BIOACTIVITIES OF *GYNURA* SPP. AND PHYTOCHEMICAL INVESTIGATIONS OF *GYNURA BICOLOR*

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ABSTRACT

Gynura bicolor and *Gynura procumbens* which belong to the botanical family of Compositae are widely used by locals as natural remedies in treating hypertension, diabetes and colon cancer. In this study, methanol, hexane, ethyl acetate and water extracts of both *Gynura* spp. were investigated for antioxidant and cytotoxic activities. Among the extracts of *G. procumbens*, methanol extract demonstrated better DPPH radical scavenging activity and inhibition of β-carotene bleaching, while hexane extract showed stronger metal chelating activity. The ethyl acetate extract of *G. procumbens* with the highest total phenolic content (TPC) exhibited moderate cytotoxicity against HT-29 and HCT 116 colon cancer cells. Among the extracts of *G. bicolor*, ethyl acetate extract with the highest TPC demonstrated the strongest ability in scavenging DPPH radicals, metal chelating, inhibition of β-carotene bleaching and cytotoxicity towards HCT 116 and HCT-15 colon cancer cells. The ethyl acetate extract induced apoptotic and necrotic cell death on HCT 116 cells determined by microscopy observation (acridine orange/ethidium bromide staining) and flow cytometry (annexin-V/PI) methods. Both *Gynura* spp. exerted no damage to CCD-18Co normal colon cells. The acute oral toxicity study indicated that methanol extracts of both *Gynura* spp. have negligible level of toxicity when administered orally and have been regarded as safe in experimental rats. Six chemical constituents, 5-*p-trans*-coumaroylquinic acid (**1**), 4-hydroxybenzoic acid (**2**), rutin (**3**), kaempferol-3-*O*-rutinoside (**4**), 3,5-dicaffeoylquinic acid (**5**) and kaempferol-3-*O*-glucoside (**6**) were isolated and identified from ethyl acetate extract of *G. bicolor*. Whilst, guanosine (**7**) and 5-*O*-caffeoylquinic acid (**8**) were successfully isolated and identified from water extract of *G. bicolor*. These eight chemical constituents were isolated from *G. bicolor* leaves for the first time, except rutin (**3**). The 3,5-dicaffeoylquinic acid (**5**), guanosine (**7**) and 5-*O*-caffeoylquinic acid

(**8**) showed selective cytotoxicity against HCT 116 cancer cells compared to CCD-18Co normal cells. Cell death and cell cycle arrest effects were observed when HCT 116 cells were treated with 3,5-dicaffeoylquinic acid (**5**) and 5-*O*-caffeoylquinic acid (**8**). The addition of cell impermeable catalase and reduced glutathione protected HCT 116 cells from cell death and cell cycle arrest effects. It was also observed that 3,5-dicaffeoylquinic acid (**5**) and 5-*O*-caffeoylquinic acid (**8**) generated extracellular hydrogen peroxide and green pigment (presumably quinone products) which contributed to cell death and cell cycle arrest. Current investigation revealed that the anti-proliferation effect of guanosine (**7**) on HCT 116 cells was resulted from cell cycle arrest associated with the activation of ERK1/2, p38 and JNK. The decreased activation of AMPK was also observed. Furthermore, the cell cycle arrest was accompanied by decreased of cyclin D1 level. These observations suggest that cell cycle arrest induced by guanosine (**7**) may be mediated through activation of ERK1/2, p38 and JNK pathways along with attenuation of AMPK pathway. The findings in present study provided scientific validation on the use of both *Gynura* spp. as natural remedies in folk medicine. Further studies on the mutagenic and toxicity effect over a longer period of time involving detection of effects on vital organ functions should be carried out to ensure that the plants are safe for human consumption.

ABSTRAK

Gynura bicolor dan *Gynura procumbens* yang tergolong dalam keluarga botani Compositae digunakan secara meluas oleh penduduk tempatan sebagai ubat semula jadi untuk merawat tekanan darah tinggi, kencing manis dan kanser kolon. Dalam kajian ini, aktiviti antioksidan dan sitotoksik ekstrak-ekstrak metanol, heksana, etil asetat dan air kedua-dua spesies *Gynura* telah disiasat. Antara ekstrak-ekstrak *G. procumbens*, ekstrak metanol menunjukkan aktiviti yang lebih baik dalam pemerangkapan radikal DPPH dan perencatan pelunturan β-karotena, manakala ekstrak heksana menunjukkan aktiviti pengkelatan logam yang lebih baik. Ekstrak etil asetat *G. procumbens* dengan jumlah kandungan fenol tertinggi (TPC) mempamerkan sitotoksik sederhana terhadap sel kanser kolon HT-29 dan HCT 116. Antara ekstrak-ekstrak *G. bicolor*, ekstrak etil asetat dengan jumlah kandungan fenol tertinggi menunjukkan keupayaan yang terkuat dalam pemerangkapan radikal DPPH, pengkelatan logam, perencatan pelunturan β-karotena dan sitotoksik terhadap sel kanser kolon HCT 116 dan HCT-15. Ekstrak etil asetat menyebabkan kematian sel apoptotik dan nekrotik terhadap sel HCT 116 di bawah pemerhatian mikroskop (pewarnaan akridin oren/etidium bromida) dan kaedah aliran sitometri (anesin-V/PI). Kajian ketoksikan oral akut menunjukkan bahawa ekstrak-ekstrak metanol kedua-dua spesies *Gynura* mempunyai tahap ketoksikan yang boleh diabaikan apabila diberikan secara oral dan telah dianggap sebagai selamat kepada tikus eksperimen. Enam sebatian kimia, asid 5-*p*-*trans*-koumaroilquinik (**1**), asid 4-hidroksibenzoik (**2**), rutin (**3**), kampferol-3-*O*-rutinosida (**4**), asid 3,5-dikafeolquinik (**5**) dan kampferol-3-*O*-glucosida (**6**) telah dipisah and dikenalpasti daripada ekstrak etil asetat *G. bicolor*. Manakala, guanosina (**7**) dan asid 5-*O*-kafeolquinik (**8**) telah berjaya dipisahkan daripada ekstrak air *G. bicolor*. Kelapan-lapan sebatian kimia ini telah dipisahkan pertama kali daripada daun *G. bicolor*, kecuali rutin (**3**). Asid 3,5-

dikafeolquinik (**5**), guanosina (**7**) dan asid 5-*O*-kafeolquinik (**8**) menunjukkan sitotoksik selektif terhadap sel kanser HCT 116 berbanding dengan sel normal CCD-18Co. Kesan kematian sel dan penyekatan kitaran sel telah diperhatikan apabila sel HCT 116 dirawat dengan asid 3,5-dikafeolquinik (**5**) dan asid 5-*O*-kafeolquinik (**8**). Rawatan dengan sel penelapan katalas dan pengurangan glutation dapat melindungi sel HCT 116 daripada kesan kematian sel dan penyekatan kitaran sel. Kajian juga menunjukkan bahawa 3,5-dikafeolquinik (**5**) dan asid 5-*O*-kafeolquinik (**8**) menghasilkan hidrogen peroksida dan pigmen hijau (kemungkinan produk quinon) yang menyumbang kapada kematian sel dan penyekatan kitaran sel. Penyiasatan ini menunjukkan bahawa kesan anti-proliferasi guanosina (**7**) terhadap sel HCT 116 berpunca daripada penyekatan kitaran sel yang berkaitan dengan pengaktifan ERK1/2, p38 dan JNK. Pengaktifan AMPK yang menurun juga telah diperhatikan. Tambahan lagi, penyekatan kitaran sel adalah diiringi oleh penurunan siklin D1. Pemerhatian ini mencadangkan bahawa penyekatan kitaran sel yang berpunca daripada guanosina (**7**) mungkin melalui pengaktifan isyarat laluan ERK1/2, p38 dan JNK bersama dengan pengecilan isyarat laluan AMPK. Penemuan-penemuan dalam kajian ini memberi fakta saintifik yang sahih dalam penggunaan kedua-dua spesies *Gynura* sebagai ubat semula jadi. Kajian-kajian lanjut mutagenik dan kesan ketoksikan pada fungsi organ penting untuk jangka yang lebih panjang perlu dijalankan untuk memastikan tumbuhan tersebut adalah selamat bagi penggunaan manusia.

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LIST OF ABBREVIATIONS

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AKT	Protein kinase B
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AO	Acridine orange
APS	Ammonium persulfate
BHA	Butylated hydroxyanisole
BSA	Bovine serum albumin
CD_3OD	Deuterated methanol
CQ	5-*O*-Caffeoylquinic acid
CRC	Colorectal cancer
DCF	Dichlorofluorescein
DCF-DA	2′,7′-Dichlorodihydrofluorescein diacetate
DCQ	3,5-Dicaffeoylquinic acid
DEPT	Distortionless enhancement by polarisation transfer
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterated DMSO
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DTT	Dithiothreitol
EB	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
EDTA-2Na	Ethylenediaminetetraacetic acid disodium
ERK	Extracellular signal-regulated kinase
ESI-MS	Electrospray ionisation-mass spectrometry
FBS	Foetal bovine serum

GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
IC_{50}	Inhibition concentration at 50 %
JNK	c-JUN N-terminal kinase
LC-MS	Liquid chromatography-mass spectrometry
LD_{50}	Lethal dose at 50 %
MMR	Mismatch repair
mTOR	Mammalian target of rapamycin
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
NMR	Nuclear magnetic resonance
$O_2^{\bullet-}$	Superoxide
$^{\bullet}OH$	Hydroxyl radical
PBS	Phosphate buffered saline
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SI	Selectivity index
SOD	Superoxide dismutase
TBST	Tris buffered saline with tween-20
TEMED	Tetramethylethylenediamine
TLC	Thin layer chromatography

LIST OF APPENDICES

CHAPTER 1: GENERAL INTRODUCTION

Nature has been providing us an endless supply of bioactive chemical constituents for a very long time. At present, medicines derived directly or indirectly from natural products are not restricted to plant sources only but microorganisms and marine organisms as well. In 1826, morphine from opium was the first natural product commercialised by Merck. Subsequently, the era of antibiotics search was first kick started by Alexander Fleming who discovered penicillin from *Penicillium rubens* in 1928. Now, the application of natural products is not limited to combat infectious pathogens alone, the application has spread to field of anticancer. Besides acting as medicine by itself, natural products are important source of chemical diversity and leads for the development of novel synthetic therapeutic agents. According to Newman and Cragg (2012), about 50 % of the current available anticancer medicines are derived directly or indirectly from natural products. In general, natural products are referred to secondary metabolites produced by organisms for adaptation, survival and protection, in contrast to primary metabolites that are important for growth and development.

To date, cancer is a common disease with high mortality rate in developing and developed countries. Lung cancer is the most common cancer with high mortality rate globally. Whilst, breast cancer is the second most common cancer followed by colorectal, prostate and stomach cancer (Ferlay *et al*., 2015). The incidence rate of cancers is still increasing every year with only a few cancers showing positive sign of reduction. This may be due to the changes in diet, lifestyle and environment. The increase of processed or fast food consumption, smoking, alcohol consumption, lack of physical exercise and polluted environment may partly explain the increasing incidence rate of cancers over time.

According to World Health Organisation's Globocan 2012 report (Ferlay *et al*., 2013), colorectal cancer (CRC) was the second most common cancer in Malaysia. Currently, CRC is mainly treated by surgery, chemotherapy and radiotherapy. In Asia, medicinal plants are often supplemented as complementary and alternative medicine to treat CRC in order to enhance the efficacy of conventional medicine, reduce side effects, prolong survival and improve the quality of life. In any cancer chemotherapy, the major concern and challenge are harmful side effects and development of multiple anticancer drugs resistance. Researchers are always paying great attention to natural products especially from medicinal plants for new leads to develop better drugs to combat CRC.

Gynura bicolor and *Gynura procumbens* belong to the botanical family of Compositae. *G. bicolor* is locally known as 'Sambung Nyawa Ungu' (Malay) and 'Hong Feng Cai' (Chinese), while *G. procumbens* is known as 'Sambung Nyawa' (Malay) and 'Feng Wei Jian' (Chinese) in Malaysia. The leaves of *G. bicolor* distinctively show reddish purple colour on the abaxial side and green colour on the adaxial side. Both *G. bicolor* and *G. procumbens* leaves have been consumed to treat CRC by some of the local people. Both plants are believed to promote health benefits such as anticancer and anti-inflammation effects. *G. bicolor* has been used for post-labour recovery, blood circulation improvement, treatment of dysmenorrhea, hemoptysis and diabetes (Li, 2006). On the other hand, *G. procumbens* has been used to treat fever, kidney disease, hypertension, diabetes and cancer (Rosidah *et al*., 2008).

Although *G. bicolor* and *G. procumbens* have been used in the treatment of CRC by locals, data on the cytotoxicity against human colon cancer cells and phytochemical investigation is still limited. The current project was carried out to provide supporting scientific evidence on the potential of both plants in prevention and treatment of CRC. Firstly, crude methanol and fractionated extracts (hexane, ethyl acetate and water extracts) of *G. bicolor* and *G. procumbens* were prepared for antioxidant and

methylthiazolyldiphenyl-tetrazolium bromide (MTT) cytotoxicity assays. The antioxidant activities of extracts were evaluated by three assays which were 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, metal chelating assay and β-carotene bleaching assay. The total phenolic content of the extracts was determined by Folin-Ciocalteau method.

The cytotoxicity of plants extracts were screened against five human colon cancer cell lines (HT-29, HCT-15, SW480, Caco-2 and HCT 116), one human breast adenocarcinoma cell line (MCF7) and one human normal colon cell line (CCD-18Co). The cells treated with cytotoxic extract were then subjected to staining and flow cytometry in order to assess the cell death and morphological changes. Acute oral toxicity was also undertaken in present study to determine the safety of *G. bicolor* and *G. procumbens* leaves for human consumption as *in vitro* trials did not always reflect the outcome of *in vivo* studies.

After evaluating the cell death induction of cytotoxic extract on selected human cancer cell line, bioassay-guided fractionation and phytochemical investigation of the plant extracts were carried out on *G. bicolor* as *G. bicolor* showed better cytotoxicity than *G. procumbens*. The isolated and identified chemical constituents were tested for their cytotoxicity on selected human cancer cell lines. The mechanisms that involved in the cell death and cell cycle arrest effect induced by the cytotoxic chemical constituents were investigated. The general procedures in the present study are outlined in Figure 1.1.

Objectives of study

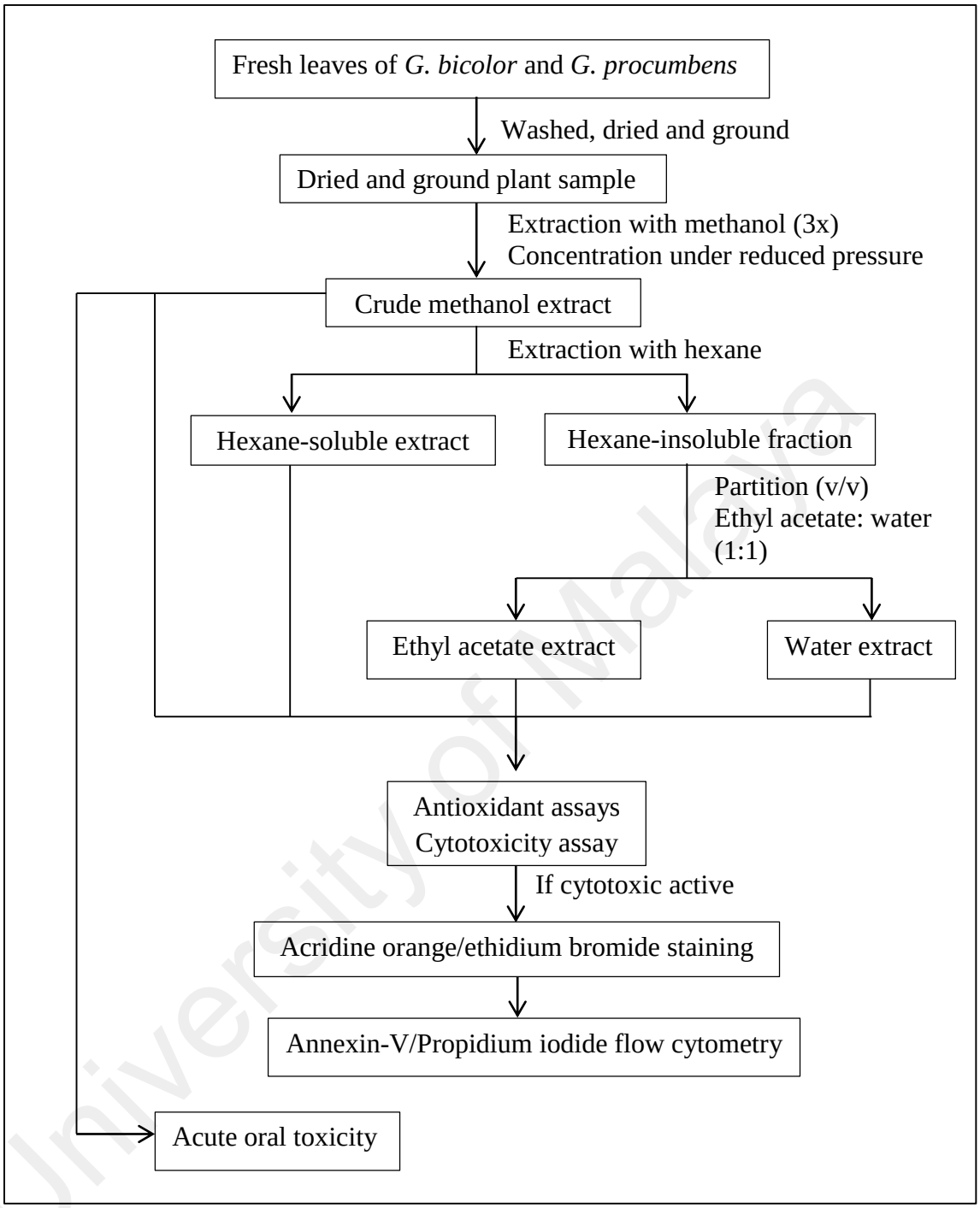
The main objectives of the present study were as follows:

i. To evaluate the cytotoxicity, antioxidant activities and acute oral toxicity of locally grown *G. bicolor* and *G. procumbens* extracts

ii. To isolate the chemical constituents from *G. bicolor* and evaluate the cytotoxic activities of the identified chemical constituents on selected human cancer cell lines

iii. To investigate the mechanisms involved in cell death and cell cycle arrest effect of 5-*O*-caffeoylquinic acid and 3,5-dicaffeoylquinic acid on selected human cancer cell line

iv. To investigate the mechanisms involved in anti-proliferation effect of guanosine on selected human cancer cell line



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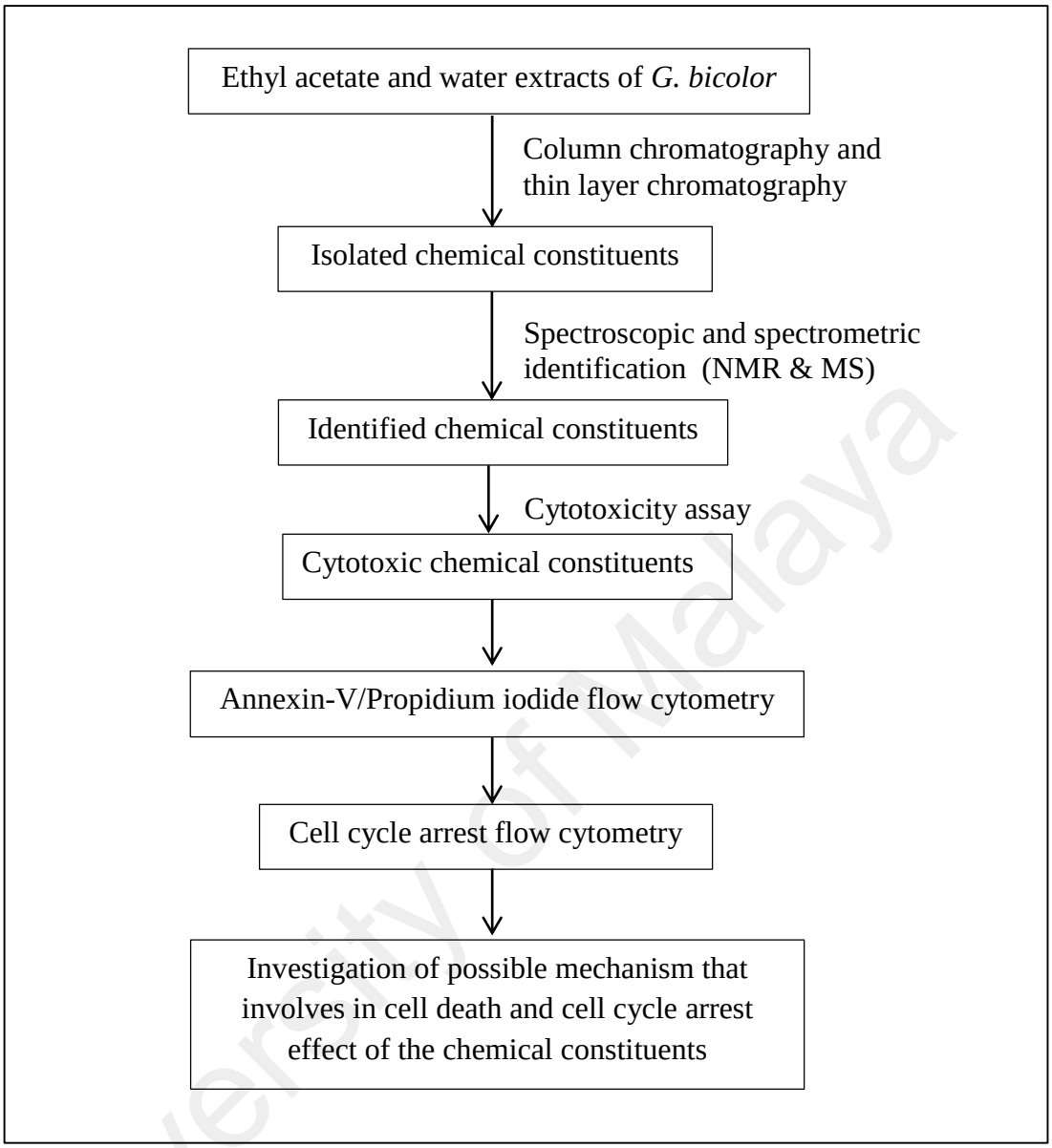


Figure 1.1: Outline of general procedures

CHAPTER 2: LITERATURE REVIEW

2.1 Plant natural products

Secondary metabolites or natural products that are produced in plant kingdom can be grouped into several main classes such as terpenes, alkaloids, flavonoids, phenolic acids and polyphenols. These secondary metabolites have wide ranges of bioactivities.

