APOPTOTIC EFFECTS AND CHEMICAL INVESTIGATION OF ACTIVE EXTRACTS OF *Curcuma mangga* RHIZOMES

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ABSTRACT

Plants have a long history of use in the treatment of many ailments including cancer. The choices of plant for drug discovery based on ethnopharmacological data rather than random approach hold greater promise of finding a good candidate for investigation. *Curcuma mangga* known "temu mangga" in Malay has been selected for investigation use as a natural remedy for various diseases in Malaysia including cancer. Crude and fractionated extracts of *C. mangga* rhizomes were initially investigated for their growth inhibitory effects on four human cancer cell lines, namely colorectal adenocarcinoma cell (HT-29), colorectal carcinoma cell (HCT-116), cervical carcinoma cell (CaSki) and lung carcinoma cell (A549), and a normal human cell (non-cancer human colorectal cell line, CCD-18Co) using sulforhodamine B (SRB) colorimetric assay. Dry rhizome powder of *C. mangga* was soaked in dichloromethane for three days and the crude dichloromethane extract (CMD) obtained was washed with n-hexane to give the hexane-soluble extract (CMDH). The hexane-insoluble residue was dissolved completely in methanol to give the fractionated methanolic extract (CMDM). All three extracts (CMD, CMDH and CMDM) generally showed good cytotoxicity effects against HT-29, HCT-116, A549 and CaSki with IC_{50} value ranging from 14.3 to 21.0 µg/mL, 15.2 to 18.3 µg/mL, 14.8 to 20.0 µg/mL and 18.7 to 21.2 µg/mL respectively. All extracts exhibited lower toxicity towards CCD-18Co (IC_{50} value ranging from 50.3 to 55.0 µg/mL) compared with chemotherapy drug (doxorubicin), with an IC_{50} value of 0.11 µg/mL. Both CMDH and CMDM were subjected to chemical investigations resulted isolation and identification of five (5) components, namely longpene A, zerumin A, coronadiene, (E)-labda-8(17),12-diene-15,16-dial and calcaratarin A. Other isolated compounds could not be identified. The isolated pure compounds showed weak

cytotoxicity effects against the selected cancer cell lines (IC_{50} values ranging from 19.1 to 34.7 µg/mL for HT-29; 17.7 to 38.6 µg/mL for HCT-116; 18.6 to 38.3 µg/mL for A549; 21.8 µg/mL to 30.2 µg/mL for CaSki). The pure compounds were less effective in preventing the proliferation of cancer cells compared with extracts. Synergism between the components maybe responsible for the observed activity. CMD was selected for further molecular investigation of its anticancer effect on HT-29 cell line for it's good cytotoxic effects against HT-29. Typical apoptotic morphological features like membrane blebbing, formation of apoptotic bodies, cell shrinkage and condensation of chromatin were observed on treated HT-29. The CMD induced cell arrest in G_2/M phase of the cell cycle after 24 hours. Externalization of phosphotidylserine from the plasma membrane was observed in a concentration- and time-dependent manner. DNA fragmentation was detected through the Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. In the Western blot analysis, the expression levels of the pro-apoptotic proteins (Bax, caspase 3, -9 and 8) were up-regulated while the anti-apoptotic proteins (Bcl-2, cIAP-2, XIAP) were down-regulated. The expression levels of cleaved PARP-1 were up-regulated. This indicated that apoptosis might have occurred through the intrinsic and extrinsic pathways. As a conclusion, the crude dichloromethane extract of *C. mangga* rhizomes has the potential to be further developed as an anticancer agent against HT-29.

Keywords: *Curcuma manga*, HT-29, apoptosis

KESAN APOPTOTIC DAN PENYELIDIKAN EKSTRAK AKTIF KIMIA DARI RIZOM *CURCUMA MANGGA*

ABSTRAK

Tumbuhan mempunyai rekod sejarah yang lama dalam rawatan untuk pelbagai jenis penyakit termasuk kanser. Pemilihan tumbuhan dalam pencarian ubat-ubatan berdasarkan data etnofarmakologi mempunyai potensi yang lebih tinggi dalam pemilihan calon yang sesuai untuk siasatan berbanding dengan pemilihan tumbuhan secara rawak. *Curcuma mangga* dikenali sebagai 'temu mangga' dalam bahasa Melayu, telah dipilih untuk kajian sebagai ubat semulajadi bagi pelbagai penyakit di Malaysia termasuk kanser. Pada peringkat permulaan, kesan kesitotoksikan ekstrak mentah dan fraksi rizom *C. mangga* terhadap empat titisan sel kanser manusia, iaitu titisan sel adenokarsinoma kolorektal (HT-29), titisan sel karsinoma kolorektal (HCT-116), titisan sel karsinoma servik (CaSki) dan titisan sel karsinoma paru-paru (A549) dan satu titisan sel normal manusia (sel kolorektal manusia, CCD-18Co) dengan menggunakan asei kolorimetri *Sulforhodamine B* (SRB). Serbuk kering rizom *C. mangga* direndam dalam diklorometana selama tiga hari dan ekstrak diklorometana (CMD) yang diperolehi telah dibasuh dengan n-heksana. Pelarut yang mengandungi ekstrak heksana disejatkan dan fraksi-fraksi heksana (CMDH) telah diperoleh. Sisa ekstrak yang tidak larut dalam heksana telah dilarutkan dalam metanol untuk menghasilkan fraksi methanol (CMDM). Ketiga-tiga ekstrak tersebut menunjukkan kesan kesitotoksikan yang baik terhadap HT-29, HCT-116, A549 dan CaSki dengan nilai IC_{50} masing-masing antara 14.3 hingga 21.0 μg/mL, 15.2 μg/mL hingga 18.3 μg/mL, 14.8 hingga 20.0 μg/mL dan 18.7 hingga 21.2 μg/mL. Ketiga-tiga ekstrak tersebut juga menunjukkan ketoksikan yang minima terhadap titisan sel kolorektal manusia normal (nilai IC_{50} antara 50.3 hingga 55.0 μg/mL berbanding dengan ubat kemoterapi (doxorubicin), yang memberi nilai IC_{50} 0.11 μg/mL). CMDH dan CMDM telah dikaji selanjutnya. Lima (5) sebatian iaitu longpene

A, zerumin A, koronadiene, (E)-labda-8(17),12-diene-15,16-dial, dan kalkaratarin A. Sebatian tulen lain yang diasingkan tidak dapat dikenalpasti. Semua sebatian di atas tidak menunjukkan kesan kesitotoksikan terhadap titisan sel-sel kanser yang dikaji (nilai IC_{50} antara 19.1 hingga 34.7 μg/mL untuk HT-29; 17.7 μg/mL hingga 38.6 μg/mL untuk HCT-116; 18.6 hingga 38.3 μg/mL untuk A549; 21.8 hingga 30.2 μg/mL untuk CaSki). Sebatian tulen di atas telah didapati kurang berkesan dalam mencegah pertumbuhan sel-sel kanser berbanding dengan ekstrak. Sinergi antara komponen mungkin telah berlaku. CMD telah dipilih untuk siasatan molekular lanjut terhadap titisan sel kanser HT-29 kerana ia mempunyai ketoksikan yang baik terhadap sel tersebut. Morfologi apoptosis seperti *blebbing* pada membran sel, pembentukan *apoptotic bodies*, pengecutan sel dan pemadatan kromatin diperhatikan terjadi apabila sel HT-29 dirawat dengan CMD. CMD telah menyebabkan pengumpulan sel dalam fasa G_2/M pada kitaran sel selepas 24 jam rawatan. *Externalization* phosphotidylserina dari membran plasma juga diperhatikan berlaku mengikut kenaikan konsentrasi dan masa. Fragmentasi DNA dikesan melalui asai *Terminal deoxynucleotidyl transferase dUTP Nick End Label* (TUNEL). Analisis *Western Blot* menunjukkan tahap ekspresi protein pro-apoptosis (Bax, caspase 3, -9 dan 8) telah meningkat manakala tahap ekspresi protein anti-apoptosis (cIAP2 dan XIAP) telah menurun. Ekspresi protein PARP-1 juga telah meningkat. Oleh itu, keseluruhan keputusan ujian menunjukkan bahawa apoptosis berlaku melalui laluan intrinsik dan ekstrinsik. Kesimpulan, ekstrak diklorometana mentah rizom *C. mangga* berpotensi untuk dimajukan sebagai agen anti-kanser terhadap kanser kolon HT-29.

Kata kunci: *Curcuma manga*, HT-29, apoptosis

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^{13}C	:	Carbon-13
°C	:	Degree celcius
Δ	:	Delta
µ	:	Micro
^{1}H	:	Proton
A549	:	Human lung carcinoma cell line
AGS	:	Human gastric cancer
AIDS	:	Acquired immune deficiency syndrome
ALS	:	Autosampler
Apaf-1	:	Apoptotic protease activating factor 1
ATCC	:	American Type Culture Collection
BIR	:	Baculovirus IAP repeat
Bp	:	Base pair
BrdU	:	Fluorescein-conjugated anti-Bromo deoxyuridine
BSA	:	Bovine serum albumin
BxPc-3	:	Human pancreatic cancer
CAN	:	Acetonitrile
CaSki	:	Human cervical carcinoma cell line
Caspases	:	Cysteinyl aspartate-specific proteinases
CCD-18Co	:	Non-cancer human colon cell line
$CDCl_3$	:	Deuterated chloroform
CDK	:	Cyclin dependent kinase
cIAP	:	Cellular IAP
CMD	:	Crude dichloromethane extract
CMDH	:	n-Hexane extract (Method B)
CMDM	:	Methanolic extract (Method B)
CME	:	Ethyl acetate extract
CMH	:	n-Hexane extract (Method A)
CMM	:	Crude methanolic extract
CMW	:	Crude water extract
CNS	:	Central nervous system
CO_2	:	Carbon dioxide
COSY	:	Correlation spectroscopy
DAD	:	Diode array detector
DAPI	:	4′,6-diamidino-2-phenylindole
DBB	:	DNA-binding domain
DCM	:	Dichloromethane
DEPT	:	Distortionless enhancement by polarization transfer
DIABLO	:	Direct IAP binding protein with low pI
DISC	:	Death-inducing signalling complex
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DR	:	Death receptor
DU-145	:	Human prostate cancer
dUTP	:	Deoxyuridine-triphosphatase
ER	:	Estrogen receptor
ESI	:	Electrospray ionisation
FACS	:	Fluorescence-activated cell sorting

FasL/FasR	:	Fas ligand
FC-AS	:	Fraction collector
FITC	:	Fluorescein Isothiocyanate
HCl	:	Hydrochloric acid
HCT 116	:	Human colorectal carcinoma cell line
HER2	:	Human epidermal growth factor receptor 2
HIR	:	High Impact Research
HPLC	:	High pressure liquid chromatography
HRP	:	Horseradish peroxidase
HSBC	:	Heteronuclear multiple-bond correlation spectroscopy
HSQC	:	Heteronuclear single-quantum correlation spectroscopy
HT-29	:	Human colorectal adenocarcinoma cell line
HtrA2	:	Omi/high temperature requirement protein A
IAF	:	Apoptosis inducing factor
IAP	:	Inhibitor of apoptosis protein
IC_{50}	:	Half maximal inhibitory concentration
J	:	Joule
KB	:	Nasopharyngeal epidermoid cell
kDa	:	Kilo Dalton
LD	:	Lethal dose
LDH	:	Lactate dehydrogenase leakage
LNCaP	:	Human prostate cancer
MCF-7	:	Human breast adenocarcinoma cell line
MEM	:	Minimum Essential Medium
MHz	:	Megahertz
MRC-5	:	non-cancer human fibroblast cell
mRNA	:	Messenger RNA
MTT	:	3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
NADH	:	Nicotinamide Adenine Dinucleotide
NCI	:	National Cancer Institute
NMR	:	Nuclear Magnetic Resonance
NOESY	:	Nuclear Overhauser effect spectroscopy
OD	:	Optical density
OMM	:	Outer mitochondrial membrane
PARP-1	:	Poly-ADP-ribose-polymerase-1
PBS	:	Phosphate buffer saline
PBST	:	Phosphate buffer saline with Tween-20
PC-3	:	Prostate cancer cell
PI	:	Propidium iodide
PS	:	Phosphatidylserine
RIPA	:	Radioimmunoprecipitation assay
RIPs	:	Ribosome-Inactivating Proteins
RNA	:	Ribonucleic acid
rpm	:	Revolutions per minute
RPMI	:	Roswell Park Memorial Institute 1640
SD	:	Standard deviation
SDS	:	Sodium dodecyl sulphate
PAGE	:	Polyacrylamide gel electrophoresis
Smac	:	Second mitochondria-derived activator
SRB	:	Sulforhodamine B

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

On 1966, Francis Peyton Roux, a tumour virologist and Nobel winner stated that the special and interesting pattern method of a tumour which can kill a man that are widespread, invasive, incontrollable and reduced growth. The definition of tumour or cancer by Roux during that time is almost similar with the new refine meaning of cancer nowadays. Currently, cancer is defined as a class of disease with ungoverned cell proliferation and spreading of mutated cells (Society, 2015). Uncontrolled and spreading of cancer can lead to death by causing malnutrition through nutrient competition with normal cells and weaken our body immune system (Lam, 2003).

The World Cancer Report (2008) estimated that 12 million new cases of cancer were diagnosed; 7 million cancer deaths and 25 million people were living with cancer in 2008 alone. It is expected by 2030 that there will be 17 million cancer deaths annually; 27 million incident cases of cancer and 75 million people are living with cancer within five years of diagnosis (Boyle & Levin, 2008). Cancer incidences are associated with aging, unhealthy lifestyle including unbalanced diets and lack of physical activities, smoking, environmental pollutants, infectious agents such as hepatitis B virus and human papillomavirus, ultra violet radiations and occupational hazards (Boyle & Levin, 2008; Kleinsmith, 2006; Lim, 2002; Mack, 2004). Over the years, tobacco smoking is found to be responsible for about 30.0%, while infectious agents contributed about 20.0% of all deaths due to cancers worldwide (Boyle & Levin, 2008).

Typically, the conventional treatments for cancer are surgery, chemotherapy and radiotherapy (Chorawala et al., 2012; Portugal et al., 2009). Surgery is most direct and efficient way to cure cancer disease by directly removing the localized tumours (Lam, 2003). However, surgery process is far less effective against cancers that spread throughout the body and it also increased the risks of bacterial infection that might

cause fatality during the surgery. Conventional chemotherapy utilized a variety of cytotoxic drugs to treat localized and metastasized cancers (Cellarier et al., 2003; Chidambaram et al., 2011; Chorawala et al., 2012). Unfortunately, the processes of cancer cell are similar to normal cell and their differences lies in their activities but not functions. Therefore, normal cell would be killed along with the cancer cells during chemotherapy. Radiotherapy is usually the last option of cancer treatment and it has the same problem like chemotherapy which is causing damage to normal cell. There is a limit for patient to undergo radiotherapy because the patient might die from radiation poisoning since exposure to radiation has cumulative effects. The side effects of conventional treatments are bone marrow suppression, depression, emesis weight loss, nausea, weakness, hair loss, and anaemia, and acute kidney failure, induction of oxidative stress and reduction of intrinsic plasma antioxidant (Ajith et al., 2008; Ajith et al., 2007; Avendaño & Menéndez, 2008; Borek, 2004; Lam, 2003; S. Sharma & K. Gupta, 1998; Sharma et al., 1997). Thus, a more specific and high efficacy anticancer drug with minimal harmful to our body is needed.

Natural product is one of the best alternatives for new anticancer agent development. Natural product is playing an important role in traditional medicines and it serves as the most fundamental and basis of earlier drugs (Butler et al., 2014). Over half of the world's population especially those from the developing country is depending on traditional plant-derived medicines for their primary health care and plant have a long history in application of cancers' treatment (Cragg et al., 2009). Herbal or plant-derived medicines typically contain different type of pharmacologically active compounds that contribute to different therapeutic effect in disease treatment included cancer (Ernst, 2005). Among the best-known plant-derived anticancer agents applied in clinical use are the vinca alkaloids, vinblastine, vincristine, taxanes, campothcin derivatives and others (Cragg & Newman, 2013; Prakash et al., 2013).

Turmeric have been documented at least for 6,000 years in history of medicine (Ravindran, 2007). *Curcuma mangga*, a species of rhizome plant under the family of Zingiberaceae, is first reported in the Andaman Island, India in year 1984 (Balakrishnan & Bhargava, 1984). *C. mangga* is locally known as "Temu mangga/kunyit putih' in Indonesia and "Khamin khao' in Thailand (Ali et al., 2010) while it is known as "Temu pauh' in Malaysia because of the pleasant mango-scent rhizomes. It can be found in Peninsular Malaysia, Thailand, Indonesia and India. *C. mangga* is one of the many plants which is quite often used in traditional medicinal in Indonesia, mainly used to treat fever, stomach-ache and chest pain. In our search for potentially active ingredients from *C. mangga*, we have set out to conduct intensive investigations with the following objectives:

1. To determine the active fractions from the rhizomes of *C. mangga* by evaluating the growth inhibitory effect of crude extract, fractionated extracts against selected cancer cells (HCT116, HT-29, A549 and CaSki) by SRB assay in a concentration- and time-dependent manner
2. To isolate and identify bioactive compounds from the biologically active fractions of *C. mangga*
3. To observe the induction of apoptosis by examining the morphological characteristics and expression level of apoptotic protein of HT-29 cells when treated with dichloromethane extract (CMD) using phase contrast microscopy. fluorescence microscopy *via* Hoechst PI double dye-staining assay and western blot.
4. To investigate the induction of apoptosis in HT-29 cells treated with CMD through the TUNEL assay using flow cytometry analysis.
5. To investigate the effect of CMD on the cell cycle of HT-29 cells.

6. To determine the percentage of cells in CMD treated through externalization of phosphotidylserine using Annexin V-FITC/PI staining and flow-cytometry analysis.

CHAPTER 2: LITERATURE REVIEW

2.1 *Curcuma mangga*

2.1.1 Taxonomy and geographical distribution

The similar and unique aroma of the unripe mango rhizome of *C. mangga* and *C. amada* makes these two species to distinguish from one another (Babu et al., 2011; Leong-Škorničková et al., 2010). However, the inflorescences produced by these two species are different where *C. mangga* produced lateral inflorescence while *C. amada* produced terminal inflorescence (Babu et al., 2011; Leong-Škorničková et al., 2010). Besides that, *C. mangga* mostly found in Indonesia while *C. amada* is native to Eastern India (Babu et al., 2011).

Figure 2.1: Rhizome of *Curcuma mangga*

2.1.2 Genetic information

Up-to-date, there are no more than ten research works on the genomic studies and genetic profiling on *Curcuma* species (Apavatjrut et al., 1996; Ardiyani, 2003; Joseph et al., 1999; Leong-Skornickova et al., 2007; Prana et al., 1978; Sirisawad et al., 2003; Skornickova & Sabu, 2005). Genetic data on *C. mangga* is limited where so far only two investigations which were done by Prana et al. (1978) and Ardiyani (2003). Prana

et al. (1978) and Škorničková et al. (2007) showed similar findings that the *C. mangga* have 42 diploid numbers but Ardiyani (2003) reported the presence of 63 diploid numbers.

2.1.3 Nutritional values

Investigation on nutritional contents and values of *C. mangga* was only done by Zanariah et al. (1997). Zanariah et al. (1997) investigated the proximate analysis, vitamin content and the amino acid profile of *C. mangga* and the results are presented in Table 2.1. From the result of proximate analysis, *C. mangga* contain highest amount of moisture (88.0g/100.0g), followed by carbohydrates (8.6g/100.0g), fats (1.2g/100.0g), fibre (1.1g/100.0g), ash (0.5g/100.0g) and the least was protein (0.4g/100.0g). The vitamin composition of *C. mangga* was mainly comprises of ascorbic acid (1.95mg/100.0g), riboflavin (0.04mg/100.0g) and thiamine (0.03mg/100.0g) whereas aspartic acid and glutamic acid were mostly found in the amino acid profile.

Table 2.1: Proximate analysis, vitamin content, and amino acid profiles of the rhizomes of *C. mangga* (Zanariah et al., 1997)

Proximate analysis result per 100.0 g		**Vitamin composition (mg/100.0 g weight)**		**Amino acid profiles (g/100.0 g weight)**	
Energy (kcal)	47.0	Thiamine	0.03	Aspartic acid	13.4
Moisture (g)	88.1	Riboflavin	0.04	Glutamic acid	14.9
Protein (g)	0.4	Ascorbic acid	1.95	Serine	5.9
Fat (g)	1.2			Glycine	6.2
Carbohydrates (g)	8.6			Histidine	1.9
Fibre (g)	1.1			Arginine	3.5

Table 2.1, continued

Proximate analysis result per 100.0 g		Vitamin composition (mg/100.0 g weight)	Amino acid profiles (g/100.0 g weight)	
Ash (g)	0.5		Threonine	1.0
			Alanine	3.0
			Proline	6.2
			Tyrosine	2.6
			Valine	6.4
			Isoleucine	3.9
			Leucine	8.2
			Phenlyalanine	5.0
			Lysine	0.7

2.1.4 Phytochemical studies

Myrcene is one of the main constituent in the essential oil of the rhizomes of C. mangga with the amount ranging from 46.5% to 81.4% (Jantan et al., 1999; Wahab et al., 2011; Wong et al., 1999). Abas et al. (2005), Liu and Nair (2011) and Malek et al. (2011) are among researchers that have reported the investigations on the chemical constituents isolated from *C. mangga*. Cucurmanggoside is one of the new labdane diterpene glucoside identified by Abas et al. (2005) from the rhizomes of *C. mangga* together with other known compounds and the chemical constituents isolated from *C. mangga* are summarized in Table 2.2.

Table 2.2: Chemical constituents of *C. mangga*

Compounds	References
cucurmanggoside	(Abas et al., 2005)
labda-8(17),12-diene-15,16-dial	(Abas et al., 2005; Liu & Nair, 2011; Malek et al., 2011)
calcaratarin A	(Abas et al., 2005; Liu & Nair, 2011)
zerumin B	(Abas et al., 2005; Liu & Nair, 2011)
scopoletin	(Abas et al., 2005)
demethoxycurcumin	(Abas et al., 2005; Kaewkrock et al., 2009; Malek et al., 2011; Tewtrakul & Subhadhirasakul, 2008)
bisdemethoxycurcumin	(Abas et al., 2005)
1,7-bis(4-hydroxypehnyl)-1,4,6-heptatrien-3-one	(Abas et al., 2005)
curcumin	(Abas et al., 2005; Malek et al., 2011)
p-hydroxycinnamic acid	(Abas et al., 2005)
(*E*)-15,16-bisnorlabda-8(17),11-dien-13-on	(Kaewkrock et al., 2009; Malek et al., 2011; Tewtrakul & Subhadhirasakul, 2008)
(*E*)-15,15-diethoxylabda-8(17),12-dien-16-al	(Kaewkrock et al., 2009)

Table 2.2, continued

Compounds	References
communic acid copallic acid 14,15,16-trinor-labdan-8,12-diol 8-methene-1,1,10-trimethyl-delcalin 1,1,10-trimethyl-decalin	(Liu & Nair, 2011)
β-sitosterol	(Malek et al., 2011)
zerumin A	

2.1.5 Anticancer activities

There are several reports in the literature on the cytotoxic activity of the rhizomes of *C. mangga* against human carcinoma cell lines (Abas et al., 2006; Hong et al., 2015; Karsono et al., 2014; Kirana et al., 2003; Liu & Nair, 2012; Liu & Nair, 2011; Malek et al., 2011; Sisimindari et al., 2004). It has been reported that a protein fraction extracted from the fresh rhizomes of *C. mangga* exhibited cytotoxic effect against Burkitt lymphoma carcinoma cell line (Raji), and human cervical carcinoma cell line (HeLa) with LC_{50} values of 41.3 μg/mL and 18.2 μg/mL, respectively. The extracted protein from oven-dried and freeze-dried rhizomes of *C. mangga* was found to exhibit weak inhibitory effect against the growth of HeLa and Raji cells. The protein obtained may have been denatured due to exposure to initially high and then low temperatures during the oven drying and freeze-drying process. Jaremko et al. (2013) reported that proteins could unfold due to high and low temperatures. It was therefore hypothesised that the extracted protein fraction might have contained Ribosome-Inactivating Proteins or RIPs, because the extracted protein fraction was able to cleave supercoiled DNA in agarose gel, which is one of RIPs characteristics (Sisimindari et al., 2004).

