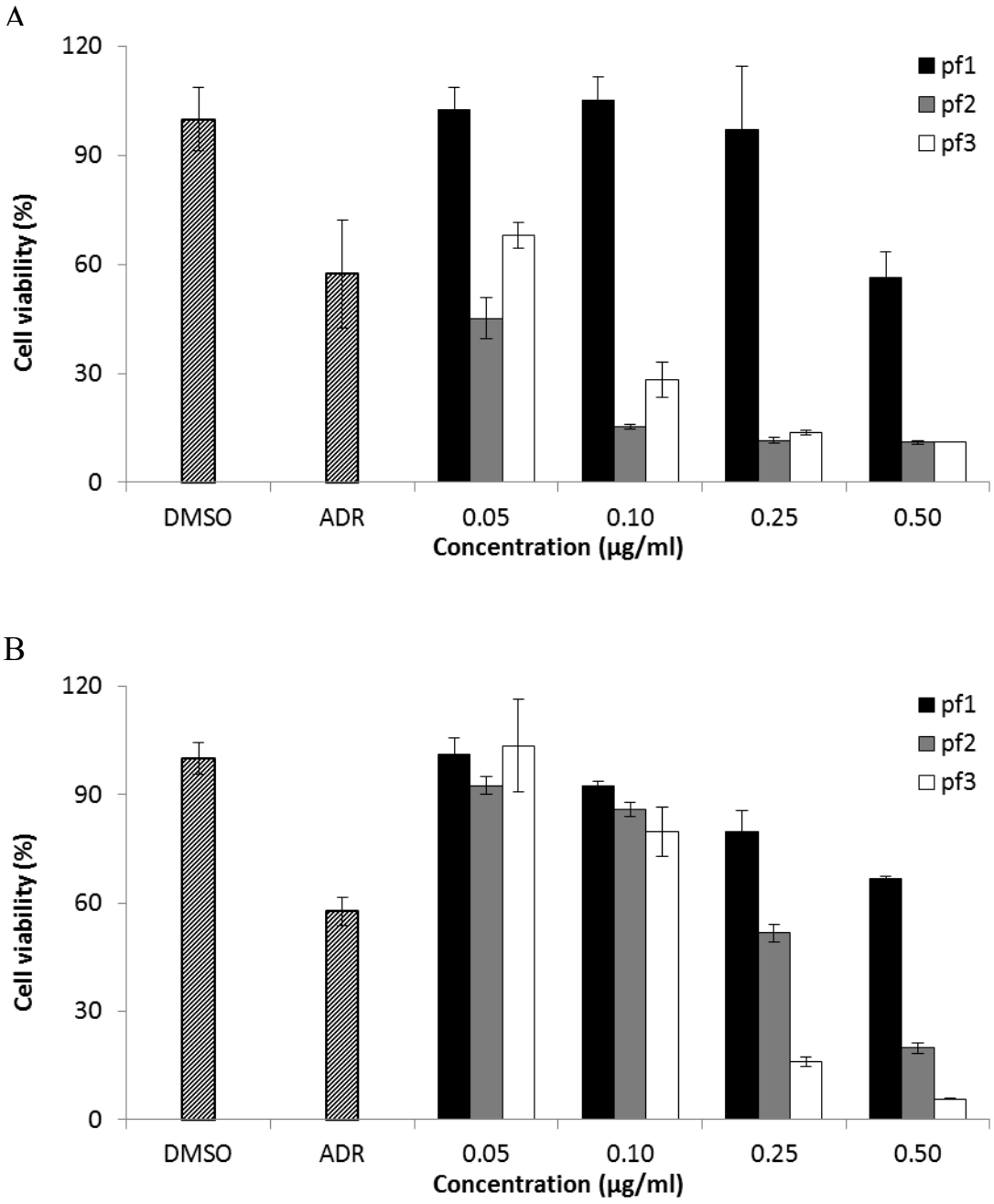


Fig. 3. Effects of each pooled fraction (pf1-pf3) on the growth of HCT116 cells. Cells were treated with indicated concentration of each pooled fraction. Cells were continuous exposed to the pooled fractions or controls at 24 h (A) or 48 h (B). Each value is the mean ± SD of triplicate of cultures.