2.1.1 Plant derived therapeutic agents

Plants have been an important source of medicines for a long time. Traditional Chinese Medicine (around 350 BC) and Indian Ayurveda (around 900 BC) had recorded the uses of many plant species for their medicinal properties in treatment of diseases and disorders. In 1826, morphine from opium was the first plant natural product which commercialised by Merck. In 1899, Bayer introduced the first semi-synthetic drug, aspirin which was derived from salicin (*Salix alba*, white willow). Subsequently, many plant derived drugs were discovered and some are widely used till today (Table 2.1).

Table 2.1: Plant derived therapeutic agents (Ji *et al*., 2009; Veeresham, 2012)

Plant source	Compound	Application
Artemisia annua (sweet wormwood)	Artemisinin	Anti-malaria
Atropa belladonna (deadly nightshades)	Atropine	Inhibitor of muscarinic acetylcholine receptor
Cephaelis ipecacuanha (ipecac root)	Emetine	Anti-protozoa
Cinchona ledgeriana (cinchona bark)	Quinine	Anti-malaria
Coffea arabica	Caffeine	Stimulant of central nervous system which targets adenosine receptor
Colchicum autumale (meadow saffron)	Colchicine	Anti-inflammatory agent for gout
Erythroxylum coca	Cocaine	Topical anesthetic
Nicotiana tabacum (tobacco)	Nicotine	Stimulant of central nervous system which targets nicotinic acetylcholine receptor
Rauwolfia serpentine (Indian snakeroot)	Reserpine	Anti-hypertension
Silybum marianum (milk thistle)	Silibinin	Treatment of liver diseases

2.1.2 Plant derived anticancer drugs

In 1955, National Cancer Institute (NCI) established anticancer drug screening and discovery program which had stimulated the discovery of many significant natural derived anticancer drugs that still in clinical use today. All of the approved plant derived anticancer drugs are mainly targeting tubulin or topoisomerase.

2.1.2.1 Vincristine and vinblastine

Vincristine and vinblastine are alkaloids isolated from the periwinkle (*Catharanthus roseus*) in Madagascar. These alkaloids bind to β-tubulin and inhibit the polymerisation of tubulins which is important for the formation of mitotic microtubule spindle. Failure in the formation of microtubule spindles disables actively dividing cancer cells to complete the mitosis process and eventually leads to cell death (Nobili *et al*., 2009; Pan *et al*., 2012).

2.1.2.2 Podophyllotoxin and etoposide

Podophyllotoxin was isolated from the root of American Mayapple (*Podophyllum peltatum*). The mechanism of action of this cytotoxic chemical constituent is similar to vincristine by binding to tubulin and interferes with the formation of mitotic microtubule spindles. Due to high toxicity, podophyllotoxins derived etoposide was synthesised. Etoposide works by inhibiting topoisomerase II that is important in changing the DNA topology by cutting and re-ligation of double stranded DNA helix during the process of DNA replication and transcription (Lodish *et al*., 2000). Cancer cells that lose the function of topoisomerase II will die of DNA breakage (Nobili *et al*., 2009; Pan *et al*., 2012).

2.1.2.3 Paclitaxel

Paclitaxel (also known as taxol) is a taxane that isolated from the bark of yew tree (*Taxus brevifolia*). Paclitaxel binds to three subunit of tubulin and prevents the depolymerisation of mitotic microtubule spindles. Paclitaxel alters the dynamic of mitotic microtubules which interferes with cell division and lead to cell death (Nobili *et al*., 2009; Pan *et al*., 2012).

2.1.2.4 Camptothecin, irinotecan and topotecan

Camptothecin is an alkaloid isolated from the bark of *Camptotheca accuminata* which inhibits the function of topoisomerase I (Liu *et al*., 2000). Topoisomerase I bind to double stranded DNA helix and induce a cut and re-ligation in single stranded DNA to unwind or relax the DNA for replication and transcription (Lodish *et al*., 2000). Disruption of topoisomerase I function results in DNA damage which lead to cell death. Camptothecin has limited therapeutic application due to severe toxicity. Subsequently, two semi-synthetic derivatives of camptothecin were developed, irinotecan and topotecan (Nobili *et al*., 2009; Pan *et al*., 2012).

2.1.3 Potential dietary plant natural products in prevention and treatment of cancer

Many dietary plants and medicinal herbs are being used for health benefits and disease such as cancer, especially in Asia. Many studies had been conducted on plants to identify the active chemical constituents that showed potential in prevention and treatment of cancer such as lycopene (tomato), resveratrol (grape), curcumin (turmeric) and epigallocatechin gallate (green tea). These plant natural products have antioxidant activity and able to induce cell death and cell cycle arrest. The induction of cell death may involve the modulation of B-cell lymphoma 2 (BCL2), B-cell lymphoma extra-large (BCLXL), BH3 interacting-domain death agonist (BID), cytochrome C, BCL2-associated X protein (BAX), BCL2-associated death promoter (BAD), caspase 3, caspase 7, caspase 9 and p53. In cell cycle arrest effect, the modulation of cyclin D1, p21, p27, p16, retinoblastoma protein (RB) and cyclin dependent kinases (CDK2, CDK4, CDK6) may involve. In addition, the modulation of signalling pathways that regulate the growth, survival and adaptation may involve such as protein kinase B (AKT), nuclear factor-kappaB (NF-κB), Wnt and mitogen-activated protein kinases

(MAPK) pathways (Cecchinato *et al*., 2007; Lee *et al*., 2011; Ramos, 2008; Russo *et al*., 2010; Surh *et al*., 2001; Tang *et al*., 2008).

2.2 Oxidative stress and cancer

Oxidative stress is the condition where antioxidants are overwhelmed by the generation of reactive oxygen or nitrogen species (ROS/RNS). It is widely believed that oxidative stress is associated with the development of cancer although the role of ROS in cancer is largely unclear. The major source of ROS is from mitochondrial electron transport chain and NADPH oxidase (NOX). NOX can catalyse the conversion of oxygen to superoxide ($O_2^{\bullet-}$) while electrons that leak from the electron transport chain can reduce oxygen to $O_2^{\bullet-}$. $O_2^{\bullet-}$ can further undergo reactions to form hydrogen peroxide (H_2O_2). Subsequently, H_2O_2 can undergo reactions to form hydroxyl radical ($^{\bullet}OH$). The $O_2^{\bullet-}$ and H_2O_2 are usually less reactive against most of the biomolecules but not the highly reactive $^{\bullet}OH$ (Halliwell, 2013). Besides ROS, $O_2^{\bullet-}$ also can produce RNS by reacting with nitric oxide ($NO^{\bullet}$) to from very reactive peroxynitrite ($ONOO^-$). Oxidative stress can cause oxidative damage to lipids, proteins and DNA. Most of the lipid and DNA oxidation products namely 4-hydroxynonenal and 8-oxo-7,8-dihydro-2'-deoxyguanosine are mutagenic that could lead to cancer (Esterbauer *et al*., 1991; Valavanidis *et al*., 2009). Therefore, it is believed that antioxidants may play an important role in cancer prevention.

2.2.1 Antioxidants

Antioxidants are agents that can react with free radicals and prevent oxidative damage to targeted molecules. In human antioxidant system, superoxide dismutase (SOD), catalase, glutathione peroxidase and peroxiredoxin are the main enzymatic antioxidants. SOD can catalyse the conversion of $O_2^{\bullet-}$ to H_2O_2 as the substrate of

catalase to form water and oxygen. In this way, the formation of highly reactive $^{\bullet}OH$ will be prevented. Besides the catalase, H_2O_2 and other peroxides can be eliminated by glutathione peroxidase and peroxiredoxin by using reduced glutathione (GSH) and thioredoxin, respectively (Gough & Cotter, 2011). GSH by itself can also act as antioxidant to convert free radicals to non-radical products with the formation of glutathione disulfide (GSSG). Besides these endogenous antioxidants, many strong exogenous antioxidants such as polyphenol, phenolic acids, vitamin C and E from plant sources may provide us with extra protections against oxidative stress. Polyphenolic compounds such as epigallocatechin gallate, cucurmin and resveratrol are powerful antioxidants (Surh, 1999). In addition to direct free radicals scavenging activity, exogenous antioxidants can indirectly reduce oxidative stress by activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor which regulates the antioxidant response element (ARE) that involves in controlling the synthesis of antioxidant enzymes (Singh *et al*., 2010). Epigallocatechin gallate, cucurmin and resveratrol have been shown to enhance the expression of antioxidant and phase II detoxifying enzymes which are mediated by Nrf2 (Kou *et al*., 2013; Na *et al*., 2008; Na & Surh, 2008).

2.3 Cancer

Over a very long period of time (about 20 to 40 years), normal cells that accumulated multiple genetic and epigenetic alternations can give rise to cancer cells (Umar *et al*., 2012). The accumulated mutations in oncogenes, tumour suppressor genes and genetic stability genes can disrupt multiple normal regulations of cell death, division, differentiation and migration. Oncogenes are the genes that drive the growth of cell while tumour suppressor genes are the genes that suppress cell growth. Genetic stability genes are the genes that minimise the mutation rate by DNA repair mechanisms

and these genes are related to the stability of chromosomes. Unlike genetic stability genes, mutated oncogenes and tumour suppressor genes are the direct factors that cause abnormal growth of neoplastic cells. Neoplastic (benign or malignant) cells that gained growth advantage over the normal cells would progressively turn into invasive tumour. In addition, it is recognised that the surrounding cells such as inflammatory cells, endothelial cells and fibroblasts in the pre-malignant site (microenvironment) are involved in the development of invasive cancer (McAllister & Weinberg, 2014; Umar *et al*., 2012).

2.3.1 Hallmarks of cancer

The hallmarks of cancer have been recognised and described as unlimited proliferation, resistance to cell death, altered metabolism, induction of angiogenesis, metastasis and evade immune surveillance (Hanahan & Weinberg, 2011; Luo *et al*., 2009). The most basic hallmarks are the unlimited proliferation and resistance to cell death. For sustaining proliferation and resisting cell death, cancer cells can have high expression of growth receptor (e.g. epidermal growth factor receptor), constitutive activation of downstream pathways [e.g. AKT, extracellular signal-regulated kinase (ERK)] without the activation of growth receptor, loss of functional growth suppressors (e.g. phosphatase and tensin homolog (PTEN), RB, p53, liver kinase B1 (LKB1), p16) and high level of telomerase (Blasco, 2005; Burkhart & Sage, 2008; Davies & Samuels, 2010; Hynes & MacDonald, 2009; Jiang & Liu, 2009; Shaw, 2009; Wajed *et al*., 2001). Agents have been developed as pro-apoptotic drugs and CDK inhibitors (cell cycle inhibitors) to attack these hallmarks, for example, ABT737, SAHBs, Alvocidib and R-roscovitine (Lee & Sicinski, 2006; McClue *et al*., 2002; Stauffer, 2007; Verdine & Walensky, 2007).

2.3.2 Cell death and cell cycle

The elucidation of mechanisms behind the cell death and cell cycle process has been carried out to define molecular mechanism of these important targets and provide insight for the development of better and more tolerable cancer therapy.

2.3.2.1 Regulated cell death

One of the pathways for cell to undergo cell death process is through apoptosis. Based on morphological features, apoptosis will lead to cell shrinkage, nuclear condensation and fragmentation, plasma membrane blebbing and apoptotic bodies formation. Based on biochemical features, apoptosis can be divided to extrinsic and intrinsic pathways.

In intrinsic pathway, BAX and BCL2 homologous antagonist/killer (BAK) induce mitochondrial outer membrane permeabilisation and lead to the release of cytochrome C. Subsequently, cytochrome C can interact with apoptotic protease activating factor 1 (APAF1) to activate caspase 9. Activated caspase 9 can activate caspase 3 that will lead to apoptosis (Kreuzaler & Watson, 2012). Activity of BAX/BAK is influenced by BCL2 and BCLXL that reside in mitochondrial membrane through the prevention of the release of cytochrome C from mitochondria. Dephosphorylated BAD is the negative regulator of BCL2 and BCLXL that allows BAX/BAK to trigger apoptosis (Lindsay *et al*., 2011).

In extrinsic pathway, upon binding of ligands to tumour necrosis factor receptors (FAS, TRAIL, TNFR), caspase 8 will be recruited and activated. The activated caspase 8 can activate caspase 3 and 7 that execute apoptosis. Moreover, caspase 8 can interact with BID to trigger the activation of intrinsic apoptotic pathway (Kaufmann *et al*., 2012). The X-linked inhibitor of apoptosis protein (XIAP) has been found as a negative regulator of apoptosis which inhibits activated caspase 3, 7 and 9 (Kaufmann *et al*.,

2012). Rat sarcoma (RAS) and phosphatidylinositol 3-kinase (PI3K) pathways can promote cell survival through AKT and ERK that inhibit pro-apoptotic BAD (Britten, 2013; Massagué, 2004). Besides that, AKT can inhibit p53 from inducing the expression of pro-apoptotic BAX. This explains the reason of constitutive activation of RAS and PI3K pathways in majority of cancer cells.

2.3.2.2 Alternative regulated cell death

In addition to apoptosis, regulated necrosis has been regarded as alternative regulated cell death and it is also called necroptosis. In the past, necrosis was generally regarded as accidental cell death. Current studies have demonstrated that necrosis can be regulated and occurs in caspase-independent manner. Loss of plasma membrane integrity at early stage of cell death is the main feature of necrosis as opposed to apoptosis where plasma membrane permeabilisation occurs at late stage. To date, key players identified in regulated necrosis are RIPK3, RIPK1 and MLKL. The molecular mechanisms of regulated necrosis are still not completely understood (Galluzzi *et al*., 2015).

2.3.2.3 Cell cycle

Cell cycle consists of four phases, including G1, S, G2 and M phase. The main machinery in cell cycle are CDKs (CDK1 – 9), cyclins (cyclin A – T) and CDK inhibitors (p16, p21 and p27) (Schwartz & Shah, 2005). Hypophosphorylated RB can induce cell cycle arrest by binding and inhibiting E2F transcription factors that promote the expression of cyclins or CDKs. When RB is hyperphosphorylated, it will dissociate from E2F and promote the cell cycle progression (Giacinti & Giordano, 2006). G1 and G2 phases are important checkpoints in cell cycle, dysregulation of these checkpoints can promote carcinogenesis. At G1 phase, many signals including metabolic, genotoxic

and mitogenic signals will decide the commitment of cell cycle arrest (G0 phase), transition to S phase (DNA synthesis phase) and cell death. The G2 phase is the checkpoint between S phase and M phase (mitosis). At G2 phase, DNA replication error will be assessed and repaired before letting the cell to enter M phase. RAS and PI3K pathways can modulate cell cycle by influencing the concentration of cyclins and CDK inhibitors. Activation of ERK pathway *via* RAS will stabilise C-MYC transcription factor that can induce the expression of cyclin D1 and suppression on the expression of p21 and p27 (Massagué, 2004). Study showed that breast cancer cell lines expressed high level of cyclin D1 and could play an important role in the development of breast cancer (Buckley *et al*., 1993). Additionally, AKT of PI3K pathway can inhibit p53 (mediated by MDM2) and FOXO transcription factors that can induce the expression of p21 and p27 (Manning & Cantley 2007; Massagué, 2004). Therefore, both activated RAS and PI3K pathways are involved in promoting cell cycle progression.

2.4 Colorectal cancer (CRC)

Colon and rectum are part of the human digestive system. Large intestine or colon consists of cecum, ascending colon, transverse colon, descending colon, and sigmoid colon. The function of colon is to absorb nutrients and mainly water. The waste as feces will pass to rectum and stored temporary before defecation. Epithelial cells that line in the mucosa (first layer that exposed to lumen) of colon and rectum may undergo changes and become hyper-proliferative to form benign tumour called adenomatous poly (or adenoma). These adenomas have risk to develop into malignant tumour (carcinoma) which can undergo metastasis and spread to other parts of the body.

Chromosomal instability is common in CRC that lead to abnormal structure and number of chromosomes. This chromosomal instability can facilitate the loss of heterozygosity (LOH). LOH is the loss of one of the two parental alleles in the

chromosomes and usually happen to tumour suppressor genes such as adenomatous polyposis coli (APC), tumour protein p53 (TP53) and SMAD family member 4 (SMAD4). The most frequently allelic losses have been found in regions of chromosome 5q, 17p and18q (Fearon & Vogelstein, 1990). Like other type of cancer, mutation of oncogenes, tumour suppressor genes and genetic stability genes are also found in neoplastic cells of CRC.

2.4.1 Mutation of tumour suppressor genes

In CRC, tumour suppressor genes mutations predominate over oncogenes mutations. One copy of tumour suppressor gene is often deleted in chromosome while the other copy is in mutant form (inactivating codon). Mutated tumour suppressor genes such as APC, AXIN2, TP53, bone morphogenetic protein receptor type 1A (BMPR1A) and SMAD4 are inheritable and can be found in somatic mutation (Vogelstein & Kinzler 2004). Mutated APC gene can lead to familial adenomatous polyposis syndrome which has very high risk of developing CRC. The APC is a protein responsible for the degradation of β-catenin through the interactions with AXIN and glycogen synthase kinase 3 (GSK3β) in Wnt signalling pathway (Lüchtenborg *et al*., 2004). High concentration of β-catenin in Wnt pathway will increase stem cell related proliferation and cell-cell adhesion (Clevers & Nusse, 2012). The p53 is a transcription factor and it influences many targeted gene expressions. It acts as sensor for stress or DNA damage (error in DNA synthesis) that will activate the regulations in cell cycle (p21) and cell death process [phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1/NOXA), p53 upregulated modulator of apoptosis (PUMA), BAX] (Speidel, 2010; Yu *et al*., 2009). Genes such as transforming growth factor beta receptor 1 (TGFBR1), transforming growth factor beta receptor 2 (TGFBR2), SMAD family member 2 (SMAD2) and BAX are often found in somatic mutation and not inheritable

(Vogelstein & Kinzler 2004). These gene products are involved in transforming growth factor beta (TGFβ) signalling pathway. When TGFβ binds to TGFβ type-2 receptor, it will form complex with TGFβ type-1 receptor and carry out the phosphorylation of TGFβ type-1 receptor. Subsequently, downstream of SMAD2 and SMAD family member 3 (SMAD3) complex will be activated and interact with SMAD4 as transcription factors that stimulate cell cytostatic effect (Ikushima & Miyazono, 2010). Therefore, mutation in TGFβ pathway will promote cell cycle progression.

2.4.2 Mutation of oncogenes

Mutation of oncogenes can take place by the alterations either in exon or promoter region and amplification. In CRC, oncogenes such as catenin beta 1 (CTNNB1), BRAF, Kirsten rat sarcoma 2 (KRAS2), neuroblastoma RAS (NRAS), neurotrophic tyrosine receptor kinase 1 (NTRK1), neurotrophic tyrosine receptor kinase 3 (NTRK3) and phosphatidylinositol 3-kinase catalytic subunit alpha (PI3KCA) are often constitutively activated due to mutation in codon (Vogelstein & Kinzler, 2004). These oncogenes mentioned above are not inheritable and only found in somatic mutation. Mutated CTNNB1 gene encodes stabilised β-catenin that will not be targeted for proteosomal degradation. This mutation causes constitutive activation of Wnt pathway which promotes CRC. NTRK1 and NTRK3 genes encode tyrosine kinase receptors that can be activated by neuron growth factor (Alberti *et al*., 2003) while PDGFRA gene (inheritable mutated oncogene found in CRC) encodes alpha-type platelet-derived growth factor receptor (another type of tyrosine kinase receptor). These tyrosine kinase receptors can transduce signals to activate the downstream RAS and RAF proteins which are encoded by KRAS2, NRAS and BRAF genes. In turn, activated RAF protein can activate the ERK through phosphorylation cascades (Berridge, 2012). ERK pathway can lead to activation of C-MYC transcription factor for cell proliferation.

PI3KCA gene encodes PI3K protein which is upstream of AKT pathway. Activated AKT can indirectly activate mammalian target of rapamycin (mTOR) pathway for protein synthesis and protect cell from cell cycle arrest and apoptosis by inhibiting FOXO and BAD, respectively (Manning & Cantley 2007).

2.4.3 Mutation of genetic stability genes

Mutated genetic stability genes can be inherited and lead to syndromes which come with high risk of developing CRC. Mismatch repair (MMR), nucleotide-excision repair and base-excision repair genes are the genetic stability genes that repair the damaged DNA and error in DNA replication. The acquisition of mutated MMR genes such as mutS protein homolog 2 (MSH2), mutS protein homolog 6 (MSH6), mutL homolog 1 (MLH1) and postmeiotic segregation increased 2 (PMS2) lead to the syndrome of hereditary nonpolyposis colon cancer (HNPCC). Protein complex of MSH2 and MSH6 can bind to mismatched DNA. This binding will recruit another protein complex of MLH1 and PMS2. The interaction of both protein complexes will lead to excision of mismatched DNA and replacement of correct match of DNA (Hewish *et al*., 2010). Mutated MMR genes have been shown to increase the rate of mutation in cancer cells with or without wild type of MMR genes (Lengauer *et al*., 1998). Patients with HNPCC will develop CRC at rate of 80 %. Nearly 100 % of patients with mutated base-excision repair gene (mutY DNA glycosylase) can develop CRC at the age of 60 (Markowitz & Bertagnolli, 2009).