Kirana et al. (2003) studied the cytotoxic activity of the ethanol extract of the rhizomes of *C. mangga* on MCF-7 human hormone-dependent breast cancer cells and HT-29 human colon cancer cells. The *C. mangga* ethanol extract was reported to

exhibit weak cytotoxic activity against both cells with IC_{50} values of 44.7 ± 2.7 µg/mL and 91.0 ± 5.9 µg/mL, respectively. In 2011, Liu and Nair reported that methanol, water and ethyl acetate extracts of the rhizomes of *C. mangga* showed mild cytotoxic effects against human lung, stomach, colon, central nervous system (CNS) and breast carcinoma cell lines with percentage of growth inhibition ranging from 9% to 46% at 200 µg/mL. In the same study, the ethyl acetate extract exhibited no activity on CNS carcinoma cell lines. In the following year, Liu and Nair (2012) found that the methanol and water extracts of the leaves of *C. mangga* exhibited slightly stronger cytotoxic activity in comparison to the rhizomes extracts with growth inhibition percentage ranging from 18% to 46% against prostate (DU-145 and LNCaP), gastric (AGS) and pancreatic (BxPc-3) human cancer cells at 100 µg/mL. These findings were in agreement with Malek et al. (2011) who reported that the methanol extract of the rhizomes of *C. mangga* showed only mild cytotoxic activity against MCF-7, nasopharyngeal epidermoid cell line (KB), lung cell line (A549), cervical cell line (Ca Ski), colon cell line (HCT 116) and HT-29 cells with IC_{50} values ranging from 22.0 ± 1.1 to 36.8 ± 3.8 µg/mL.

In the same communication, Malek et al. (2011) reported that the hexane fraction exhibited good cytotoxic activity against MCF-7, KB, A549, Ca Ski, and HT-29 cells with IC_{50} values ranging from 8.1 ± 0.2 to 17.9 ± 0.3 µg/mL while the ethyl acetate fraction showed moderate cytotoxic activity against MCF-7, KB, A549, HCT116, and HT-29 cells with IC_{50} values ranging from 18.5 ± 0.1 to 47.1 ± 0.5 µg/mL. The methanol extract and its hexane and ethyl acetate fractions were not toxic to non-cancer human fibroblast cell line MRC-5, which was in agreement with Kirana et al. (2003) who reported that the ethanol extract of *C. mangga* has low toxicity toward SF 3169 skin fibroblasts.

Studies by Widowati et al. (2011) reported that the ethanol extract of the rhizomes of *C. mangga* showed no antiproliferative activity against human breast ductal carcinoma cell line, T47D. Again, in 2013, Widowati and colleagues investigated the antiproliferative activity of the aqueous ethanol of the rhizomes of C. mangga and found the extract showed no antiproliferative activity against the same cell line, T47D. The results obtained by Widowati et al. (2013) were in agreement with those obtained by Liu and Nair (2011) and Malek et al. (2011). Both T47D and MCF-7 cells are classified under the same cluster (Luminal A), where both cells shared the same characteristics such as lack of expression of human epidermal growth factor receptor 2 (HER2), expressed estrogen receptor (ER), amenable to hormone therapy and chemotherapy and expressed low Ki67, which is proliferation marker in breast cancer (Holliday & Speirs, 2011). The lack of antiproliferative activity reported by Widowati et al. (2011) may be due to low exposure time of the extract to the cells as they treated T47D cells for 24 hours, while other researchers treated cancer cell lines for at least 72 hours (Abas et al., 2006; Kirana et al., 2003; Malek et al., 2011). Thus, it can be deduced that the antiproliferative activity exhibited by the extracts of the rhizomes of *C. mangga* is time dependent, where the growth inhibition is very low within 24 hours of treatment and highest after 72 hours.

Karsono et al. (2014) reported that 70% aqueous ethanol extract exhibited reduction of prostate cancer (PC-3) cell viability from 100.0% (control) to 25.7% at concentration ranged between 50 – 200 μg/mL after 24 hours treatment. In the following year, Hong et al. (2015) reported that the hexane (CMH) and ethyl acetate (CME) extract of *C. mangga* rhizome exhibited cell inhibition on HT-29 after 24 hours treatment with IC_{50} values of 39.3 ± 6.0 μg/mL and 32.2 ± 2.7 μg/mL respectively. Both studies also showed the almost similar pattern of results on the cell cycle distribution where the

accumulation of cell at G_0/G_1 and cell reduction in S phase either treated with aqueous or non-aqueous extract. Cell accumulation at G_2/M and S phase disappeared at the highest dose (200 μg/mL) in studies by Karsono et al. (2014) while there was still cell accumulation at G_2/M and S phase even at the highest dose of 40 μg/ml shown by Hong et al. (2015). Excessive use of high concentration of extracts in Karsono et al. (2014) might have caused the cells to experience shock and then died instantly which explained why cell accumulation occurred in the G_0 phase. However, these findings suggested the possibility of the type of chemical constituents in the extract of *C. mangga* which induced cell cycle arrest in both cells.

2.2 Cancer

2.2.1 Cell cycle

A living organism such as human originated from a single cell which then develop into a fertilized egg, and continues to grow and develop to form a human being housed 50 trillion living cells in the body (Boerner et al., 2002). Duplication of the numerous materials is one of the criteria for making two cells from a single cell where the duplication of hereditary molecules stored in the DNA of chromosomes, is one the best example to describe the process of a cell division (Pardee, 2002). In order for a cell to duplicate its DNA and other cell constituents and divided into two daughter cells, cells must enter into four individual phases called G_1 phase, S phase, G_2 phase and M phase in order to complete the task. Combination of these phases known as cell cycle.

Cell cycle is generally illustrated with a circular diagram (Figure 2.2). Cell cycle can be divided into two phases called interphase (G_1 phase, S phase and G_2 phase) and mitosis (M phase). Interphase is the period when the cell begins to accumulate material and nutrients, and doubling of the genetic molecules before undergoes mitosis where the

mitophase is the period when separation from one original cell ("mother cell") and split into two daughter cells with identical genome and DNA information (Behl & Ziegler, 2014; King & Cidlowski, 1998; Shackelford et al., 1999). Interphase occurs approximately 95% and the mitosis only takes 5% for a complete cell cycle.

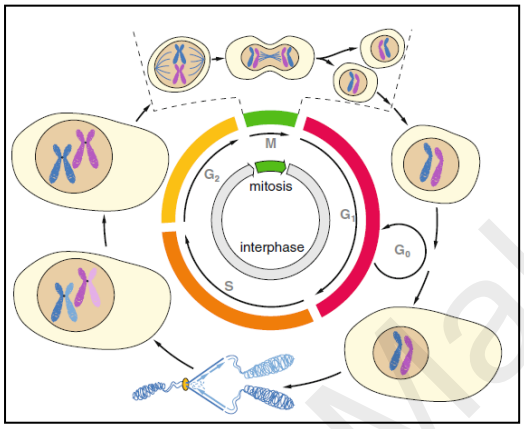


Figure 2.2: Cell cycle phases divided into four phases: G_0/G_1, S, G_2 and M phase (Behl & Ziegler, 2014)

A sensor mechanism, called checkpoints is used to maintain the correct order of events by monitoring the progression and transition of one phase to another phase in cell cycle. The G in G_1 and G_2 phase stands for gap that act as a guardian to monitor the condition of the extracellular and intracellular condition to ensure everything is in order before the next cell cycle is initiated (Behl & Ziegler, 2014; King & Cidlowski, 1998; Pardee, 2002). The event occurs in each phase are summarized in Table 2.3.

Table 2.3: Summary events in cell cycle phase (Behl & Ziegler, 2014; King & Cidlowski, 1998; Shackelford et al., 1999)

Phase	Events occur
Interphase	
G_1 (or known as pro-mitotic post synthetic) phase	Cell begins to grow, formation of cytoplasm and organelles; synthesis of mRNA, histone protein and the enzyme of the DNA replication machinery
S or synthesis phase	Doubling of genomic data and packaging of genomic DNA
G_2 (or known as premitotic or post-synthetic) phase	Round up and increase in general cell size; synthesis of RNA
Mitosis phase	Two daughter cells are split from a single cell by separating the doubled DNA arranged in chromosome, as well as the cellular nucleus.

2.2.2 Hallmark of cancers

Hanahan and Weinberg (2000) suggested that cancer cells normally manifest six essential alterations to the cell physiology that progress towards malignant growth which are self-sufficient growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Figure 2.3). However, some new emerging hallmark of cancers had been discovered and discussed by recent researchers.

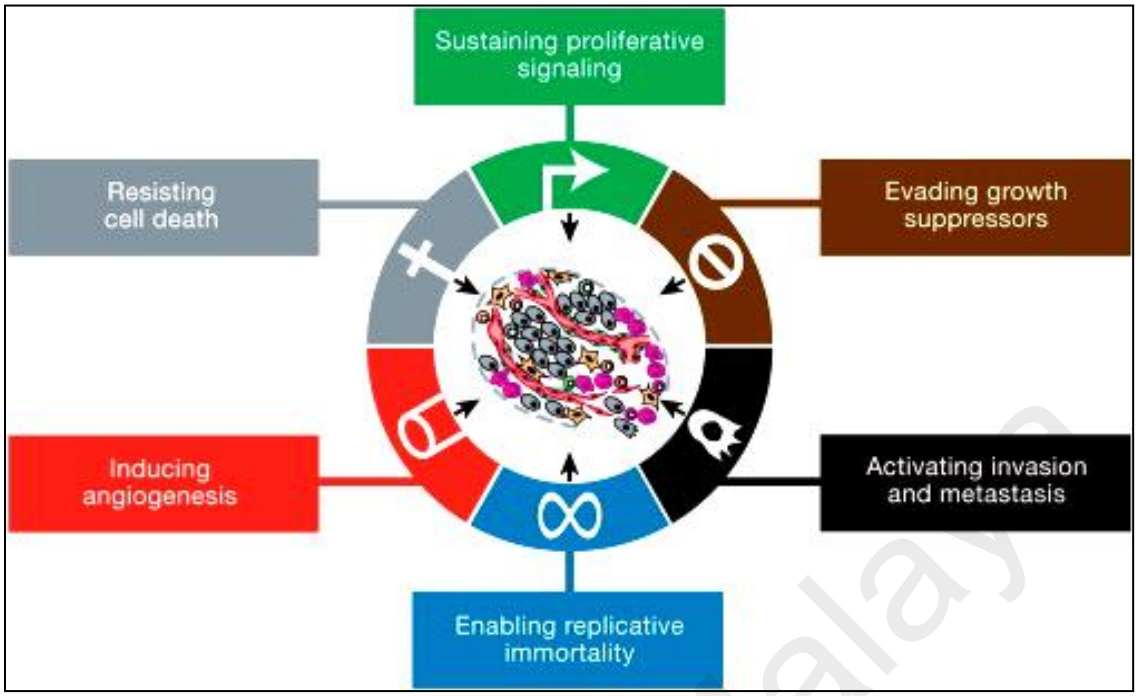


Figure 2.3: Hallmarks of cancer (Hanahan & Weinberg, 2000)

2.2.3 Carcinogen

Carcinogen is defined as an agent that can cause cancer. There are multiple factors that can caused cancer such as radiation, chemicals, viruses and others which resulted in the most cases of the human cancers. Carcinogens can be classified into several classes based on the mechanisms by which these agents react on the cell or tissue. Genotoxic carcinogens, metabolized carcinogens and physical carcinogens are the three main classes of carcinogens. Genotoxic carcinogens like organic chemicals (hydrazine, ethylene dibromide) are direct-acting carcinogen and have the ability to cause damage directly to the DNA, protein and cellular constituents due to the reactivity of their functional groups (Gooderham & Carmichael, 2002). Alternatively, metabolized carcinogens like 2-acetylaminofluorene require the metabolic activation in order to exert their carcinogenic properties through hydroxylation of the amide nitrogen to produce a metabolite that has a higher carcinogenic effect than the parent molecule (Gooderham & Carmichael, 2002). Ultraviolet radiation is one of the best example of physical

carcinogens that will increase the risk of carcinogenesis by exposing the radiation energy to the biological materials and cause changes on the bonds that are holding them which subsequently lead to chemical changes and possibly biological effects (Schwartz, 2002).

Carcinogens in the diet that trigger the initial stage include moulds and aflatoxins (for example, in peanuts and maize), nitrosamines (in smoked meats and other cured products), rancid fats and cooking oils, alcohol, and additives and preservatives (Sugimura et al., 2002). The complementary of unhealthy lifestyle (smoking, poor exercise, imbalance diet) and stressful working environment (contaminated area, high exposure to UV radiation and chemical waste) can cumulatively lead to DNA damage and the progression of cancer (Hecht, 2002; Schwartz, 2002; Vainio et al., 2002). Genetic or specific of inherited trait is another important factor that will lead to the progression of cancer besides with the exposure to carcinogens (Evans, 2002). Thus, patients that has familial adenomatous polyposis condition are more susceptible to colon cancer.

2.2.4 Carcinogenesis

A normal cell is regulated with multi-signalling pathways in response toward the environmental or external factors that either promote or suppress cell growth. Loss of regulations in a cell could lead a normal cell to transform into a neoplastic cancer cell in multistage process and this process is known as carcinogenesis (Devi, 2005). Carcinogenesis can be divided into three main stages which are initiation, promotion and progression. Initiation is the first step in carcinogenesis whereby the cellular genome begins to mutate when induced by carcinogens. The mutated genetic information will then be carried over to the progeny cell. Alteration of a typical cell to a

cancer cell is controlled by a DNA sequence called oncogenes. The mutated cell in the initiation stage is less harmful but a prolonged transformed cell which is repeatedly exposed to carcinogens will stimulate the proliferation of the initiated cell. In addition, expression of the initial mutation is determined by the interaction among the oncogenes and the temporarily changes of specific gene expression can be also caused by a few factors like lipid metabolites, cytokines and certain phorbol esters. The final stage of the carcinogenesis is progression and during this stage, mutations and chromosomal aberration and the increase of maglinant sub-population occured. This process could be accelerated by prolonged exposure to carcinogenic stimuli which will lead cells to further proliferation and growth into a tumour. Heterogeneity of the cell population will increase as the tumour size and further lead to more mutation (Devi, 2005).

2.3 Apoptosis in cancer

Cell death is one of the important processes to maintain the balance of physiology for most of the metazoan species (Reed, 2002). During development and aging, many cells have undergone cell death in order to secure the development and functionality of the body such as the formation of the organs and separation of toes during embryo development (Elmore, 2007; Schulze-Osthoff, 2008). Programmed cell death normally occurred through an ordered sequences of process known as “apoptosis”, a term originated from Greek with meaning of falling of leaves from trees in autumn (Lawen, 2003; Reed, 2002; Schulze-Osthoff, 2008; Wong, 2011; Wu et al., 2001). Apoptosis plays important role in our body such as maintaining the cell populations in tissue, human defence system, normal embryo development and maintenance of body homeostasis (Elmore, 2007; Rastogi & Sinha, 2010; Reed, 2002). The leverage between cell death and cell division need be balance and be controlled regularly because

excessive or less activities in either cell death or division will bring consequence effects to the body. Excessive of cell death will lead to acquired immune deficiency syndrome (AIDS) and neurodegenerative diseases such as Alzheimer and Parkinson syndrome while reduced of apoptosis will lead to cancer, persistent viral infection, or autoimmune disorders (Wu et al., 2001).

Another mode of cell death is necrosis which is defined as catabolic, passive and degraded processes that caused cell injury (Elmore, 2007; Hung & Chow, 1997; Wu et al., 2001). Differences between necrosis and apoptosis in the context of morphological and biochemical characteristics can be distinguished even though some of the characteristics overlap among these two-cell death modes. Typical characteristics of necrosis are initial cell swelling and loss of cell membrane function resulted in increased permeability and intracellular edema; lyses of nuclear chromatin into ill-defined plumps; dilation of organelles and lysosomal degradation that cause cell erupted and cytosolic constituents are released into the extracellular environment that will provoke an inflammatory responses (Bjelaković et al., 2005; Elmore, 2007; Hung & Chow, 1997; Proskuryakov et al., 2003; Schulze-Osthoff, 2008).

In contrast, apoptosis is an anabolic, innate and genetically steering process that eliminates the injured single cell (Bjelaković et al., 2005). The morphological changes of apoptotic cell are characterized by pyknosis, membrane blebbing, formation of apoptotic bodies and cell shrinkage while the biochemical changes include DNA fragmentation and externalization of phosphotidylserine at the cell surface (Hung & Chow, 1997; Lawen, 2003; Rastogi & Sinha, 2010; Wong., 2011; Wu et al., 2001).

2.3.1 Mechanism of apoptosis

The mechanism of apoptosis commonly comprises of multiple molecules that possesses up- and down-regulatory effects. The factors that decide the fate of both pro- and anti-apoptotic molecules are the trigger factors and cell type. It is less possible that the apoptosis of a cell controlled by the changes of a solitary element.

Typically, a group of proteolytic enzymes known as cysteinyl aspartate-specific proteinases (caspases) that play important roles in the process of apoptosis. Caspases play an important role in the mechanism of apoptosis (Rastogi & Sinha, 2010; Wong., 2011) and can be divided into initiator caspases (caspase-8, -9 and -10) and effector caspases (caspases-3, -6 and -7). All caspases are produced in the cells as catalytically inactive zymogen known as procaspases and are required to undergo proteolytic activation during apoptosis (Riedl & Shi, 2004). The effector caspases are activated by initiator caspases through cleavage at specific internal aspartate residues that split into the large (~p20) and small subunits (~p10) where both of these subunits are tightly associated with each other to form a caspase monomer (Riedl & Shi, 2004). In contrast, initiator caspases are auto-activated and under apoptotic condition, the activation of initiator caspases will trigger a cascade of downstream caspase activation which is strictly regulated and usually requires the assembly of a multi-component complex (Riedl & Shi, 2004). The cascade effects by the activated caspase occur through two paths, which are extrinsic pathway or/and intrinsic pathway.

2.3.2 External pathway/ Death receptor pathway

External pathway or known as extrinsic death receptor pathways (Figure 2.4) initiate apoptosis through the involvement of transmembrane receptor-mediated interactions which often refers to binding of death ligands to the death receptor (Elmore, 2007;

Wong, 2011). Members of tumor necrosis factor (TNF) receptor gene superfamily are one the example of death receptor that involved in the extrinsic death receptor pathway. Common similarities shared among these members of the TNF receptor family are the domains with enriched extracellular cyteine and consists of a cytoplasmic domain of about 80 amino acids known as death domain. The function of death domain is transmitting the death signal from the cell surface to the intracellular signalling pathways and the top characterized ligands and corresponding death receptor included FasL/FasR, TNF α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Elmore, 2007). The formation of an adapter protein and the whole ligand-receptor-adaptor protein complex, also known as death-inducing signalling complex (DISC) is the result of binding death ligand to the death receptor. The assembly and activation of pro-caspase 8 is initiated by DISC followed by the initiation of apoptosis by activated form of caspase 8 by cleaving the downstream or other exercutioner caspases (Elmore, 2007; Lawen, 2003; Rastogi & Sinha, 2010; Riedl & Shi, 2004; Wong, 2011). Link between caspase-8 and caspase-3 and the link caspase and the mitochondria pathway through Bid.

2.3.3 Internal pathway/ Mitochondrial Pathway

Internal pathway or known as intrinsic mitochondrial pathways (Figure 2.4) initiates apoptosis through a varied collection of non-receptor-mediated stimuli that generate intracellular signals such as hypoxia, permanent damaged genetic materials, high oxidative stress and concentration of cytosolic calcium ions that act directly on targets within the cell and are mitochondrial events either in negative or positive pattern (Elmore, 2007; Wong, 2011). One of the most significant characteristics of the intrinsic mitochondrial pathway is the release of the pro-apoptotic protein, cytochrome c from the mitochondria into the cell cytoplasm due to the high permeability of the

mitochondria caused by the intracellular stimuli (Wong, 2011). The intrinsic pathway is regulated by a group of protein under the family Bcl-2 that consists of pro-apoptotic protein (e.g. Bax, Bad, Bak) and anti-apoptotic protein (e.g. Bcl- 2, Bcl-W). The function of the anti-apoptotic proteins is to prevent the release of mitochondrial cytochrome c into cytoplasm. In contrast, the pro-apoptotic proteins enhance and promote the release of mitochondrial cytochrome c into the cytoplasm (Wong, 2011). Other apoptotic protein molecules such as apoptosis inducing factor (IAF), direct IAP binding protein with low pI (DIABLO), second mitochondria-derived activator (Smac) and Omi/high temperature requirement protein A (HtrA2) are released as well from the intermembrane space of the mitochondria. The cytochrome c release from the mitochondria will bind with Apaf-1 and caspase-9 to form a complex known as apoptosome that will activate the effector caspase, caspase-3. Additionally, Smac/DIABLO or OMI/HtrA2 promote the activation of caspase by disrupting the interaction of inhibitor of apoptosis proteins (IAPs) with the caspases (caspase-3 or -9) through binding to IAPs (Elmore, 2007; Wong, 2011).

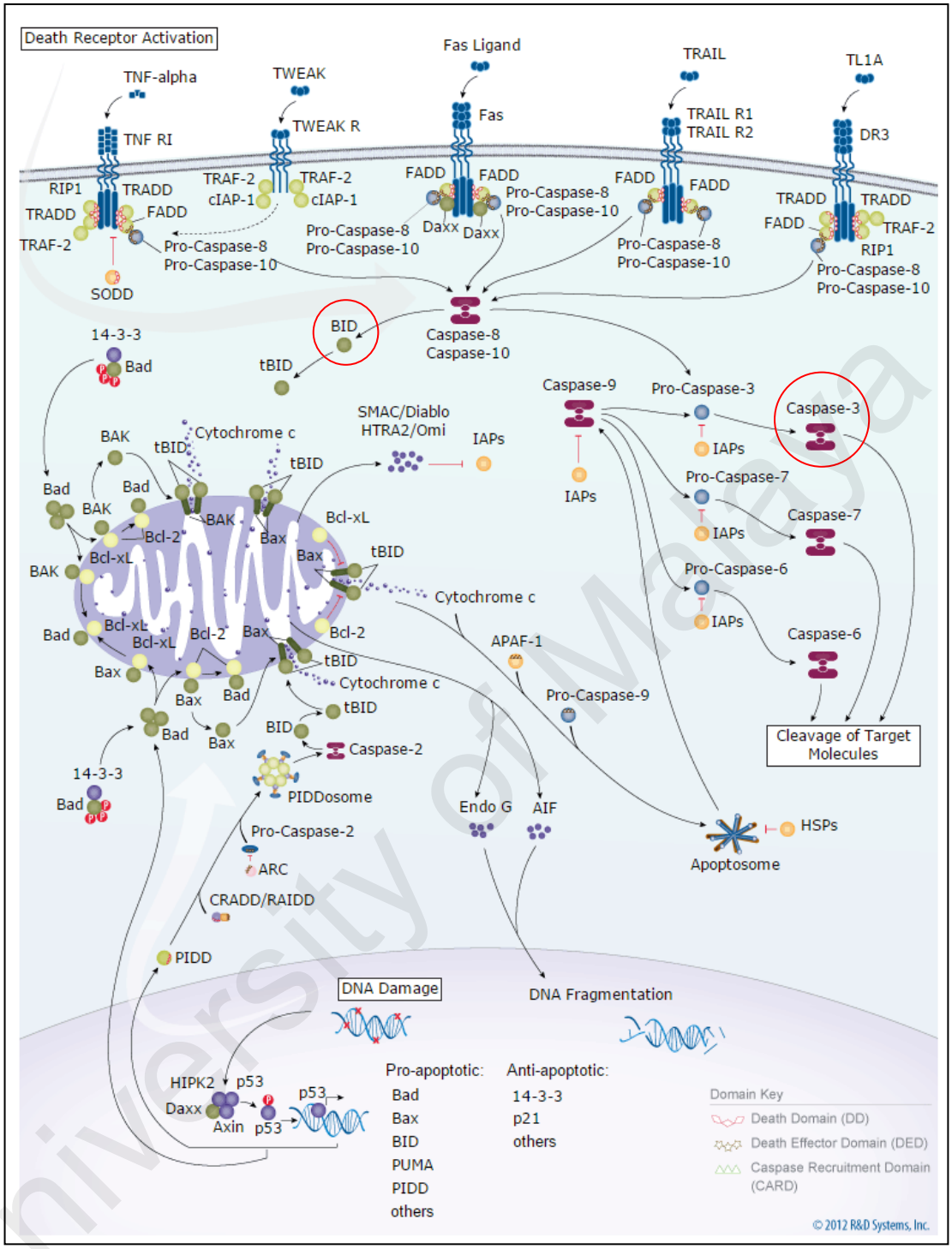


Figure 2.4: Extrinsic and intrinsic pathways (R&D System, 2012)

2.4 Bioassay investigation

2.4.1 Cytotoxicity screening

Evaluation or screening of compounds for their potential cytotoxicity at cellular level *via* short term *in vitro* cytotoxicity assays with cultured cells has been widely used as these assays are inexpensive, rapid, sensitive and reproducible and could further reduce the use of animals for LD_{50} and other similar tests (Borenfreund et al., 1988; Chiba et al., 1998; Fotakis & Timbrell, 2006; Weyermann et al., 2005). The common methods that used for determine cell viability after exposure to toxic compounds are methyl tetrazolium (MTT) assay, lactate dehydrogenase leakage (LDH) assay and neutral red assay (Fotakis & Timbrell, 2006). However, a better and novel approach of cytotoxicity screening method was discovered and it is known as the sulforhodamine B (SRB) assay. Therefore, the modified SRB cytotoxicity assay described by Houghton et al. (2007) was utilised to determine the cytotoxic activity of the extracts and isolated compounds from *C. mangga*.