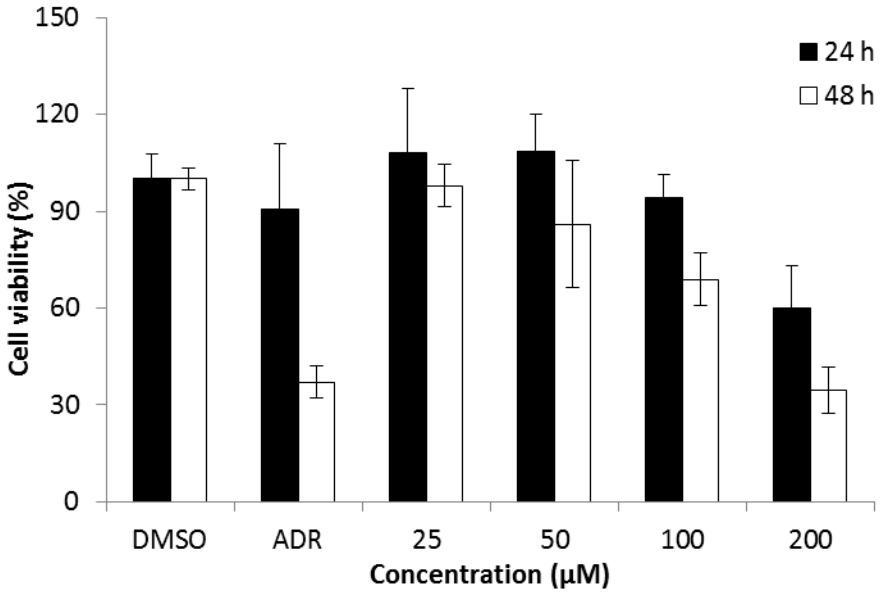


Fig. 4. Effect of kaempferol on the growth of HCT116 cells. Cells were treated with indicated concentration of kaempferol for 24 and 48 h. Each value is the mean ± SD of triplicate of cultures.

2. Apoptosis analysis

To further verify into the nature of growth inhibition by pooled fractions, FACS analysis was performed. To distinguish the live cell from dead population and demonstrate the apoptosis induced by pooled fractions from *M. oleifera* leaf was observed by anti-cleaved caspase-3 antibody and propidium iodide (PI) double staining method. After 24 h treatment of ADR (1.0 μg/ml), kaempferol (100 μM), or 0.25 μg/ml of pf1~pf3, the percentage of cells undergoing apoptosis in the HCT116 cells is presented in Fig. 5. The results showed that pf1 and pf2 cause a significant increase of 83.0% and 20.9% caspase-3 positive cells (as an apoptotic cells), respectively. Notably, pf1 treatment can induce the largest level of cleaved caspase-3-positive cells, compared with other pooled fractions. Even though

kaempferol treatment showed significantly increased cleaved caspase-3-positive cells, the treatment with pooled fractions was able to induce more cleaved caspase-3-positive cells than that with kaempferol.

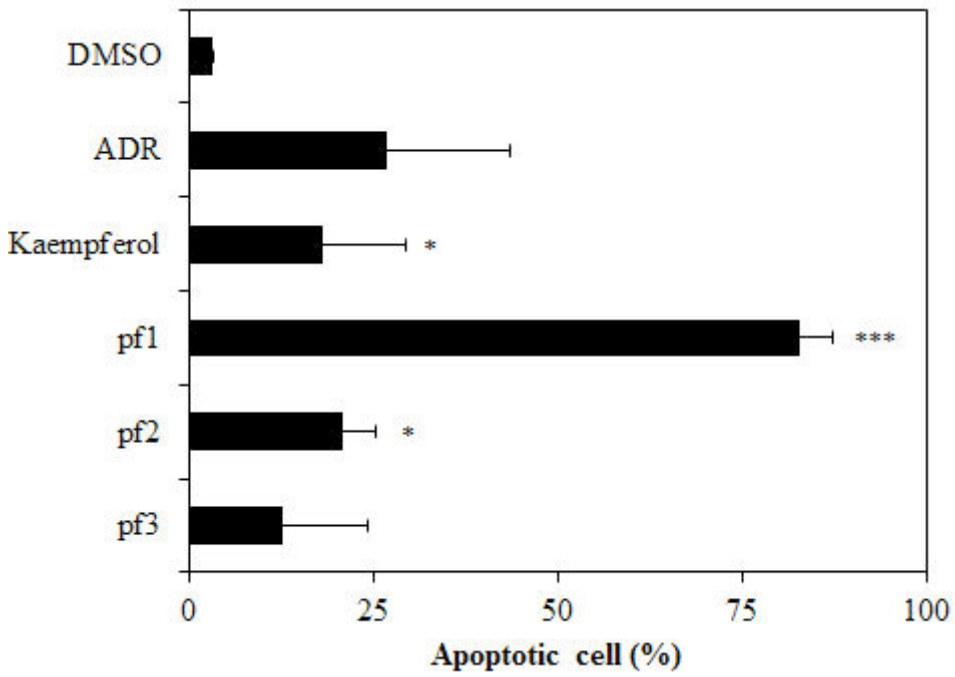


Fig. 5. Apoptosis was quantitated as the percentage of cleaved caspase-3-positive cells. Values are mean ± SD of three independent experiments. *$P<0.05$, ***$P<0.005$, significantly different from the negative control as treatment with 0.5% of DMSO (vehicle).