2.4.4 Current main drugs used for CRC treatment

The most common types of treatment used in CRC are surgery, radiotherapy and chemotherapy. In chemotherapy, most frequent used drugs are 5-fluorouracil (5-FU), capecitabine, irinotecan and oxaliplatin. 5-FU is an antimetabolite based drug that

targets DNA, RNA and protein synthesis of the cells. 5-FU (an analogue of uracil) can be converted to fluorouridine triphosphate (FUTP), fluorodeoxyuridine monophosphate (FdUMP) and fluorodeoxyuridine triphosphate (FdUTP). FUTP is the active metabolite that can be incorporated into RNA to disrupt RNA processing and function. FdUMP is the metabolite that inhibits thymidylate synthase to produce deoxythymidine monophosphate (dTMP) while FdUTP can be incorporated into DNA. Both FdUMP and FdUTP are the active metabolites which disrupt the DNA synthesis (Longley *et al*., 2003). The mutation status of TP53 and MMR genes of the cancer cells have been associated with the sensitivity to 5-FU (Adamsen *et al*., 2011). Due to variable bioavailability and rapid degradation, 5-FU needs to be administered intravenously. To overcome this problem, capecitabine is developed as an oral drug that will be metabolised to 5-FU by three enzymes. Carboxylesterase will convert capecitabine to 5'-deoxy-S-fluorocytidine and then to 5'-deoxy-S-fluorouridine (5-DFUR) by cytidine deaminase. Tumour cells that usually contain higher amount of thymidine phosphorylase can convert 5-DFUR to 5-FU, this can provide some selectivity against cancer cells (Walko & Lindley, 2005). Diaminocyclohexane (DACH) ligand containing oxaliplatin is the third-generation platinum based drugs such as *cis*-platin and carboplatin. Oxaliplatin is developed as alternative platinum based drug which offers lower toxicity and resistance compared to *cis*-platin (Hellberg *et al*., 2009; Virag *et al*., 2012). One of the mechanisms of action of these drugs is forming DNA adduct which can interfere with DNA replication and transcription. This DNA adduct can induce DNA strand breaks that associated with cell death (Raymond *et al*., 2002). Studies showed that oxaliplatin exerted cytotoxicity against both MMR-proficient and -deficient cells but *cis*-platin exhibited maximal cytotoxicity against MMR-proficient cells only (Fink *et al*., 1997; Nehmé *et al*., 1999).

2.5 Human cancer cell lines

Human tumour derived cell lines are valuable tools that are easy to acquire and maintain for endless usage in cancer research. The genetic alterations of cancer cell lines have been characterised to understand their malignant phenotypes, cancer development, cancer cell biology, responses to certain agents and drug resistance.

2.5.1 HCT 116 cell line

HCT 116 cell line is derived from poorly differentiated (lack of gland-like structure or normal specialised structure) colorectal carcinoma with Duke's D stage (Ahmed *et al*., 2013). The Duke's staging system is divided into four stages – A, B, C and D. Duke's A being the early stage and Duke's D is the late stage. In general, late stage and more aggressive tumour are often associated with poorly differentiated cells (Ueno *et al*., 2012). This cell line is popular for transfection and development of isogenic cell lines. Isogenic cell lines are pair of cell lines with similar genetic backgroup except one targeted gene which is altered by knock-in or knock-out techniques. This way can lead to insights on the functions of a specific gene. As example, HCT 116 -/- (homozygote, knock-out TP53 gene) is compared with parental HCT 116 +/+ (homozygote, wild-type TP53 gene) under the treatments of 5-FU. It revealed that p53 is required to activate the expression of p21 in HCT 116 +/+ while HCT 116 -/- cannot activate the expression of p21, as determined by western blot (Sur *et al*., 2009). High clonogenicity and lack of differentiation capacity have been shown to be the characteristics of this cell line (Yeung *et al*., 2010). This cell line acquires heterozygous mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as p16) gene, one allele with nonsense mutation and one methylated wild-type allele. Mutations of genes p14 (heterozygous, frameshift), CTNNB1 (heterozygous, deletion), KRAS (heterozygous, missense), PIK3CA (heterozygous, missense), MLH1

(homozygous, nonsense) and breast cancer gene 2 (BRCA2) a tumour suppressor gene that help repair damaged DNA also have been found in this cell line (Burri *et al*., 2001; Forbes *et al*., 2011; Ikediobi *et al*., 2006; Okamoto *et al*., 1994).

2.5.2 HCT-15 cell line

HCT-15 cell line is derived from well- to moderate differentiated Dukes' stage C colorectal adenocarcinoma (Dexter *et al*., 1979). Like most of the human cancer cell lines, this cell line has being xenograft tumour model in nude mice for research. This cell line acquires some mutations which include APC membrane recruitment protein 1 (AMER1) (homozygous, nonsense), APC (heterozygous, nonsense/frameshift), BRCA2 (heterozygous, frameshift), KRAS (heterozygous, missense), MSH6 (heterozygous, frameshift), PIK3CA (heterozygous, missense) and TP53 (heterozygous, missense) (Forbes *et al*., 2011; Ikediobi *et al*., 2006).

2.5.3 HT-29 cell line

HT-29 cell line is derived from Dukes' stage C colorectal adenocarcinoma (Ahmed *et al*., 2013). Under standard culture conditions, these cells will grow to multilayer with undifferentiated morphology. However, these cells appear to have intermediate differentiation capacity when culture in three dimensions conditions (Yeung *et al*., 2010). Most of the CRC specimens often have elevated expression of cyclooxygenase-2 (COX-2). The COX-2 and its main product prostaglandin E_2 which regulates inflammation and cell proliferation have been associated with the development of CRC (Greenhough *et al*., 2009). This cell line is a useful model for COX-2 related research because of high constitutive expression of COX-2 (Shao *et al*., 2000; Yazawa *et al*., 2005). It is also used as a model to study absorption and transport of potential therapeutic compounds in intestinal cells because of inducible differentiation capacity.

HT-29 cells can differentiate into enterocytes (polarised, brush border on the apical surface and connected by tight junctions) which mimic the intestinal cells when exposed to inducers such as forskolin, cholera toxin and hypertonic salt solution (Ophir *et al*., 1995). HT-29 cells are shown to be able to adapt and resist against 5-FU cytotoxicity through the activation of survival autophagy and the lack of wild type p53 protein. Thus, HT-29 cells can serve as a model for 5-FU resistance research (Sui *et al*., 2014). This cell line acquires some mutations which include APC (heterozygous, nonsense/frameshift), PIK3CA (heterozygous, missense), SMAD4 (homozygous, nonsense), TP53 (homozygous, missense) and BRAF (heterozygous, missense) (Forbes *et al*., 2011; Ikediobi *et al*., 2006).

2.5.4 SW480 cell line

SW480 cell line is derived from Dukes' stage B primary colorectal adenocarcinoma (Ahmed *et al*., 2013). This cell line is usually compared with SW620 cell line for the study of CRC progression and metastasis. SW620 cell line is derived from metastatic (lymph node) CRC of the same patient (Ghosh *et al*., 2011). This cell line acquires some mutations which include KRAS (homozygous, missense), APC (heterozygous, missense), TP53 (homozygous, missense), C-MYC (amplification) and SMAD4 (putative splicing mutation) (Gayet *et al*., 2001; Lüchtenborg *et al*., 2004; Plowman *et al*., 2006; Rochette *et al*., 2005; Woodford-Richens *et al*., 2001).

2.5.5 Caco-2 cell line

Caco-2 cell line is derived from colorectal adenocarcinoma (Gartel *et al*., 2000). Under standard culture conditions, these cells will grow to monolayer. Upon confluency, the cells can undergo differentiation to form polarised cells with apical membrane and basolateral membrane. The polarised cells can form microvilli structures on the apical

membrane surface which are the characteristics of normal enterocytes (Djelloul *et al*., 1997; Gartel *et al*., 2000). These properties of the cells have been used as *in vitro* model to study mechanisms of absorption and transport of compounds in intestine (Farrell *et al*., 2011). Basically, there are two routes of transportation – (1) paracellular where the compounds go across the monolayer through spaces between cells, (2) transcellular (active or passive) where the compounds go across monolayer through cell membrane. This cell line is also expressing many important drug efflux transporters such as P-glycoprotein, breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 2 (MRP2) (Taipalensuu *et al*., 2001). This *in vitro* model offers a way to predict the *in vivo* oral bioavailability of the compounds. As the expression of COX-2 in this cell line has been found to be constitutive (Kamitani *et al*., 1998), therefore, caco-2 cells are also suitable for COX-2 related CRC research. This cell line acquires some mutations which include TP53 (heterozygous, deletion), SMAD4 (homozygous, missense), APC (homozygous, missense) and CTNNB1 (heterozygous, missense) (Djelloul *et al*., 1997; Ilyas *et al*., 1997; Lüchtenborg *et al*., 2004; Woodford-Richens *et al*., 2001).

2.5.6 MCF7 cell line

MCF7 is one of the most frequent used breast adenocarcinoma cells besides MDA-MB-231 cells. In 1970, Sister Catherine Frances suffered from metastatic breast cancer where the breast cancer had spread to pleura and chest wall. Herbert D. Soule successfully developed MCF7 cell line from the pleural effusion of Sister Catherine Frances (Lee *et al*., 2015). MCF7 cell line is a popular and valuable model for the study of hormone receptor-positive breast cancer as the cells express estrogen, androgen, progesterone and glucocorticoid receptors (Lee *et al*., 2015). These cells depend on estrogen for *in vitro* optimal growth and *in vivo* tumour formation (Sommers *et al*.,

1990). MCF7 cells have contributed to the knowledge of estrogen receptors activation and growth stimulation, this is fundamentally important because most of the breast cancers are estrogen receptors positive. This cell line acquires mutation in CASP3 gene which is one of the effector of apoptosis. The CASP3 gene of MCF7 has a deletion that blocks the translation of CASP3 mRNA to caspase 3 (Jänicke *et al*., 1998). This cell line depends on other effectors such as caspase 6, 7 and 9 to carry out the apoptosis (Liang *et al*., 2001). MCF7 cell also acquires mutation in CDKN2A (homozygous, deletion) and PIK3CA (heterozygous, missense) (Forbes *et al*., 2011; Musgrove *et al*., 1995).

2.6 The Compositae family

Compositae (also known as Asteraceae) is the largest family of flowering plants and about 10 % of all flowering species is in this family (Rahman *et al*., 2008). In 1816, Cassini was the first who morphologically described this family by grouping it into tribes. Now, based on morphological and molecular data, this family is divided into two main groups, non-Asteroideae and Asteroideae (subfamily). Within these groups there are 12 subfamilies with 43 tribes. There are 11 subfamilies in non-Asteroideae group, which are Barnadesioideae, Stifftioideae, Mutisioideae, Wunderlichioideae, Gochnatioideae, Hecastocleidoideae, Carduoideae, Pertyoideae, Gymnarrhenoideae, Cichorioideae and Corymbioideae. Asteroideae is an important subfamily of Compositae with 20 tribes, including Senecioneae, Calenduleae, Gnaphalieae, Astereae, Anthemideae, Inuleae, Athroismeae, Feddeeae, Helenieae, Coreopsideae, Neurolaeneae, Tageteae, Chaenactideae, Bahieae, Polymnieae, Heliantheae, Millerieae, Madieae, Perityleae and Eupatorieae (Funk *et al*., 2009; http://www.compositae.org). The Senecioneae tribe is a major tribe of Compositae which consists of 170 genera with

3,500 species in the world. Senecioneae is distributed in South Africa, Central America, the Andes and Southeast Asia (Funk *et al*., 2009; Shi *et al*., 2011).

2.6.1 The *Gynura* genus

The genus of *Gynura* is one of the genera of Senecioneae tribe. In 1825, Cassini started to give descriptions on *Gynura* plant materials. To date, 44 species had been described in this genus of *Gynura*. The distribution of *Gynura* species is observed in Africa, Asia (especially Southeast Asia) and northern Australia. Many *Gynura* species had been cultivated for food, medicinal and horticultural purposes (Vanijajiva & Kadereit, 2011).

Scientific classification

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Compositae

Subfamily: Asteroideae

Tribe: Senecioneae

Genus: *Gynura*

2.6.2 Medicinal *Gynura* species

2.6.2.1 *Gynura bicolor*

G. bicolor is very popular in Asia especially Taiwan and Japan. It is also widely cultivated in China, Myanmar, and Thailand (Shi *et al*., 2011; Vanijajiva & Kadereit, 2011). The leaves of *G. bicolor* show reddish purple colour on the abaxial side and

green colour on the adaxial side. The aerial parts are often consumed and believed to confer a wide range of benefits such as anti-cancer, anti-hyperglycemic, antioxidant and anti-inflammatory effects (Hayashi *et al*., 2002; Li *et al*., 2009b; Lu *et al*., 2012; Wu *et al*., 2013). Traditionally, it has been used for post-labour recovery, blood circulation improvement, treatment of dysmenorrhea and hemoptysis (Li, 2006).

2.6.2.2 *Gynura procumbens*

The native geographic distribution of *G. procumbens* is tropical West Africa, India, China, Myanmar, Thailand, Malaysia, Philippines, Indonesia and Papua New Guinea (Vanijajiva & Kadereit, 2011). Traditionally, *G. procumbens* is used to treat fever, kidney disease, hypertension, diabetes and cancer. Previous studies showed that *G. procumbens* has wide range of bioactivities such as anti-herpes simplex virus (Nawawi *et al*., 1999), anti-hyperglycaemic (Akowuah *et al*., 2002; Algariri *et al*., 2013), anti-inflammatory (Iskander *et al*., 2002), anti-hyperlipidaemic (Zhang & Tan, 2000), antioxidant (Rosidah *et al*., 2008), anti-hypertension capabilities (Hoe *et al*., 2007; Kim *et al*., 2006) and cytotoxicity (Hew *et al*., 2013; Nurulita *et al*., 2012).

2.6.2.3 *Gynura pseudochina*

G. pseudochina is distributed in tropical Africa, China, Bhutan, India, Myanmar, Nepal, Sri Lanka, Thailand, and Indonesia (Shi *et al*., 2011). Traditionally, it is used against uterine hemorrhages, dysentery, inflammation, fever, herpes viral infection and breast tumour (Siriwatanametanon *et al*., 2010; Siriwatanametanon & Heinrich, 2011; Windono *et al*., 2012). Phytochemical analysis revealed the presence of pyrrolizidine alkaloids (senecionine and senkirkine), flavonol (rutin), phenolic acids (3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and chlorogenic acid). Siriwatanametanon and Heinrich (2011) showed that flavonol and phenolic acids are

responsible for NF-κB inhibitory effects of *G. pseudochina*. NF-κB inhibition was suggested to be one of the mechanisms which involved in anti-inflammation effects.

2.6.2.4 *Gynura divaricata*

G. divaricata is distributed in China and Vietnam (Vanijajiva & Kadereit, 2011). This plant is called 'Bai Bei San Qi' in Tradition Chinese Medicine. Traditionally, it is used to treat bronchitis, pulmonary tuberculosis, pertussis, sore eye, toothache, rheumatic arthralgia and diabetes (Chen *et al*., 2009). Some bioactivity studies showed that *G. divaricata* exhibited hypoglycemic (Jiang *et al*., 2009), α-glucosidase and α-amylase inhibitory (Chen *et al*., 2014; Wu *et al*., 2011), cytotoxic (Chen *et al*., 2009) and antioxidant activities (Wan *et al*., 2011a). Phytochemical investigations revealed the presence of pyrrolizidine alkaloids (Roeder *et al*., 1996), cerebrosides (Chen *et al*., 2009), flavonoids (quercetin and kaempferol glycosides), terpenoids (epi-friedelinol, β-sitosterol, stigmasterol), nucleosides (adenosine and uridine) (Hu *et al*., 2006) and phenolic acids (caffeoylquinic acid derivatives) (Chen *et al*., 2014; Wan *et al*., 2011b).

2.6.2.5 *Gynura japonica*

G. japonica which also known as *G. segetum* is distributed in Nepal, China, Taiwan, Japan, Thailand and Philippines (Shi *et al*., 2011; Vanijajiva & Kadereit, 2011). In Traditional Chinese Medicine, it is called 'Ju San Qi'. *G. japonica* was described by Li Shih Chen in literary work "A compendium of Chinese medicinal herbs" during Ming Dynasty (1590 A.D.) (Lin *et al*., 2003). It was used to treat blood circulation problem, hemoptysis, dissipate blood stasis, also for hemostasis, detumescence and antiplatelet aggregation effects (Lin *et al*., 2003; Roeder, 2000; Yang *et al*., 2009). Phytochemical studies revealed the presence of pyrrolizidine alkaloids (senecionine and senlciphylline) (Liang & Roeder, 1984; Yang *et al*., 2009), stigmastane steroids,

triterpenoids (friedelan-3-ol, friedelan-3-one, α-amyrin, β-amyrin, cycloarta-24(31)-en-3β-ol, lupeol and α-tocospirone), triterpenes (isoarborinol and arborinol), α-tocopheroids (α-tocospiro A and α-tocospiro B), benzoids (4-hydroxybenzaldehyde, methyl 4-hydroxybenzoate and 4-hydroxybenzoic acid), zhebeiresinol, lumichrome, cerebrosides and phenolic acids (syringic acid, vanillic acid, trans-*p*-hydroxycinnamic acid) (Lin *et al*., 2004; Zhu *et al*., 2013).

2.6.2.6 *Gynura elliptica*

G. elliptica which is called 'Lan Yu Mu Er Cai' in Chinese, is distributed in Taiwan, China and Philippines (Vanijajiva & Kadereit, 2011). According to Li (2006), it is used for flu, diuretic, antitoxic, anti-inflammatory, encephalitis and antiplatelet aggregation activity. Lin *et al*. (2000) isolated gynunone, gynunol, pyrrolizidine alkaloids (senecionine and senkirkine), chromanones (6-acetyl-2,2-dimethylchroman-4-one and 6-hydroxy-2,2-dimethylchroman-4-one), vanillin and syringaldehyde from the methanol extract of *G. elliptica* root.

CHAPTER 3: ANTIOXIDANT CAPACITY, CYTOTOXICITY AND ACUTE ORAL TOXICITY OF *G. BICOLOR* AND *G. PROCUMBENS*

3.1 Introduction

G. bicolor and *G. procumbens* are widely used in Malaysia as culinary cooked vegetables and believed to confer a wide range of benefits such as anticancer, anti-inflammatory, and possibly antihypertensive effects. There is limited information regarding the cytotoxic activity of both plants on human cancer cell lines, especially *G. bicolor*. The present study aimed to evaluate the total phenolic content, antioxidant activity, cytotoxic effect and acute oral toxicity of *G. bicolor* and *G. procumbens* leaves. The crude methanol and its fractionated extracts (hexane, ethyl acetate, and water) of both *Gynura* spp. were prepared prior to the bioactivity assessments. Folin-Ciocalteu's method was used for the measurement of total phenolic content of extracts, while three established testing systems were carried out to evaluate the antioxidant capacity, namely 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, metal chelating assay and β-carotene bleaching assay. These antioxidant assays have been widely used to determine the antioxidant capacities as these assays require standard equipments and deliver reproducible fast results. More than one antioxidant assays were performed in the present study because a single antioxidant assay is not sufficient to measure the various modes of action of antioxidants in a test sample. As *G. bicolor* and *G. procumbens* are believed to have anticancer properties especially against colon cancer by the locals, the cytotoxic activities of *G. bicolor* and *G. procumbens* extracts were investigated against five human colon cancer cell lines with varying molecular characteristics (HT-29, HCT-15, SW480, Caco-2 and HCT 116), a human breast adenocarcinoma cell line (MCF7), and a human normal colon cell line (CCD-18Co). To the best of our knowledge, this is the first report on the cytotoxic effect of *G. bicolor* on

colon and breast adenocarcinoma cell lines. The cells treated with cytotoxic extract were then subjected to acridine orange (AO) / ethidium bromide (EB) staining and annexin-V/ propidium iodide (PI) flow cytometry in order to assess the cell death and morphological changes. In view of the increasing popular consumption of *G. bicolor* and *G. procumbens*, acute oral toxicity assay was carried out in the present study to ensure that both *Gynura* spp. are safe for human consumption. Hence, the resulting information will certainly provide some scientific validation on the traditional use of locally grown grown *G. bicolor* and *G. procumbens* leaves.

The specific objectives of the present study were as follows:

i. To evaluate the cytotoxicity and antioxidant activities of locally grown *G. bicolor* and *G. procumbens* extracts
ii. To determine the safety of *G. bicolor* and *G. procumbens* extracts for consumption
iii. To assess the type of cell death and morphological changes induced by cytotoxic extract on selected cancer cells

3.2 Literature review

G. bicolor is locally known as 'Hong Feng Cai' (Chinese) and 'Sambung Nyawa Ungu' (Malay) in Malaysia. It is a spreading to erect parennial herb which can grow to 4 m in height with fleshy, glabrous and woody base stem. The leaves are lanceolate to elliptic with dark green on the upper surface and dark purple on the lower surface. This plant can propagate through stem and flowering but the latter is a rare event in herb plantation. The florets are in dark red to orange-yellow (Davies, 1978; Vanijajiva & Kadereit, 2011). The appearance of *G. bicolor* is shown in Figure 3.1.

Previous study by Hayashi *et al*. (2002) demonstrated that hot water extract of *G. bicolor* at 1 mg/ml could induce apoptosis in HL60 leukemic cells. It was suggested that

the hot water extract may contain anthocyanidins (pelargonidin, delphinidin, malvidin and malvidin-3-glucoside) and contributed to the cytotoxicity. The study by Lu *et al*. (2012) reported the antioxidant activities of *G. bicolor* juice evaluated by DPPH, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and superoxide radicals scavenging assays. The anti-inflammation activity of *G. bicolor* extracts had been demonstrated on human umbilical vein endothelial cells by decreasing the production of interleukin-6, tumour necrosis factor alpha (TNF-α), prostaglandin E_2 and cyclooxygenase-2 (COX-2) (Chao *et al*., 2015). Lastly, Li *et al*. (2009b) reported that the ethyl acetate and butanol extracts of *G. bicolor* could decrease the blood sugar level on normal and diabetic induced mice.