2.4.1.1 SRB cytotoxicity assay

There were two main techniques used to determine the cytotoxic effect of the natural products. These techniques include the reagents 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT). These reagents used in tetrazolium-based assays are dependant to the metabolic reduction by mitochondria in viable cells to produce a coloured formazan product. However, if the activities of the mitochondria were inhibited by cellular levels of NADH and glucose, or disrupted by other factors, it would affect and afford varies and inconsistent results. (Houghton et al., 2007).

SRB assay was first developed in year 1990 by Skehan et al. and this cytotoxicity screening method was eventually adapted by the National Cancer Institute (NCI) for the routine use of *in vitro* antitumor screen (Papazisis et al., 1997; Skehan et al., 1990). SRB is an anionic bright pink aminoxanthene protein dye with two sulfonic groups and it has the molecular formula of $C_{27}H_{30}N_2O_7S_2$ and molecular weight of 558.66 (Voigt, 2005). The SRB dye electrostatically and pH-dependently binds to the protein basic amino acid residues. In other word, the SRB assay relies on the measurement of whole protein content of the cell based on the binding of SRB dye on the basic amino acid of the cell (Vichai & Kirtikara, 2006). Compared to MTT assay, the SRB assay produces higher sensitivity and linearity results which are suitable for the study of chemosensitivity for sub-confluent monolayer and multilayer cell clusters that contain high cell numbers. SRB assay was also more stable than MTT assay which allowed the plates to be stored for up to months (Keepers et al., 1991). The other advantages of using SRB assay are inexpensive, rapid detection and a better signal-to-noise ratio (Keepers et al., 1991; Papazisis et al., 1997; Skehan et al., 1990; Vichai & Kirtikara, 2006; Voigt, 2005).

2.4.2 Apoptosis detection assay using fluorescence-activated cell sorting (FACS)

2.4.2.1 Terminal dUTP Nick End-Labeling (TUNEL) assay

DNA laddering is a technique used to visualize the endonuclease cleavage products of apoptosis (Wyllie, 1980). TUNEL assay was first described and established by Gavrieli and co-worker in year 1992. TUNEL assay is one of the few methods used to detect DNA fragmentation through the incorporation of the enzyme terminal deoxynucleotidyl transferase (TdT) to the labelled deoxyuridine-triphosphatase (dUTP) into free 3'-hydroxyl termini while resulted from the breakage of genomic DNA into

high molecular weight single stranded DNA and low molecular weight double stranded DNA (Ito et al., 2006; Kressel & Groscurth, 1994; Kumari et al., 2008; Loo, 2002; Martinez et al., 2010; Taatjes et al., 2008). Pre-fixation of cells with crosslinking agents such as ethanol or formaldehyde is important to prevent the extraction of small DNA fragments (Huang et al., 2005; Wlodkowic et al., 2011). Typically, the labelling of double strands breaks procedure (Figure 2.5) is completed with only fluorescein-conjugated anti-Bromo deoxyuridine (BrdU) antibody, or combined with another colour fluorochrome of DNA binding staining dye such as propidium iodide. The double staining in TUNEL assay allowed the users to distinguish apoptotic and non-apoptotic cells as well as cell distribution in these sub-populations (Huang et al., 2005). The advantages of TUNEL assay are high sensitivity, cheap and easy to handle and therefore, this assay is considered as a general method for detection of DNA fragmentation and identifying apoptotic cell under the appropriate condition (Loo, 2002; Wlodkowic et al., 2011).

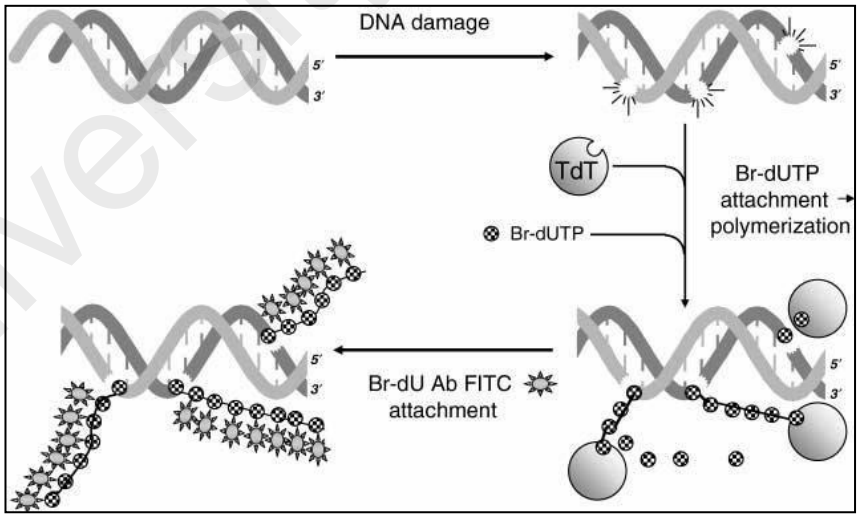


Figure 2.5: Schematic illustration of DNA strand-break labelling by TdT-mediated Br-dUTP attachment to 3'OH ends and polymerization, followed by immunocytochemical (FITC) detection of BrdU (Huang et al., 2005)

2.4.2.2 Cell cycle analysis

Cell cycle analysis is used to determine the stage when the cells are growth arrested. The DNA content is different in different type of phases. The cell cycle analysis depends on the intensity of stain binding to the DNA which directly reflects the content of DNA within the cell (Darzynkiewicz, 2010; Nunez, 2001; Pozarowski & Darzynkiewicz, 2004; Rabinovitch, 1993). The stained materials or cells are then analyzed using flow cytometer to measure the emitted fluorescence by stained materials. The measured fluorescence is converted into electronic pulse which is proportional to the amount of DNA content (Nunez, 2001). There are many types of different dye which have high affinity towards DNA such as propidium iodide (PI), 4′,6-diamidino-2-phenylindole (DAPI) and Hoechst dye. For PI, addition of RNAse A is important to digest the RNA and prevent false positive outcome during the staining process because PI can bind to both DNA and double stranded RNA (Darzynkiewicz, 2010; Nunez, 2001; Pozarowski & Darzynkiewicz, 2004). DAPI and Hoechst dye bind to the minor groove of DNA and thus addition of RNAse is not required. Other parameters should be of concerned to improve the analyzing of DNA content within the cells which include cell numbers for analysis and type of fixation agent such as ethanol or formaldehyde (Darzynkiewicz, 2010). When the stained materials analyzed using flow cytometer, the doublet which might be form when cells clumped together after cell fixation should be excluded as this would produce false high DNA content in the G_2/m phase. The doublet is actually two singlet cell clumped together in the G_0/G_1 phase (Darzynkiewicz, 2010; Nunez, 2001; Pozarowski & Darzynkiewicz, 2004).

CHAPTER 3: METHODOLOGY

3.1 Extraction and fractionation of plant sample

3.1.1 Plant material

The rhizomes of *C. mangga* were obtained from Yogjakarta, Indonesia in July 2014. A voucher specimen (voucher number: HI 1331) was deposited in the Herbarium of Institute of Biological Sciences, Faculty of Science, University of Malaya.

3.1.2 Extraction and fractionation of plant materials

Two methods (Method A and Method B) were employed for the extraction and fractionation procedure. The difference between these methods was the solvent used in the extraction of the crude extract. However, Method B was selected as a final choice in the plant extraction and fractionation and this will be further discussed in Section 3.3.1. Both methods are summarized in Section 3.1.2a and 3.1.2b.

3.1.2a Method A: Crude methanolic extract

The dried, ground and powdered rhizome *C. mangga* (1.0 kg) was soaked in methanol for three days at room temperature. The solvent containing extract was then decanted, dried with anhydrous sodium sulphate and evaporated using a rotary evaporator (Buchi, Model: R-210), yielded 106.4g of dark brown methanol extract (CMM). The crude methanol extract was then extracted with hexane until the solvent was colourless. The extracting solvent was subsequently dried with anhydrous sodium sulphate and evaporated to obtain a yellowish-brown extract (CMH).

The insoluble residue was further fractionated using a mixture of ethyl acetate and water (ratio 1;1) and two layers of liquid were obtained with the ethyl acetate at the top layer. This ethyl acetate layer was separated from the aqueous layer using a separating funnel. The aqueous layer was extracted repeatedly with fresh ethyl acetate until the extracting solvent become colourless. All ethyl acetate layers obtained were combined and dried with anhydrous sulphate, then evaporated to obtain a sticky dark brown fraction (CME). The aqueous layer was evaporated to remove any extracting solvent and then freeze dried to obtain a gummy light brownish yellow extract (CMW). The yield of crude methanolic extracts, hexane, ethyl acetate and water fractions are shown in Table 4.1. The percentage of the fractions was calculated based on the yield of crude methanolic extracts.

3.1.2b Method B: Crude dichloromethane extract

Dried powdered rhizomes of *C. mangga* (1.0kg) were soaked in dichloromethane (DCM) for three days at room temperature. The solvent was removed, desiccated with anhydrous sodium sulphate and evaporated using a rotary evaporator to yield a yellowish-brown crude DCM extract (CMD). The CMD was then extracted with n-hexane until the solvent became colourless. The solvent-containing extract was then dried with anhydrous sodium sulphate and evaporated using a rotary evaporator to give a yellowish oily hexane fraction (CMDH). The hexane-insoluble residue was then further dissolved in methanol which was then dried with anhydrous sodium sulphate. After filtration, the filtrate was evaporated to give a dark yellowish brown methanolic fraction (CMDM).

The extraction procedure is shown in Figure 3.1. The yield of crude DCM extracts (CMD), hexane (CMDH) and methanolic fractions (CMDM) are shown in Table 4.1. The percentage of the fractions was calculated based on the weight of the DCM extracts.

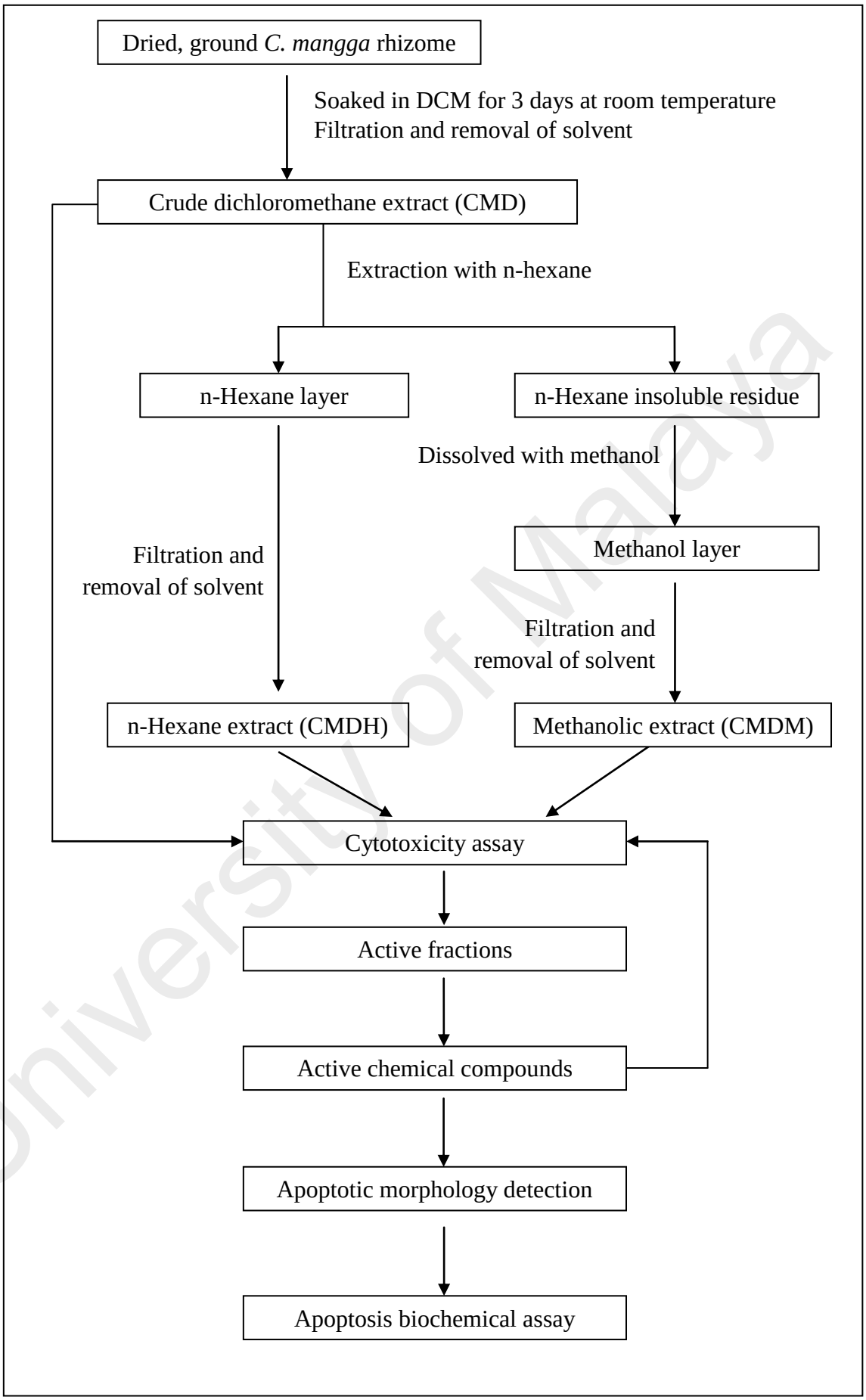


Figure 3.1: A summary of the bioassay-guided chemical investigation of rhizome *C. mangga* from Method B

3.2 Cytotoxicity screening

3.2.1 Sulforhodamine B (SRB) assay

The cytotoxic effects of the CMD, CMDH, CMDM and isolated pure compounds were investigated performed with SRB assay as described by Houghton et al. (2007) with modification against the following selected human cancer cell lines, namely HT-29 (human colorectal adenocarcinoma cell line), HCT-116 (human colorectal carcinoma cell line), A549 (human lung carcinoma cell line). CaSki (human cervical carcinoma cell line) and CCD-18Co (non-cancer human colon cell line)

3.2.1.1 Cell culture and reagents

All cell lines were purchased from American Type Culture Collection (ATCC, USA). Type of basic media and supplements for each cell lines are shown in Table 3.1 and all supplements were purchased from Sigma-Aldrich, USA. All cells were grown in 25cm^3 tissue culture flasks (Corning, USA). The cell lines were incubated and maintained in incubator (Esco, Model: CCL-170B-8) under condition of humidified 5.0% CO_2 atmosphere at 37°C. When the cells achieved 80-90% confluency, they were detached by accutase (Sigma-Aldrich) and sub-cultured into new sterile culture flasks for further propagation.

Table 3.1: Basic medium and different supplements for different cancer cell lines.

Cell lines	Basic medium	Supplement
HCT-116 HT-29	McCoy's 5A (Sigma-Aldrich)	• 10.0 % fetal bovine serum • 1.0 % amphotericin B • 1.0 % penicillin/streptomycin
A549	F-12K (Gibco)	• 10.0 % fetal bovine serum • 1.0 % amphotericin B • 1.0 % penicillin/streptomycin • 220 mg/L sodium pyruvate
Ca Ski	*RPMI-1640 (Nacalai Tesque)	• 10.0 % fetal bovine serum • 1.0 % amphotericin B • 1.0 % penicillin/streptomycin • 110 mg/L sodium pyruvate
CCD-18Co	*Eagle's MEM (Sigma-Aldrich)	• 10.0 % fetal bovine serum • 1.0 % amphotericin B • 1.0 % penicillin/streptomycin • 1.0 % non-essential amino acid • 110 mg/L sodium pyruvate

*RPMI 1640 : Roswell Park Memorial Institute 1640
*Eagle's MEM: Eagle's Minimum Essential Medium

3.2.1.2 **Procedures of SRB assay**

The confluent cells were detached and cells at density of 3.0×10^4 cells/mL were seeded into 96-well plates. Adherent of cells in the wells were observed after 24 hours of incubation. The media was then discarded. Stock solution of various concentrations (ranging from 1.0 µg/mL – 100.0 µg/mL in DMSO) with the medium was then added to the wells containing cells. The final concentration of DMSO in all wells did not exceed 0.1%. The treated cells were incubated for 24, 48 and 72 hours at 37°C in incubator. The untreated well served as the negative control. With some modifications, the SRB assay was performed according to Houghton et al. (2007). After incubation period (24,

48 and 72 hours), 50.0 µl of 40.0% (v/v) ice cold trichloroacetic acid (TCA) was added to each well and kept at 4.0°C for an hour. The medium containing TCA was discarded and the plates were added with 50.0 µL of distilled water to remove the excess TCA in the wells. The washing process was repeated 4 times. Next, 50.0 µL of 0.4% (w/v) SRB dye (Sigma) was added into well and the plates were incubated at room temperature for 30 minutes. The excess SRB dye was removed and the plates were washed with 50.0 µL of 1% (v/v) acetic acid for 5 times. 100.0 µL of 10mM of Tris Base was added to the wells in the plate and the plate agitated for 500 rpm in the Thermo Shaker, BioSan, Latvia for 5 minutes to solubilise the bound SRB dye in every well. The dye absorbance (optical density, OD) was then determine using a microplate reader (Synergy H1 Hybrid, Biotek) at wavelengths 570 nm and 630 nm equipped with Gen5 Data Analysis software (Biotek). The procedure of SRB is shown in Figure 3.2.

The experiment was conducted in triplicates. For each sample, the mean data from triplicates were expressed in terms of cell inhibition percentage relative to the negative control. The percentage of cell inhibition values was obtained by calculation of absorbance of the control ($OD_{control}$) and of the samples (OD_{sample}) using equation as shown below:

$$\text{Percentage of inhibition} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\%$$

*OD = Optical density

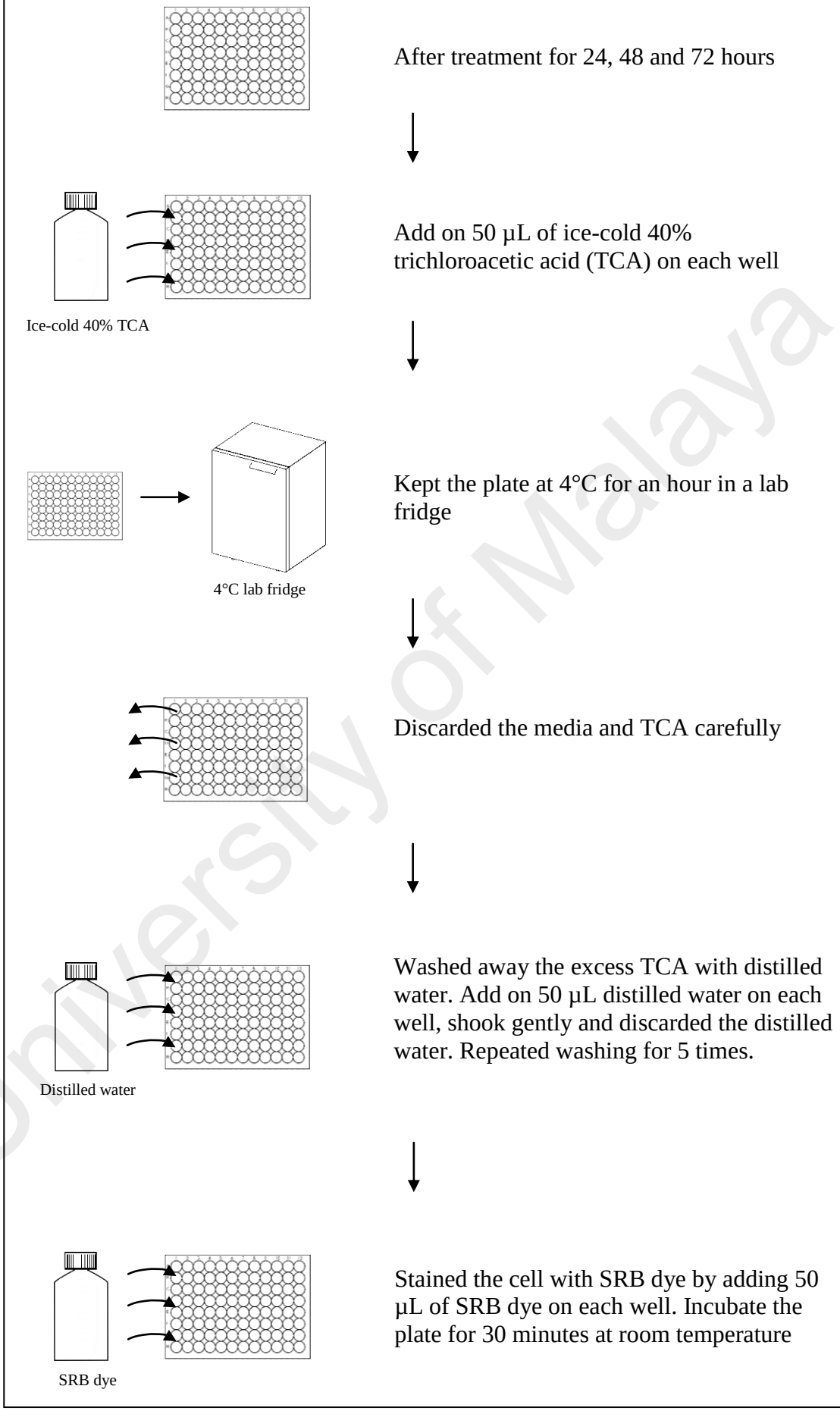
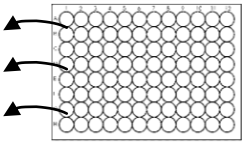
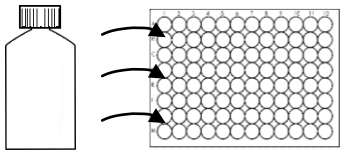


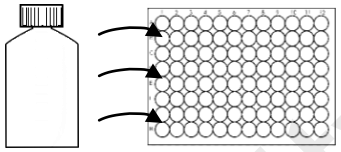
Figure 3.2: The procedure of SRB assay



Discarded the SRB dye carefully

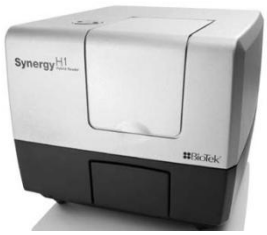


Washed away the u dye with 1% acetic acid. Add on 50 µL acetic acid on each well, shook gently and discarded the acid. Repeated washing for 5 times.