Isolation and Purification

To clarify phytochemical components in the bioactive pf2 and pf3 fraction from methanol *M. oleifera* leaves extract, those two fractions were therefore selected further isolation and identification analysis.

By TLC analysis, either compound A or B was separated on ODS plate, using MeOH: H_2O (60:40) as a mobile phase, and sprayed with 50% H_2SO_4 and charred at 90-100 °C. Those were revealed the presence of yellow spot at R_f of 0.393 and 0.464, respectively (Fig. 6). Then, compound A and B were subjected further to the optimized preparative column chromatography.

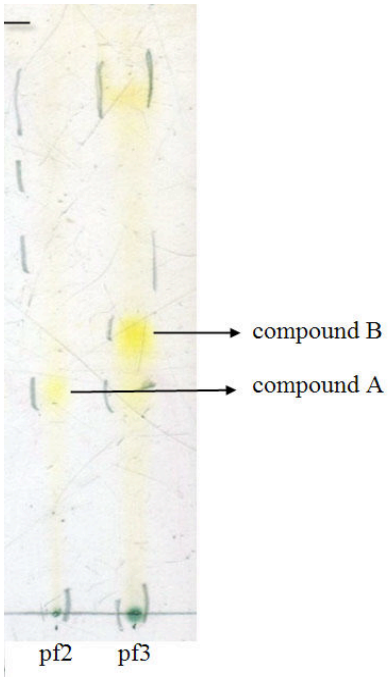


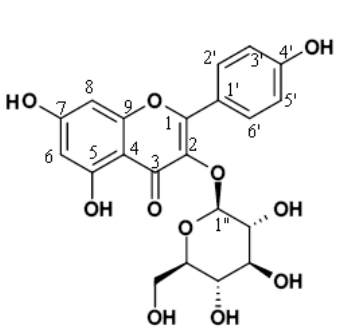
Fig. 6. TLC plate of pf2 and pf3

The structures elucidation of isolated compounds were determined from their MS and ^{1}H NMR spectral data, and compared with the literatures. The ^{1}H NMR spectra of A and B showed the characteristic signals of flavonoid glycosides. The molecular formula of A and B were indicated as $C_{21}H_{20}O_{11}$ and $C_{21}H_{20}O_{12}$, respectively by MS, which gave molecular ion peak at m/z 447 and 463 $[M-H]^-$. In addition to the compound A and B mass and ^{1}H NMR spectra showed signals corresponding to a sugar moiety. Thus, the structure of A and B was determined as kaempferol 3-o-glucoside (astragalin) and quercetrin 3-o-glucoside (isoquercetin),

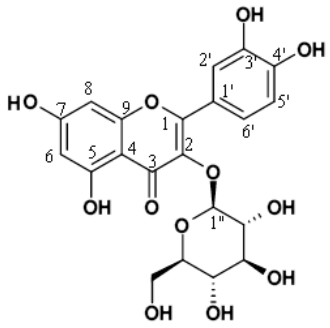
respectively (Fig. 7). However, there are others compound also contained in the pf2 and pf3 that represent in the TLC spots.

Astragalin: yellow amorphous powder, Molecular formula: $C_{21}H_{20}O_{11}$. LC-MS: m/z 447 $[M-H]^-$. ^1H NMR (600 MHz, CD_3OD): δ ppm 7.92 (d, *J*=8.8, 2H, H-2′, 6′), 6.75 (d, *J*=8.8, 2H, H-3′, 5′), 6.21 (d, *J*=2.1, 1H, H-8), 6.03 (d, 2.1, 1H, H-6), 5.08 (d, *J*=7.2, 1H, H-1″ of Glc). (45)

Isoquercetin: yellow amorphous powder, Molecular formula: $C_{21}H_{20}O_{12}$, LC-MS: m/z 463 $[M-H]^-$. ^1H NMR (600 MHz, CD_3OD) δ ppm 7.70 (d, *J*=2.3, 1H, H-2′), 7.58 (dd, *J*=2.3, 8.5, 1H, H-6′), 6.86 (d, *J*=8.5, 1H, H-5′), 6.35 (d, *J*= 2.1, 1H, H-8), 6.17 (d, *J*=2.1, 1H, H-6), 5.23 (d, *J*=7.7, 1H, H-1″ of Glc). The peaks assigned in ^1H NMR corresponded to those Refs. (46)



Astragalin



Isoquercetin

Fig. 7. Structure identification of compound A and B isolated from the active ethanol fraction (pf2, pf3)