Figure 3.1: The appearance of *G. bicolor*

G. procumbens is locally known as ‘Sambung Nyawa’ in Malay and ‘Jian Wei Feng’ in Chinese. It is a scrambling to climbing herb with glabrous, fleshy to woody stem that can grow up to 5 m in range. The green leaves are elliptic to rhomboid and

glabrous. This plant can be propagated through flowering and stem cutting. The florets are yellow or orange-red (Vanijajiva & Kadereit, 2011). The appearance *G. procumbens* is shown in Figure 3.2.

Previous study reported that ethanol extract of *G. procumbens* could reduce the level of blood glucose, cholesterol and triglyceride in streptozotocin-induced diabetic rats when oral administered at 150 mg/kg (Zhang & Tan, 2000). Akowuah *et al*. (2002) showed the butanol fraction of *G. procumbens* which contained kaempferol glycosides and quercetin glycosides exhibited anti-hyperglycaemic effect in streptozotocin-induced diabetic rats when oral administered at 1g/kg. The report by Iskander *et al*. (2002) indicated that the ethyl acetate fraction of *G. procumbens* (0.75 mg/ear) could inhibit the inflammation in rats' ear induced by croton oil and the results were comparable to hydrocortisone (6 mg/ear). The water extract of *G. procumbens* was reported to inhibit the plaque formation of herpes simplex virus by 15 % at 100 μg/ml (Nawawi *et al*., 1999). The water extract and partially purified fraction also showed anti-hypertension capabilities on spontaneously hypertensive rats through nitric oxide production and angiotensin converting enzyme inhibition, respectively (Hoe *et al*., 2007; Kim *et al*., 2006). Lastly, ethyl acetate fraction of *G. procumbens* showed cytotoxicity on MCF7 and T47D breast cancer cells with IC_{50} values of 270 μg/ml and 64 μg/ml, respectively (Nurulita *et al*., 2012). In addition, protein fraction demonstrated cytotoxic effect on MDA-MB-231 breast cancer cells with IC_{50} value of 3.8 μg/ml (Hew *et al*., 2013).

Figure 3.2: The appearance of *G. procumbens*

3.3 Materials and methods

3.3.1 Chemicals and reagents

Gallic acid, butylated hydroxyanisole (BHA), DPPH, potassium ferricyanide, Folin-Ciocalteu phenol reagent, AO, EB, methylthiazolyldiphenyl-tetrazolium bromide (MTT), RPMI 1640 medium, McCoy's 5A medium, Eagle's Minimum Essential Medium (EMEM), sodium bicarbonate, *cis*-platin, carboxymethyl cellulose, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), foetal bovine serum (FBS), accutase, penicillin/streptomycin (100X), amphotericin B (250µg/ml) were purchased from Sigma-Aldrich company. Methanol, hexane and ethyl acetate were purchased from Fisher Scientific Company. FITC Annexin V Apoptosis Detection Kit I was purchased from BD Biosciences Company.

3.3.2 Plant samples collection and identification

Fresh leaves of *G. bicolor* and *G. procumbens* were collected from Seremban, Negeri Sembilan, Malaysia in February 2011. The identification of plant species was done by Dr Yong Kien Thai of Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia. Voucher specimens of *G. bicolor* (herbarium no: KLU47744) and *G. procumbens* (herbarium no: KLU47743) were deposited at the herbarium of Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

3.3.3 Preparation of extracts

The fresh leaves of *G. bicolor* and *G. procumbens* were washed, dried and ground to fine powder. The powder of plant sample was extracted with methanol at room temperature for three days. The methanol-soluble extract was filtered and evaporated with rotary evaporator (Buchi) to obtain dark green crude methanol extract. The insoluble sample was again extracted with methanol for two more times. The collected crude methanol extract was further extracted with hexane to obtain hexane-soluble extract and hexane-insoluble residue. Hexane-insoluble residue was further partitioned with ethyl acetate and water (1:1) to yield ethyl acetate and water extracts. The water soluble extract was then freeze-dried to obtain water extracts. The crude methanol and its fractionated extracts (hexane, ethyl acetate and water) of both *Gynura* spp. were kept in the dark at 4 °C for no more than one week prior to evaluation of total phenolic content, antioxidant effect, cytotoxic activity and acute oral toxicity.

3.3.4 Determination of total phenolic content

The phenolic content of extracts was determined by Folin-Ciocalteu method which was modified to accommodate the 96-well plate as described previously by

Sulaiman and Ooi (2012). Firstly, 25 µl of Folin-Ciocalteu reagent was added to 10 µl of extract solution (4 – 20 mg/ml in methanol or distilled water) in each well of 96-well plate. The mixture was incubated for five minutes at room temperature before adding 25 µl of 20 % (w/v) sodium carbonate. This was followed by addition of distilled water to make a final volume of 200 µl per well. The reaction mixture was further incubated for 30 minutes at room temperature before the absorbance reading was taken at 760 nm using a microplate reader (Thermo Scientific Multiskan GO). A standard curve was plotted using gallic acid (0 - 1000 mg/l). The absorbance value of extracts after subtraction of control (y) was translated into total phenolic content [mg/l of gallic acid equivalents (GAEs)] using gallic acid standard curve with the following formula: Total phenolic content = (y - 0.0496) / 0.001; R^2 = 0.9855. All the extracts were assayed in triplicate. The results were expressed as milligram of gallic acid equivalents per gram of extract (mg of GAEs/g of extract).

3.3.5 DPPH radical scavenging activity

DPPH radical scavenging activity of extracts was measured according to the method described by Sulaiman and Ooi (2012). Briefly, 150 µl of DPPH solution (0.3 mM) was added to 50 µl of extract (at various concentrations) in each well of 96-well plate before incubation for 30 minutes at room temperature. As for the blank, 50 µl of distilled water or methanol was added to the DPPH solution instead of extract. The absorbance value was read at 515 nm using a microplate reader (Thermo Scientific Multiskan GO) after incubation. BHA was used as a positive reference standard in the present study. All the extracts and positive reference standard were assayed in triplicate. The scavenging ability of the extract was expressed as IC_{50} value, the concentration at which 50 % of DPPH radicals were scavenged. The IC_{50} value was obtained by

extrapolating from the graph of DPPH radical scavenging activity (%) versus concentration of extract.

3.3.6 Metal chelating assay

The metal chelating assay was performed in 96-well plate based on the protocol described by Sulaiman and Ooi (2012). Briefly, 50 µl of extract (at various concentrations) was incubated with 5 µl of ferrous chloride tetrahydrate (2 mM) and 130 µl of deionised water in each well of 96-well plate. After 5 minutes incubation at room temperature, the reaction was initiated by addition of 15 µl ferrozine (5 mM). After 10 minutes, the absorbance value was measured at 562 nm using a microplate reader (Thermo Scientific Multiskan GO). EDTA was used as a positive reference standard for the experiment. All the extracts and positive reference standard were assayed in triplicate. The chelating effect of extracts was expressed as IC_{50}, which is the concentration at which 50 % of metal ions were chelated. The lower IC_{50} value indicates the stronger metal chelating ability of the extract.

3.3.7 β-Carotene bleaching assay

The β-carotene bleaching activities of extracts were measured as described by Murugan and Iyer (2012). Briefly, 5 µl linoleic acid and 42 µl Tween 20 were added into β-carotene solution (1 mg/ml in chloroform). After evaporation of chloroform, 10 ml of oxygenated distilled water was added and the mixture was vigorously shaken to form emulsion. The 200 µl of emulsion was then added to 50 µl extract (at various concentrations) in each well and incubated at 50 ^{o}C in dark. The absorbance value was read at 0 and 120 minutes at 470 nm. BHA was used as a positive reference standard in the present study. The rate of β-carotene bleaching (R) was calculated according to the equation: $R = [\ln (A_0/A_t)]/t$, where ln is natural logarithm, A_0 is absorbance reading at 0

minute, At is absorbance reading at 120 minutes. The antioxidant activity (%) was calculated in percentage of inhibition relative to control using equation: $[(R_{control} - R_{sample})/ R_{control}] \times 100$ %.

3.3.8 MTT cytotoxicity assay

Human colon cancer cell lines (HT-29, HCT-15, SW480, Caco-2 and HCT 116), one human breast adenocarcinoma cell line (MCF7) and one human normal colon cell line (CCD-18Co) were purchased from American Type Culture Collection (ATCC). HCT-15, SW480 and MCF7 cells were maintained in RPMI 1640 medium; HT-29 and HCT 116 cells in McCoy's 5A medium; Caco-2 and CCD-18Co cells in EMEM medium. All media were supplemented with 10 % FBS, 2 % penicillin/streptomycin (100X) and 1 % amphotericin B. Caco-2 cells were maintained in EMEM medium which was supplemented with 20 % FBS instead of 10 %. The cells were cultured at 37 ^{o}C in CO_2 incubator.

The MTT cytotoxicity assay was carried out as described previously by Mosmann (1983). All the extracts were firstly dissolved in DMSO (with the exception of water extract which dissolved in distilled water) to form stock solutions (20 mg/ml) before testing. Briefly, 3 x 10^3 cells were seeded into 96-well plate for 24 hours before treatment with various concentrations (1, 10, 25, 50, 75, 100, 200 µg/ml) of the extract. The final concentration of DMSO in each well was 0.5 % in 200 µl medium. Untreated cells were used as negative controls. Following 24, 48 and 72 hours of incubation, 20 µl of MTT (5 mg/ml) was added into each well and further incubated for another four hours. The medium was then removed and replaced with 200 µl DMSO. The absorbance was measured at 570 nm with 650 nm as background using a microplate reader (Thermo Scientific Multiskan GO). All the extracts were assayed in triplicate.

Cis-platin was used as a positive reference standard. IC_{50} value is the concentration of extract or positive reference standard that inhibits 50 % of the cells growth.

3.3.9 AO/EB double staining

Briefly, HCT 116 cells were seeded into 6-well plate (1 x 10^5 cells/well) for 24 hours before treatment with 20 µg/ml of cytotoxic ethyl acetate extract of *G. bicolor*. Cells treated with 0.5 % DMSO were used as untreated control. After incubation of 24 hours, 160 µl of AO/EB solution (one part of 100 µg/ml AO and one part of 100 µg/ml EB in PBS) was loaded into each well which containing 2 ml of medium. The cells were immediately visualised under inverted fluorescence microscope (Olympus IX73) with blue excitation mirror unit at 200x magnification. *Cis*-platin (20 µg/ml) was used as a positive reference in the present study.

3.3.10 Annexin-V/PI flow cytometry

Briefly, HCT 116 cells (2 x 10^5 cells) in 3 ml of medium were seeded in 60 mm culture dish. After 24 hours, HCT 116 cells were treated with 20 µg/ml of cytotoxic ethyl acetate extract of *G. bicolor*. Cells with 0.5 % of DMSO were used as untreated control. The cells were harvested using accutase after 24 hours of treatment. The cells were then washed and adjusted to 1 x 10^6 cells/ml in binding buffer from FITC Annexin V Apoptosis Detection Kit I. This was followed by addition of 10 µl annexin-V conjugated with FITC and 10 µl PI from the kit to 100 µl cells solution. After 15 minutes incubation, 10,000 cells were analysed by flow cytometer (BD FACSCanto II) using FACSDiva software. Cells treated with 20 µg/ml of c*is*-platin were used as positive control.

3.3.11 Acute oral toxicity assay

The study was performed on healthy male Sprague-Dawley rats (aged 8-12 weeks; mean body weight 209 g) which obtained from Laboratory Animal Center, Faculty of Medicine, University of Malaya. The weight variation in the rats did not exceed ± 20 % standard deviation (SD) of the mean weight. The animal research protocol was approved by Institutional Animal Care and Use Committee, University of Malaya (UM IACUC) before commencing the study [Ethics number: ISB/29/06/2012/SKS (R)].

The acute oral toxicity of *G. bicolor* and *G. procumbens* methanol extracts was conducted using the procedure described by OECD (2002) guideline 423 with some modification. The crude methanol extracts were firstly suspended in 0.3 % carboxymethyl cellulose suspension (vehicle). The male rats were randomly assigned to three treatment groups and one control group, with three rats for each group. The treatment groups were dosed at 300, 2000 and 5000 mg of crude methanol extract per kilogram of body weight, while the control group was administered with vehicle only. Rats were housed in stainless steel, wire-mesh cages in an experimental animal room (ventilated, 25 ^{o}C, 50-60 % humidity, 12 hours light/dark cycle). The rats had free access to water and food and were acclimatised to the room condition for five days before starting the experiment. The rats were fasted for 12 hours prior to dosing (access to water only). After fasting, the body weights of rats were recorded. The rats were dosed using a stainless steel ball-tipped gavage needle attached to an appropriate syringe. The volume of administration is 1 ml/100 g of body weight. After dosing, food was withheld for four hours before providing food to the rats. All rats were observed for mortality, signs of toxicity and behavioral changes at four hours after dosing and daily for 14 days. Individual weights were recorded from day 1 to 14. The experiment was performed twice for each dose.

3.3.12 Statistical analysis

The antioxidant data in present study were subjected to one-way analysis of variance (ANOVA) and the significance of the difference between the means was determined by Duncan's multiple range tests at 95 % least significant difference ($p < 0.05$). The IC_{50} values for cytotoxic activity were obtained by nonlinear regression using GraphPad Prism statistical software.

3.4 Results and discussion

3.4.1 Extraction yield of *G. bicolor* and *G. procumbens*

The yield of dried powder samples and methanol extracts of *G. bicolor* and *G. procumbens* are shown in Table 3.1. *G. procumbens* contained higher moisture content compared to *G. bicolor* as shown by the yield of dried powder of *G. procumbens* (7.92 %) and *G. bicolor* (9.55 %). The methanol extract yield was higher in *G. bicolor* (9.90 %) compared to *G. procumbens* (8.78 %) which was based on the weight of dried powders. The yield of fractionated extracts from methanol extracts in Table 3.2 and 3.3 showed that both plant species contained high amount of non-polar constituents as shown by the yield of hexane extracts of *G. bicolor* (38.30 %) and *G. procumbens* (34.00 %). The results showed that *G. procumbens* contained higher amount of semi-polar constituents than *G. bicolor* as shown by the yield of ethyl acetate extracts of both plants.

Table 3.1: Yield of methanol extracts of *G. bicolor* and *G. procumbens*

Plant	Sample / Extract	Weight (g)
G. bicolor	Fresh leaves	8049.20
	Dried and ground powder	768.70 (9.55 %)
	Methanol extract	76.10 (9.90 %)
G. procumbens	Fresh leaves	8469.70
	Dried and ground powder	670.80 (7.92 %)
	Methanol extract	58.90 (8.78 %)

Table 3.2: Yield of fractionated extracts of *G. bicolor*

Extract	Yield of extract (g) [extracted from 61.10 g of methanol extract]	Percentage (%)
Hexane	23.40	38.30
Ethyl acetate	1.40	2.29
Water	11.9	19.48

Table 3.3: Yield of fractionated extracts of *G. procumbens*

Extract	Yield of extract (g) [extracted from 41.23 g of methanol extract]	Percentage (%)
Hexane	14.00	34.00
Ethyl acetate	2.50	6.10
Water	7.00	17.00

3.4.2 Total phenolic content of *G. bicolor* and *G. procumbens* extracts

Phenolic compounds such as phenolic acids, flavonoids and tannins are the major determinant of antioxidant potentials in plants and they could be a natural source of antioxidants, anti-mutagenic and anti-tumour activities (Pellegrini *et al*., 2003). In the present study, Folin-Ciocalteu method was used to determine the total phenolic content of the extracts as it is rapid, reproducible, simple and convenient. In this assay, blue compound will be formed when Folin-Ciocalteu reagent is reduced. Phenolic

compounds are well known for electron and hydrogen atom transfer activity; therefore, Folin-Ciocalteu method is widely used to determine general phenolic content of plant samples (Huang *et al*., 2005).

As shown in Table 3.4, total phenolic content of ethyl acetate extract of *G. bicolor* was significantly higher than other extracts ($p < 0.05$). The total phenolic content of *G. bicolor* extracts in descending order was ethyl acetate > hexane > methanol > water, with the values varied from 0.28 to 10.87 mg of GAEs/g of extract. The ethyl acetate extract has the highest phenolic content with 10.87 mg of GAE/g of extract, followed by hexane (0.91 mg of GAE/g of extract), methanol (0.75 mg of GAE/g of extract) and water (0.28 mg of GAE/g of extract) extracts. Hexane extract usually contains high level of oil, wax and chlorophyll while water extract contains high sugar content. This could be the reason for lower phenolic content in both of the extracts. Phenolic compounds such as flavonols and poly-acylated anthocyanins had been identified in *G. bicolor* leaves in previous studies reported by Lu *et al*. (2010) and Shimizu *et al*. (2010a). These phenolic compounds may be present in the ethyl acetate extract and contribute to the high phenolic content of ethyl acetate extract of *G. bicolor* in the current study.

Overall, extracts of *G. procumbens* showed higher total phenolic content compared to extracts of *G. bicolor*. Similar with *G. bicolor*, ethyl acetate extract of *G. procumbens* showed the highest phenolic content (172.68 mg of GAE/g of extract) among all extracts tested (Table 3.5). The current results are in agreement with that obtained in a previous study by Rosidah *et al*. (2008) which reported ethyl acetate extract of *G. procumbens* had the highest phenolic content although the extraction method used by Rosidah *et al*. (2008) was different from that used in the current study. Similar phenolic content ranging from 67.18 to 67.59 mg of GAE/g of extract was observed in methanol and water extracts. The non-polar hexane extract which lacked of

polar and semi-polar phenolic constituents showed significantly the lowest phenolic content (22.03 mg of GAE/g of extract) among all the extracts tested.

Table 3.4: Total phenolic content of *G. bicolor* extracts

Extract	Concentration of total phenolics (mg of GAEs/g of extract)
Methanol	0.75 ± 0.02^{b}
Hexane	0.91 ± 0.04^{b}
Ethyl acetate	10.87 ± 0.22^{c}
Water	0.28 ± 0.02^{a}

GAEs, gallic acid equivalents; Values are expressed as mean ± standard deviation (n = 3), means with different letters (a-c) in the same column were significantly different ($p < 0.05$, ANOVA).

Table 3.5: Total phenolic content of *G. procumbens* extracts

Extract	Concentration of total phenolics (mg of GAEs/g of extract)
Methanol	67.59 ± 3.62^{b}
Hexane	22.03 ± 1.19^{a}
Ethyl acetate	172.68 ± 2.73^{c}
Water	67.18 ± 0.77^{b}

GAEs, gallic acid equivalents; Values are expressed as mean ± standard deviation (n = 3), means with different letters (a-c) in the same column were significantly different ($p < 0.05$, ANOVA).

3.4.3 DPPH radical scavenging activity of *G. bicolor* and *G. procumbens* extracts

Radical scavenging activity is very important due to the deleterious role of free radicals in food and biological systems (Bursal & Koksal, 2011). DPPH radical scavenging assay is a discolouration assay which evaluates the decrease of purple colouration by the addition of the antioxidant to a DPPH solution in methanol. The lower IC_{50} value indicates stronger ability of the extract to act as DPPH scavenger as lesser scavengers were required to achieve 50 % scavenging reaction.

In the present study, *G. bicolor* extracts were investigated through the free radical scavenging activity *via* their reactions with the stable DPPH radicals (Table 3.6). Ethyl acetate extract appeared to be the major contributor for antioxidant capacity of *G. bicolor* in DPPH assay as DPPH scavenging activity of ethyl acetate extract was significantly higher than other extracts ($p < 0.05$). The descending order of DPPH scavenging activity of *G. bicolor* extracts was ethyl acetate > methanol > hexane > water. The ethyl acetate extract exhibited the lowest IC_{50} value (0.53 mg/ml), followed by methanol, hexane and water extracts with IC_{50} values of 4.93, 11.15 and 13.57 mg/ml, respectively. This indicates that the ethyl acetate extract may contain antioxidative substances which have hydrogen-donating activity to scavenge DPPH radicals and able to terminate the chain reaction of free radicals.

Methanol extract of *G. procumbens* showed the highest DPPH radical scavenging activity (IC_{50} value of 1.13 mg/ml), followed by water (IC_{50} value of 1.51 mg/ml), hexane (IC_{50} value of 2.44 mg/ml) and ethyl acetate (IC_{50} value of 4.16 mg/ml) extracts, as shown in Table 3.7. To my surprise, ethyl acetate extract of *G. procumbens* which had highest phenolic content showed the lowest scavenging activity against DPPH radical. The hydroxyl group of phenolic constituents from ethyl acetate extract were expected to donate electron or hydrogen to convert DPPH radical to non-radical products (Bendary *et al*., 2013). Thus, semi-polar phenolic constituents of *G. procumbens* may not play a major role in scavenging DPPH-related radicals. The combination of semi-polar phenolics, polar phenolics and other constituents were found to be more effective in scavenging DPPH radical as obtained for methanol extract in the current study.

3.4.4 Metal chelating activity of *G. bicolor* and *G. procumbens* extracts

The metal ion chelating ability plays a significant role in antioxidant mechanism because it prevents the generation of reactive oxygen species (ROS) *via* Fenton reaction and the consequent oxidative damage (Srivastava *et al*., 2006). In the present study, the metal chelating ability of extracts was investigated by assessing the ability of the antioxidants to compete with the indicator ferrozine to complex with ferrous ion (Fe^{2+}) in solution. Metal chelating activity of *G. bicolor* ethyl acetate extract was significantly higher than other extracts ($p < 0.05$). The metal chelating activity of *G. bicolor* extracts in descending order was ethyl acetate > methanol > hexane > water, as shown in Table 3.6. The methanol, hexane and ethyl acetate extracts showed comparable metal chelating activity with IC_{50} values in the range of 3.80 - 4.90 mg/ml, while water extract on the other hand showed inhibitory only at 28.37 mg/ml. All the extracts showed low metal chelating capability as compared to EDTA, which was the positive reference standard. This could be possibly due to lack of chemical compounds that could act as metal chelators in the *G. bicolor* extracts.

Metal chelating activity of hexane extract of *G. procumbens* was significantly higher than the other extracts tested (Table 3.7). The metal chelating activity of *G. procumbens* extracts in descending order was hexane > methanol > ethyl acetate > water. This indicated that non-polar constituents of *G. procumbens* appeared to be the main contributor in chelating metal ions and preventing oxidative stress. However, all the extracts also demonstrated low metal chelating activity compared to EDTA, the positive standard reference.