Solubilised the bound SRB dye by adding 100 µL of Tris base on each well

Shake the plate for 5-10 minutes at 500 rpm to ensure homogenous solubilisation of bound SRB dye by Tris base.



Read the absorbance in a microplate reader with working wavelength 570 nm and 630 nm.

Figure 3.2, continued

3.3 Isolation of the chemical compounds

3.3.1 High pressure liquid chromatography (HPLC)

Fractionated hexane (CMH) and ethyl acetate (CME) extracts from Method A and, fractionated hexane (CMDH) and hexane-insoluble (CMDM) extracts from Method B were initially injected into an Agilent 1260 infinity HPLC system comprising of a quaternary pump equipped with a 1260 autosampler (ALS), a 1290 thermostat, a 1260 thermostatted column compartment (TCC), a 1260 diode array detector (DAD VL+), a 1260 fraction collector (FC-AS) and Agilent OpenLAB CDS Chemstation software for analytical HPLC analysis. Binary eluent of chromatographic-grade acetonitrile (ACN) and ultrapure water were utilized in the analytical analysis under the following gradient conditions: 0 to 5 minutes of linear gradient from 30 to 40% ACN; 5 to 15 minutes of a linear gradient from 40 to 70% ACN; 15 to 30 minutes of a linear gradient from 70 to 100% ACN; 35 to 40 minutes of isocratic 100% ACN; and 40 to 45 minutes of linear gradient from 100 to 30% ACN at a flow rate of 1.0 ml/min. An analytical column, ZORBAX Eclipse XDB-C18 (4.6 × 250 mm, 5 μm) was used in the analytical HPLC analysis and the temperature was set to 30°C. The extracts were prepared to a concentration of 5 mg/mL in methanol and filtered through a 0.45 mm membrane filter (Sartorius). The sample (5.0 μL) was injected onto the column and the peaks were detected by monitoring the UV absorbance at 214 and 280 nm.

Next, the extracts were prepared at 50 mg/mL in methanol and further injected into the Agilent Semi Prep XDB-C18 column (9.4 × 250 mm, 5 μm) with sample injection volume of 100 μL at a flow rate of 5.00 mL/min. The selected peaks in the resulting chromatogram were repeatedly collected using a fraction collector and the retention times of similar fractions from each round were analyzed with software to ensure no overlap with other fractions. Similar fractions from each round were then combined, the

mobile phases were evaporated using a rotary evaporator at 40°C, and the fractions were weighed.

The isolation of pure compounds from the fractionated CMH and CME extracts were halted due to low solubility in methanol during sample preparation for analytical and semi-preparative analysis. Besides that, the chromatography profile during semi-preparative analysis was unrepeatable and certain peaks in the chromatogram disappeared after 10 to 15 rounds. The CMH and CME extracts easily caused blocking of the semi-preparative column even though the column had been well flushed/washed with a solvent mixture of 70% methanol and 30% chloroform, and ACN. Therefore, CMDH and CMDM extracts from Method B was selected for further semi-preparative analysis. Eight peaks were observed in the HPLC analysis from each CMDH and CMDM extracts, and the eluents of the peaks from each extract were collected and then pooled to give eight fractions, based on the similarity of spots on TLC. Fractions that showed a single spot on TLC were subjected to analytical HPLC analysis to determine their purity.

3.3.2 Nuclear Magnetic Resonance (NMR) and q-Time-Of-Flight (qTOF) mass spectrometry

The Bruker Advance III (Germany) 600 MHz NMR spectrometer which was used to obtain 1D NMR spectrum (such as ^{1}H, ^{13}C and DEPT) and 2D NMR spectrum (such as HSQC, HSBC, COSY and NOESY), was located at the High Impact Research (HIR) central building, University of Malaya. Deuterated chloroform (Merck) with tetramethylsilane (TMS) as internal standard was used to dissolve the pure compounds. The NMR chromatograms were analyzed with ACD/NMR Processor Academic Edition software.

The spectrometer of Bruker Impact II Ultra-High Resolution (UHR) QqTOF was used to determine the mass spectra of pure compounds. The acquisition parameters were as follow: Electrospray ionisation (ESI) as source, negative ion polarity, scanning from 50 m/z to 2,000 m/z, 180°C of dry heater and 5.0 l/min for dry gas.

3.4 Apoptotic cell morphology assessment

3.4.1 Phase contrast microscopy

Cells at a density of 3.0×10^4 cells/mL were seeded into sterile 24-well plate and incubated overnight to ensure the cells adhered on the surface of the plate. The cells were treated the with three concentrations (20.0 µg/mL, 30.0 µg/mL and 40.0 µg/mL) of the CMD extract and incubated for 24, 48 and 72 hours in a CO_2 incubator. Different morphological changes of the treated cells such as cell shrinkage, cell detachment, rounding and blebbing of membrane were observed. The image of the morphological changes of the treated cells was captured at 40X magnifications under phase contrast microscope (Zeiss Axio Vert, A1).

3.4.2 Fluorescence microscopy

The double staining method that using two types of dyes (Hoechst 33342 and propidium iodide) for observing the changes of nuclear morphology of cells using fluorescence microscopy. The procedures in cell seeding and treatments were as described in the previous experiment in Section 3.4.1 except that the cells treated only for 24 hours in the experiment. After the incubation period, both media and harvested cells were further centrifuged at 1500 rpm for five minutes. The cell pellets were washed with ice-cold phosphate buffer saline (PBS) solution and re-suspended with

PBS solution. The cell suspensions were then incubated with Hoechst 33342 in the dark at 37°C and humidified 5% CO_2 incubator for seven minutes. Then PI was added into the cell suspension and further incubated in the dark under for 15 minutes at room temperature. Ices were used to deactivate and stabilize the activity of Hoechst dye. The cell samples were loaded on a slide and the morphology of cells was examined under fluorescent microscope (Leica, Germany DM16000B). The image of nuclear morphological changes of the cells was captured at 40X magnifications. The general procedure in performing the double staining on cell using dye Hoechst 33342 and PI was illustrated in Figure 3.3.

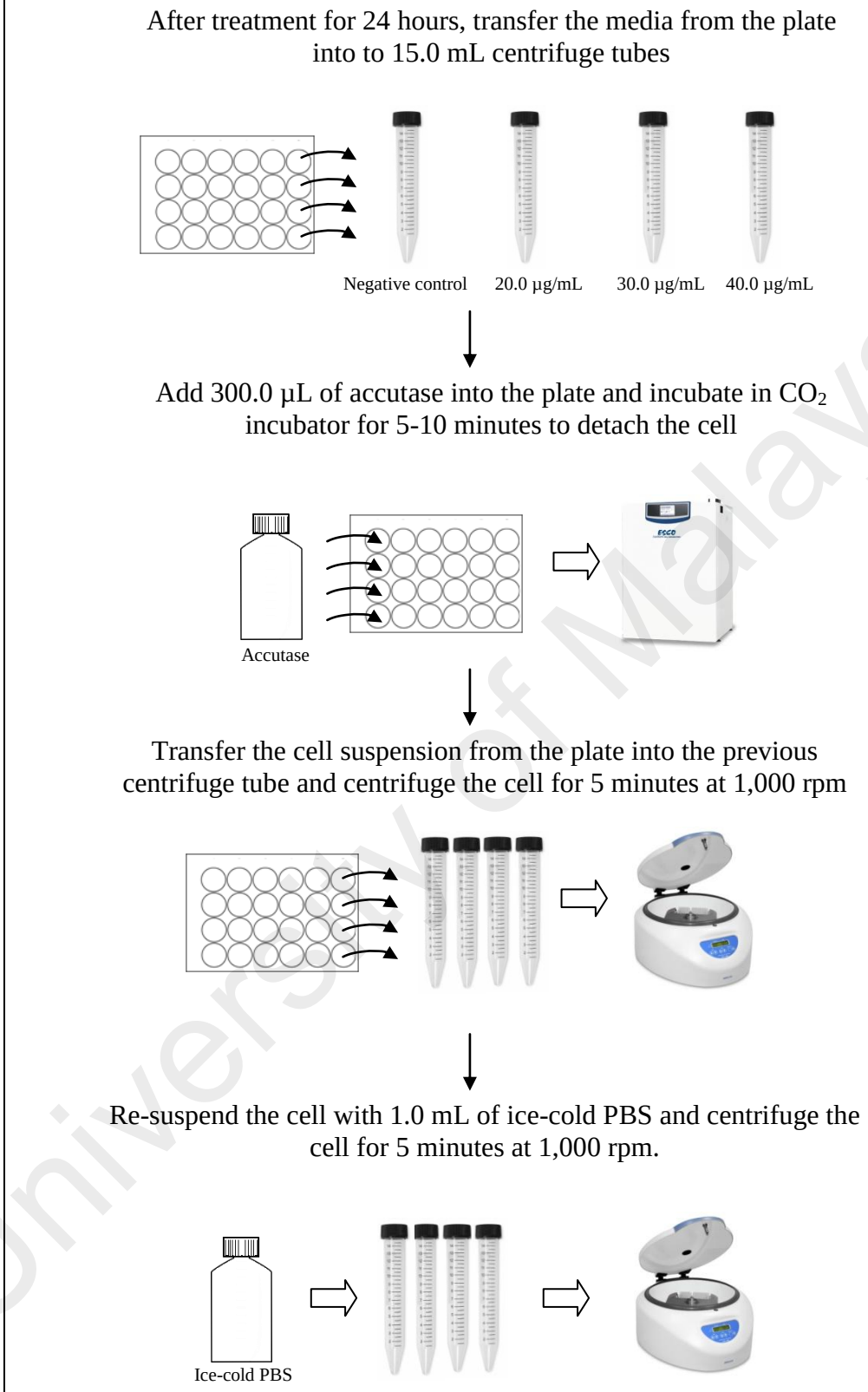
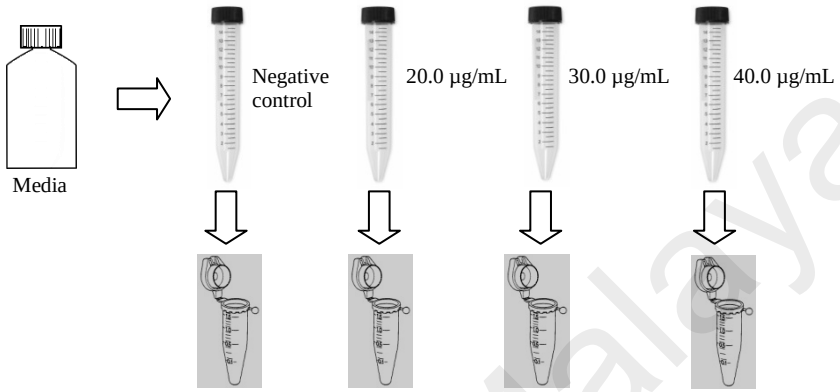


Figure 3.3: A summary of the sample preparation for Hoechst 33342 and propidium iodide double staining microscopy

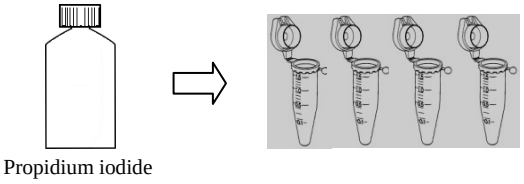
Re-suspend the cell with 43.9 µL of media and transfer the suspended cell into 1.5 mL centrifuge tube containing 4.8 µL of 100 µg/mL Hoechst 33342 dye for staining the cell in the dark



1.5 mL centrifuge tube containing 4.8 µL of Hoechst dye

Stain the cell and incubate in CO_2 incubator for 7 minutes in dark condition

Stain the cells with 1.3 µL of 100 µg/mL propidium iodide for 15 minutes under dark condition in room temperature



Put the stained cells on ice to inactivate the staining process of Hoechst dye and the stained cells are ready for observation under the fluorescence microscope

Figure 3.3, continued

3.5 Apoptosis biochemical assay

3.5.1 Detection of externalization of phosphatidylserine (PS) through binding of Annexin V to PS

Detection of early and late apoptosis by binding of Annexin V to phosphatidylserine (PS) was analyzed using a flow cytometer. Briefly, 1.2×10^5 cells/mL was plated into 12-well plate and treated with 3 different concentrations (20.0, 30.0 and 40.0 µg/mL) for 24, 48 and 72 hours in triplicates. The cells were harvested into 15.0 mL centrifuge tubes and washed with ice cold PBS. Cell pellet was re-suspended with Annexin-V binding buffer and transferred into 1.5 mL centrifuge tube. For negative and positive control, 400 µL of binding buffer was used to re-suspend cell pellet from each control and each control would divide into another four tubes by transferring 100 µL cell suspension into new tubes. Suspended cells were stained with Annexin-V for 15 minutes by adding 10 µL of Annexin V. After 15 minutes, the cells were centrifuged and re-suspended with 500 µL of binding buffer. Propidium iodide was added into the cell suspension prior analyzed with flow cytometer. Detection of apoptosis was performed using Accuri C6 flow cytometer. For each measurement, 10, 000 events were counted where cell population was distributed into different quadrants and each quadrant was analysed with quadrant statistics. Lower left quadrant represented viable cells; lower right quadrant represented early apoptotic cell; and upper right quadrant represented late apoptotic or necrotic cells.

3.5.2 Detection of DNA fragmentation

Detection of DNA fragmentation was performed using the APO-BrDU TUNEL Assay Kit (Invitrogen). HT-29 cells at a density of 5×10^5 cells/mL were grown on 6-wells plate overnight, and treated with three different concentrations for 24, 48 and 72

hours at 37°C and 5% CO_2. The untreated cells were grown as a negative control. The cells were harvested, washed with PBS and fixed with 1% (w/v) paraformaldehyde. The cells were centrifuged, washed and fixed with ice-cold 70% ethanol. Labelling of DNA was performed based on the instructions given by the manufacture, and then the percentage of DNA fragmentation in cells were analysed by flow cytometer (Accuri C6).

3.5.3 Cell cycle analysis

The cell cycle analysis was performed according to Ho et al. (2013) with slight modifications. Briefly, HT-29 cells were plated at cell number of 3.0×10^5 cells/mL and treated with crude CMD extract for 24 hours in triplicates. The cells were harvested, washed and then fixed with 70% ethanol. The cell fixation took overnight time in -20°C freezer. The next day, the fixed cells were centrifuged to become pellet, washed with ice-cold PBS and re-suspended with staining buffer containing 50 mg/mL of propidium iodide, 0.1% Triton-X-100, 0.1% sodium citrate, and 100 mg/mL of RNase. The cell suspension was incubated in the dark condition for 30 minutes at room temperature. The stained cell was then analyzed with Accuri C6 flow cytometer.

3.5.4 Western blot analysis

3.5.4.1 Antibodies and chemicals

The usage of different type of antibodies in the Western blot included the primary antibodies for DR5, cleaved PARP-1 (cleaved p25), and antibodies against polyclonal XIAP and polyclonal cIAP2 were obtained from GeneTex whereas antibodies in counter with monoclonal Bax, monoclonal p21, monoclonal p53 and β-actin were obtained from Thermo Scientific. Antibodies against polyclonal caspase-3, monoplonal

caspase-8, polyclonal caspase-9, monoclonal CDK-4, monoclonal cyclin D1 and polyclonal p27 were obtained from Cell Signalling. The horseradish peroxidase (HRP)-labelled anti-mouse and anti-rabbit secondary antibodies were obtained from Thermo Scientific. Radioimmunoprecipitation assay (RIPA) buffer and stripping buffer were purchased from Thermo Scientific. Bovine serum albumin (BSA) premix sets and Bradford reagent were bought from Bio-rad.

3.4.4.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) procedures

In order to examine the changes in expression levels changes of the apoptotic proteins, the HT-29 cells (2.0×10^6 cells/mL) were plated in a $25cm^3$ tissue culture flask and treated with crude CMD extract (40 µg/mL) for different time intervals (0, 3, 6, 12, 18 and 24 hours) in an incubator with 5% of CO_2 at temperature of 37°C. The treated cells were washed with ice cold 1X PBS and harvested using cell scraper. The harvested cells were lysed with 200µL of RIPA buffer containing 25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40 (Tergitol-type NP-40), 1% sodium deoxycholate and 0.1% SDS (Sodium Dodecyl Sulphate) for 30 minutes on ice. The lysates were then centrifuged at 14,000 rpm for 30 minutes at 4°C and the supernatants were collected. Protein concentrations in the supernatant were determined using Bradford method (Bio-rad Laboratories). Briefly, a standard curve with absorbance reading against protein concentration (mg/mL) was plotted by using seven different concentrations (2.0, 1.5, 1.0, 0.75, 0.50, 0.25, 0.125mg/mL) of pre-mix BSA solution. The protein concentration of the samples was obtained by determine the x-intercept (protein concentration) value from the standard curve.

For protein denaturation, the protein samples were boiled at 100°C for five minutes. Before the protein denaturation, the proteins were mixed with denaturation buffer (1M Tris-HCl, pH 6.8, 25% SDS, β-mercaptoethanol, 100% glycerol, 1% bromophenol blue) at the ratio of 3 parts of proteins to 1 part of denaturation buffer. Equal amount of proteins (50 µg) was loaded onto the 12% SDS-PAGE gel for gel electrophoresis and 20 ampere currents at maximum (When the usage of current exceeds 20 amperes, the SDS gel tend to melt and affect the protein separation) was applied to a single gel electrophoresis (e.g. 40 amperes for two gels electrophoresis). A pre-stained protein ladder (Thermo Scientific) was loaded onto the gel as well to identify the approximate size of the protein running in the gel during the electrophoresis. The proteins in the gel were then electroblotted onto a nitrocellulose membrane (Merck) using wet transfer method for 2 hours by applying 90V current supply. The nitrocellulose membranes were then blocked with Blocking One (Nacalai Tesque, Inc) for one hour. The membranes were then probed with a specific primary antibody (1: 1,000 dilutions in blocking buffer) overnight at 4°C by rocking the membrane with a rocker. Next, the unbound antibody in the membranes were removed by washing with PBS containing 1X of Tween-20 (PBST. Then, the membranes were incubated by HRP-conjugated secondary antibody with the dilutions of 1: 10,000 in blocking buffer on the rocker at room temperature for one hour. After incubation with the secondary antibody, the membranes were again washed with PBST for 3 times to remove unbound secondary antibody. The visualization of the protein bands was performed using enhanced chemiluminescence (WesternBright ECL, Advansta) and the pictures of the visualized protein bands were taken on Fusion-FX7 gel imaging (Vilber, Germany) with its build-in Fusion-CAPT software. The membranes were then rocked vigorously with stripping buffer for 15 minutes to remove all the bound substances on the membrane. The stripped membranes were washed, blocked with blocking buffer and incubated with

another specific primary antibody if necessary. The concentration of the cell lysate as well as subcellular constituents were determined by using the β-actin. Software ImageJ was performed to quantity the densitometric values of the bands and the results were normalized with the loading control sample (β-actin). The normalized results were then expressed in fold change comparative to control.

3.6 Statistical analysis

All results were expressed as mean ±SD and all the experiments were performed at least twice using sample triplicate. All raw data were statistically processed with IBM SPSS Statistics 22.0 software. T-test was performed to determine the significant differences between the treated cell and non-treated cell. $P < 0.05$ was considered statistically significant.

CHAPTER 4: RESULTS

4.1 Extraction of plant materials

Two different extraction methods were performed on *C. mangga* rhizomes as described in Section 3.1.2a and 3.1.2b. In Method A, the initial extracting solvent (Section 3.1.2a) was methanol followed by partitioning with n-hexane, ethyl acetate and water consecutively on Method B (Section 3.1.2b) the initial extracting solvent was dichloromethane, followed by fractionation with n-hexane and methanol.

The yield of crude methanolic extract (25.0% based on 1.0 kg powdered sample) from Method A was three times higher that of the crude dichloromethane extract from Method B (7.50 %) (Table 4.1). The percentage of fractionated extracts was calculated according to the weight of crude extracts. The initial fractioning solvent on the crude extracts for both methods were similar (n-hexane) but the percentage yield based on crude extracts were different. For Method A, the percentage yield of fractionated n-hexane extract (CMH) based on crude methanolic extract (CMM) was 33.6%. In contrast, the percentage yield of fractionated n-hexane extract (CMDH) based on crude dichloromethane extract (CMD) from Method B was 60.53% which was almost twice amount that the percentage yield of CMH.

Table 4.1: Yield of crude and fractioned extracts of *C. mangga* rhizome from Method A and Method B

Method	Extracts	Yield (g)	Percentage (%)
Method A	CMM	250.4	25.0
	CMH	84.1	33.6
	CME	70.9	28.3
	CMW	17.5	7.0
Method B	CMD	75.0g	7.50
	CMDH	45.4	60.53
	CMDM	28.3	37.73

4.2 Cytotoxicity assay of plant extracts and fractions

The cytotoxic activity of the extracts on the various human cancer cell lines (HT-29, HCT 116, A549 and CaSki) and normal colon cell line (CCD-18co) was investigated. The cells were treated with several concentrations of plant extracts/fractions and subjected to SRB assay. This assay estimates cytotoxicity based on the total protein content, which is assumed to be proportional to the number of cells (Houghton et al., 2007). Cytotoxicity of a test sample which is expressed as IC_{50} and can be obtained by evaluating from the graph of percentage inhibition against concentration (Figure 4.1). IC_{50} is the concentration of the sample which can cause 50% cell death when the cells are exposed to the test sample over a period of time. The IC_{50} values in µg/mL of all crude and fractionated extracts from Method A and B against various cell lines are summarized in Table 4.2 and Table 4.3, respectively.