DISCUSSION

In the present study, we extract *M. oleifera* leaves with 100% methanol at 50-60°C by using Soxhlet extractor. It has been reported that the Soxhlet extraction technique is a good extraction method giving a high recovery yield of extract. Moreover, this method offers good recovery of bioactive constituents which are thermostable compounds (47). Various solvents have been used in the extraction process of *M. oleifera* leaves. When *M. oleifera* leaves are extracted by shaking and reflux method with either methanol or ethanol, there is a little difference in total phenolic and flavonoid content of extracts (48). The various extraction methods have been studies for determination of major constituents of leaf, total phenolic and total flavonoid content, including maceration, percolation and Soxhlet extraction method. When 70% ethanol is used as the solvent of extraction, maceration method gives the highest total phenolic and flavonoid content, follows by Soxhlet extraction and percolation. However, the Soxhlet extraction provides the highest antioxidant activity using DPPH assay, compared to maceration (49).

To fractionate the components in the extracts from *M. oleifera* leaves, we perform a Sephadex LH-20 column chromatography. This separation method is based on differences in the size of the molecules and widely used for isolation of small molecules from plant natural products (50). In this study, the results shows that the components from early collected are the large molecules that larger than the largest pores of the swollen beads (above the exclusion limit). Those are not able to penetrate into the gel beads and therefore, pass through the bed in the 70% ethanol phase and elute from the column first, follow by smaller molecules. Note that, the elution step using 70% ethanol should remove all components that have been retained on the column. The entire separation process takes place using 425

ml (2.3 times of one total column volume) of solvent passing through the Sephadex LH-20 column to remove all expected components. The limitation of this fractionation is that each collected fraction yield is very small and not usable in further biological assays. Thus, each collected fraction is combined into 4 pooled fractions (pf1, pf2, pf3 and pf4), based on their chromatogram pattern at OD 210 and 260 nm (Fig 3). The pf1 is weighted and give the largest amount, comparing with other pooled fractions. Each pooled fraction has the components related in size of molecule. For example, components in pf1 have larger size molecules than that in pf2, pf3 and pf4, respectively. Due to the tiny yield (less than 1 mg) of last pooled fraction (pf4), it is unable to evaluate for the cell proliferation assays. Low amounts of active compounds are frequently obtained from a plant extract (51). To assess extensive biological studies, it needs to have larger amounts of active compounds. This generally involves the need for a re-fractionation of the *M. oleifera* leaves extract, which should be performed in the scale up of fractionation system.

When HCT116 cells are carried out for determining the proliferation, pf2 and pf3 are able to reduce cell growth more than pf1. Our results may suggest that components in pf2 or pf3, which are smaller molecules than that found in pf1, have the inhibitory effect on HCT116 cell proliferation and having a greater effect than that in pf1. On the contrary, pf3 is not able to demonstrate clear inhibition of apoptosis, it exhibits a strong inhibitory activity on cell growth. The pf1 represents the strongest induction of apoptotic effect. Comparing with the kaempferol, each pooled fraction obtains the strong anti-proliferative effect and apoptotic induction effect. In the previous studies, the ethanol extract of *M. oleifera* leaves gives various phytochemicals, such as glucosinolates, flavonoids, and phenolic acid (52). Seeram and coworkers (2015) have reported that pomegranate juice is more potent

than its individual ingredients (punicalagin, ellagitannin and ellagic acid) on human colon (HT29, HCT116, SW480, SW620), prostate (RWPE-1, 22Rvl) and oral (KB, CAL27) tumor cells. It is also indicated that the synergistic and/or additive effects from the other components presenting in pomegranate juice are antioxidant and antiproliferative activities (53). Accordingly, among the three pooled fractions, pf1~pf3, from *M. oleifera* leave extracts, each pooled fraction has many small molecules of components. Those components may synergistically increase the effects of antiproliferation and apoptosis induction on HCT116 cells, comparing to the individual component, kaempferol. From all of the above data, it can be concluded that HCT116 cell growth are effectively inhibited and its apoptosis is generally induced induced by pooled fractions. This may partially result from the different components that contain in each pooled fraction. However, to use *M. oleifera* as the anticancer of colon, the further study is needed especially on the molecular signaling mechanism.

It should be noted that pf1 is not chosen for the isolation study. Since pf1 is eluted firstly from the column, it may contain a large amounts of various impurities, for instance, fatty acids, chlorophylls and other large molecules. These large molecules may interfere the activities of biological components. Normally, the bioactive compounds are a small molecule (54). For this reason, the pf2 and pf3 are selected to identify the major components. Actually, Sephadex LH-20 is a gel filtration matrix that able to remove large molecules of contaminants, yielding small biomolecules in a single step (54).