Table 3.6: The IC_{50} values of *G. bicolor* extracts in DPPH radical scavenging activity and metal chelating assay

Extract	IC_{50} value (mg/ml)	
	DPPH radical scavenging activity	Metal chelating assay
Methanol	4.93 ± 0.15^{c}	4.10 ± 0.10^{b}
Hexane	11.15 ± 0.21^{d}	4.90 ± 0.00^{c}
Ethyl acetate	0.53 ± 0.01^{b}	3.80 ± 0.10^{b}
Water	13.57 ± 0.23^{e}	28.37 ± 0.40^{d}
BHA*	0.03 ± 0.00^{a}	-
EDTA*	-	0.04 ± 0.00^{a}

*Positive reference standard. Values are expressed as mean ± standard deviation (n = 3), means with different letters (a-e) in the same column were significantly different ($p < 0.05$, ANOVA).

Table 3.7: The IC_{50} values of *G. procumbens* extracts in DPPH radical scavenging activity and metal chelating assay

Extract	IC_{50} value (mg/ml)	
	DPPH radical scavenging activity	Metal chelating assay
Methanol	1.13 ± 0.01^{b}	4.59 ± 0.09^{c}
Hexane	2.44 ± 0.07^{d}	1.99 ± 0.08^{b}
Ethyl acetate	4.16 ± 0.04^{e}	11.37 ± 0.31^{d}
Water	1.51 ± 0.02^{c}	16.50 ± 0.36^{e}
BHA*	0.03 ± 0.00^{a}	-
EDTA*	-	0.04 ± 0.00^{a}

*Positive reference standard. Values are expressed as mean ± standard deviation (n = 3), means with different letters (a-e) in the same column were significantly different ($p < 0.05$, ANOVA).

3.4.5 β-Carotene bleaching activity of *G. bicolor* and *G. procumbens* extracts

The antioxidant activity of extracts was evaluated with coupled oxidation of β-carotene bleaching and linoleic acid. In β-carotene bleaching assay, oxidation of linoleic acid releases linoleic acid peroxide as free radicals that oxidise β-carotene and cause discolouration, which can be monitored spectrophotometrically. The presence of

antioxidants can reduce the β-carotene bleaching and neutralise the free radicals formed (Ikram *et al*., 2009).

As shown in Table 3.8, the antioxidant activity of *G. bicolor* extracts at highest concentration tested (20 mg/ml) in descending order was methanol > ethyl acetate > hexane > water. At the lower concentrations (4 and 8 mg/ml), ethyl acetate extract showed the highest antioxidant activity among all the extracts at 37.21 and 54.66 %, respectively. The result indicates that the antioxidative components in the methanol and ethyl acetate extracts of *G. bicolor* may hinder the extent of β-carotene bleaching by neutralising free radicals formed in the system.

Generally, antioxidant activity of *G. procumbens* extracts in β-carotene bleaching assay increased in a concentration-dependent manner (Table 3.9). Similar to *G. bicolor*, methanol and ethyl acetate extracts demonstrated significant higher antioxidant activity compared to hexane and water extracts. At 4 mg/ml, antioxidant activity of methanol extract was significantly higher than the other extracts tested (43.49 %). At 20 mg/ml, antioxidant activity of ethyl acetate extract was significantly higher than the other extracts (90.41 %). Methanol and ethyl acetate extracts may contain constituents that inhibited the co-oxidation of linoleic acid and β-carotene.

Table 3.8: The antioxidant activity (%) of *G. bicolor* extracts measured by β-carotene bleaching method

Extract	Concentration of extract (mg/ml)				
	4.0	8.0	12.0	16.0	20.0
Methanol	28.75 ± 6.08^{bv}	43.43 ± 6.33^{cw}	66.38 ± 4.96^{dx}	69.01 ± 2.49^{dx}	69.57 ± 3.84^{cx}
Hexane	29.69 ± 2.26^{bv}	33.06 ± 1.02^{bv}	36.43 ± 3.40^{bv}	45.88 ± 3.32^{cw}	61.33 ± 0.43^{bx}
Ethyl acetate	37.21 ± 0.90^{cv}	54.66 ± 4.46^{dw}	53.76 ± 4.47^{cw}	38.7 ± 1.27^{bv}	63.64 ± 5.00^{bx}
Water	11.69 ± 1.47^{av}	18.63 ± 0.60^{aw}	18.30 ± 2.30^{aw}	13.30 ± 2.97^{av}	46.15 ± 4.32^{ax}
BHA*	69.51 ± 1.46^{dv}	78.84 ± 1.53^{ew}	83.52 ± 1.10^{ex}	88.06 ± 1.44^{ey}	89.71 ± 1.54^{dy}

*Positive reference standard; Values are expressed as mean ± standard deviation (n = 3), means with different letters (a-e) in the same column were significantly different, means with different letters (v-z) in the same row were significantly different ($p < 0.05$, ANOVA).

Table 3.9: The antioxidant activity (%) of *G. procumbens* extracts measured by β-carotene bleaching method

Extract	Concentration of extract (mg/ml)				
	4.0	8.0	12.0	16.0	20.0
Methanol	43.49 ± 0.46^{dv}	50.27 ± 0.94^{cw}	52.70 ± 1.11^{cx}	61.05 ± 0.54^{cy}	80.10 ± 1.13^{cz}
Hexane	33.44 ± 0.39^{cv}	37.72 ± 0.93^{bw}	34.02 ± 0.86^{bv}	43.12 ± 1.35^{bx}	61.26 ± 0.66^{by}
Ethyl acetate	22.87 ± 1.45^{bv}	52.95 ± 1.47^{dw}	89.52 ± 0.90^{dy}	85.17 ± 1.31^{dx}	90.41 ± 1.80^{dy}
Water	18.04 ± 2.52^{aw}	16.85 ± 1.27^{aw}	15.56 ± 1.82^{aw}	7.39 ± 1.21^{av}	29.98 ± 0.00^{ax}
BHA*	69.51 ± 1.46^{ev}	78.84 ± 1.53^{ew}	83.52 ± 1.10^{ex}	88.06 ± 1.44^{ey}	89.71 ± 1.54^{dy}

*Positive reference standard; Values are expressed as mean ± standard deviation (n = 3), means with different letters (a-e) in the same column were significantly different, means with different letters (v-z) in the same row were significantly different ($p < 0.05$, ANOVA).

3.4.6 Cytotoxic activity of *G. bicolor* and *G. procumbens* extracts

It is generally known that the genetic background of cell lines could affect the sensitivity toward anticancer agent. In present study, the cytotoxic activity of extracts was evaluated against five human colon cancer cell lines with varying molecular characteristics (HT-29, HCT 116, HCT-15, SW480, Caco-2) and one human breast cancer cell line (MCF7) using the MTT cytotoxicity assay which measures the mitochondrial oxidoreductase activity of viable cells. The human normal colon cell line (CCD-18Co) was used to determine the specificity for cancerous cells. According to the United States National Cancer Institute plant screening program, a plant extract is considered cytotoxic if the IC_{50} value is 20 µg/ml or less after incubation between 48 to 72 hours (Lee & Houghtan, 2005).

The results of cytotoxicity screening of *G. bicolor* extracts against the selected human cell lines, expressed as IC_{50} value which averaged from three experiments, are summarised in Table 3.10. All the extracts did not show any cytotoxic effect (with IC_{50} values > 100 µg/ml against all the tested human cell lines), except ethyl acetate extract with cytotoxic sensitivity differed between the various human cancer cell lines. Among the tested cells, cytotoxicity of ethyl acetate extract was found to be most active against HCT 116 and HCT-15 cells, with IC_{50} values of 16.0 and 12.8 µg/ml after 24 hours of incubation; 20.9 and 18.7 µg/ml after 48 hours of incubation, respectively. HCT 116 and HCT-15 are colon cancer cell lines which have wild type p53 and both are mismatch repair (MMR) -deficient, while HT-29, SW480 and Caco-2 cells have mutant p53 and MMR-proficient (Mariadason *et al*., 2003). Based on the current result, it was speculated that the cytotoxicity of ethyl acetate extract was mediated by wild type p53 and MMR-deficient of colon cell lines. On the other hand, MCF7 cells which also expressing wild type p53 (Lu *et al*., 2001) showed lower cytotoxicity than HCT 116 and HCT-15 cells, this could be due to different tissue origin of cells. Generally, the tested

human cancer cell lines exhibited time-dependent response, by which IC_{50} values obtained were increased over time (24, 48 and 72 hours). The cytotoxic effect of ethyl acetate extract was reduced when the treatment duration increased. The tested carcinoma cell lines seem to be able to overcome the treatment inhibitory effect. The ethyl acetate extract may contain chemical constituents which stimulate the repair mechanisms of the cells to adapt and grow. Another explanation could be due to the stability of the active chemical constituents in ethyl acetate extract by which the chemical constituents may be degraded over time. As shown in Table 3.10, the cytotoxic effect of ethyl acetate extract on normal colon cells (CCD-18Co) was not detected (IC_{50} >100 µg/ml). This indicated that ethyl acetate extract was selectively toxic against cancerous cell lines. Thus, the active chemical constituents in the ethyl acetate extract may lead to valuable constituents that have the ability to kill cancerous cells but exert no damage to normal cells. The cytotoxic effect of ethyl acetate extract may be due to the presence of anthocyanins and flavonols which had been identified in previous studies (Hayashi *et al*., 2002; Lu *et al*., 2010; Shimizu *et al*., 2010a). Anthocyanins were reported to exhibited anti-proliferation effect on variety of carcinoma cell lines (Wang & Stoner, 2008). Flavonols such as quercetin and kaempferol were reported to have the ability to suppress the growth of HT-29, SW480 and HCT 116 cancer cells (Kim *et al*., 2010; Li *et al*., 2009a; Shan *et al*., 2009). As compared to other *Gynura* species, cytotoxic cerebroside (gynuraoside) which had been identified in ethyl acetate extract of *G. divaricata* (Chen *et al*., 2009) may be present in *G. bicolor* as well and thus contributed to the cytotoxic activity of the *G. bicolor* ethyl acetate extract.

To the best of our knowledge, this is the first report on the cytotoxicity of *G. procumbens* extracts on human colon cancer cells (HT-29, HCT 116, HCT-15, SW480, Caco-2) and human normal colon cells (CCD-18Co). All *G. procumbens* extracts were

considered not actively cytotoxic (IC_{50} > 20 µg/ml). However, ethyl acetate extract showed moderate cytotoxicity against the six tested cancer cell lines at 72 hours treatment (Table 3.11). At 24 hours treatment, ethyl acetate extract was more selective towards HT-29 and HCT 116 cells than other cancer cell lines with IC_{50} values of 35.7 and 42.6 µg/ml, respectively. Furthermore, in comparison with normal colon cells CCD-18Co, the selectivity index (SI) values of ethyl acetate extract against HT-29 and HCT 116 cells were > 5.6 and > 4.7, respectively. At this point, it is difficult to hypothesise the sensitivity of both cancer cell lines towards ethyl acetate extract because both cell lines have very different genetic makeup according to Catalogue Of Somatic Mutations In Cancer (COSMIC) database (Forbes *et al*., 2011). Ethyl acetate extract showed low cytotoxicity against CCD-18Co normal colon cells (IC_{50} values > 200 µg/ml), thus suggesting its medicinal efficacy which may selectively kill cancer cells. Previous phytochemical analysis revealed that *G. procumbens* leaves contained kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, rutin and quercetin-3-*O*-rhamnosyl(1-6)galactoside (Akowuah *et al*., 2002; Rosidah *et al*., 2008). Therefore, flavonols such as quercetin and kaempferol may be present in ethyl acetate extract and contributed to the cytotoxicity towards HT-29 and HCT 116 cells.

Table 3.10: Cytotoxic activity (IC_{50} values) of *G. bicolor* extracts against selected human cell lines

Extract	Treatment duration (hour)	Cytotoxicity (IC_{50}) in µg/ml						
		HT-29	**HCT 116**	**HCT-15**	**SW480**	**Caco-2**	**MCF7**	**CCD-18Co**
Methanol	24	>100	>100	>100	>100	>100	>100	>100
	48	>100	>100	>100	>100	>100	>100	>100
	72	>100	>100	>100	>100	>100	>100	>100
Hexane	24	>100	>100	>100	>100	>100	>100	>100
	48	>100	>100	>100	>100	>100	>100	>100
	72	>100	>100	>100	>100	>100	>100	>100
Ethyl acetate	24	39.7 ± 1.7	**16.0 ± 4.5**	**12.8 ± 5.3**	26.3 ± 1.2	32.7 ± 1.5	36.5 ± 3.4	>100
	48	49.5 ± 8.6	**20.9 ± 0.2**	**18.7 ± 1.9**	31.7 ± 5.7	46.3 ± 8.7	34.8 ± 0.8	>100
	72	37.3 ± 2.5	29.2 ± 0.9	21.2 ± 1.2	47.8 ± 3.6	55.2 ± 6.4	46.3 ± 4.2	>100
Water	24	>100	>100	>100	>100	>100	>100	>100
	48	>100	>100	>100	>100	>100	>100	>100
	72	>100	>100	>100	>100	>100	>100	>100
Cis-platin*	24	>12.5	12.0 ± 0.7	6.2 ± 0.4	>12.5	>12.5	11.2 ± 0.5	>12.5
	48	10.1 ± 0.2	4.0 ± 0.3	3.9 ± 0.2	6.8 ± 0.5	4.3 ± 0.4	4.2 ± 0.4	>12.5
	72	6.4 ± 0.6	2.9 ± 0.1	1.7 ± 0.4	3.2 ± 0.6	1.9 ± 0.2	2.7 ± 0.3	>12.5

*Positive reference standard. Values are expressed as mean ± standard deviation (n = 3).

Table 3.11: Cytotoxic activity (IC_{50} values) of *G. procumbens* extracts against selected human cell lines

Extract	Treatment duration (hour)	Cytotoxicity (IC_{50}) in µg/ml						
		HT-29	**HCT 116**	**HCT-15**	**SW480**	**Caco-2**	**MCF7**	**CCD-18Co**
Methanol	24	>100	>100	>100	>100	>100	>100	>200
	48	>100	>100	>100	>100	>100	>100	>200
	72	>100	>100	>100	>100	>100	>100	>200
Hexane	24	>100	>100	>100	>100	>100	>100	>200
	48	>100	>100	>100	>100	>100	>100	>200
	72	>100	>100	>100	>100	>100	>100	>200
Ethyl acetate	24	35.7 ± 1.2	42.6 ± 1.7	60.0 ± 6.6	55.7 ± 4.7	>100	81.5 ± 3.4	>200
	48	57.9 ± 3.9	47.9 ± 3.6	62.0 ± 2.0	57.3 ± 4.0	56.7 ± 8.6	61.6 ± 0.0	>200
	72	62.4 ± 8.4	66.1 ± 1.4	78.7 ± 1.2	65.2 ± 1.8	66.7 ± 5.5	58.3 ± 4.8	>200
Water	24	>100	>100	>100	>100	>100	>100	>200
	48	>100	>100	>100	>100	>100	>100	>200
	72	>100	>100	>100	>100	>100	>100	>200
Cis-platin*	24	>12.5	12.0 ± 0.7	6.2 ± 0.4	>12.5	>12.5	11.2 ± 0.5	>12.5
	48	10.1 ± 0.2	4.0 ± 0.3	3.9 ± 0.2	6.8 ± 0.5	4.3 ± 0.4	4.2 ± 0.4	>12.5
	72	6.4 ± 0.6	2.9 ± 0.1	1.7 ± 0.4	3.2 ± 0.6	1.9 ± 0.2	2.7 ± 0.3	>12.5

*Positive reference standard. Values are expressed as mean ± standard deviation (n = 3).

3.4.7 Apoptosis and necrosis evaluation by AO/EB staining and annexin-V/PI flow cytometry on ethyl acetate extract of *G. bicolor*

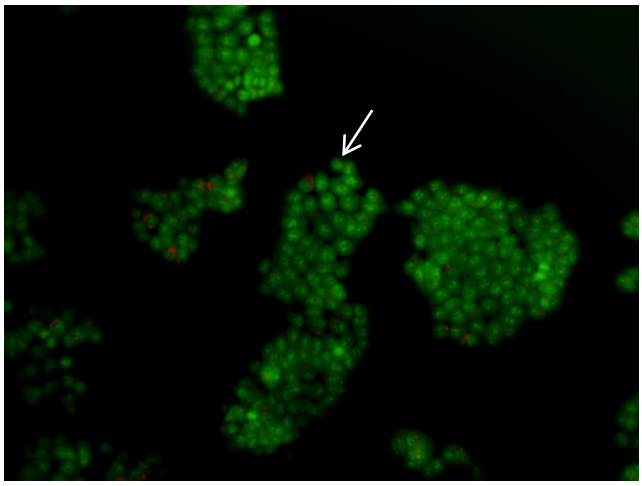
In cell death induction, apoptosis is usually more desired than necrosis as apoptosis is a normal well regulated process and does not often result in inflammation; while necrotic cell death is caused by external injury and almost always results in inflammation (Amaravadi & Thompson, 2007). However, necrotic cell death can be useful to manage apoptosis resistant tumours (Amaravadi & Thompson, 2007; Okada *et al*., 2004).

In the present study, AO/EB staining was used to assess the type of cell death and morphological changes induced by cytotoxic ethyl acetate extract of *G. bicolor*. AO can penetrate viable and non-viable cells and makes the nuclei appear green, while non-viable cells which lost the membrane integrity will take up EB that makes the nuclei appear red. Hence, viable cells will have green organised structure nuclei; early apoptotic cells will have green nuclei with condensed chromatin; late apoptotic cells will have orange to red nuclei with condensed chromatin (EB overwhelms AO); necrotic cells will show orange to red organised structure nuclei.

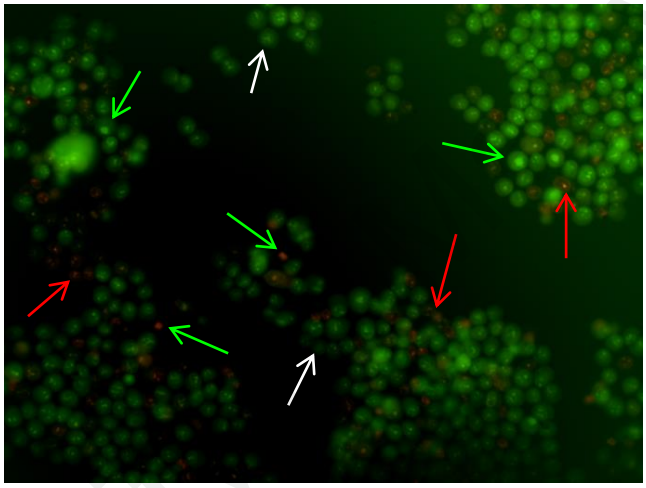
As shown in Figure 3.3, apoptotic and necrotic HCT 116 cells were detected when treated with ethyl acetate extract of *G. bicolor*. The ethyl acetate extract-treated HCT 116 cells were rounding up compared with untreated control cells. Chromatin condensation is one of the hallmarks of apoptosis. Green and red nuclei with condensed chromatin were observed in HCT 116 cells after 20 µg/ml of ethyl acetate extract treatment, indicating ethyl acetate could induce HCT 116 cells to undergo early and late apoptosis. Whilst, red nuclei with non-condensed chromatin observed in Figure 3.3 (B) indicated the necrotic cell death in HCT 116 cells as lack of chromatin condensation.

In order to further assess and confirm the type of cell death induced by cytotoxic ethyl acetate extract of *G. bicolor*, flow cytometry was used to analyse FITC annexin-V and PI stained cells as it can quantitatively determine the percentage of cells within a population that are undergoing early and late apoptosis or necrosis. During early apoptosis, phosphatidylserine (PS) is translocated from inner to outer leaflet of plasma membrane. The exposed PS could be detected by FITC conjugated annexin-V. In the event of late apoptosis and necrosis, loss of membrane integrity could be detected by PI.

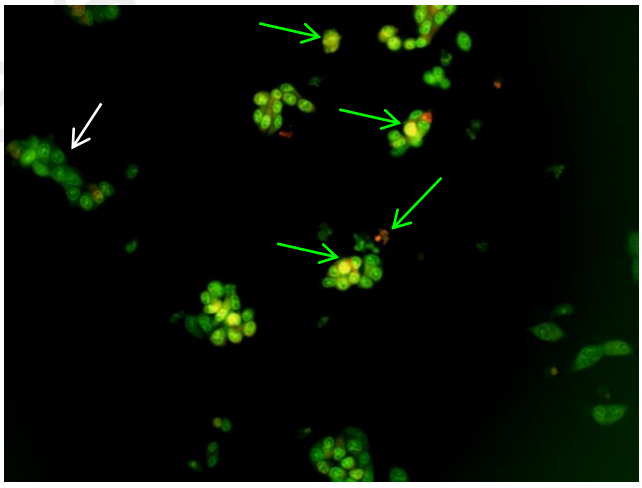
According to Figure 3.4, untreated HCT 116 cells showed negative staining of both annexin-V and PI (Q3; annexin V-/PI-), indicating viable cells. After treatment with 20 µg/ml of ethyl acetate extract, HCT 116 cells demonstrated an increase of necrotic cells (Q1; annexin V-/PI+) from 0.3 % (untreated control) to 57.2 %. Cells stained with both annexin-V and PI (Q2; annexin V+/PI+), which representing late apoptotic cells, were found to increase from 4.3 % (untreated control) to 10.5 % after treatment with the ethyl acetate extract. Taken together, the results clearly showed that the ethyl acetate extract appeared to induce late apoptosis and necrosis in HCT 116 cells.