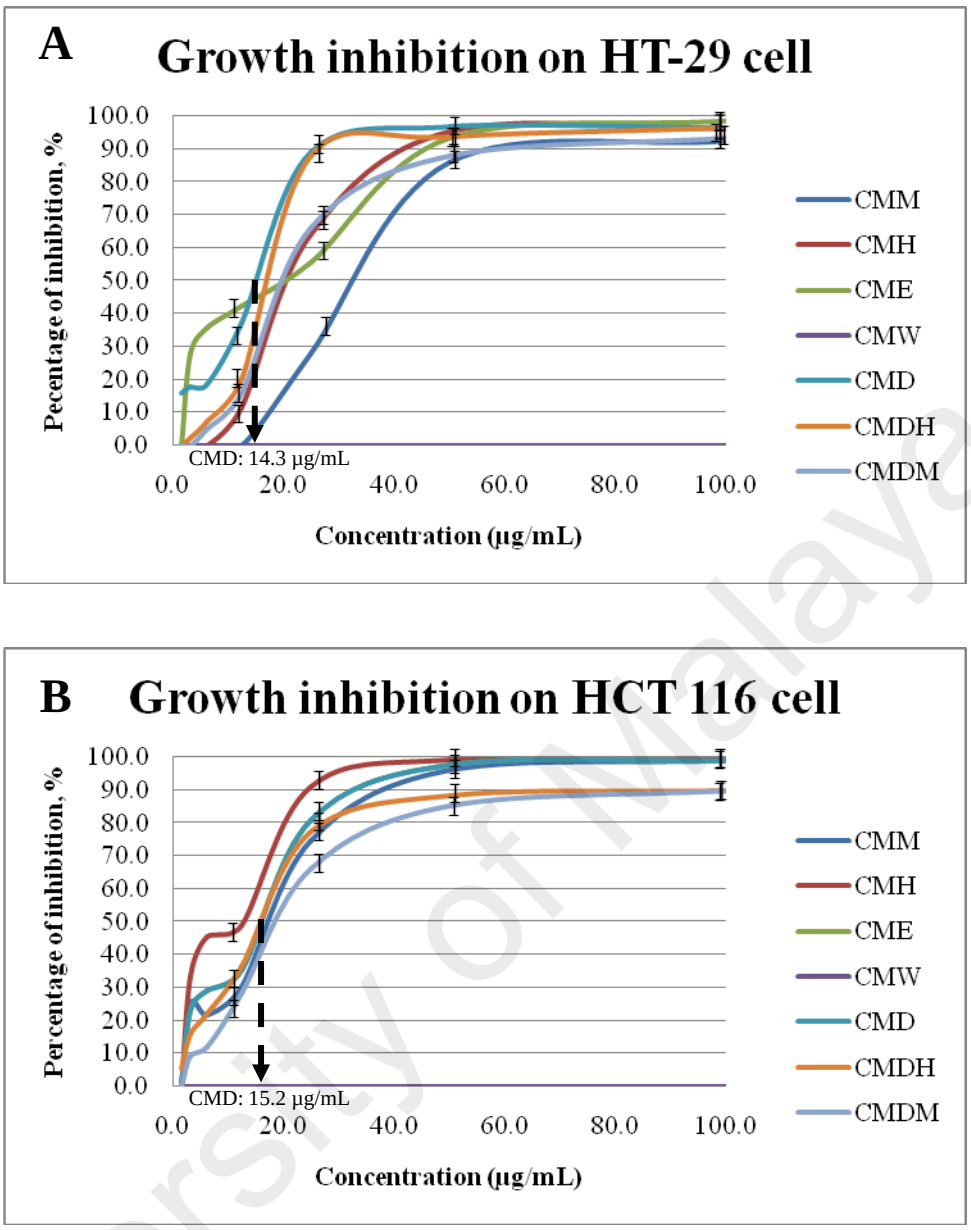
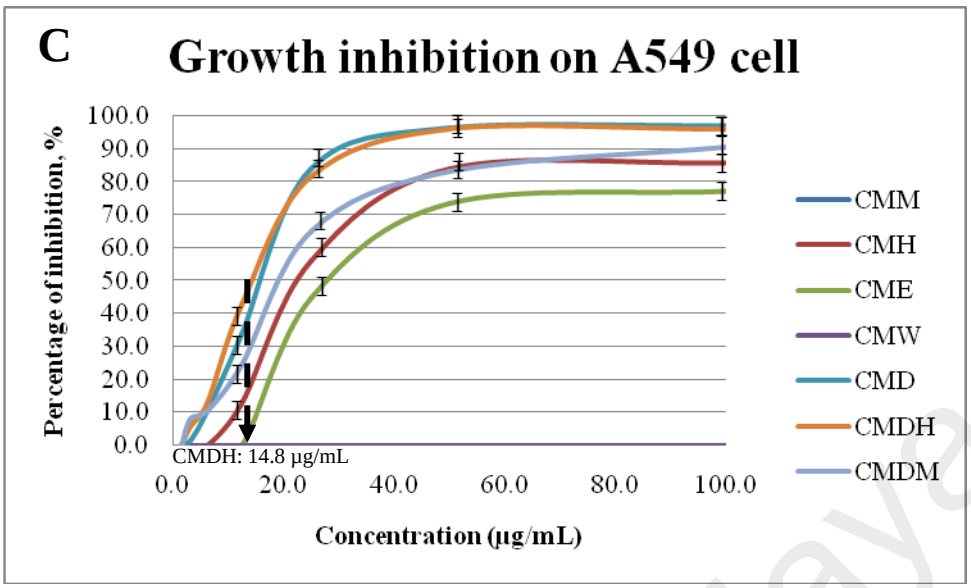


Figure 4.1: Percentage of growth inhibition on various cancer cell lines (A: HT-29; B: HCT 116; C: A549; D: CaSki) by all extracts from Method A (CMM, CMH, CME and CMW) and Method B (CMD, CMDH and CMDM). Three replicates were conducted in each sample.



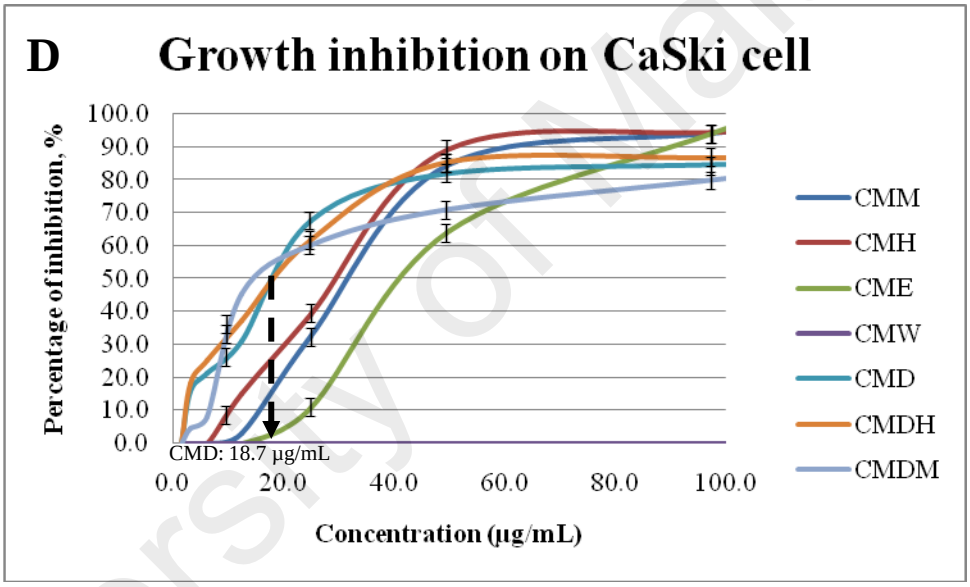


Figure 4.1, continued

Table 4.2: Cytotoxic activities (IC_{50} values) of extracts from Method A on various cancer cell lines and human normal cell (CCD-18Co) in comparison to doxorubicin

Cell lines	Time (hours)	IC_{50} values (µg/mL) of extract/fraction from Method A				Doxorubicin (µg/mL)
		CMM	CMH	CME	CMW	
HT-29	24	42.1 ± 1.3	39.3 ± 2.4	32.2 ± 5.1	>100	0.36 ± 0.4
	48	32.6 ± 0.6	17.9 ± 1.2	32.1 ± 5.4		
	72	34.4 ± 1.1	21.7 ± 0.8	15.6 ± 4.4		
HCT-116	24	29.9 ± 1.3	15.8 ± 1.7	13.4 ± 0.8	>100	0.28 ± 0.1
	48	28.3 ± 0.9	13.1 ± 0.8	17.7 ± 0.7		
	72	18.1 ± 0.7	16.1 ± 0.2	16.6 ± 0.5		
A549	24	>100	30.8 ± 4.3	66.7 ± 3.1	>100	0.63 ± 0.1
	48		33.3 ± 2.5	45.7 ± 2.5		
	72		23.2 ± 1.1	31.7 ± 2.3		
CaSki	24	58.1 ± 3.1	57.9 ± 4.4	65.3 ± 3.7	>100	0.23 ± 0.2
	48	57.8 ± 2.2	55.1 ± 1.1	61.1 ± 3.2		
	72	33.2 ± 2.4	31.9 ± 0.4	41.9 ± 1.4		
CCD-18Co	72	45.8 ± 1.3	22.5 ± 0.9	22.8 ± 0.9	>100	0.11 ± 0.1

The IC_{50} value shows 50% reduction in cell viability caused by several concentrations of extracts according to SRB assay. Doxorubicin was used as positive control. Mean ± standard deviation of three replicates from three independent experiments is expressed in each value.

Table 4.3: Cytotoxic activities (IC_{50} values) of extracts from Method B on various cancer cell lines and human normal cell (CCD-18Co) in comparison to doxorubicin

Cell lines	Time (hours)	IC_{50} values (µg/mL) of extract/fraction from Method B			Doxorubicin (µg/mL)
		CMD	CMDH	CMDM	
HT-29	24	47.0 ± 1.4	48.0 ± 1.8	49.0 ± 0.8	0.36 ± 0.4
	48	14.2 ± 1.0	14.0 ± 0.7	18.0 ± 0.5	
	72	14.3 ± 0.2	16.3 ± 0.5	21.0 ± 0.4	
HCT-116	24	20.9 ± 1.3	22.7 ± 0.7	22.6 ± 1.7	0.28 ± 0.1
	48	17.7 ± 0.4	18.1 ± 0.6	15.7 ± 0.5	
	72	15.2 ± 0.8	17.8 ± 0.7	18.3 ± 0.8	
A549	24	18.6 ± 0.8	16.0 ± 1.7	19.4 ± 0.7	0.63 ± 0.1
	48	18.6 ± 0.8	16.0 ± 1.7	19.0 ± 1.2	
	72	16.5 ± 0.4	14.8 ± 0.6	20.0 ± 0.7	
CaSki	24	33.4 ± 0.9	35.6 ± 0.5	42.8 ± 0.8	0.23 ± 0.2
	48	23.5 ± 0.5	23.7 ± 0.7	35.1 ± 0.7	
	72	18.7 ± 0.7	19.5 ± 0.3	21.2 ± 0.4	
CCD-18Co	72	50.3 ± 0.5	52.4 ± 1.1	55.0 ± 2.4	0.11 ± 0.1

The IC_{50} value shows 50% reduction in cell viability caused by several concentrations of extracts according to SRB assay. Doxorubicin was used as positive control. Mean ± standard deviation of three replicates from three independent experiments is expressed in each value.

4.3 Isolation and identification of chemical compounds

Fractionated extracts from both methods were subjected to HPLC for isolation of pure compounds. However, the fractionated extracts from Method A was halted from further isolation chemical compounds because both CMH and CME extracts caused clogging in the column after 10 to 15 rounds. After the column was washed and back-flushed, the chemical profile of the extracts was found to be inconsistent and the retention of the peaks in the chromatogram shifted. Meanwhile, CMDH and CMDM extracts did not display the same problem as found in CMH and CME extract during the semi-preparative isolation. Therefore, only CMDH and CMDM were further investigated.

4.3.1 CMDH and CMDM from Method B

The active hexane-soluble fraction (CMDH) was subjected to HPLC, as described in Chapter 3 (Section 3.3.1). After repeated semi-preparative analysis, eight compounds were isolated. Only four compounds were successfully identified using NMR and qTOF mass spectrometry and the mass spectral and NMR data were found to be consistent with those reported in the literatures (Itokawa et al., 1988; Nakamura et al., 2008; Xu et al., 1995; Xu et al., 2015). These compounds were longpene A (peak corresponding to H1), zerumin A (peak corresponding to H2), coronadiene (peak corresponding to H3) and (*E*)-labda-8(17),12-dien-15,16-dial (peak corresponding to H7). Meanwhile, the active hexane-insoluble fraction (CMDM) was also subjected to HPLC and 8eight compounds were isolated after repeated semi-preparative analysis. Only one compound was identified as calcaratarin A (peak corresponding to M8) which was consistent with published data (Kong et al., 2000). The HPLC profile of CMDH and CMDM is shown in Figure 4.2, and the structures of the isolated compounds are illustrated in Figure 4.3. Longpene A is a rare type of norditerpene with an *ent*-labdane skeleton (Xu et al., 2015). Zerumin A, (*E*)-labda-8(17),12-dien-15,16-dial and calcaratarin A are labdane diterpenes and only differ by functional groups attached on the basic skeleton structure. Coronadiene is a labdane-type trinorditerpene. All isolated compounds has been reported previously in *C. mangga* except longpene A. Although longpene A was first reported by Xu et al. (2015), it has never been reported to exist in *C. mangga*. The 1H and 13C NMR data for longpene A is shown in Table 4.4 and 4.5 respectively. The yield of compounds collected from semi-preparative analysis is shown in Table 4.6.

A

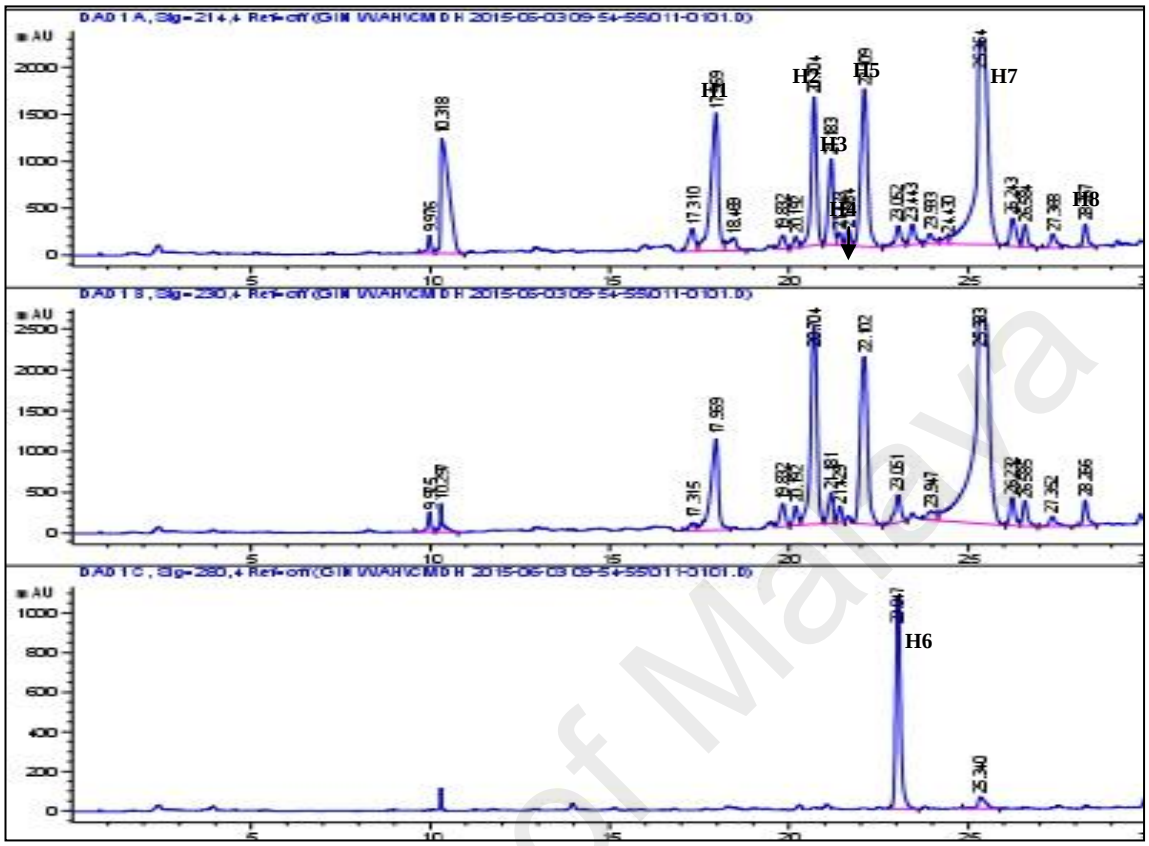


Figure 4.2: HPLC profiles of CMDH and CMDM. (A) Eight peaks were detected in HPLC profile of CMDH. Seven peaks (H1-H5, H7, and H8) were detected in wavelength 214 nm while one peak (H6) was detected in wavelength 280 nm. Only H1, H2, H3 and H7 were identified. (B) Eight peaks were also detected in HPLC profile of CMDM. Seven peaks (M1-M4, M6, M7 and M8) were detected under wavelength 214 nm while one peak (M6) was detected in wavelength 280 nm. Only one compound (M8) was identified.

B

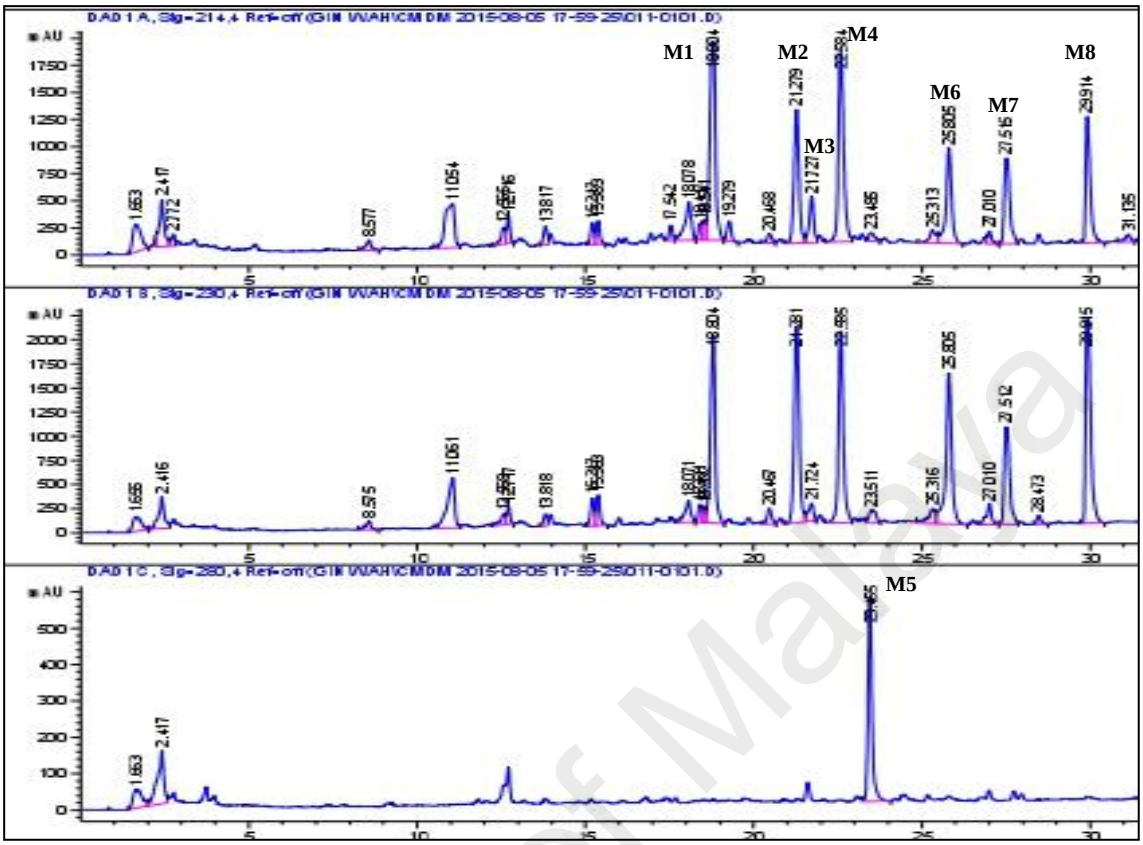
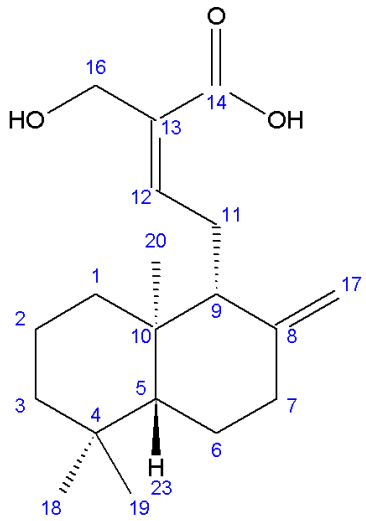
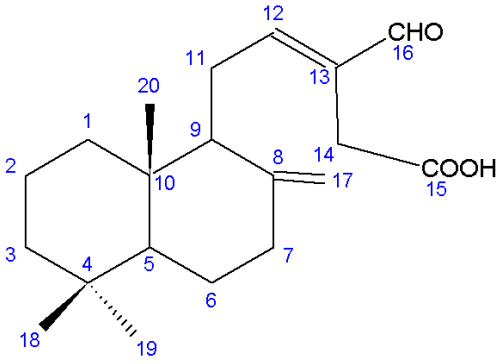


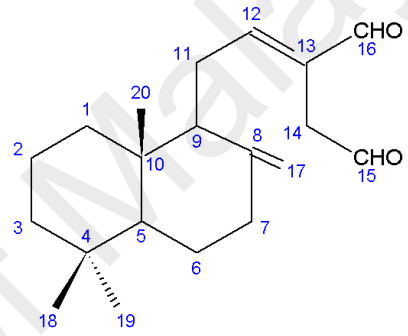
Figure 4.2, continued

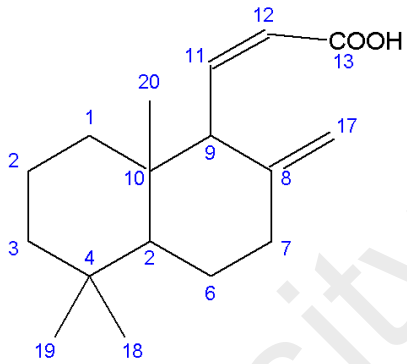




Zerumin A

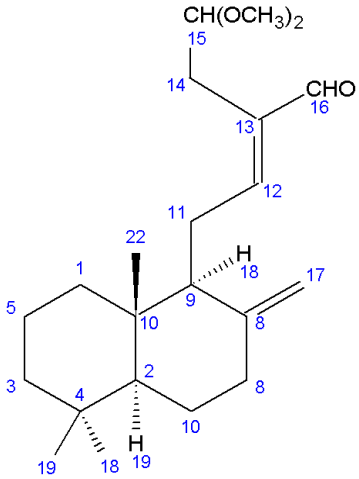
Longpene A





(E)-labda-8(17),12-dien-15,16-dial

Coronadiene



Calcaratarin A

Figure 4.3: The chemical structure of isolated compounds were elucidated and identified as following compounds: Longpene A (Xu et al., 2015); Zerumin A (Xu et al., 1995); H3: Coronadiene (Nakamura et al., 2008); (E)-labda-8(17),12-dien-15,16-dial (Itokawa et al., 1988); and Calcaratarin A (Kong et al., 2000).

Table 4.4: ^{1}H NMR data of longpene A in $CDCl_3$

Position	Type of Proton	Chemical Shift, δ (Multiplicity, *J* in Hz)	
		Longpene A (600 MHz)	**Literature review* (400 MHz)**
12	C=CH	6.97 t (6.9)	6.97 t (6.7)
16	CH2–OH	4.38 s	4.39 s
17	$C=CH_a$	a 4.42 s	a 4.42 s
	$C=CH_b$	b 4.84 s	b 4.84 s
18	CH_3	0.89 s	0.89 s
19	CH_3	0.82 s	0.83 s
20	CH_3	0.74 s	0.74 s

*(Xu et al., 2015)

Table 4.5: ^{13}C NMR data of longpene A in $CDCl_3$

Position	Type of carbon	Chemical Shift, δ in ppm	
		Longpene A (150 MHz)	**Literature review* (100 MHz)**
1	CH_2	39.3	39.2
2	CH_2	19.3	19.3
3	CH_2	42.0	42.0
4	Cq	33.6	33.6
5	CH	55.4	55.4
6	CH_2	24.1	24.1
7	CH_2	37.9	37.9
8	Cq	148.1	148.0
9	CH	56.7	56.7
10	Cq	39.3	39.6
11	CH_2	23.9	23.8
12	CH	150.2	150.1
13	Cq	129.5	129.5
14	C=O	172.4	172.2
16	CH_2	57.3	57.2
17	CH_2	107.9	107.9
18	CH_3	33.6	33.6
19	CH_3	21.8	21.7
20	CH_3	14.5	14.4

*(Xu et al., 2015)

Table 4.6: Yield of compounds after 100 runs of collection from semi-preparative analysis

Compounds/fractions	Weight of yield (mg)
Longpene A	14.2
Zerumin A	18.2
Coronadiene	8.6
H4	4.8
H5	18.2
H6	9.2
(*E*)-labda-8(17),12-dien-15,16-dial	52.3
H8	6.0
M1	15.9
M2	14.1
M3	2.9
M4	23.0
M5	6.3
M6	10.2
M7	11.4
Calcaratarin A	28.1

4.4 Cytotoxicity assay of isolated pure compounds

All compounds (including identified and unidentified compounds) were tested for cytotoxicity screening against the same cell lines that were tested for the crude extracts. The cytotoxic effects of the compounds on the selected cell lines are expressed as IC_{50} values are shown in Table 4.7.