From the gel filtration chromatogram, it is found that some molecule may bind non-specifically to Sephadex LH-20 gel. Since the component obtained from pf2 and pf3 is astragalin and isoquercetin, respectively but the molecular weight of astragalin and isoquercetin is 448.37 and 464.4, respectively. Thus, some

components from pf2 and pf3 are not always eluted by size exclusion. This may be due to the weak bond interaction, hydrophobic or hydrophilic interaction, between those molecules and gel matrix in the chromatography column.

It is not surprising that astragalin and isoquercetin were obtained from *M. oleifera* leaves as it showed in previous studies (55). Astragalin and isoquercetin are a flavonoid glycoside and are obtained from other leaves such as *Diospyros kaki* leaves, mulberry leaves, *Sapium sebiferum* leaves (56-59). Many studies of astragalin and isoquercetin have focused on in vitro antioxidant activity and are not directly applicable to the endpoint of human colon cancer prevention (60, 61). Since active pooled fractions (pf2, pf3) are strong potent inhibitor of HCT116 cell growth. The isolated compounds (astragain, isoquercetin) were also obtained from our work, biological studies should be performed to find whether isolated compounds may be capable to inhibit colon cancer cells growth. There is still much work needed to search for novel and effective anticancer aiming on small compounds to further reveal in colon cancer prevention.

REFERENCES

1. Misuna, S., Senawong, T., Chungjatupornchai, W. (2009): Cytotoxicity of cancer cells by Thai medicinal plant extracts possessing the activity of histone deacetylase inhibitor, The 12th National Graduate Research Conference, , SDP1, 1-8.

2. GLOBOCAN, Global Cancer Facts and Figures (2011): Colon and rectum 2008. American cancer society, Atlanta, Georgia, 2nd Edition, 1-57.

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

4. Sultana, S., Asif, H.M., Nazar, H.M.I., Akhtar, N., Rehman, J.U. and Rehman, R.U. (2014): Medicinal plants combating against cancer-a green anticancer approach. Asian Pac. J. Cancer Prev., **15**, 4385-4394.

5. Khuhaprema,T., Attasara, P. Sriplung, H., Wiangnon, s., Sangrajrang, S. (2013): Cancer in Thailand 2007-2009, Volumn VII, p232.

6. Shaw, C. (2011): Treatment of cancer. Nutition and cancer. Wiley-Blackwell, A John Wiley & Sons, p398.

7. National Cancer Institute, USA 2015. <http://www.cancer.gov/cancertopics/treatment/types> (Retrieved 2015-05-10).

8. Nichenametla, S.N., Taruscio, T.G., Barney, D.L., Exon, J.H. (2006): A review of the effects and mechanisms of polyphenolics in cancer. Cri. Rev. in Food Sci. and Nutri., **46**, 161-183.

9. Kleinsmith, L.J. (2006): Principles of cancer biology. Pearson education, Inc. p312.

10. Sreelatha, S., Jeyachitra, A. and Padma, P.R. (2011): Antiproliferation and induction of apoptosis by *Moringa oleifera* leaf extract on human cancer cells. Food Chem. Toxico., **49**, 1270-1275.

11. Sharma, J., Kaur, L., Kanuja, N., Nagpal, M. and Bala, R. (2013): Natural polymers-promising potential in drug delivery. Int. J. PharmTech Res., **5**, 684-699.

12. Charoensin, S. (2014): Antioxidant and anticancer activities of *Moringa oleifera* leaves. J. of Med. Plant Res.. **8(7)**. 318-325.

13. Anwar, F., Latif, S., Ashraf, M. and Gilani, A.H. (2007): *Moringa oleifera*: a food plant with multiple medicinal uses. Phytother. Res., **21**, 17-25.

14. Amaglo, N.K., Bennett, R.N., Curto, R.B.L., Rosa, E.A.S., Turoco, V.L., Giuffrida, A., Curto, A.L., Crea, F., Timpo, G.M. (2010): Profiling selected phytochemicals and nutrients in different tissues of the multipurpose tree *Moringa oleifera* L., grown in Ghana. Food Chem., **122**, 1047-1054.

15. Makkar, H.P.S., Becker, K. (1996): Nutional value and antinutitional components of whole and ethanol extracted *Moringa oleifera* leaves. Animal Feed Sci. Tech., **63**, 211-228.

16. Sanchez-Machodo, D.I., Nunez-Gastelum, J.A., Reyes-Moreno, C., Ramirez-Wong, B., Lopez-Cervantes, J. (2010): Nutritional quality of edible parts of *Moringa oleifera.* Food Analysis Methods, **3**, 175-180.