(A) Untreated control



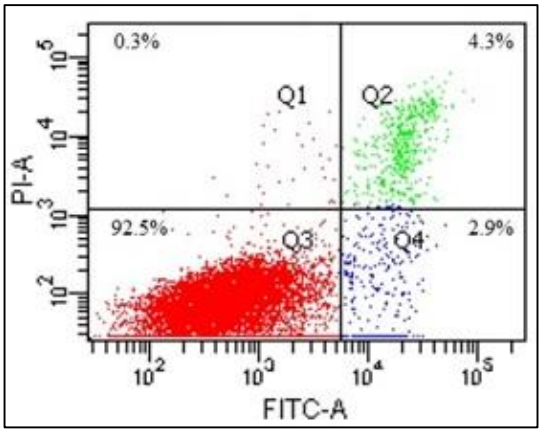
(B) 20 µg/ml of *G. bicolor* ethyl acetate extract treatment



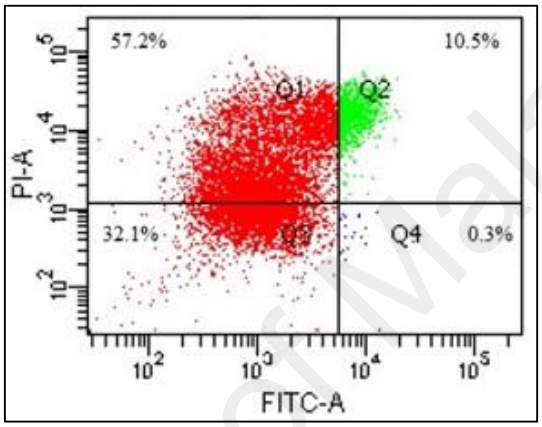
(C) 20 µg/ml of *cis*-platin treatment

Figure 3.3: HCT 116 cell death evaluation by AO/EB staining

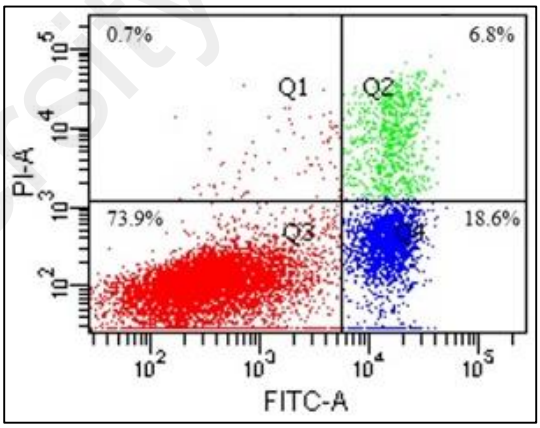
(A) Untreated control of HCT 116 cells at 24 hours. (B) HCT 116 cells treated with 20 µg/ml of *G. bicolor* ethyl acetate extract for 24 hours. (C) HCT 116 cells treated with 20 µg/ml of *cis*-platin for 24 hours. White arrows indicate live cells, green arrows indicate apoptotic cells and red arrows indicate necrotic cells. Magnification is 200x. Experiments were repeated twice with similar outcome.



(A) Untreated control



(B) 20 µg/ml of *G. bicolor* ethyl acetate extract treatment



(C) 20 µg/ml of *cis*-platin treatment

Figure 3.4: HCT 116 cell death evaluation by annexin-V/PI flow cytometry
(A) Untreated control of HCT 116 cells at 24 hours. (B) HCT 116 cells treated with 20 µg/ml of *G. bicolor* ethyl acetate extract for 24 hours. (C) HCT 116 cells treated with 20 µg/ml of *cis*-platin for 24 hours. Experiments were repeated twice with similar outcome.

3.4.8 Acute oral toxicity of *G. bicolor* and *G. procumbens* methanol extracts

Acute oral toxicity test was carried out in the present study to evaluate the safety of *G. bicolor* and *G. procumbens* methanol extracts for human consumption. Throughout the 14 days of observation period after oral administration, there were no deaths reported and no changes observed on the outer appearance (skin, fur, eyes, and mucous membranes) of treated rats which were dosed at 300, 2000 and 5000 mg of crude methanol extract of both plants per kilogram of body weight. The treated rats did not show any signs of toxicity (loss of appetite, vomiting, constipation, diarrhoea, dysphagia, hematemesis, hematochezia, tremors, convulsions, salivation and coma) and behavioral changes (hyperactivity, hypoactivity). The overall condition of treated rats was similar to untreated control rats. As shown in Table 3.12 and 3.13, the mean body weight of all treatment groups gradually increased over the observation period. Both plant methanol extracts were classified in category 5 according to Globally Harmonised Classification System for Chemical Substances and Mixtures (GHS) of OECD. Test sample in category 5 has relatively low acute oral toxicity and the expected lethal dose at 50 % (LD_{50}) is more than 5000 mg/kg. This is in agreement with Kennedy *et al*. (1986) which stated that test sample with LD_{50} more than 5000 mg/kg is considered as non-toxic. The current result of acute oral toxicity was in agreement with that of no *in vitro* toxicity was observed against the normal cell line and confirmed the safety of both plants for consumption. Previous study by Rosidah *et al*. (2009) also showed the same results for methanol extract of *G. procumbens* prepared using Soxhlet extraction.

Table 3.12: The effect of methanol extract of *G. bicolor* on rat body weight

Treatment dose (mg/kg)	Body weight (g)													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Control	183 ± 6	183 ± 10	187 ± 12	188 ± 10	192 ± 16	197 ± 16	195 ± 13	197 ± 12	198 ± 14	200 ± 17	205 ± 23	210 ± 20	212 ± 18	215 ± 18
300	218 ± 17	223 ± 16	223 ± 17	227 ± 16	230 ± 16	233 ± 18	233 ± 16	240 ± 18	243 ± 18	248 ± 19	249 ± 18	253 ± 17	256 ± 20	259 ± 21
2000	221 ± 24	223 ± 23	226 ± 24	228 ± 22	230 ± 23	231 ± 21	234 ± 22	237 ± 20	238 ± 22	241 ± 24	244 ± 23	246 ± 24	249 ± 25	252 ± 25
5000	200 ± 28	203 ± 27	204 ± 28	208 ± 26	210 ± 28	213 ± 28	218 ± 24	220 ± 25	223 ± 25	228 ± 23	230 ± 24	233 ± 24	238 ± 24	240 ± 25

Values are expressed as mean ± standard deviation (n =6).

Table 3.13: The effect of methanol extract of *G. procumbens* on rat body weight

Treatment dose (mg/kg)	Body weight (g)													
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Control	183 ± 6	183 ± 10	187 ± 12	188 ± 10	192 ± 16	197 ± 16	195 ± 13	197 ± 12	198 ± 14	200 ± 17	205 ± 23	210 ± 20	212 ± 18	215 ± 18
300	224 ± 24	225 ± 25	226 ± 26	230 ± 27	230 ± 27	233 ± 24	235 ± 25	239 ± 22	240 ± 24	243 ± 20	246 ± 22	251 ± 20	256 ± 22	259 ± 20
2000	195 ± 13	193 ± 16	193 ± 13	228 ± 45	200 ± 15	200 ± 15	203 ± 13	202 ± 10	207 ± 10	210 ± 10	213 ± 13	215 ± 10	220 ± 10	222 ± 13
5000	223 ± 21	226 ± 20	229 ± 20	231 ± 19	235 ± 20	238 ± 18	239 ± 20	243 ± 20	244 ± 20	247 ± 19	249 ± 20	253 ± 21	253 ± 21	257 ± 21

Values are expressed as mean ± standard deviation (n = 6).

3.5 Conclusion

The ethyl acetate extract with highest total phenolic content among the extracts of *G. bicolor* generally showed stronger ability in scavenging DPPH radicals, metal chelating and β-carotene bleaching inhibition. The current study clearly demonstrated for the first time that the ethyl acetate extract of *G. bicolor* strongly possessed cytotoxic activity against the human cancer cell lines and induced apoptotic and necrotic cell death, especially towards the HCT 116 and HCT-15 colon cancer cells. On the other hand, among the extracts of *G. procumbens*, methanol extract demonstrated better DPPH radical scavenging ability and β-carotene bleaching inhibition while hexane extract showed better metal chelating activity. It appears that crude methanol extract of *G. procumbens* works better as antioxidant compared to the fractionated extracts. The ethyl acetate extract of *G. procumbens* exhibited moderate cytotoxicity against cancer cells while low cytotoxicity against normal cells.

The acute oral toxicity indicated that both *G. bicolor* and *G. procumbens* have negligible level of toxicity when administered orally and regarded as safe in experimental rats. Taken together, the results indicated the potential benefits of *G. bicolor* and *G. procumbens* in prevention and treatment of colon cancer.

CHAPTER 4: PHYTOCHEMICAL INVESTIGATION OF *G. BICOLOR* LEAVES AND CYTOTOXICITY EVALUATION OF THE CHEMICAL CONSTITUENTS AGAINST HCT 116 CELLS

4.1 Introduction

Colorectal cancer (CRC) is the third most common cancer in the world and second highest mortality in the United States. Researchers are increasingly turning their attention to natural products for new leads to develop better drugs against CRC. Natural products have been a source of medicine by providing endless supply of bioactive chemical constituents.

As presented in Chapter 3, the ethyl acetate extract of *G. bicolor* possessed cytotoxicity and induced apoptotic and necrotic cell death on HCT 116 human colon cancer cells. Hence, in the present study, effort had been put into phytochemical investigation on the leaves and cytotoxicity evaluation of the chemical constituents. The cytotoxic ethyl acetate extract was further fractionated to explore potential cytotoxic chemical constituents. To our best knowledge, information on chemical constituents from *G. bicolor* water extract is limited. In order to add knowledge regarding chemical constituents in *G. bicolor*, the fractionation and isolation of chemical constituents from water extract of *G. bicolor* was carried out as well.

The specific objectives of the present study were as follows:

i. To isolate chemical constituents from cytotoxic ethyl acetate extract of *G. bicolor* through bioassay guided fractionation using chromatographic techniques

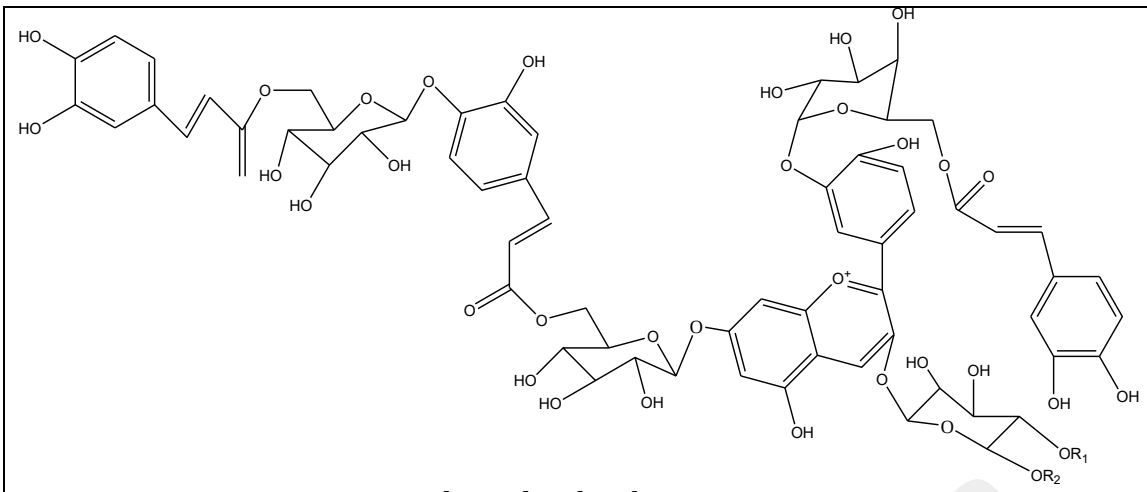
ii. To isolate chemical constituents from water extract of *G. bicolor* using chromatographic techniques

iii. To identify and elucidate the structures of the chemical constituents using spectroscopic methods

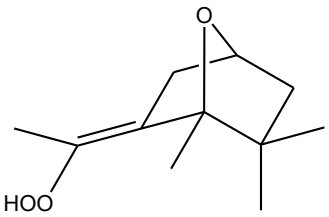
iv. To evaluate the cytotoxic activities of the identified chemical constituents against HCT 116 human colon cancer cells

4.2 Literature review

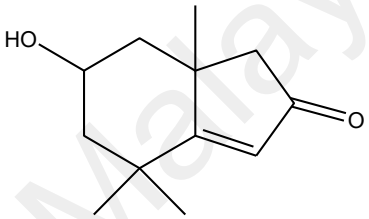
Previous phytochemical analysis of *G. bicolor* leaves revealed that the purple colour of the leaves was due to the presence of anthocyanins. Three major stable poly-acylated anthocyanins, namely bicolnin, bicolmalonin and rubrocinerarin were identified by Shimizu *et al*. (2010a) from leaves. The base structure of these anthocyanins was found to consist of a cyanidin (aglycone of anthocyanin), glucose moieties and caffeoyl moieties. Previous studies by Chen *et al*. (2012) and Lu *et al*. (2010) reported the isolation of quercetin, kaempferol, quercitrin, isoquercitrin, rutin, ficusic acid, loliolide, dehydrovomifoliol, vomifoliol, boscialin, (6*S*,9*S*)-roseoside, benzyl-β-D-glucopyranoside and 2-phenylethyl-β-D-glucopyranoside from *G. bicolor*. The volatile compounds of *G. bicolor* leaves were investigated by Lu *et al*. (2004) and Shimizu *et al*. (2009). The major volatiles were mono- and sesquiterpenes such as α-pinene, α-copaene, (*E*)-caryophyllene, α-humulene and bicyclogermacrene. In addition, Shimizu *et al*. (2010b) also isolated gynuradienol, hydroperoxy-gynuradiene, gynurenol, eudesm-11-en-4α-ol, intermedeol, alismol, alismoxide, (2*E*,6*E*)-3-isopropyl-6-methyl-10-oxoundeca-2,6-dienal and 1-tridecene-3,5,7,9,11-pentayne from the root of *G. bicolor*. The structures of chemical compounds isolated from *G. bicolor* in previous studies are shown in Figure 4.1.



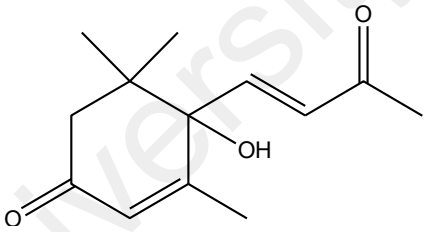
Poly-acylated anthocyanins



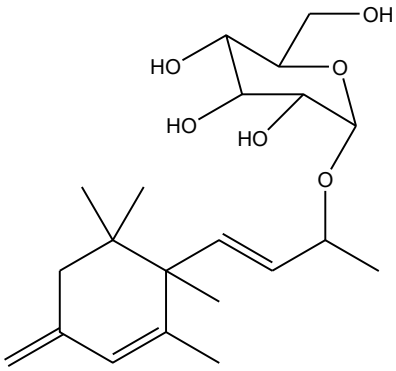
Ficusic acid



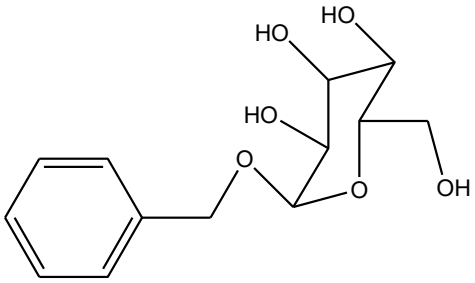
Loliolide



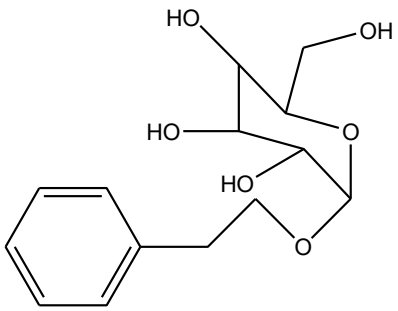
Dehydrovomifoliol



(6*S*,9*S*)-roseoside



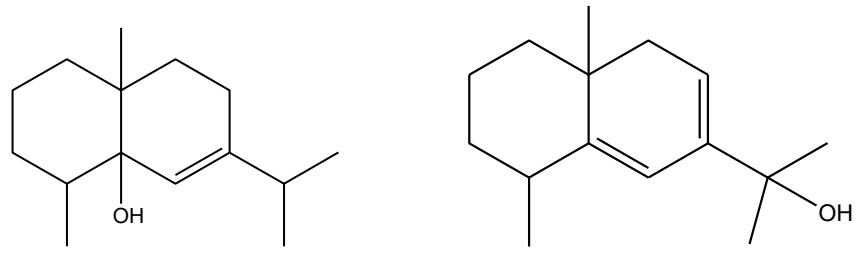
Benzyl-β-D-glucopyranoside



2-phenylethyl-β-D-glucopyranoside

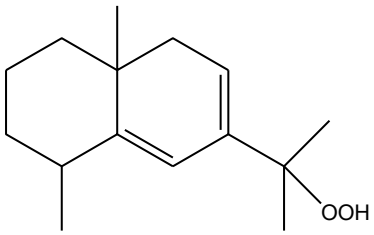
Figure 4.1, continued

Figure 4.1, continued

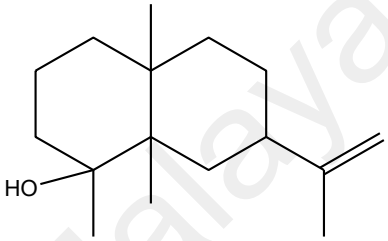


Gynurenol

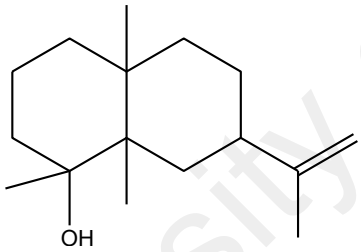
Gynuradienol



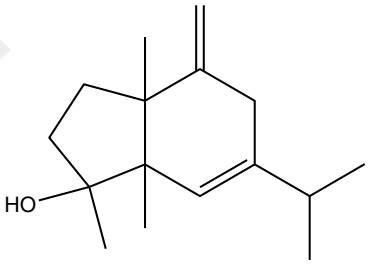
Hydroperoxy-gynuradiene



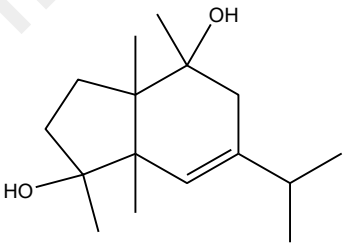
Eudesm-11-en-4α-ol



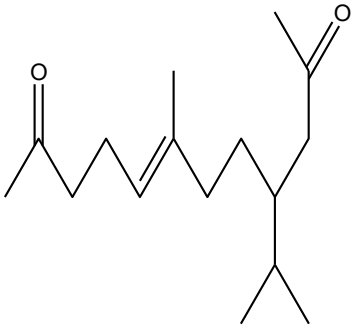
Intermedeol



Alismol



Alismoxide



(2*E*,6*E*)-3-isopropyl-6-methyl-10-oxoundeca-2,6-dienal

Figure 4.1: Compounds isolated from *G. bicolor* in previous studies

4.3 Materials and methods

4.3.1 Chemicals and reagents

Methylthiazolyldiphenyl-tetrazolium bromide (MTT), McCoy's 5A medium, Eagle's Minimum Essential Medium (EMEM), sodium bicarbonate, *cis*-platin, dimethyl sulfoxide (DMSO), foetal bovine serum (FBS), penicillin/streptomycin (100X), amphotericin B (250 µg/ml), deuterated methanol (CD_3OD) and deuterated DMSO (DMSO-d_6) were purchased from Sigma-Aldrich company. Methanol, hexane, ethyl acetate and acetonitrile solvents were purchased from Fisher Scientific Company.

4.3.2 Preparation of extracts

The fresh leaves of *G. bicolor* (20.0 kg) were washed, dried and ground to fine powder (1.8 kg, 9.0 %). The powder was extracted six times with methanol at room temperature yielding a dark green crude methanol extract (277.0 g, 15.4 %). The crude methanol extract (277.0 g) was further extracted with hexane to obtain hexane-soluble extract (85.1 g, 30.7 %) and hexane-insoluble residue. Hexane-insoluble residue was further partitioned with ethyl acetate and water (1:1) to yield ethyl acetate (15.0 g, 5.4 %) and water extracts (135.7 g, 49.0 %). The percentage yield of fractionated extracts was based on the weight of crude methanol extract used for fractionation.

4.3.3 Fractionation and purification of chemical constituents

The purification of chemical constituents was carried out using one or a combination of several chromatographic techniques such as column chromatography and thin layer chromatography (TLC). The choice of techniques depends mostly on the solubility and polarities of the compounds to be purified and isolated.

The ethyl acetate and water extracts of *G. bicolor* were fractionated and purified by a combination of column chromatography utilising MCI-gel CHP 20P (Supelco),

Chromatorex ODS (100–200 mesh, Fuji Silysia Chemical LTD) and Sephadex LH-20 (Amersham Biosciences). TLC was performed on pre-coated silica gel 60 F_{254} plates (0.2 mm thick, Merck) with solvent systems of chloroform:methanol:water (9:1:0.1; 8:2:0.2; 7:3:0.5; 6:4:0.8 v/v). Bands were visualised under UV at 254 nm and 365 nm and derivatised with 10 % sulfuric acid followed by heating.

Firstly, the ethyl acetate extract of *G. bicolor* (15.0 g) was fractionated by MCI-gel CHP 20P column as shown in Figure 4.2. Elution was performed using gradient aqueous methanol from 40 to 100 % (v/v) in increment of 10 % methanol. The gradient elution yielded ten fractions, namely E1 (5.9 g), E2 (1.8 g), E3 (2.1 g), E4 (1.7 g), E5 (1.9 g), E6 (0.6 g), E7 (0.5 g), E8 (0.4 g), E9 (0.1 g) and E10 (0.6 g). As shown in Table 4.1, fraction E9 exhibited the strongest cytotoxicity against HCT 116 cells with IC_{50} value of 39.7 $\pm$ 1.5 µg/ml after 72 hours of incubation. Thus, fraction E9 was further fractionated with Chromatorex ODS. Elution was performed using gradient aqueous methanol from 40 to 100 % (v/v) with increment of 5 % methanol. The gradient elution yielded six sub-fractions, E9.1 (3.7 mg), E9.2 (1 mg), E9.3 (4 mg), E9.4 (2.6 g), E9.5 (8.7 mg) and E9.6 (54.7 mg). The results of MTT cytotoxicity assay showed that E9.5 was the most cytotoxic active sub-fraction with IC_{50} value of 26.3 $\pm$ 5.7 µg/ml against HCT 116 cells (Table 4.2). However, further fractionation was not done on sub-fraction E9.5 because of the limited amount (8.7 mg) and there was multiple chemical constituents (at least five bands) detected on TLC plate. Hence, liquid chromatography-mass spectrometry (LC-MS) was carried out on E9.5 sub-fraction to detect any known chemical constituents (Figure 4.6 – 4.11).