Table 4.7: Cytotoxic activities of identified compounds on various cancer cell lines and human normal cell (CCD-18Co) and doxorubicin after 72 hours of incubation

Compounds	IC_{50} in µM				
	HCT-116	HT-29	A549	CaSki	CCD-18Co
Longpene A	>100	>100	>100	>100	>100
Zerumin A	76.7 ± 3.8	61.9 ± 1.6	58.5 ± 2.2	>100	>100
Coronadiene	87.1 ± 3.1	86.2 ± 5.7	>100	>100	>100
(*E*)-labda-8(17),12-dien-15,16-dial	39.0 ± 0.7	63.2 ± 1.0	>100	72.1 ± 5.6	60.1 ± 4.2
Calcaratarin A	>100	>100	>100	>100	>100
Doxorubicin	0.7 ± 0.7	0.5 ± 0.2	1.2 ± 0.2	0.4 ± 0.4	0.2 ± 0.2

The IC_{50} value shows 50% reduction in cell viability caused by several concentrations of extracts according to SRB assay. Doxorubicin was used as positive control. Mean ± standard deviation of three replicates from three independent experiments is expressed in each value.

4.5 CMD extract induced morphological changes in HT-29 cells

Cell undergoing apoptosis showed several morphological characteristics which included cell shrinkage, cell blebbing and others. These can be observed under the phase contrast microscope. CMD has the highest cytotoxicity effect on HT-29 cells, so HT-29 cells were selected for this experiment. After treatment with CMD extract with various concentrations (20, 30 and 40 µg/mL) for 24, 48 and 72 hours, it was observed there were fewer cells and less interaction between the remaining cells. Other apoptotic morphological changes of the treated cells included cytoplasm vacuolization, membrane blebbing, cell shrinkage, nuclear disintegration and formation of apoptotic bodies. Cell numbers were gradually decreased and the apoptotic morphological changes were observed more frequently as the cell were treated with higher concentration as well as longer incubation time with CMD extract. The observed morphological changes in treated cells under phase contrast microscopy are shown in Figure 4.4.

The examination of morphological alteration of nuclear was performed with double staining with Hoechst 33342 and propidium iodide (PI). Dull blue colour was observed in untreated cell was indicated viable and healthy cell. In contrast, the vibrant blue and pink fluorescence with condensed/fragmented nuclei were observed in a population of the treated cells after 24 hours of treatment. The bright blue with fragmented and condensed nuclei indicated cells undergoing early apoptosis while cells with pink fluorescence undergo late apoptosis. As the concentration of plant extract increased on the treated cells, more bright blue and pink fluorescence cells were observed. Some necrosis-like cell death (red colour) was observed in treated cells after 24 hours treatment. The observed morphological changes in treated cells with fluorescence microscopic are shown in Figure 4.5. This technique provides a method to distinguish between early and late apoptosis, and necrosis of treated cells the morphological changes observed from the phase contrast microscopy and fluorescence microscopy

might suggest that the cytotoxic effect of CMD extract on HT-29 cells was related to the induction of apoptosis. Further tests were done to validate the obtained results.

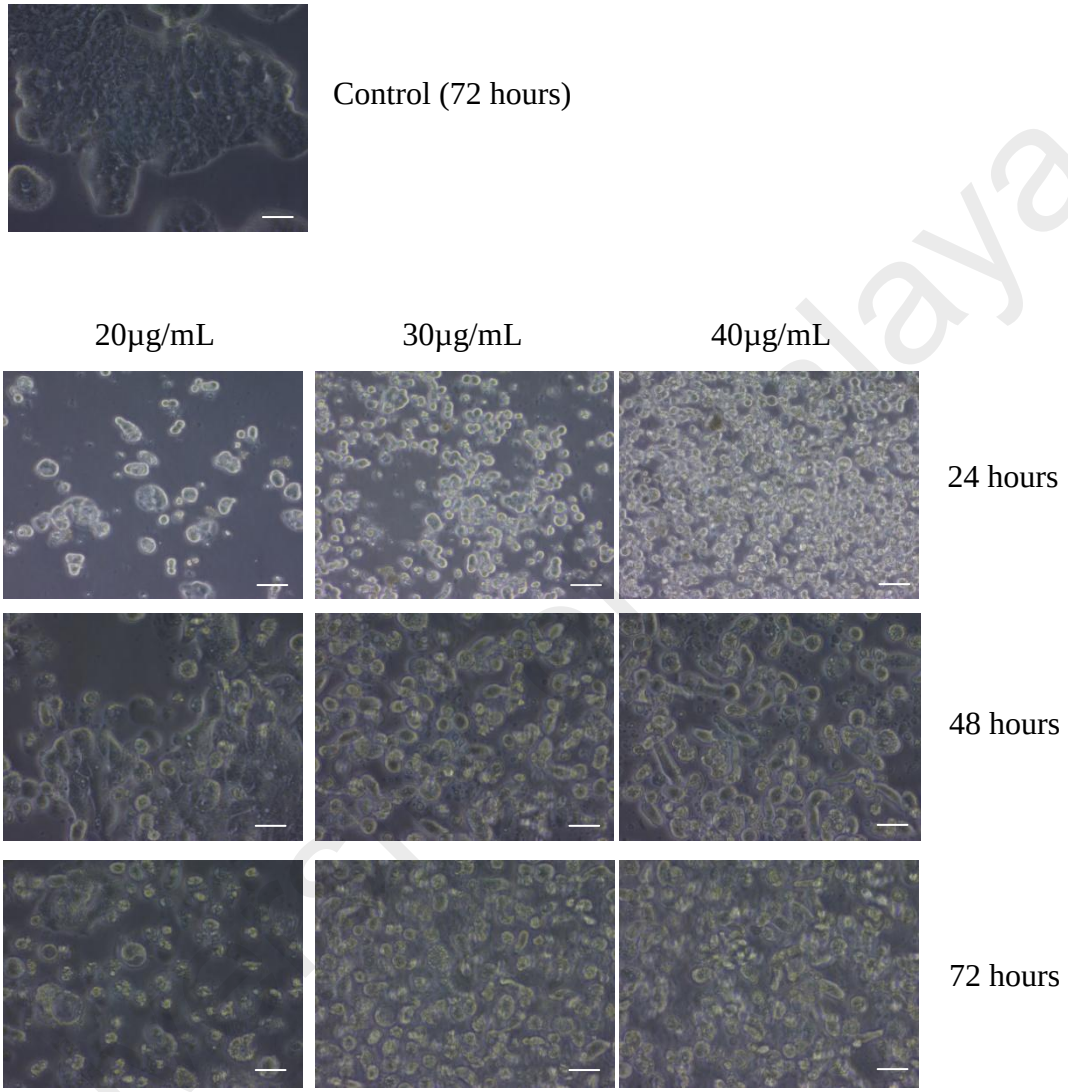


Figure 4.4: Morphological changes in phase contrast microscopy. HT-29 cells were treated with CMD extract at the specified concentrations for 24, 48 and 72 hours, and then observed under an inverted phase contrast microscope at magnification of 40× with setting of scale bar equal to 1µm. The control cells are evenly spread with a normal structure of nuclei. The characteristics of apoptotic morphology were observed in treated cells (cell size reduction, development of vacuoles, fragmented nuclei and detachment of apoptotic bodies. Untreated cells in 0.5% DMSO used as the control. Three replicates were conducted in each sample.

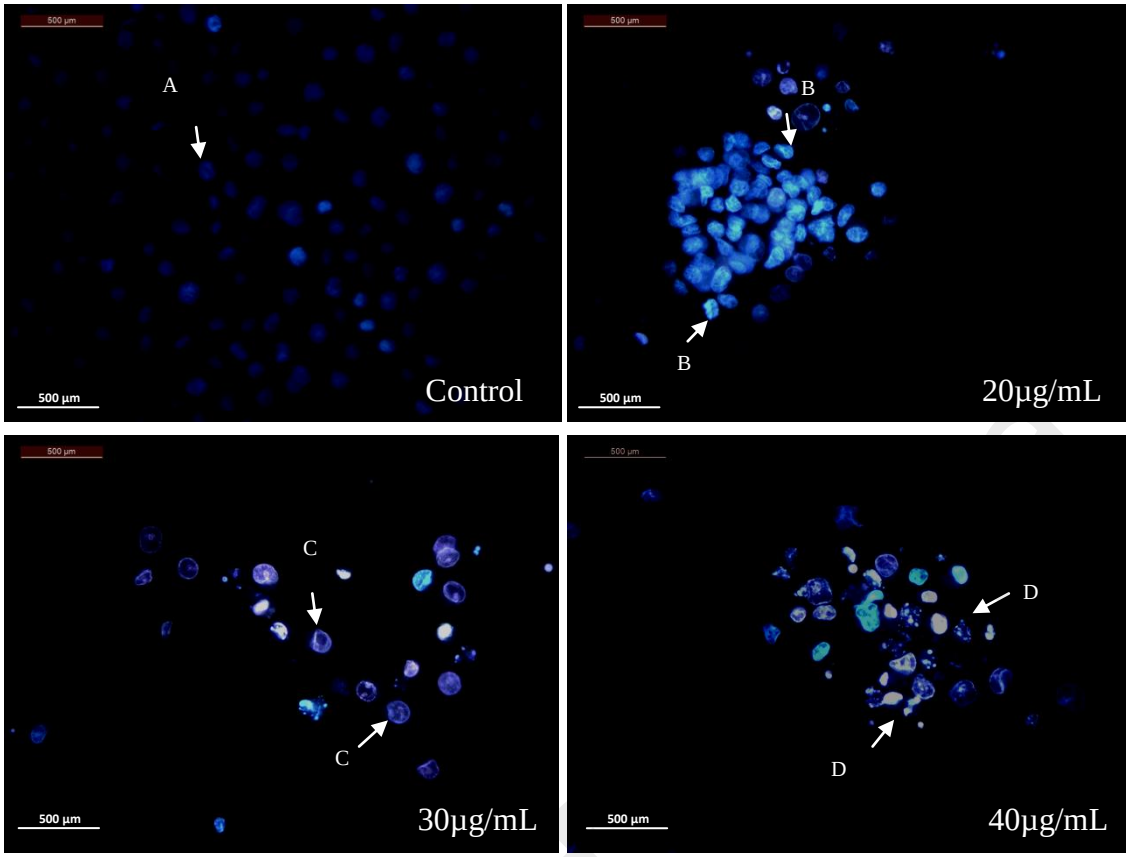


Figure 4.5: Nuclear morphological changes under fluorescence microscopy. HT-29 cells under treatment of CMD extract at the specified concentrations for 24 hours. Next, the treated were observed under fluorescence microscope at magnification of 40× with setting of scale bar equal to 1µm. Arrows labelled with the following alphabets shows: (A) normal nuclei and viable cell; (B) Condensed/fragmented chromatin of early apoptotic cells; (C) highly condensed/fragmented chromatin of late apoptotic cells; (D) dead cells/necrosis (pink). Untreated cells in 0.5% DMSO used as the control.

4.6 Effects of CMD on induction of early and late apoptosis in HT-29 cell lines

The biochemical hallmarks of cancer include DNA fragmentation, externalization of phosphatidylserine and cleavage of intracellular substrates (Ouyang et al., 2012a; Saraste & Pulkki, 2000). In viable cells, phosphatidylserine is present in the inner leaflet of the plasma membrane. However, during apoptosis, phosphatidylserine translocates to the outer leaflet of the plasma membrane and binds to the annexin receptor of the macrophages to initiate phagocytosis (Weinberg, 2013). The cell population of HT-29 in the early and late stage of apoptosis increased significantly ($p<0.05$) as the

concentration and exposure time to crude CMD extract were increased. Figure 4.6 shows the detection of early and late apoptosis induced by crude CMD extract. After 48 hours treatment, the population of early apoptotic cells (Annexin-V single positive cells) were increased from 6.4% ± 0.30, 12.4% ± 0.70, and 16.2% ± 0.20 at 20, 30 and 40 µg/mL respectively, while the population of late apoptotic cell (Annexin-V/PI double positive cells) increased from 10.3% ± 0.20, 24.3% ± 0.40 and 42.7% ± 0.40 at 20, 30 and 40 µg/mL respectively. The percentage of late apoptotic cells was significantly increased and greater than the percentage of early apoptotic cells in concentration of 30 and 40 µg/mL.

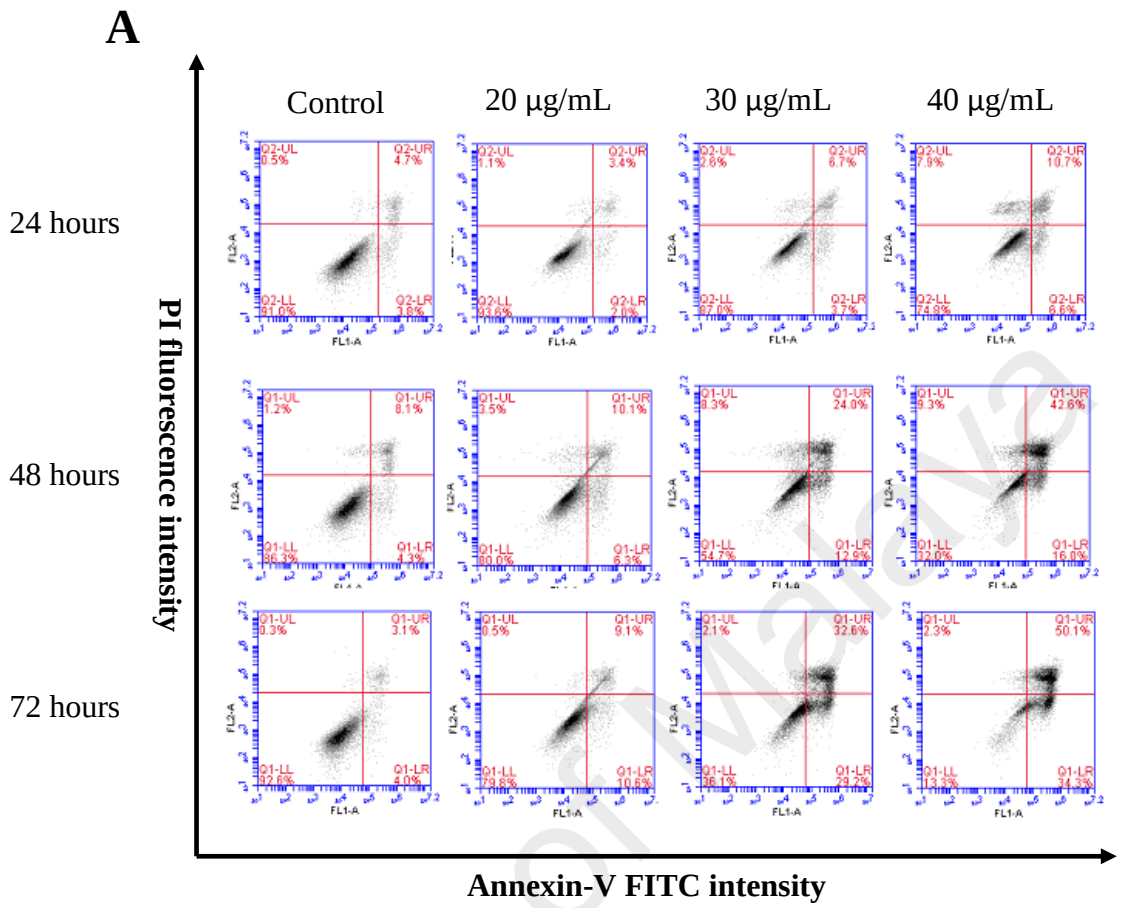


Figure 4.6: CMD induced externalization of PS in HT-29. (A)A density plot of HT-29 cells treated with increasing concentrations of CMD for 24, 48, and 72 hours were analysed using flow cytometric after the staining with AnnexinV-FITC and PI. The separation of different type of cell population are according to the four quadrants in a dot plot which are bottom left (viable cell), bottom right (early apoptotic cells), top right (late apoptotic cells) and top left (necrotic cells). (B)The events of early and late apoptotic of HT-29 cells with total 10,000 cells were quantified as percentage in bar chart. Values given are expressed as mean ± SD of triplicates obtained from three independent experiments. Significant of statistical differences with comparison of the control ($p<0.05$) are expressed in the asterisk (*). Untreated cells in 0.5% DMSO used as the control.

B

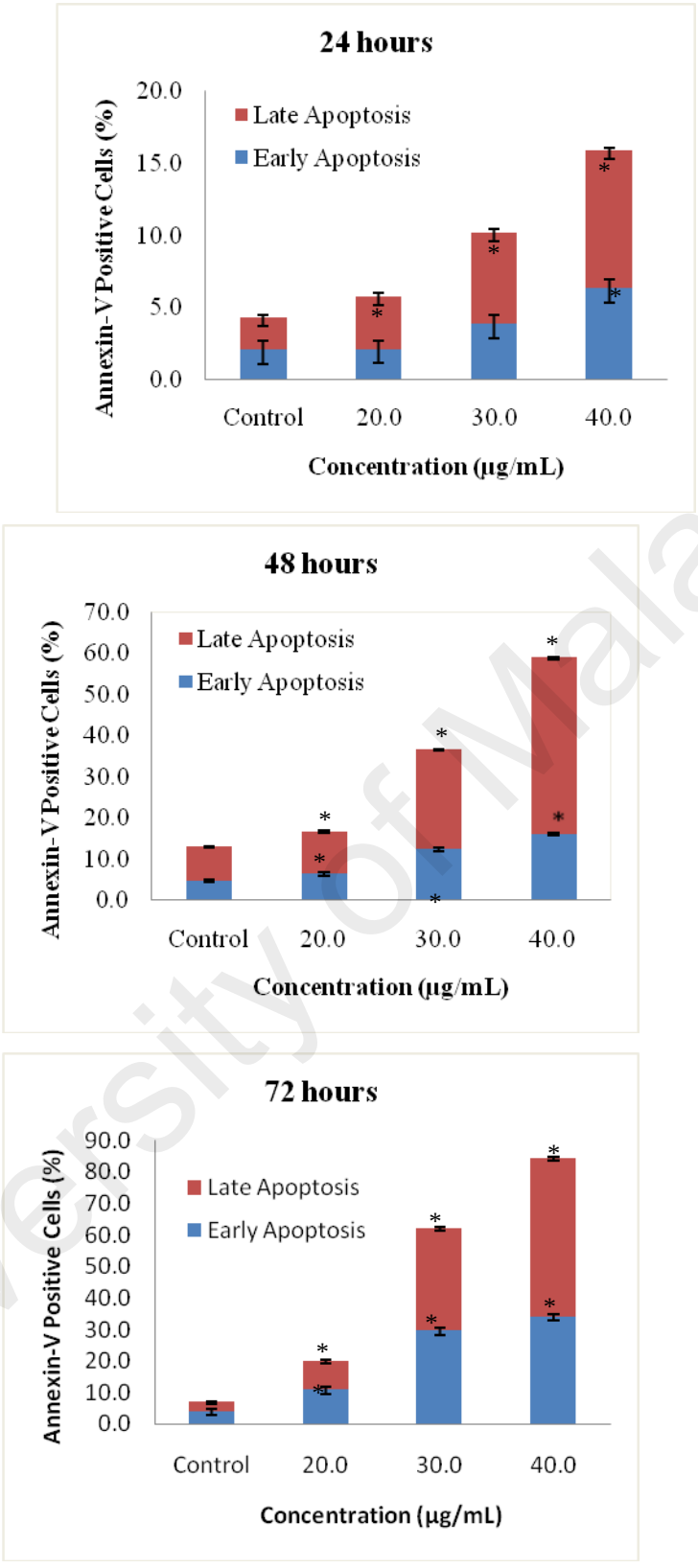


Figure 4.6, continued

4.7 Effects of CMD on DNA fragmentation in HT-29 cells

Figure 4.7 showed DNA fragmentation in HT-29 induced by CMD extract. The upper quadrant represents the percentages of DNA fragmentation in HT-29 after treatment with CMD extract. The DNA fragmentation in HT-29 cells increased significantly ($p<0.05$) in a time- and concentration dependent manner. Based on the results, the DNA fragmentation increased from 0.8 ± 0.01% (control), to 16.2 ± 0.6%, 69.5 ± 0.3% and 72.9 ± 0.6% when treated with increasing concentration of CMD (20-40 µg/mL) after 72 hours of incubation. It was observed that DNA fragmentation in HT-29 were drastically increased when the cells were treated at concentration of 30 and 40 µg/mL compared to lowest concentration, 20 µg/mL. The outcome from the TUNEL assay supported the hypothesis that CMD induced apoptosis in HT-29 cells.