17. Moyo, B., Masika, P.J., Hugo, A., Muchenje, V. (2011): Nutritonal characterization of Moringa (*Moringa oleifera* Lam.) leaves. Af. J. of Biotech., **10(60)**, 12925-12933.

18. Araica, B.M., Sporndly, R., Sanchez, N.R., Sporndly, E. (2011): Moringa (*Moringa oleifera*) leaf meal as a source of protein in locally produced concentrates for dairy cows fed low protein diets in tropical areas. Livestock Sci., **137**, 10-17.

19. Soliva, C.R., Kreuzer, M., Foidl, N., foidl, G., Machmuller, A., Hess, H.D. (2005): Feeding value of whole and extracted *Moringa oleifera* leaves for ruminants and their effects on ruminal fermentation in vitro. Animal Feed Sci. and Techno., **118**, 47-62.

20. Iqbal, S. and Bhanger, M.I. (2006): Effect of season and production location on antioxidant activity of *Moringa oleifera* leaves grown in Pakistan. J. Food Compost.Anal., **19**, 544-551.

21. Makkar, H.P.S. and Becker, K. (1996): Nutritional value and antinutritional components of whole and ethanol extracted *Moringa oleifera* leaves. Animal Feed Sci. Tech., **63**, 211-228.

22. Khalafalla, M.M., Abdellatef, E., Dafalla, H.M., Nassrallah, A.A., Aboul-Enein, K.M., Lightfoot, D.A., El-Deeb, F.E. and El-Shemy, H.A. (2010): Active principle from *Moringa oleifera* Lam leaves effective against two leukemias and a hepatocarcinoma. Afri. J. Biotech., **9**, 8467-8471.

23. Karthivashan, G., Fard, M.T., Arulselvan, P., Abas, F. and Fakurazi, S. (2013): Identification of bioactive candidate compounds responsible for oxidative challenge from hydro-ethanolic extract of *Moringa oleifera* leaves. J. Food Sci., **78**, C1368-C1375.

24. Siddhuraju, P., Becker, K. (2003): Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of Drumstick tree (*Moringa oleifera* Lam.) Leaves. J. of Agri. and Food Chem., **51**, 2144-2155.

25. Sreelatha, S., Padma, P.R. (2009): Antioxidant Activity and Total Phenolic content of *Moringa oleifera* leaves in two stages of Maturity. Plant Foods Hum. Nutr., **64**, 303-311.

26. Shih, M.C., Chang, C.M., Kang, S.M., Tsai, M.L. (2011): Effect of different parts (leaf, stem and stalk) and seasons (summer and winter) on the chemical compositions and antioxidant activity of *Moringa oleifera*. Inter. J. of Molecular Sci., **12**, 6077-6088.

27. Razis, A.F.A., Ibrahim, M.D., Kntayya, S.B. (2014): Health benefits of *Moringa oleifera*. Asian Pacific Journal of Cancer Prevention, **15**, 8571-8576.

28. Peixoto, J.R.O., Silva, G.C., Costa, R.A., Fontenelle, J.L.S., Vieira, G.H.F., Filho, A.A.F. and Vieira, R.H.S.F. (2011): In vitro antibacterial effect of aqueous and ethanolic Moringa leaf extracts. Asian Pac. J. Trop. Med., **4**, 201-204.

29. Chuang, P., Lee, C., Chou, J., Murugan, M., Shieh, B. and Chen, H. (2007): Anti-fungal activity of crude extracts and essential oil of *Moringa oleifera* Lam. Bioresour. Technol., **98**, 232-236.

30. Verma, V.K., Singh, N., Saxena, P., Singh, R. (2012): Anti-ulcer and antioxicant activity of *Moringa oleifera* (Lam) leaves against aspirin and ethanol induced gastric ulcer in rats. Inter. Res. J. of Pharm., **2(2)**, 46-57.

31. Tende, J.A., Ezekiel, I., Dikko, A.A.U., Goji, A.D.T. (2011): Effect of ethanolic leaves extract of *Moringa oleifera* on blood glucose levels of streptozocin-induced diabetics and normoglycemic wister rats. British J. of Pharm. and Toxic., **2(1)**,1-4.

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

33. Bose, C.K. (2007): Possible role of *Moringa oleifera* Lam. root in epithelial ovarian cancer. Med. Gen. Med., **9**, 26.

34. Berkovich, L., Earon, G., Ron, I. Rimmon, A., Vexler, A., Lev-Ari, S. (2013): *Moringa oleifera* aqueous leaf extract down-regulates nuclear factor-kappaB and increases cytotoxic effect of chemotherapy in pancreatic cancer cells. Complementary and Alt. Med., **13**, 212.

35. Tiloke, C., Phulukdaree, A., Chuturgoon, A.A. (2013): The antiproliferative effect of *Moringa oleifera* crude aqueous leaf extract on cancerous human alveolar epithelial cells. Complementary and Alt. Med., **13**, 226.