Further fractionation of other ethyl acetate fractions was performed with gradient aqueous methanol from 0 to 100 % (v/v) in increment of 5 % methanol, or otherwise stated. As illustrated in Figure 4.3, fraction E2 (1.8 g) was fractionated by Sephadex LH-20 and thirteen sub-fractions (E2.1 - E2.13) were obtained. Sub-fraction

E2.5 (90.3 mg) was fractionated by Chromatorex ODS to obtain another six sub-fractions (E2.5.1 - E2.5.6). The sub-fraction E2.5.2 (48.4 mg) was chromatographed on Sephadex LH-20 with gradient aqueous methanol from 0 to 100 % (v/v) in increment of 10 % methanol and yielded constituent (**1**) (9.4 mg). Sub-fraction E2.10 (69.9 mg) was fractionated by Chromatorex ODS and yielded constituent (**2**) (7.6 mg), constituent (**3**) (15.2 mg) and constituent (**4**) (10.8 mg).

As shown in Figure 4.4, fraction E3 (2.1 g) was fractionated by Chromatorex ODS and elution was performed using gradient aqueous methanol from 0 to 100 % (v/v) in increment of 10 % methanol, which yielded eight sub-fractions (E3.1 - E3.8). Sub-fraction E3.4 (169 mg) was further fractionated by Sephadex LH-20 and yielded constituent (**2**) (14.2 mg) again. Sub-fraction E3.5 (1.2 g) was fractionated by Sephadex LH-20 to yield constituent (**5**) (107 mg). Sub-fraction E3.6 (575 mg) was chromatographed on Sephadex LH-20 and eight sub-fractions (E3.6.1 - E3.6.8) were obtained. E3.6.3 (23.2 mg) and E3.6.4 (71.3 mg) were fractionated by MCI-gel CHP 20P and afforded constituent (**4**) (46.2 mg) again. Sub-fraction E3.6.5 (184.2 mg) was fractionated by MCI-gel CHP 20P and yielded constituent (**6**) (22.2 mg).

As illustrated in Figure 4.5, the water extract of *G. bicolor* (135.7 g) was fractionated by MCI-gel CHP 20P with gradient aqueous methanol from 0 to 100 % (v/v) in increment of 10 % methanol and resulted sixteen fractions (W1 – W16). Constituent (**7**) (49.9 mg) was obtained as a precipitate from W4 fraction. Fraction W6 (1.6 g) was chromatographed on Sephadex LH-20 with gradient aqueous methanol from 0 to 100 % (v/v) in increment of 10 % methanol and yielded constituent (**8**) (51.2 mg).

4.3.4 Identification of chemical constituents

All the chemical constituents were identified using Bruker DRX 300 NMR spectrometer operating at 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR and DEPT-

135. The mass of the constituents were determined using Flexar FX15 UHPLC (Perkin Elmer) coupled to 3200 QTRAP (AB Sciex) or Accela ™ UHPLC coupled to LTQ Orbitrap (Thermo Fisher Scientific) fitted with an electrospray interface. The samples were chromatographed with Zorbax C_{18} (Agilent) or Hypersil GOLD RP C_{18} (Thermo Scientific). The solvent consisted of acetonitrile (A) and water with 0.1 % formic acid (B). The data were processed using Cliquid software or Qual browser.

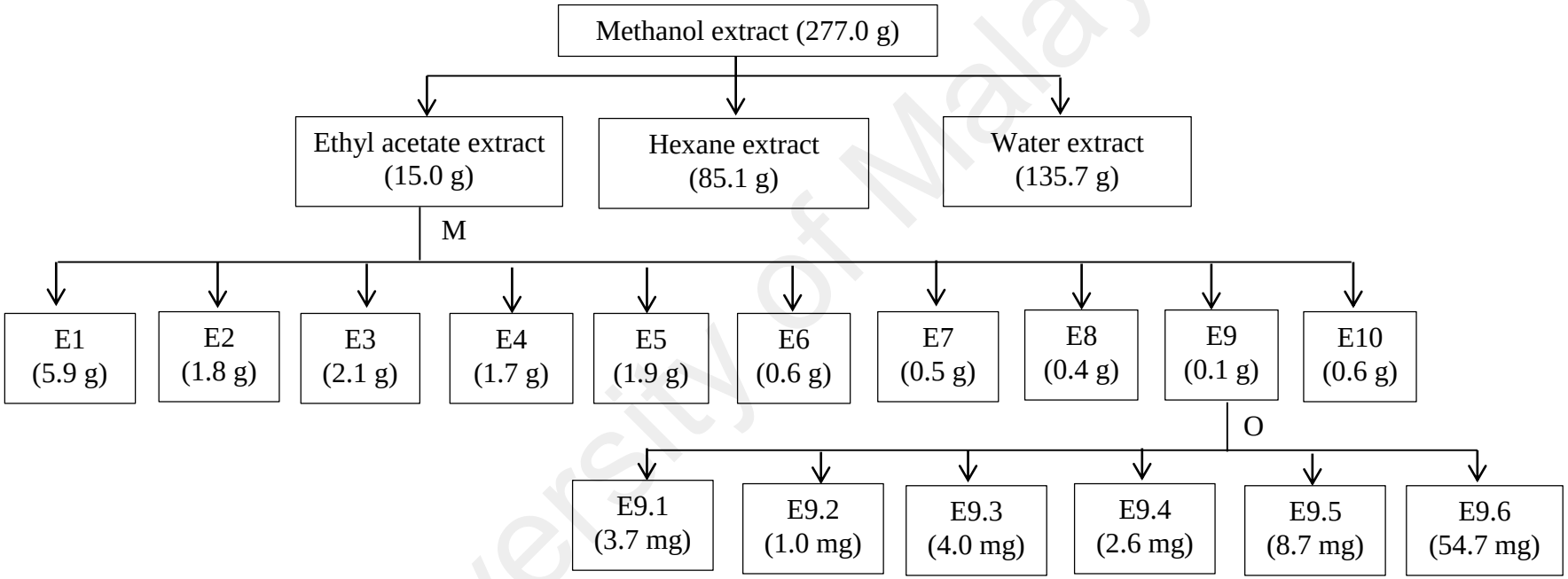


Figure 4.2: Fractionation and purification of *G. bicolor* extracts
M = MCI gel CHP 20P; O = Chromatorex ODS

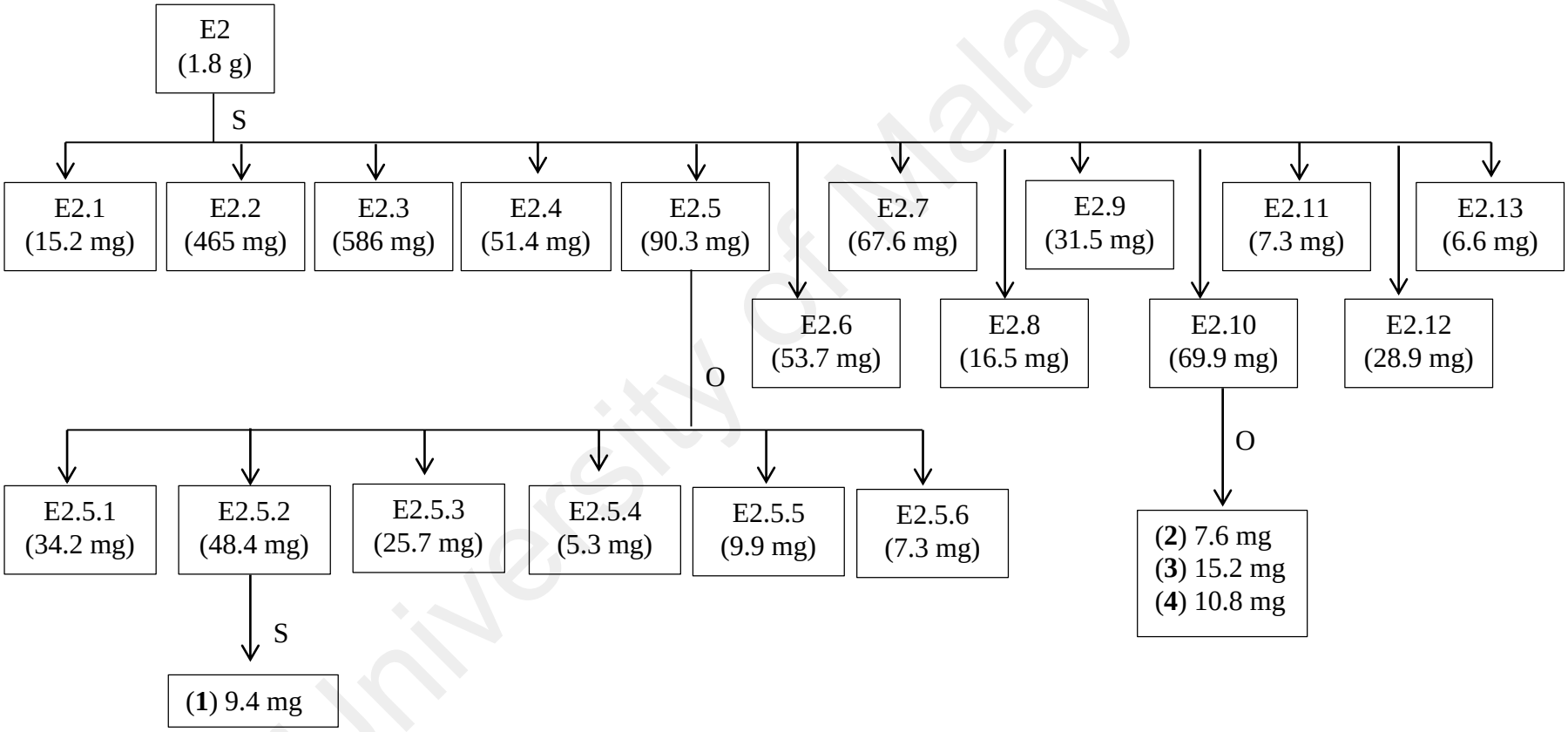


Figure 4.3: Purification and isolation of chemical constituents from fraction E2 of *G. bicolor* ethyl acetate extract

S = Sephadex LH20; O = Chromatorex ODS

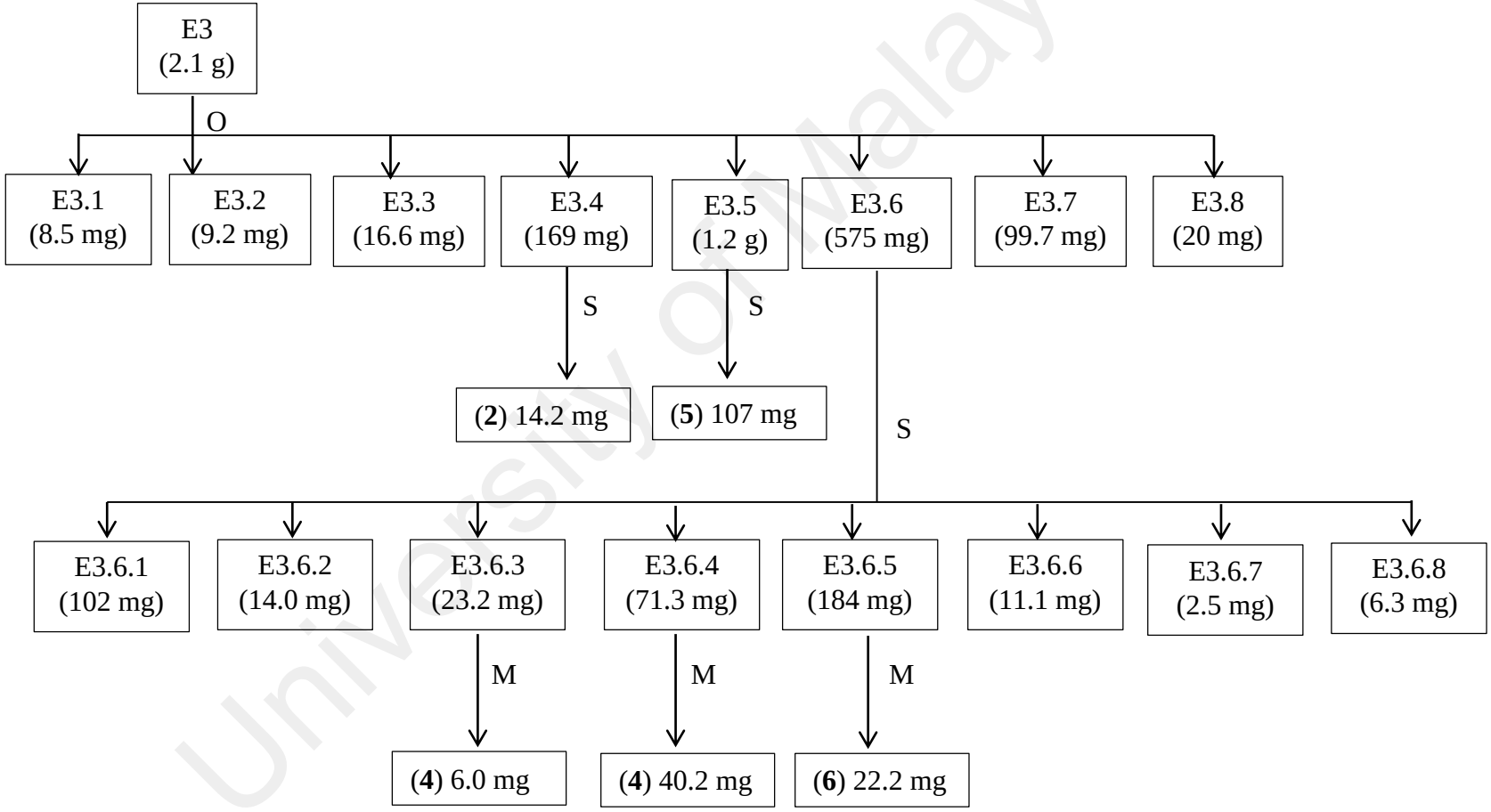


Figure 4.4: Purification and isolation of chemical constituents from fraction E3 of *G. bicolor* ethyl acetate extract
M = MCI gel CHP 20P; S = Sephadex LH20; O = Chromatorex ODS

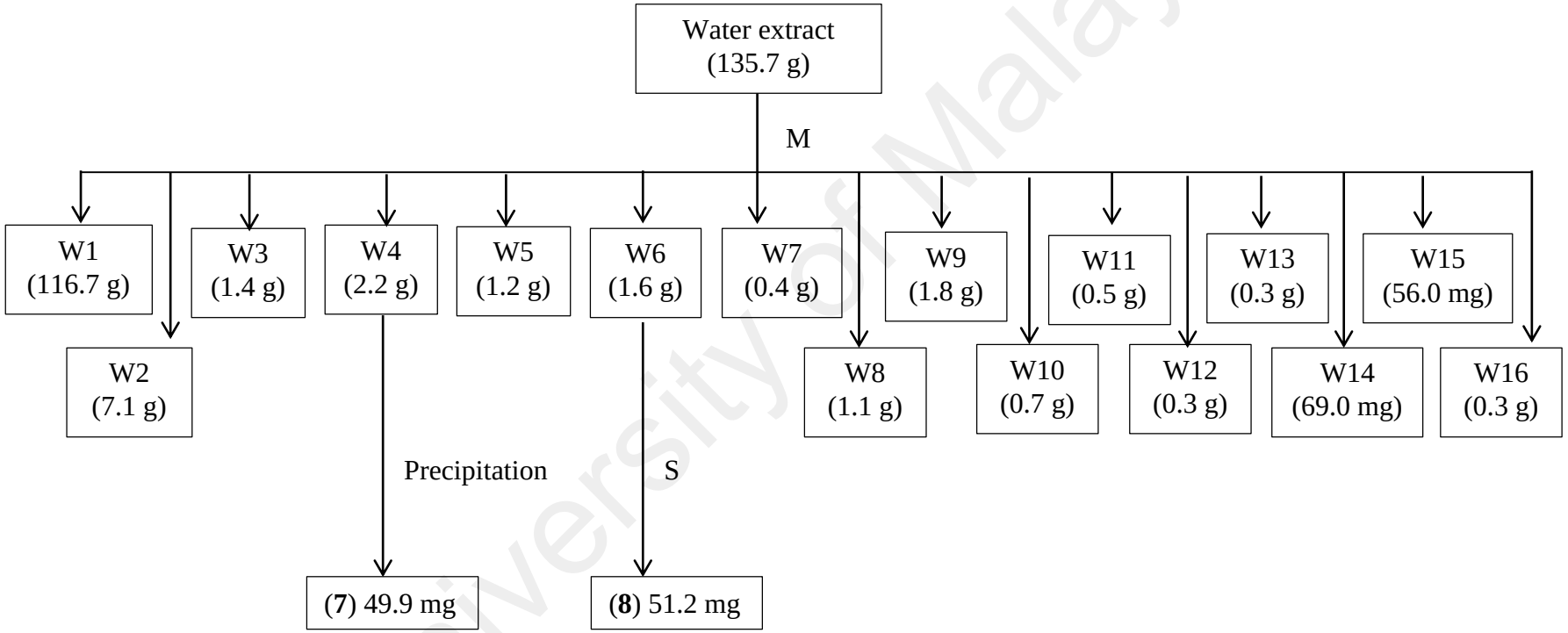


Figure 4.5: Purification and isolation of chemical constituents from water extract of *G. bicolor*
M = MCI gel CHP 20P; S = Sephadex LH20

4.3.5 MTT cytotoxicity assay

The cell culture and MTT cytotoxicity assay were carried out as described in section 3.3.8. Selectivity index (SI) was determined in present study to investigate the specificity of the chemical constituents to cancerous cells compared to normal cells. The SI of the tested constituent is defined as ratio of cytotoxicity on normal colon cells to colon carcinoma cells, which is IC_{50} on CCD-18Co cells/IC_{50} on HCT 116 cells. The isolated constituents which did not show cytotoxic effect on HCT 116 colon carcinoma cells (with IC_{50} values > 100 µg/ml) were not tested against CCD-18Co normal colon cells.

4.4 Results and discussion

4.4.1 Bioassay guided fractionation of *G. bicolor* ethyl acetate extract

Bioassay guided fractionation of the cytotoxic ethyl acetate extract of *G. bicolor* was carried out in current study. As shown in Table 4.2, sub-fraction E9.5 exhibited the strongest cytotoxic activity against HCT 116 cells. Due to the limited amount of sample E9.5 (8.7 mg), LC-MS was carried out to detect the presence of chemical constituents. The LC-MS data showed five major peaks, the retention time and *m*/*z* $[M-H]^-$ are showed in Table 4.3 and Figure 4.6 – 4.11. The MS spectral data showed that chemical constituents at peak 1 and 4 may be isomers (compare Figure 4.7 and 4.10). Chemical constituents at peak 2 and 5 also showed similar fragmentation pattern but different retention time suggesting isomeric constituents (compare Figure 4.8 and 4.11). Unfortunately, none of these five constituents were able to be tentatively identified.

Table 4.1: Cytotoxicity of fractions E1-E10 against HCT 116 cells

Fractions	IC_{50} (μg/ml)
E1	>100
E2	>100
E3	>100
E4	>100
E5	>100
E6	>100
E7	>100
E8	71.0 ± 9.3
E9	**39.7 ± 1.5**
E10	72.0 ± 11.8

HCT 116 cells were treated with ethyl acetate fractions of *G. bicolor* for 72 hours. Values are expressed as mean ± standard deviation (n = 3).

Table 4.2: Cytotoxicity of sub-fractions E9.1-E9.6 against HCT 116 cells

Fractions	IC_{50} (μg/ml)
E9.1	>100
E9.2	>100
E9.3	>100
E9.4	42.3 ± 20.0
E9.5	**26.3 ± 5.7**
E9.6	36.7 ± 2.1

HCT 116 cells were treated with ethyl acetate sub-fractions of *G. bicolor* for 72 hours. Values are expressed as mean ± standard deviation (n = 3).