A

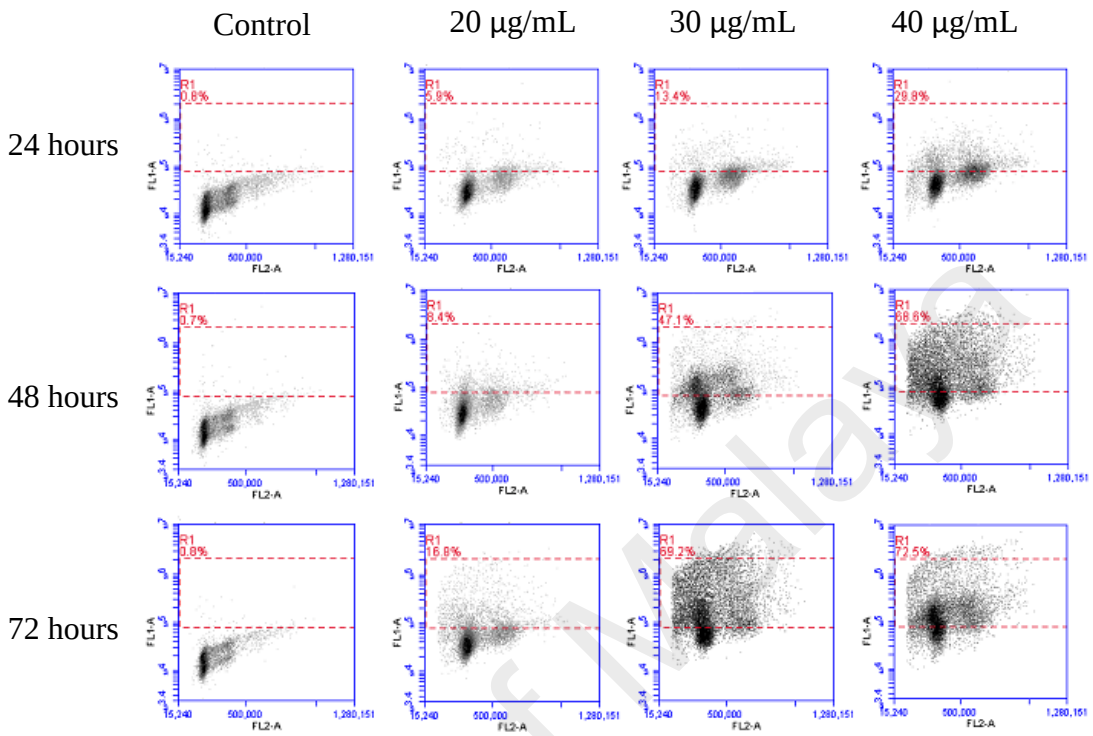
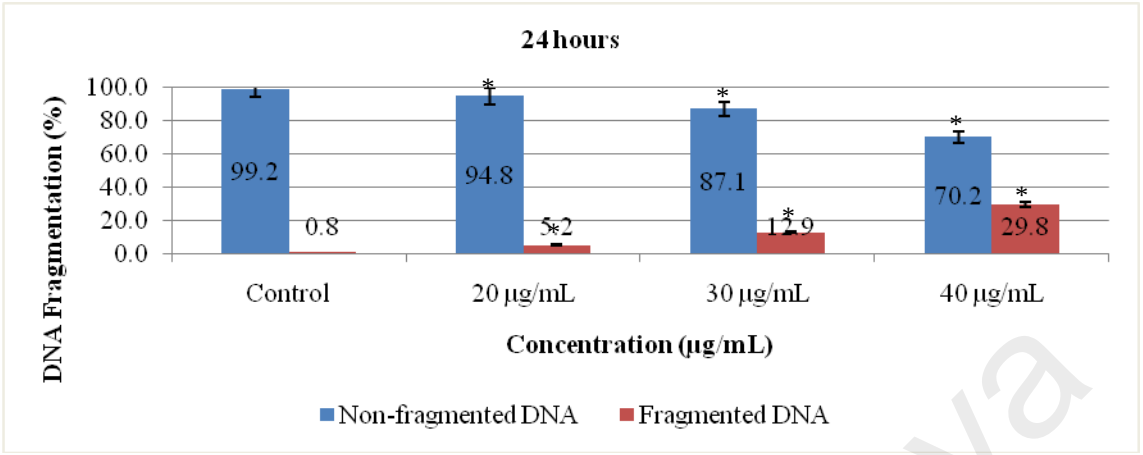
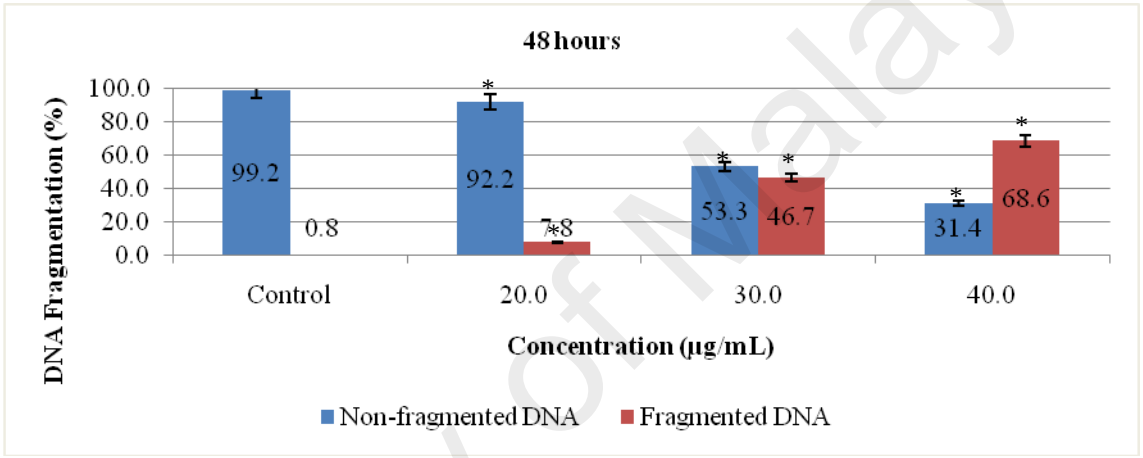


Figure 4.7: CMD induced DNA fragmentation in HT-29. (A) Selected concentration of CMD extract for the treatment of HT-29 cells (24, 48 and 72 hours) were analysed for DNA fragmentation by performing TUNEL assay kit. Two quadrants (upper and lower) in a dot plot were used to distinguished the non-fragmented/fragmented DNA. The upper quadrant indicated the fragmented DNA and lower quadrant indicated viable cells (non-fragmented). (B) The result of DNA fragmentation of HT-29 cells are expressed in percentages and illustrated in bar diagram. Values given are expressed as mean ± SD of triplicates obtained from three independent experiments. Significant of statistical differences with comparison of the control ($p<0.05$) are expressed in the asterisk (*). Untreated cells in 0.5% DMSO used as the control.

B





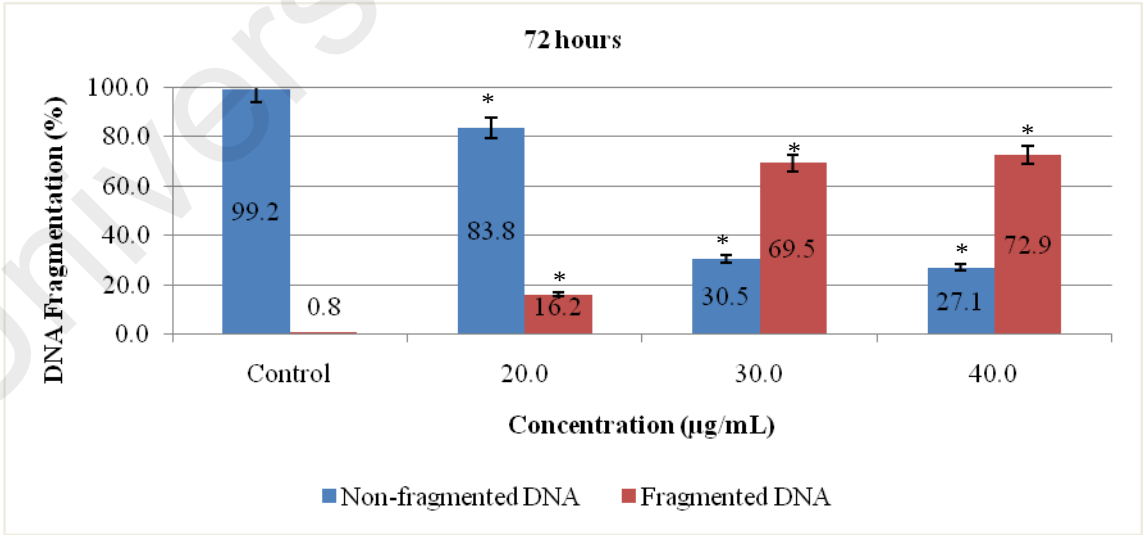


Figure 4.7, continued

4.8 Effects of CMD on the cell cycle arrest and the cell cycle regulatory proteins in HT-29 cells

The regulation of cell cycle development is commonly linked with cell proliferation. The initiation of molecular mechanisms cell cycle arrests on HT-29 cells were examined by further test. After 24 hours treatment, there was an increase and accumulation of cells in the G_2/M phase at all concentration as shown in Figure 4.8. Decreased of cell population in G_0/G_1 phase and increased of cell accumulation in S phase with increased of concentration. The treated cells when treated with 20, 30 and 40 µg/mL of CMD extract accumulated in the G_2/M phase from 12.76 ± 0.01% (control), 26.10% ± 0.02%, 39.66 ± 0.06% and 44.16 ± 0.02% respectively. The treatment of HT-29 cells with CMD extract over more than 24 hours induced more cell death and caused insufficient cell numbers for data interpretation. Further investigation on the molecular mechanisms underlying cell cycle arrest in HT-29 treated with CMD extract involve the study of proteins which play important roles in regulation of cell cycle progression using the western blot analysis. As shown in Figure 4.9, CMD treatments drastically increased the levels of cyclin-dependant kinase inhibitor, p21 and p27 in HT-29 cells. Consistent with the activation of cyclin-dependent kinase inhibitor was then significantly decrease in the levels of cyclin-dependant kinases Cdk4, and the cell cycle regulatory protein, cyclin D1 in HT-29 cells after 18 hours treatment with CMD extract. Three replicates were conducted for the western blot analysis on the proteins that regulates cell cycle progression.

A

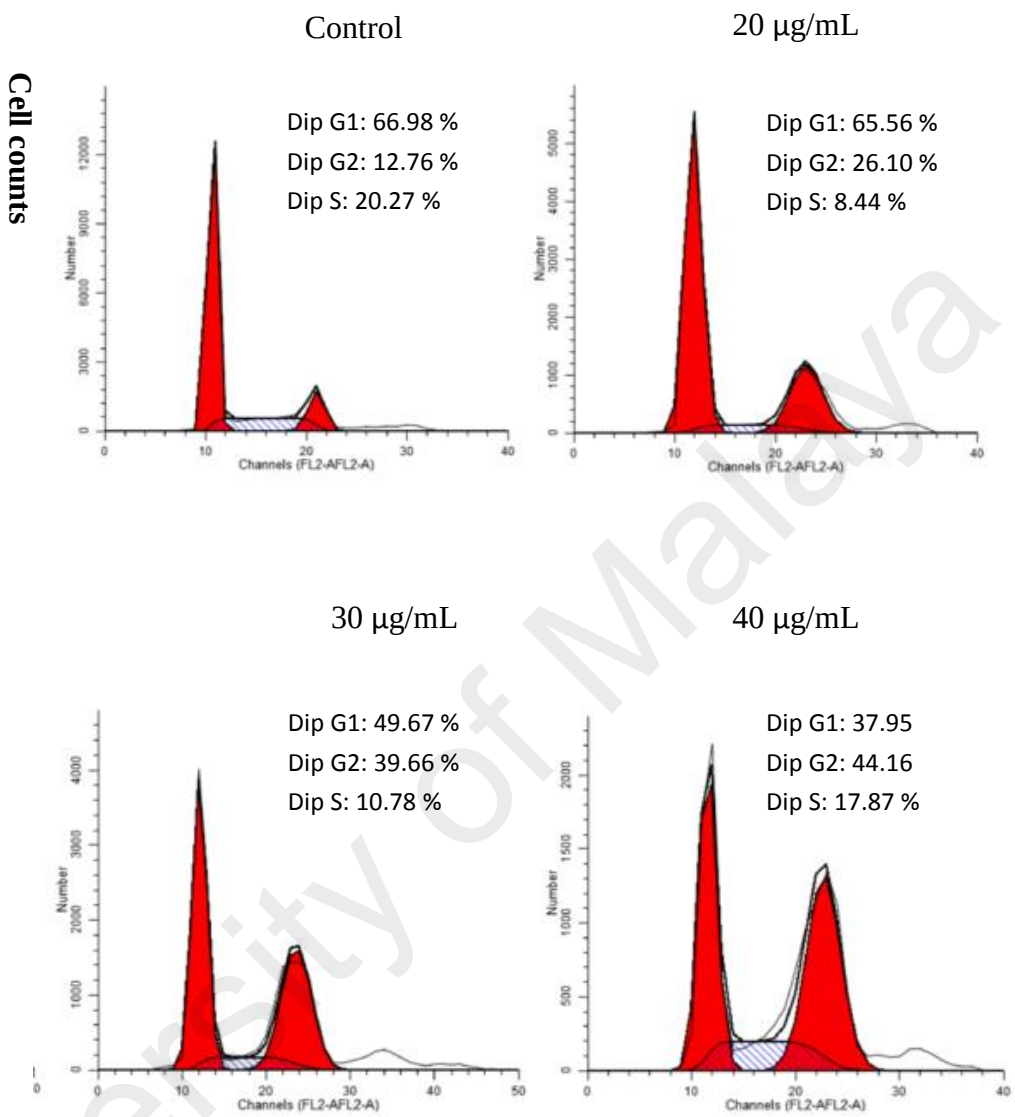


Figure 4.8: CMD induced cell cycle arrest in HT-29. (A) HT-29 were treated with the selected concentrations of CMD for 24 hours and the DNA of the cells were stained using PI. Flow cytometry was used for analysing the content of DNA in HT-29 cells and the DNA distribution in cell cycle (G_0/G_1, S and G_2/M phases) was quantified with software called the ModFit. (B) The result of accumulation of HT-29 cells in each phase are expressed in percentages and illustrated in bar diagram. Values given are expressed as mean ± SD of triplicates obtained from three independent experiments. Significant of statistical differences with comparison of the control ($p<0.05$) are expressed in the asterisk (*). Untreated cells in 0.5% DMSO used as the control.

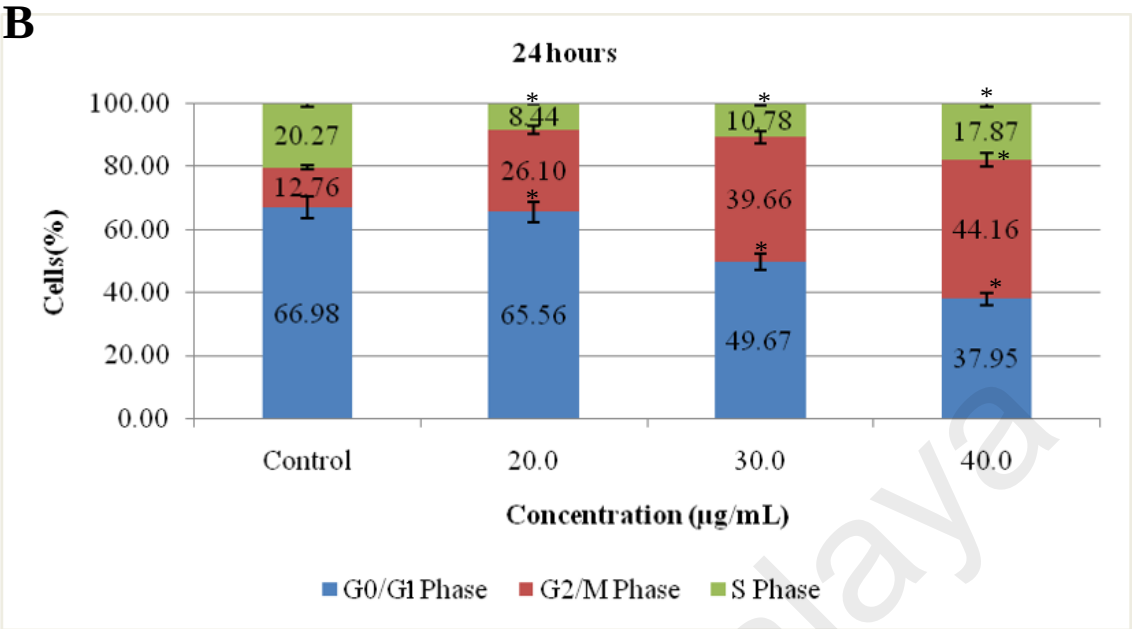


Figure 4.8, continued

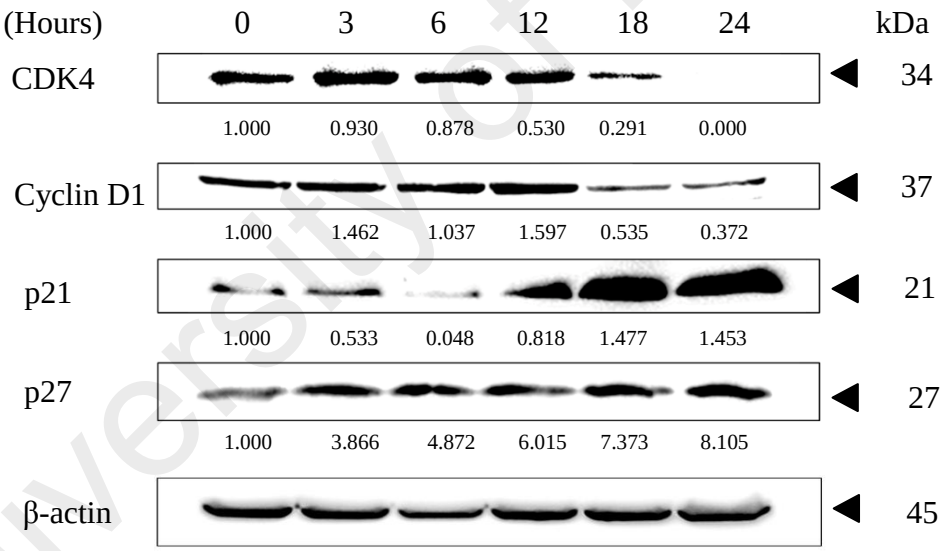


Figure 4.9: Effects of CMD on cell cycle regulatory proteins in HT-29. Cells were incubated in the absence or presence of CMD (40.0 µg/mL) for the indicated times, and followed by protein extraction. Changes in levels of the cyclin D1, cyclin dependent kinase (Cdk) 4 and Cdk inhibitors ($p21^{Cip1}$ and $p27^{Kip1}$) were analyzed by Western blot. Untreated cells in 0.5% DMSO served as the control. β-actin was used as the loading control. The results from representative experiments were expressed relative to the proteins level at 0 hr after normalization to β-actin signals.

4.9 Effects of CMD on the level of pro-apoptotic and anti-apoptotic proteins in HT-29 cells

The expression level of pro-apoptotic and anti-apoptotic proteins in HT-29 were altered after treatment with crude CMD extract as shown in Figure 4.10. The expression level of pro-apoptotic protein Bax was drastically increased in HT-29 in a time-dependent manner after incubation with CMD extract. The expression level of Bax increased approximately 6-fold compared to the negative control (at zero-hour treatment), between 12 to 24 hours of treatment. The expression level of anti-apoptotic proteins such as xIAP and cIAP-2 were altered in a time-dependent manner after incubation with CMD extract. The expression level of xIAP was initially increased but was downregulated after 12 hours treatment with CMD extract while the expression level of cIAP-2 decreased after 6 hours of treatment and remained constant up to 24 hours. Three replicates were conducted for the western blot analysis on both pro- and anti-apoptotic proteins.

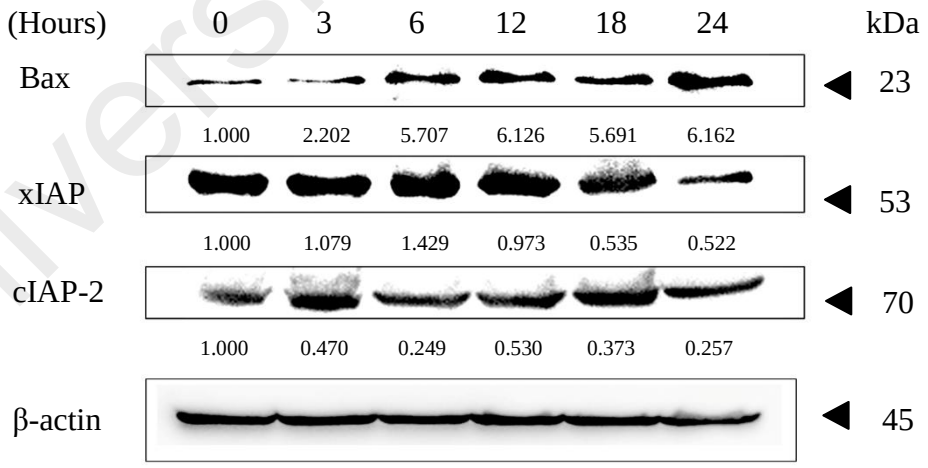


Figure 4.10: Effects of CMD on the levels of pro- and anti-apoptotic proteins in HT-29. HT-29 cells were treated with CMD (40.0 μg/mL) for specified time points, and followed by protein extraction and western blot analysis. CMD extract increased the level of pro-apoptotic protein (Bax) and inhibit the anti-apoptotic proteins (XIAP and cIAP-2). The results from representative experiments were expressed relative to the protein level at 0 hr after normalization to β-actin signals.

4.10 Cleavage of PARP-1 by CMD via the activation of caspases in HT-29 cells

The expression level of all tested procaspase precursors (caspase-8, caspase-9 and caspase-3) were downregulated in a time-dependent manner in HT-29 after incubation with CMD extract. Activation of caspase 3 was confirmed when the level of procaspase-3 precursor was downregulated which suggested the cleavage and activation of enzymes. The activation of caspase 3 was further confirmed with the cleavage of the intact poly-ADP-ribose-polymerase-1 (PARP-1) into a smaller 25 kDa fragment of PARP during apoptosis. The cleavage of PARP-1 via activation of caspases in HT-29 is shown in Figure 4.11.

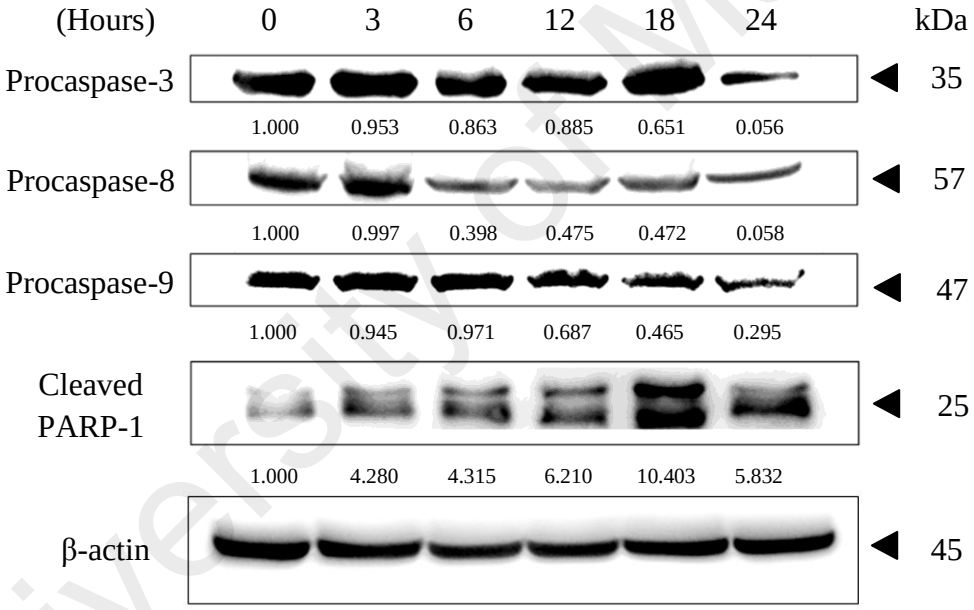


Figure 4.11: Induction of CMD on activation of caspases and cleavage of PARP-1 in HT-29 cells. HT-29 cells were treated with CMD (40.0 μg/mL) for specified time points, and followed by protein extraction and western blot analysis. CMD extract reduced the level of procaspases (caspase-3, -8 and -9) and increased level of expression of cleaved PARP-1. The results from representative experiments were expressed relative to the protein level at 0 hr after normalization to β-actin signals.

CHAPTER 5: DISCUSSION

5.1 Cytotoxicity screening of extracts and isolated compounds

A plant extract is considered to have cytotoxic effect if the IC_{50} is 20 µg/mL or lower after 48 to 72 hours of treatment (Marchetti et al., 2015). The hexane-soluble fraction (CMH) from Method A exhibited good cytotoxic effect on HT-29 and HCT-116 cells after 72 hours of treatment of cells (IC_{50} values of 21.7 ± 0.8 µg/mL and 16.1 ± 0.2 µg/mL respectively), but it was not active against A549 and CaSki cells (IC_{50} values of 23.2 ± 1.1 µg/mL and 31.9 ± 0.4 µg/mL respectively) after 72 hours of treatment. CME also showed good cytotoxic effect on HT-29 and HCT-116 cells (with IC_{50} values of 15.6 ± 4.4 µg/mL and 16.6 ± 0.5 µg/mL respectively) but poor cytotoxic effect on A549 and CaSki cells (with IC_{50} values of 31.7 ± 2.3 µg/mL and 41.9 ± 1.4 µg/mL respectively) after 72 hours of treatment. Crude methanolic extract (CMM) only showed good cytotoxicity effect on HCT-116 cells with the IC_{50} values of 18.1 ± 0.7 µg/mL, but displayed poor or no growth inhibitory activities against other cell lines (IC_{50} values of more than 30.0 µg/mL). Many of the results obtained for extracts from Method A was not consistent with results from Malek et al. (2010). The differences might be due to the type of assay used for evaluating the effects induced by the extract. However, the water extract did not exert any damage on all cell treated lines with IC_{50} values more than 100.0 µg/mL which was consistent with the result of Malek et al. (2010).