36. Purwal, L., Pathak, A.K., Jain, U.K. (2010): *In vivo* anticancer activity of the leaves and fruits of *moringa oleifera* on mouse melanoma. Pharmacologynoline, **1**, 655-665.

37. Li, W., Du, B., Wang, T., Wang, S. and Zhang, J. (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. Chem. Biol. Interact., **177**, 121-127.

38. Robards, K., Prenzler, P.D., Tucker, G., Swatsitang, P. and Glover, W. (1999): Phenolic compounds and their role in oxidative processes in fruits. Food Chem., **66**, 401-436.

39. Xiao, H.B., Krucker, M., Albert, K. and Liang, X.M. (2004): Determination and identification of isoflavonoids in *Radix astragali* by matrix solid-phase dispersion extraction and high-performance liquid chromatography with photodiode array and mass spectrometric detection. J. Chromatogr. A, **1032**, 117-124.

40. Ma, Z., Zhang, W., Wang, L., Zhu, M., Wang, H., Feng, W. and Ng, T.B. (2013): A novel compound from the mushroom *Cryptoporus volvatus* inhibits porcine reproductive and respiratory syndrome virus (PRRSV) in vitro. PLoS One, **8**, e79333.

41. Kubota, S., Fukumoto, Y., Ishibashi, K., Soeda, S., Kubota, S., Yuki, R., Nakayama, Y., Aoyama, K., Yamaguchi, N. and Yamaguchi, N. (2014): Activation of the pre-replication complex is blocked by mimosine through reactive oxygen species-activated Ataxia telangiectasia mutated (ATM) protein without DNA damage. J. Biol. Chem., **289**, 5730–5746.

42. Aoyama, K., Fukumoto, Y., Ishibashi, K., Kubota, S., Morinaga, T., Horiike, Y., Yuki, R., Takahashi, A., Nakayama, Y. and Yamaguchi, N.

(2011): Nuclear c-Abl-mediated tyrosine phosphorylation induces chromatin structural changes through histone modifications that include H4K16 hypoacetylation. Exp. Cell Res., **317**, 2874-2903.

43. Aoyama, K., Yuki, R., Horiike, Y., Kubota, S., Yamaguchi, N., Morii, M., Ishibashi, K., Nakayama, Y., Kuga, T., Hashimoto, Y., Tomonaga, T. and Yamaguchi, N. (2013): Formation of long and winding nuclear F-actin bundles by nuclear c-Abl tyrosine kinase. Exp. Cell Res., **319**, 3251-3268.
44. Kubota, S., Fukumoto, Y., Aoyama, K., Ishibashi, K., Yuki, R., Morinaga, T., Honda, T., Yamaguchi, N., Kuga, T., Tomonaga, T. and Yamaguchi, N. (2013): Phosphorylation of KRAB-associated protein 1 (KAP1) at Tyr-449, Tyr-458, and Tyr-517 by nuclear tyrosine kinases inhibits the association of KAP1 and heterochromatin protein 1α (HP1α) with heterochromatin. J. Biol. Chem., **288**, 17871-17883

45. Aishan, H., Baba, M., Iwasaki, N., Kuang, H.X., Okuyama, T. (2010): The constituents of *Urtica cannabina* used in Uighur medicine. Pharmaceutical Biology, **48**(5), 577-583.
46. Kazuma, K., Noda, N., Suzuki, M. (2003): Malonylated flavonol glycosides from the petals of *Clitoria ternatea*. Phytochemistry, **62**, 229-237.

47 Heinrich, M., Barnes, J., Gibbons, S. and Williamson, E.M. (2004): Methods in natural product chemistry. Fundamentals of Pharmacognosy and Phytotherapy, Elsevier Science Limited, p107.

48 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.

49 Vongsak, B., Sithisarn, P., Mangmool, S., Thongpraditchote, S., Wongkrajang, Y. and Gritsanapan, W. (2013). Maximizing total phenolics,

total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method. Indus. Crops and Products, **44**, 556-571.

50. Amarowicz, R., Kolodziejczyk, P.P. and Pegg, R.B. (2003): Chromatographic separation of phenolic compounds from Rapeseed by Sephadex LH-20 column with ethanol as the mobile phase. J. Liq. Chromatogr. Relat. Technol., **26**, 2157-2165.

51 Rates, S.M.K. (2001): Plants as source of drugs. Toxicon, **39**, 603-613.

52. Mbikay, M. (2012): Therapeutic potential of *Moringa oleifera* leaves in chronic hyperglycemia and dyslipidemia: a review. Front. Pharmacol., **3**, 1-12.