On the other hand, all crude extract and active fractions from Method B showed good cytotoxic effect on all tested cell lines with IC_{50} values less than 22.0 µg/mL. Crude dichloromethane extract (CMD) showed the strongest cytotoxic effect on HT-29 cell with IC_{50} values of 14.3 ± 0.2 µg/mL compared to other tested cell lines. The hexane-soluble fraction (CMDH) from Method B showed the strongest cytotoxic effect on A549 cell with IC_{50} value of 14.8 ± 0.6 µg/mL while the hexane-insoluble fraction

(CMDM) showed the highest cytotoxic effect on HCT-116 cell with IC_{50} value of 15.7 ± 0.5 µg/mL. In summary, the overall cytotoxic performances of crude extract and active fractions from Method B displayed better cytotoxic effects against all tested cancer lines. In addition, extracts from Method B exerted lower toxicity on normal colon cell line, CCD-18Co (with IC_{50} values greater 50.0 µg/mL) compared to the chemotheraphy drug, doxorubicin. Extracts from Method A showed higher toxicity with IC_{50} values less than 50.0 µg/mL except the water extract (CMW) in comparison to extracts in Method B. In general, extracts from Method A showed cytotoxic effects on selective tested cancer cell lines while extracts from Method B showed cytotoxic effects on all tested cancer cell lines.

Pure compounds are considered active when the IC_{50} values *in vitro* assay is 50 µM or less (Boik, 2001). Based on the results of cell lines after 72 hours incubation shown in Table 4.7, compound (*E*)-labda-8(17),12-dien-15,16-dial exhibited good cytotoxic effect on HCT116 after 72 hours treatment displayed IC_{50} values of 39.0 ± 0.7 µM respectively. Meanwhile, compounds zerumin A and (*E*)-labda-8(17),12-dien-15,16-dial showed moderate cytotoxicity on HT-29 with IC_{50} value of 61.9 ± 1.6 µM and 63.2 ± 1.0 µM respectively. Zerumin A exhibited moderate cytotoxicity against A549 cells with IC_{50} value of 58.5 ± 2.2 µM after 72 hours treatment. Longpene A, zerumin A, coronadiene, (*E*)-labda-8(17),12-dien-15,16-dial and calcaratarin A showed weak cytotoxic effect against CaSki. Other unidentified compounds showed weak or no cytotoxicity against all tested cell lines. As mentioned previously, zerumin A and (*E*)-labda-8(17),12-dien-15,16-dial degraded over time and the cytotoxic effects were also reduced with time.

Typically, pure compounds had higher potency in anticancer effects than the crude or fractionated extracts. Based on the cytotoxicity screening results, the crude and fractionated extracts displayed better cytotoxic effect than the isolated pure compounds. Besides that, the cytotoxicity screening results by crude and fractionated extracts were consistent even though the extracts were stored for more than three months (as shown in Table 4.3). This suggests that the compounds were more stable in the extract form and synergism between the compounds could explain for the good cytotoxic effects shown by the crude and fractionated extracts against tested cancer cells. Synergism or potentiation is the effect of combination which is greater than the sum of individual effects (Rasoanaivo et al., 2011). Thearubigins, polymeric polyphenols found in black tea, did not inhibit any growth of tumor, but when combined with genistein from soybean, the combination would suppress the human prostate tumor growth as well as causing cell arrest in G_2/M phase (HemaIswarya & Doble, 2006). Another example of synergism effect was shown by a combination of four thiazolidinone compounds which exhibited higher cytotoxicity than the individual compounds against paclitaxel-sensitive lung carcinoma (H460) and P-gp over-expressing paclitaxel-resistant cell (H460/TaxR) with minimal toxicity against normal fibroblasts cell (HemaIswarya & Doble, 2006). Then the cytotoxic effect of crude and fractionated extracts in comparison to the individual compounds might be due to the combined synergism effect of the individual compounds.

Hence, the crude dichloromethane extract (CMD) was selected for further investigation on the cell death mechanism of HT-29 since CMD exhibited the lowest IC_{50} value on HT-29.

5.2 Apoptotic morphology of HT-29 cells induced by CMD

Cells undergoing apoptosis have characteristics of morphological and biochemical features. The morphological characteristics of apoptosis include membrane blebbing, a reduction in cell size (pyknosis), cell rounding, chromatin condensation, nuclear fragmentation, presence of apoptotic bodies, and cell detachment due to loss of cellular adhesion (Duprez et al., 2009; Ola et al., 2011a; Ouyang et al., 2012b; Wong, 2011). All these morphological changes can be observed in Figure 4.4 and 4.5 and thus, these results support the induction of apoptosis in HT-29 cells by CMD.

5.3 Early- and late apoptosis event in HT-29 cells induced by CMD

One biochemical event of apoptosis is the externalization of phosphotidylserine. Annexin V-PI staining was performed to detect early and late apoptosis in treated HT-29 cells. Annexin V dye can easily bind to the externalized phosphatidylserine (PS) on the outer membrane of apoptotic cells (Elmore, 2007). The presence of PS on the cell surface is one of the most significant characteristics in apoptosis due to the negative charge of phospholipid with its ability to modify the interactions with other lipids that might disrupt the lipid supporting structures (Demchenko, 2012). During early apoptosis, cell membrane asymmetry is disrupted causing the exposure of PS residues to the outer cell membrane leaflet and Annexin V is strongly associated with PS residues (van Engeland et al., 1997; van Engeland et al., 1998). Therefore, the application of Annexin V conjugated to FITC enables apoptotic cells to be identified and quantified on a single-cell basis by flow cytometry. Studies on detection of early and late apoptosis induced by CMH and CME extract also showed percentages of cell population undergo early and late apoptosis increased in a concentration- and time-dependant manner (Hong et al., 2015). The percentages of early and late apoptotic cells induced by CMD extract

were lower in comparison to those of CMH and CME after 24 hours of treatment. After 48 and 72 hours treatment, the percentages of early and late apoptotic cells induced by CMD extract were higher than shown by both CME and CMH extract which was reported by Hong et al. (2015). Up-to-date, this is the second report on detection of externalization of PS by active fraction from *C. mangga*. However, the results from this study further support the apoptotic events in HT-29 induced by CMD extract.

5.4 DNA fragmentation in HT-29 cells induced by CMD

Cleavage of chromosomal DNA is one of the biochemical hallmark of cell apoptosis. Cleavage occurs in the internucleosomal regions of double-stranded DNA and the DNA strands are cleaved into approximately 200 base pair (bp) long DNA fragments by endogenous DNases (Maiese et al., 2012; Saraste & Pulkki, 2000). A chromosome is mainly made up with DNA and histones in which the DNA strands tightly wrap around the core of histones and the primary function of histomes is controlling the folding of DNA strand into a chromatin. It has been suggested by Lee et al. (1996) that the endonucleases catalyze the DNA fragmentation during the relaxation of chromatin structure as the histones structure was modified when treated with anticancer agent. This suggested that the mechanism of CMD extract in DNA fragmentation of HT-29 could be related to changes in histones associated with the DNA strands. TUNEL assay is used to observe this biochemical hallmark, which involves *in situ* labeling of DNA strand breaks (DSBs) by 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdU) incorporation and detection using a dye-labeled anti-BrdU antibody (Darzynkiewicz et al., 2008; Gavrieli et al., 1992). The outcomes from the TUNEL assay showed DNA fragmentation occurred in HT-29 cells when treated with CMD extract. This indicated

that CMD extract caused individual of apoptosis in HT-29 cells. This is the first report of induction of DNA fragmentation by *C. mangga* rhizomes extract.

5.5 Cell cycle arrest and changes of cell-cycle regulatory proteins

One of the significant hallmark of cancer that showed ungoverned cell growth/proliferation in cancer is unregulated cell cycle. Mutation on key checkpoint genes is responsible for the disruption of molecular mechanisms on regulation of cell cycle in cancer (Novak & Tyson, 2003). Cell cycle are separated by two phases that comprises of M phase (mitosis) and S phase (DNA synthesis), divide by G_1 and G_2 phase which served as the separation gaps (Han et al., 2012). A sequence of cyclin-dependant kinases (Cdks) are responsible to the regulation of phase transition in cell cycle through binding to their respective regulatory proteins like cyclins as shown in Figure 5.1. The binding of Cdks with their respective cyclins would trigger different downstream biochemical events that involved the phosphorylation of the targeted proteins (Vermeulen et al., 2003). The presence of endogenous CDKs inhibitor such as $p21^{Cip}$ and $p27^{Kip}$ or the degradation of cyclins is negatively regulating the activities of CDK-cyclin complexes (Vermeulen et al., 2003). High level of cyclin D1 is maintained throughout the G/M phase to G_0/G_1 phase and the level of cyclin D1 is automatically reduced when it reached S phase since it interfere with the DNA synthesis processes (Stacey, 2003). Phosphorylation of the retinoblastoma (Rb) proteins and the release of E2F transcription factor into the nucleus activate the transcription of cell cycle related genes which will be inhibited through the binding of p21 and p27 into CDK4/cyclin D and CDK2/cyclin E complexes respectively (Giacinti & Giordano, 2006; Munoz-Alonso et al., 2005). When DNA damage is present or the DNA synthesis during the S phase is incomplete, the G_2/M checkpoint halts cells from entering the M phase where the cells can undergo mitosis (Kastan & Bartek, 2004). Therefore, the G_2/M phase is

one of the decisive phase for the fate of the damaged cells, which either activate the repairing of damaged DNA, or activate apoptosis if the DNA damage is irreparable (Blagosklonny & Pardee, 2002; Huang et al., 2008; Molinari, 2000). Hong et al. (2015) reported that the CMH and CME extracts of *C. mangga* rhizome induced cell cycle arrest in G_0/G_1 phase and G_2/M phase in HT-29 after 72 hours of treatment. However, in this study, CMD extract induced higher percentage of cell arrest in G_2/M phase compared to those displayed by CMH and CME extracts. This might be due to the presence of different active compounds in the CMD, CMH and CME extracts.

The cell cycle arrest at the G_2/M phase is consistent with the downregulation of Cdk4 and cyclin D1 after 18 hours of treatment. CMD extract as shown by the Western blot analysis in Figure 4.9. Downregulation Cdk-4 and cyclin D1 is necessary to inhibit the progression of HT-29 cells into the G_0/G_1 phase. The increase in the level of p21 and p27 protein was also observed in the Western blot analysis. These two proteins responsible in deactivation of CDK-4 and cyclin D1 protein.

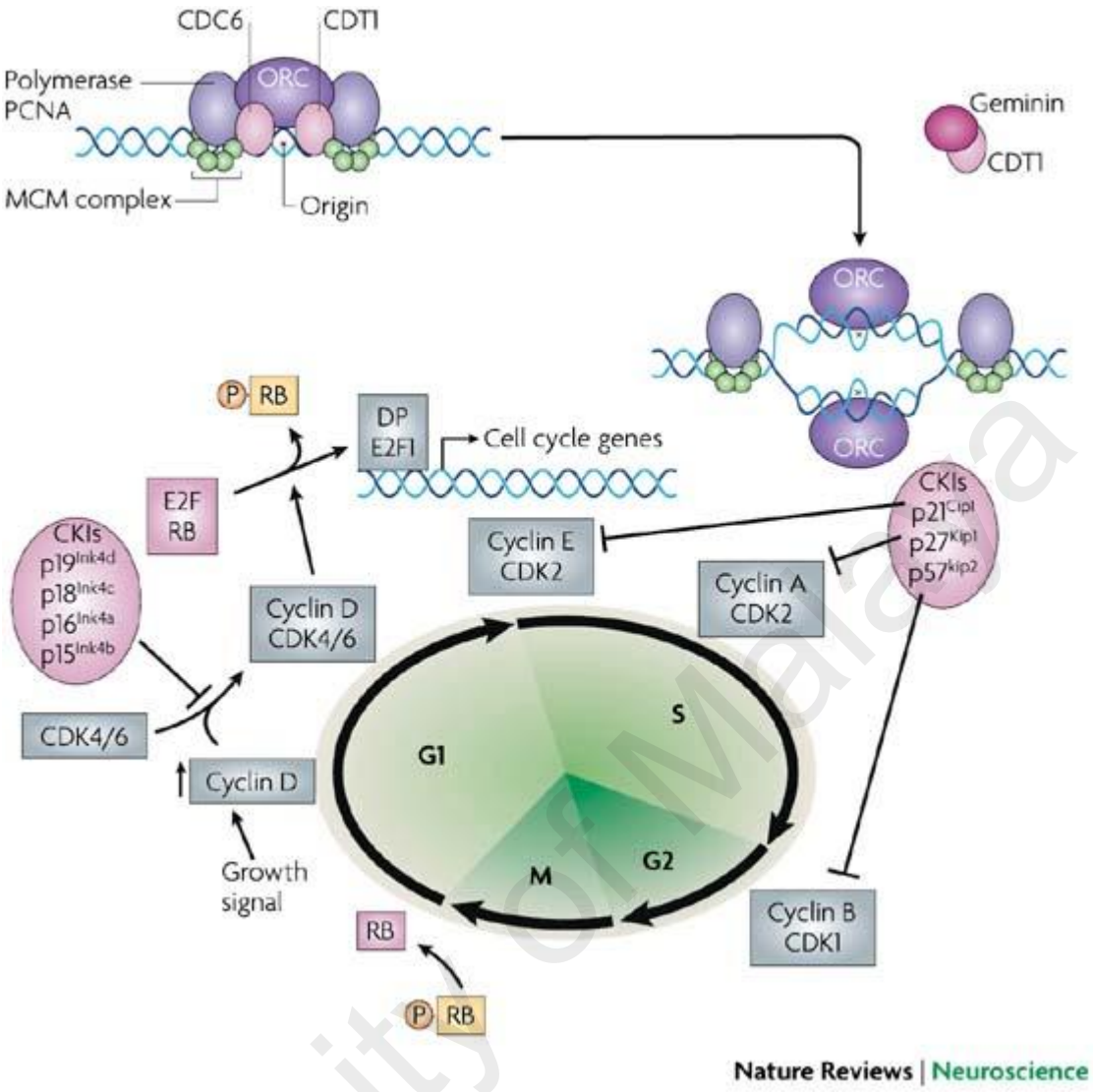


Figure 5.1: The transition of one phase to another phase in cell cycle is tightly controlled and regulated through the binding of a series of cyclin-dependant kinases (Cdks) to their respective cyclins (Herrup & Yang, 2007)

5.6 Changes of level of pro- and anti-apoptotic proteins in HT-29 cells

In the Western blot analysis, it was found that the expression level of pro-apoptotic and anti-apoptotic proteins in HT-29 were altered after treatment with crude CMD extract. In a typical intrinsic apoptotic pathway, the signals that trigger cell deaths are produced and originated from within the cell itself. The mitochondrion is a key organelle that acts as an initiator and executioner of the intrinsic apoptotic pathway besides its classic function as a site for cellular ATP generation (Harris & Thompson,

2000; Zeestraten et al., 2013). Bcl-2 family members responsible for both positive and negative stimuli and the activation of the intrinsic apoptotic pathway is determined by the integration among them (Aggarwal & Takada, 2005). Bcl-2 family members can be divided into two groups: pro-apoptotic (Bax, Bak, Bad) and anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w) proteins (Lindsay et al., 2011; Ola et al., 2011b). Bax and Bak are the proteins that play an important role in promoting the release of cytochrome c from the mitochondria into the cytosol by elevating the permeability of the outer mitochondrial membrane (OMM) through the oligomerization of Bax and Bak (Hunter et al., 2007; Lindsay et al., 2011; Pietenpol & Stewart, 2002). Release of cytochrome c then binds with adaptor Apaf-1 and procaspase 9 to form the apoptosome which later lead to the activation of caspase-9 and -3 (Baliga & Kumar, 2003; Gross, 2001). In contrast, the anti-apoptotic proteins bind with pro-apoptotic proteins and antagonize their functions by inhibiting the pore formation of OMM through balancing the mitochondrial membrane potential (Harris & Thompson, 2000; Jain et al., 2013; Tamm et al., 2001). Therefore, the upregulation of Bax in HT-29 treated with CMD extract promote the permeability of OMM and thus the formation of pore on OMM that lead to release of cytochrome c, formation of apoptosome and downregulation of procaspase-9 (activation of caspase-9) as shown in Figure 4.11.

There are eight types of proteins that can be found in the human inhibitor of apoptosis protein (IAP) family members which is characterized with one to three Baculovirus IAP Repeat (BIR) domain(s) at the N-terminal that approximately consists of 70 amino acids (Berthelet & Dubrez, 2013; de Almagro & Vucic, 2012; Wei et al., 2008). The IAPs such as X-linked IAP (XIAP), cellular IAP (cIAP)-1, cIAP-2, 81dimmers81g and others facilitated the regulation of cell death in multiple ways, ranging from inhibition of apoptosis through inhibiting the activities of caspases to cell cycle and inflammation (de Almagro & Vucic, 2012; Wei et al., 2008). XIAP is one the

best characterized IAP and the most potent endogenous caspase inhibitor that directly binds and inhibit caspases (Graber & Holcik, 2011). XIAP has three BIR domains (BIR1, BIR2. BIR3) that has high affinity but uneven functions on caspases. For example, the BIR3 domain of XIAP binds to the active site of monomeric caspase-9 to form a heterodimer that prevent cell death. XIAP also binds to caspase-3/-7 through the BR2 and the linker regions between BIR1 and BIR2 by blocking active binding site of caspases (Wei et al., 2008). However, XIAP can be inhibited by IAP antagonists such as second mitochondria-derived activator of caspases/direct IAP binding protein with low Pi (Smac/DIABLO) and high-temperature-regulated A2/Omi (HtrA2/Omi). These IAP antagonists are cleaved into active forms during the release of apoptogenic proteins including cytochrome c, Smac/DIABLO and HtrA2/Omi from the mitochondria into the cytosol (Wei et al., 2008). The released active Smac/DIABLO binds to the BIR3 domain of XIAP through its IAP-binding motif to prevent the further binding of XIAP on caspase-9, which then accelerate the XIAP autoubiquitination and degradation (Li et al., 1997; Wei et al., 2008). On the other hand, cIAP-2 inhibits cell apoptosis through its E3 ligase activity (Hu & Yang, 2003; Rodrigue-Gervais et al., 2014). It has been demonstrated that the cIAP-2 inhibited programmed cell death by directly suppressing the pro-apoptotic activity of caspases and also aiming the pro-apoptotic components of the tumor necrosis factor alpha (TNF-α) signalling pathway for ubiquitin degradation (Conte et al., 2006; Guicciardi et al., 2011). In the study, it can be observed that the levels of both XIAP and cIAP -2 were downregulated in HT-29 after treatment with CMD extract and subsequently resulted in the activation of caspases-8, -9 and -3.

5.7 Cleavage of PARP-1 through the activation of caspases

Based on the results of this study, the levels of all tested caspases were activated which eventually lead to the cleavage of PARP-1 (Figure 4.11). PARP-1 is an abundant and chromatin-associated enzyme that play important role in poly-ADP-ribosylation that maintain the genomic integrity by binding to single- or double-stranded DNA break to activate and initiate auto-ribosylation (D'Amours et al., 2001; Ko & Ren, 2012; Los et al., 2002). Therefore, frequent activity of DNA repair in cancer is due to the upregulation of PARP-1(Ko & Ren, 2012). In contrast, PARP-1 also plays an important role in cell death which included apoptosis and necrosis. Necrosis cell death is only initiated when the energy level is extremely depleted (D'Amours et al., 2001). On the other hand, active executioner caspases (caspase-3 or -7) cleave PARP-1 into two different size of fragments: a 24/25 kDa of DNA-binding domain (DBB) and an 89 kDa catalytic fragment during execution stage in apoptosis (Chaitanya et al., 2010; Tian et al., 2000). After the cleaving of PARP-1 by caspases, the DBB binds irreversibly to the DNA strand breaks which then inhibit the DNA repairing enzyme and thus attenuate the DNA repairing (Chaitanya et al., 2010) which lead to apoptosis cell death. Therefore, cleaving of PARP-1 by caspase is one of the hallmark of apoptosis. Effector or executioner caspases are usually inactive dimmers in the cytosol and are only activated by active initiator caspases so that the effector caspases become active and it is then able to cleave PARP-1 which halt the DNA repairing process and eventually apoptosis cell death (Alenzi et al., 2010). In this study, initiator caspases (caspase-8 and -9) were activated in HT-29 treated with CMD extract that provide a strong support for the activation of caspase-3 and PARP-1. Besides that, the level of activated caspase-8 is higher than the activated caspase-9 suggesting that the extrinsic apoptotic pathway is the main cell-signalling pathway in HT-29 induced by CMD extract rather than the intrinsic pathway. Based on the western blot and the DNA fragmentation analysis, all

procaspases were downregulated (caspases are activated) which lead to cleavage of PARP-1, DNA fragmentation and apoptosis cell death.

CHAPTER 6: CONCLUSIONS

Four compounds were successfully isolated and identified from CMDH and these four compounds were longpene A, zerumin A, coronadiene and (*E*)-labda-8(17),12-dien-15,16-dial. Only one compound, calcaratarin A was isolated from fraction CMDM. The compound (*E*)-labda-8(17),12-dien-15,16-dial exhibited good cytotoxic effect on HCT 116 with an IC_{50} value of 39.0 ± 0.7 µM after 72 hours of incubation. Zerumin A showed moderate cytotoxic effect on A549 with IC_{50} value of 58.5 µM after 72 hours treatment. All the identified compounds have no growth inhibitory effect against HT-29 and CaSki while other unidentified compounds displayed no toxicity towards all cell lines. Interestingly, the CMD extract from which CMDH and CMDM were derived showed better inhibitory effects in comparison to the pure compounds. Synergism between the compounds may have been responsible for this effect.

Apoptotic morphologies were observed in HT-29 treated with CMD in concentration- and time-dependent manner. Cell shrinkage, membrane blebbing, cytoplasm vacuolization, formation of apoptotic bodies and nuclear disintegration were observed in phase contrast microscopy while chromatin condensation was observed in fluorescence microscopy. These morphological changes in HT-29 treated by CMD is first evidence of induction of apoptosis in HT-29 by CMD extract. Externalization of phosphotidylserine and DNA fragmentation in HT-29 were detected after treatment with CMD extract for 24, 48 and 72 hours. The CMD extract increased the population of early- and late apoptosis, and DNA fragmentation in HT-29 in concentration- and time-dependent manner. Besides that, cell population were arrested in G_2/M phase was detected as well in HT-29 after treatment with CMD extract for 24 hours. These findings further validated the induction of apoptosis in HT-29 after treated with CMD extract.

Western blot analysis was further performed to analyze the damage in protein expression in HT-29 induced by CMD extract. The CMD extract increased the level of expression of cell cycle regulatory proteins (cyclin D1, Cdk4, p21, p27) in HT-29 that was correlated to the G_2/M phase arrest in the cell cycle analysis. Besides that, CMD extract also increased the expression level of pro-apoptotic protein, Bax as well as decreased the expression level of anti-apoptotic proteins (XIAP and cIAP-2). The level of expression of activated caspase-8 and -9 suggested that apoptosis in HT-29 was induced by CMD extract via extrinsic and intrinsic signalling pathway. Activation of caspase-8 and -9 would lead to activation of caspase-3 that caused cleavage of PARP-1 and then cell death. These western blot analyses suggested that the extrinsic-, intrinsic signalling pathway as well as the cell cycle progression were altered in HT-29 after treatment with CMD extract which that contributed to cell apoptosis in HT-29.

In conclusion, the active crude and fractions of *C. mangga* rhizomes exhibited good cytotoxic effects against various cancer cell lines (IC_{50} values: 14.3 ± 0.2 µg/mL on HT-29; 15.2 ± 0.8 µg/mL on HCT 116; 16.5 ± 0.4 µg/mL on A549 and 18.7 ± 0.7 µg/mL on CaSki) after 72 hours of incubations and the effects were even better than the isolated pure compounds. The CMD extract induced typical apoptotic morphological and biochemical characteristics in HT-29. Apoptosis may have occurred mainly through the extrinsic pathway through caspase-8 and to a lesser extent through the mitochondrial pathway through the up-regulation of Bax, cytochrome c release and caspase-9. Activation of caspase-8 and 9 triggers the activation of caspase-3 which led to the DNA fragmentation and PARP-1 cleavage. This eventually led to cell death *via* apoptosis. Therefore, the CMD extract of C. *mangga* rhizomes has the potential to be developed into an anticancer agent for the treatment of colon cancer (HT-29). The result observed in this study thus supported the traditional use of *C. mangga* rhizomes in cancer-related diseases.

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