53. Seeram, N.P., Adams, L.S., Henning, S.M., Niu, Y., Zhang, Y., Nair, M.G. and Heber, D. (2005): *In vitro* antiproliferative, apoptotic and antioxidant activities of punicalgin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J. of Natritional Biochem., 16, 360-367.

54 Maxwell, M.A.B. and Williams, J.P. (1967): The purification of lipid extracts using sephadex LH-20. J. of Chromato., **21**, 62-68.

55 Vongsak, B., Sithisarn, P. and Gritsanapan, W. (2013): Simultaneous determination of crypto-chlorogenic acid, isoquercetin, and astragalin contents in *Moringa oleifera* leaf extracts by TLC-densitometric method. Evidence-Based Comp. and Alter. Med., **2013**, 1-7.

56 Xue, Y., Miyakawa, T., Hayashi, Y., Okamoto, K., Hu, F., Mitani, N., Furihata, K., Sawano, Y. and Tanokura, M. (2011): Isolation and tyrosinase inhibitory effects of polyphenols from the leaves of Persimmon, *Diospyros kaki*. J. Agric. Food Chem., **59**, 6011-6017.

57 Katsube, T., Imawaka, N., Kawano, Y., Yamazaki, Y., Shiwaku, K. and Yamane, Y. (2006): Antioxidant flavonol glycosides in mulberry (*Morus alba* L.) leaves isolated based on LDL andtioxidant activity. Food Chem., **97**, 25-31

58 Fu, R., Zhang, Y., Peng, T., Guo, Y. and Chen, F. (2015): Phenolic composition and effects on allergic contact dermatitis of phenolic extracts *Sapium sebiferum* (L.) Roxb. leaves. J. of Ethnopharm., **162**, 176-180.

59 Choi, J., Kang, H.J., Kim, S.Z., Kwon, T.O., Jeong, S. and Jang S.(2013): Antioxidant effect of astragalin isolated from the leaves of *Morus alba* L. against free radical-induced oxidative hemolysis of human red blood cells. Arch. Pharm. Res., **36**, 912-917.

60 Jung, S.H., Kim, B.J., Lee, E.H. and Osborne, N.N.(2010). Isoquercitrin is the most effective antioxidant in the plant Thuja orientalis and able to counteract oxidative-induced damage to a transformed cell line (RGC-5 cells). Neurochem. Inter. **57**, 713-721.

61 Valentova, K., Vrba, J., Bancirova, M., Ulrichova, J.and Kren, V. (2014) Isoquercitrin: Pharmacology, toxicology, and metabolism. Food and Chem. Toxi. **68**, 267-282.

LIST OF PUBLICATIONS

Tragulpakseerojn, J., Yuki, R., Honda, T., Morii, M., Apirakaramwong, A., Yamaguchi, N., Pamonsinlapatham, P., Yamaguchi, N.: Apoptotic activities of the extract from *Moringa oleifera* leaves on human HCT116 colon cancer cells. *Fundamental Toxicological Sciences*, **1**(4), 143-149 (2014)

THESIS COMMITTEE

This thesis was examined by following members authorized by the Graduate School of Pharmaceutical Sciences, Chiba University.

Examiners

Masami Ishibashi, Ph.D. (Pharm. Sci.) Professor of Chiba University
(Graduate School of Pharmaceutical Sciences) *Chief examiner*

Toshihiko Toida, Ph.D. (Pharm. Sci.) Professor of Chiba University
(Graduate School of Pharmaceutical Sciences)

Toshihiko Murayama, Ph.D. (Pharm. Sci.) Professor of Chiba University
(Graduate School of Pharmaceutical Sciences)

ACKNOWLEDGEMENTS

This thesis could not be accomplished without the great support by my graduate advisors, Professor Naoto Yamaguchi and co-advisors, Professor Masami Ishibashi.

I would like to present my sincere gratitude to the member of thesis committee, Professor Toshihiko Toida and Professor Toshihiko Murayama who devoted their time to suggest me to write this research. Without their guidance and reviews, this thesis cannot complete.

I was grateful thanks to the Excellent International Student Scholarship between Chiba University and Silpakorn University (J.T.) and the Thailand Research Fund (TRF) (P.P., MRG5680119), and grants-in-aid for Scientific Research C (N.Y., 25460356) from the Japanese Ministry of Education, Culture, Sports, Science and Technology. T.H. and M.M. are Research Assistants of the Program for Leading Graduate Schools (Leading Graduate School Chiba University Nurture of Creative Research Leaders in Immune System Regulation and Innovative Therapeutics) from the Japanese Ministry of Education, Culture, Sports, Science and Technology for their support.

I always keep in mind about people who gave me knowledge and many tools. This thesis has been finished because of many helps from laboratory members.