Table 4.3: LC-MS data of sub-fraction E9.5

Peak	Retention time (minutes)	$[M-H]^-$ (*m/z*)
1	7.11	303
2	8.77	345
3	9.39	657
4	10.0	303
5	10.55	345

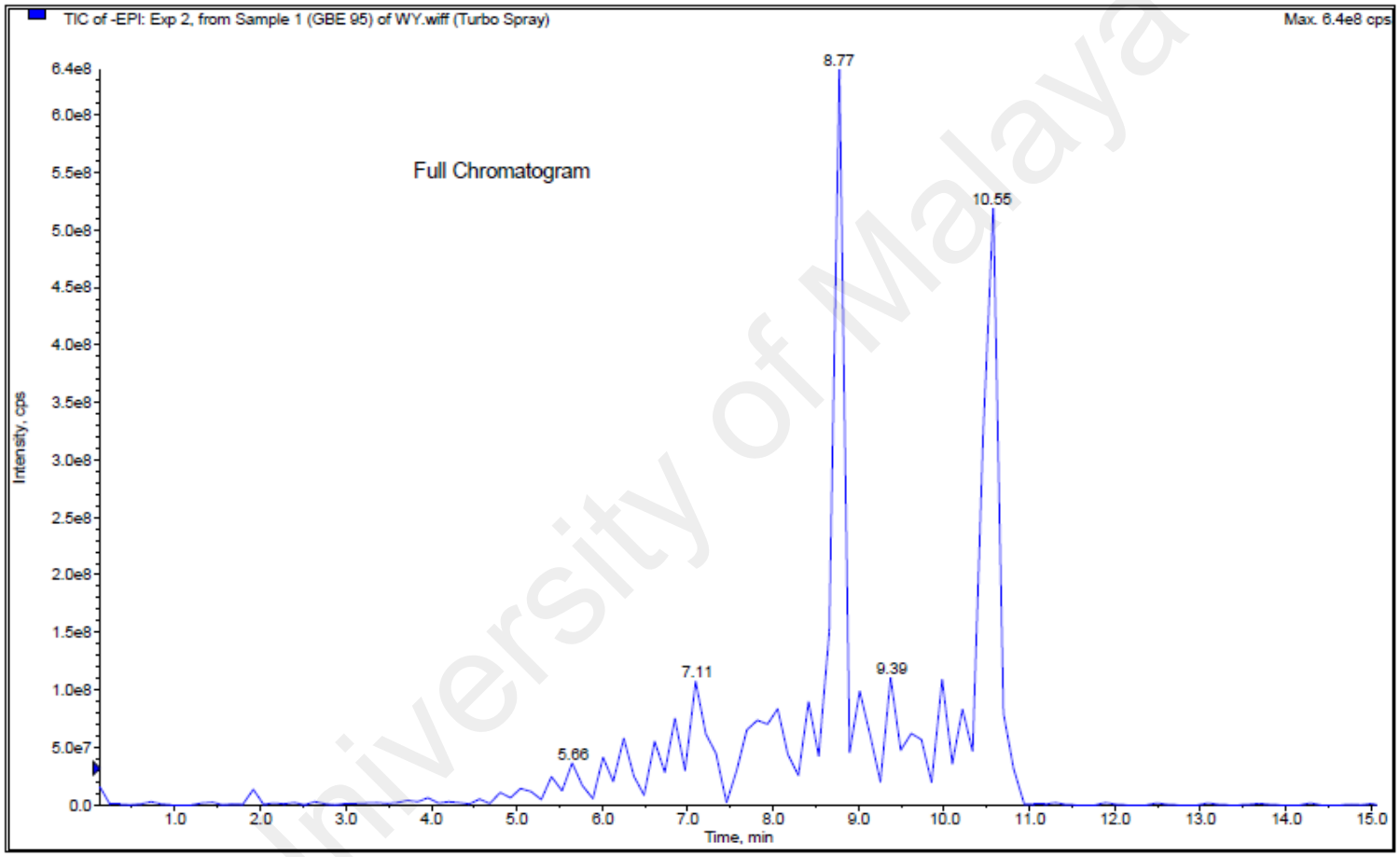


Figure 4.6: Liquid chromatogram of E9.5 sub-fraction

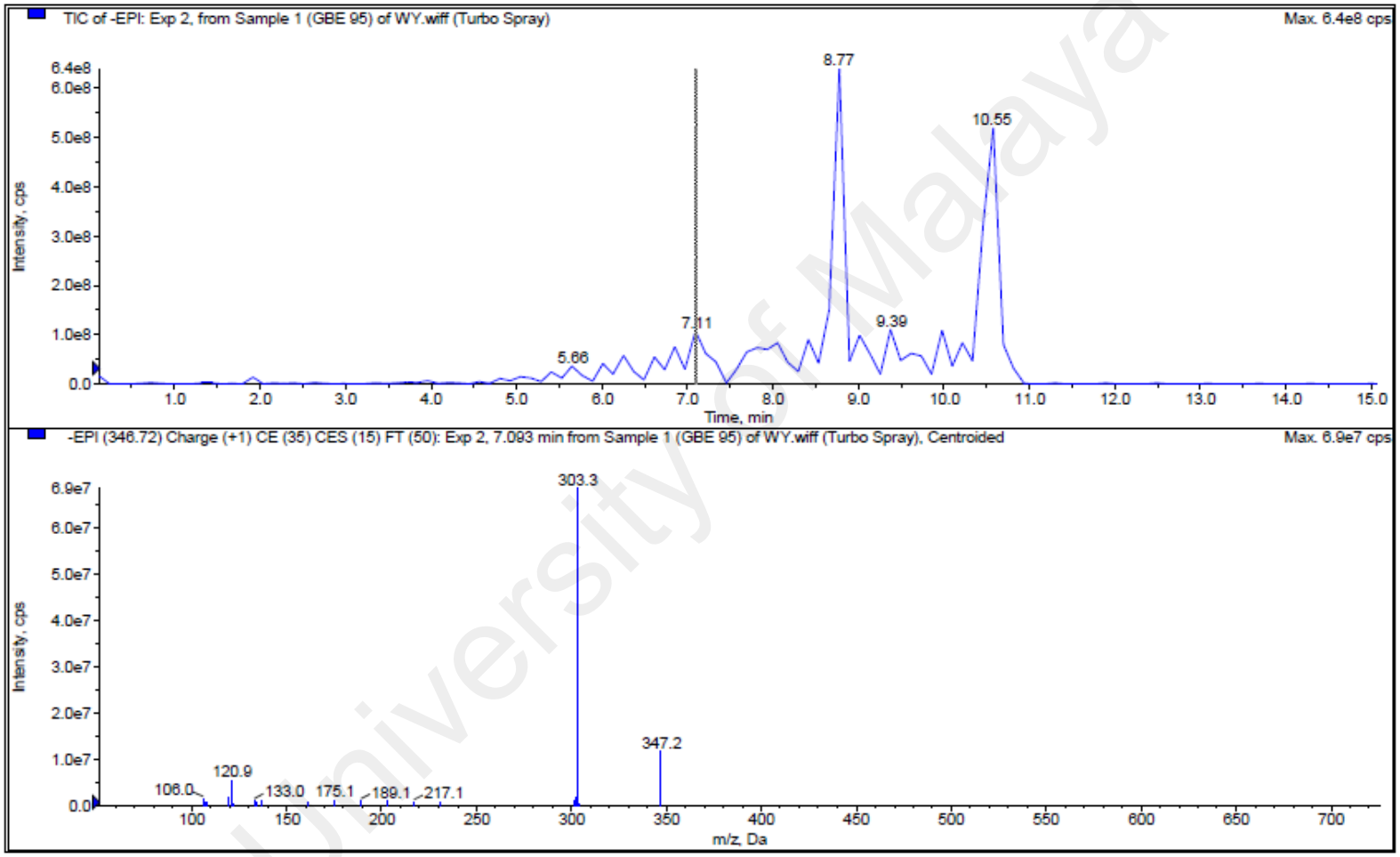


Figure 4.7: MS of peak 1 of E9.5 sub-fraction

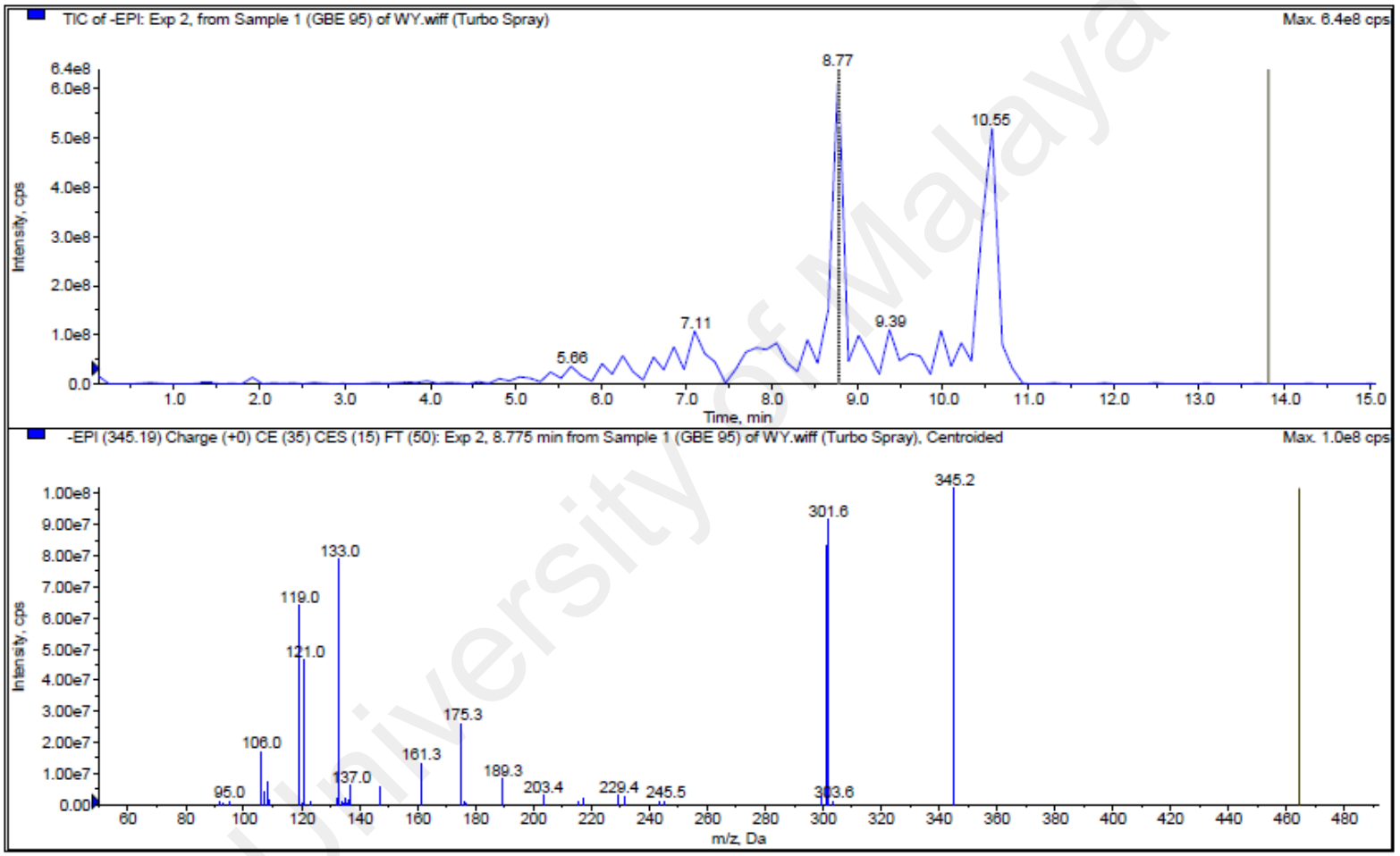


Figure 4.8: MS of peak 2 of E9.5 sub-fraction

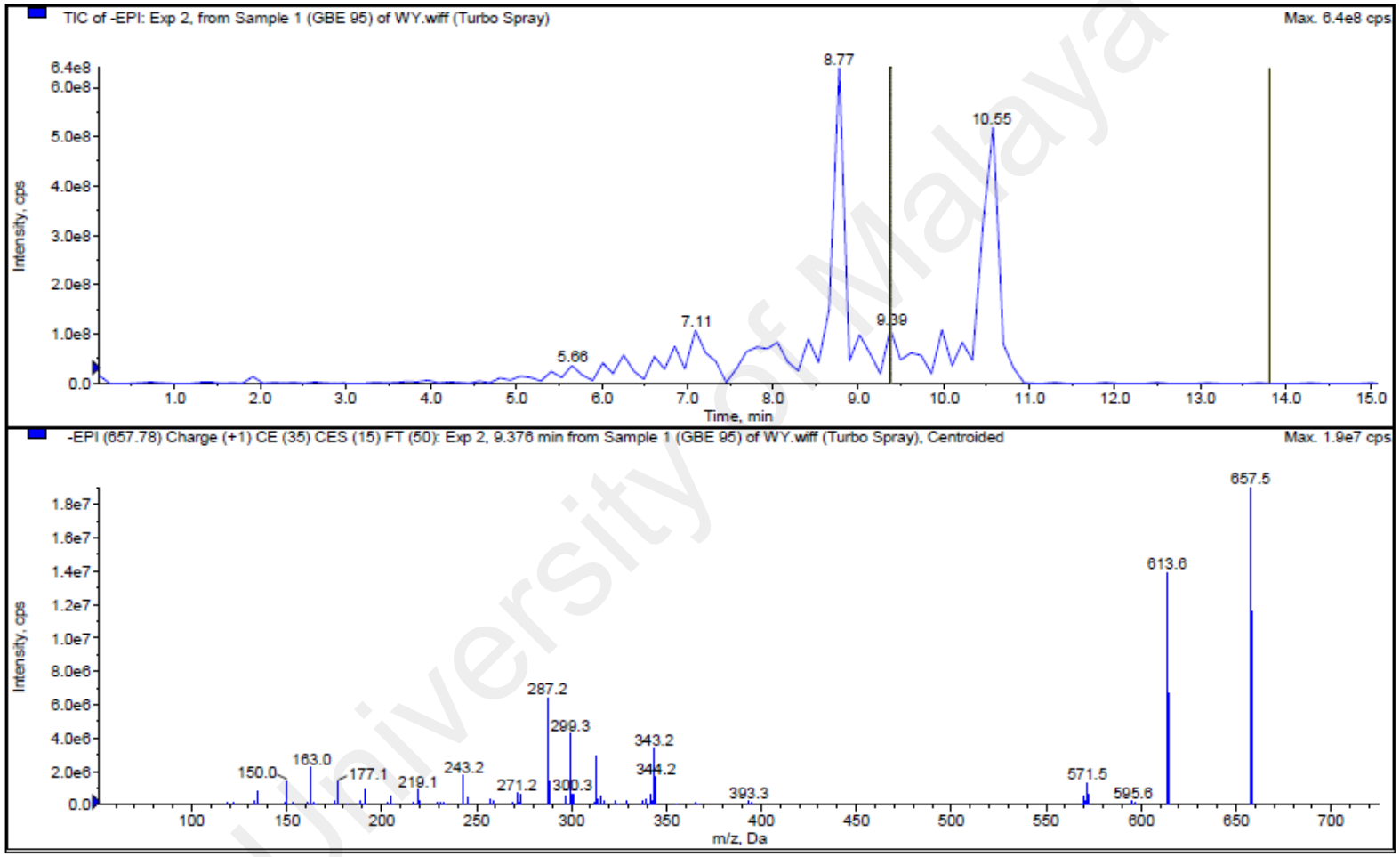


Figure 4.9: MS of peak 3 of E9.5 sub-fraction

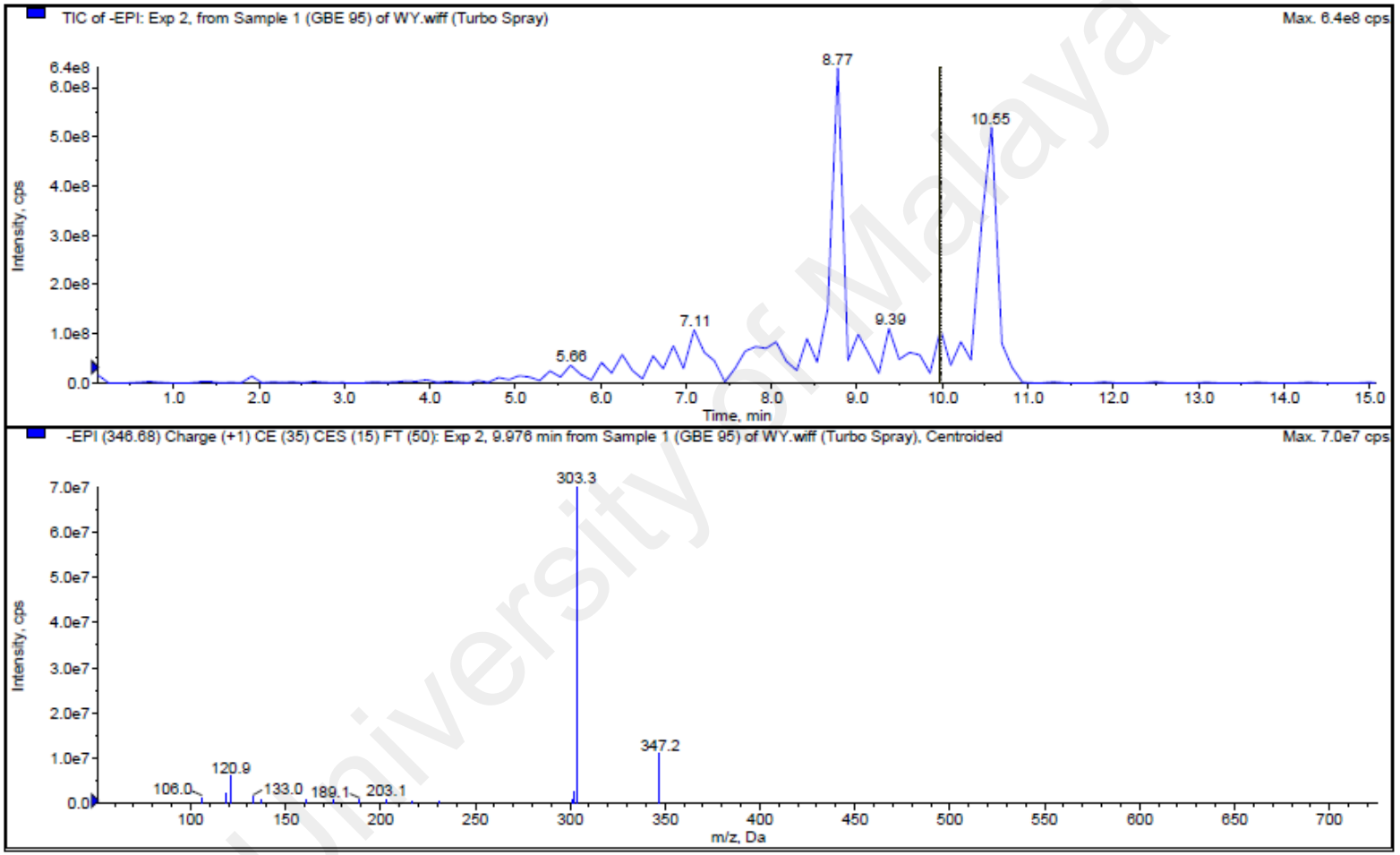


Figure 4.10: MS of peak 4 of E9.5 sub-fraction

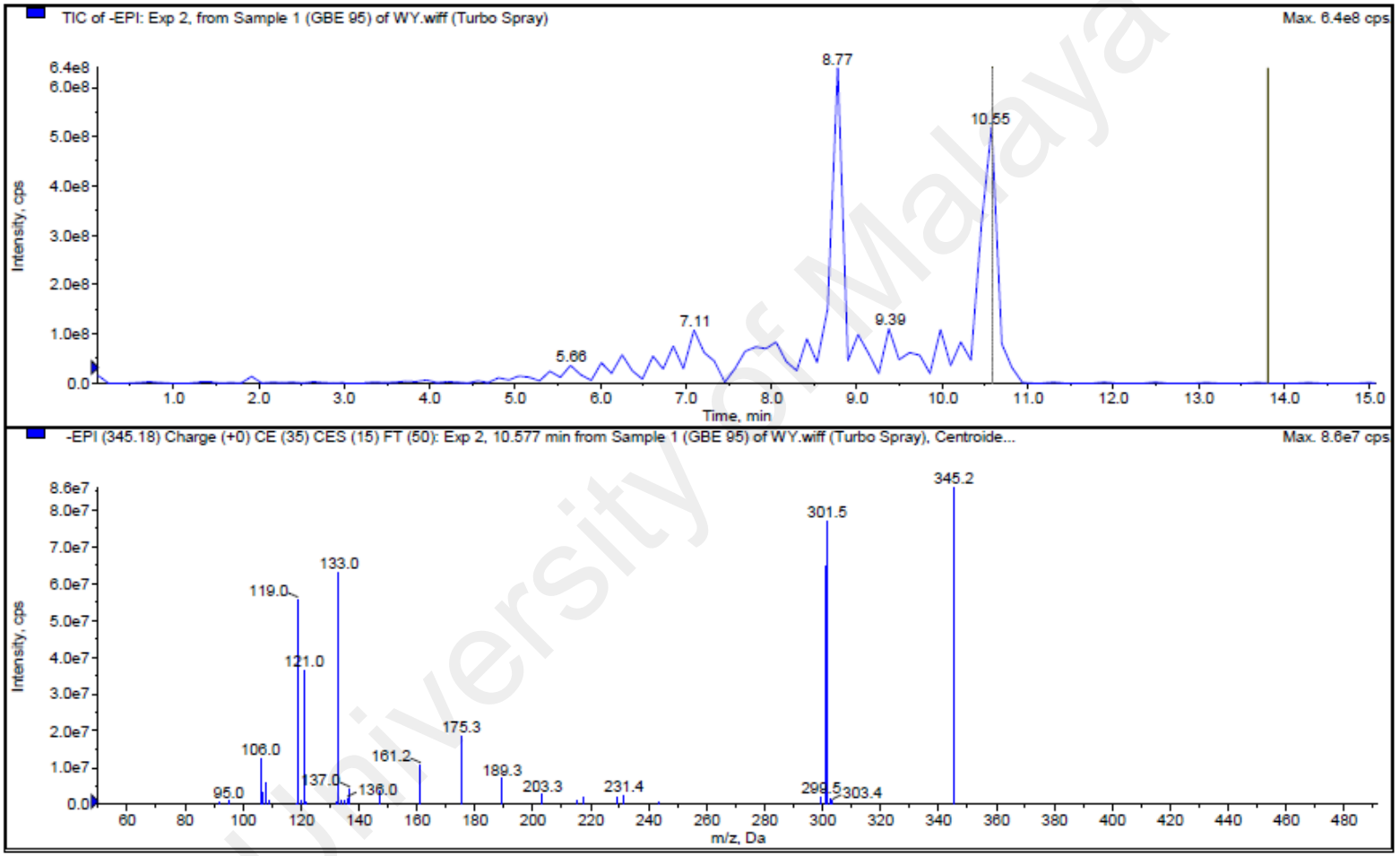


Figure 4.11: MS of peak 5 of E9.5 sub-fraction

4.4.2 Identification of isolated chemical constituents

The fractionation and purification of ethyl acetate and water extracts of *G. bicolor* were carried out and led to eight isolated chemical constituents. The structures of constituents (**1**) to (**8**) are shown in Figure 4.12. The ^{1}H NMR spectra of constituents (**1**) and (**8**) displayed chemical shift in 3.7 - 4.2 ppm region, suggesting two oxygen bearing methine protons (H-3, 4) while chemical shift in 2 - 2.2 ppm region suggested two pairs of methylene protons (H-2, 6). In ^{13}C NMR spectrum, chemical shifts of signals in 71.0 - 73.0 ppm region suggested three oxygenated carbon (C-3, 4, 5), 38.1 - 38.8 ppm region suggested two methylenes (C-2, 6) and a carbon signal at 177.1 ppm (C-7) indicated the presence of a carboxylic acid. The presence of a quaternary carbon at 76.1 ppm (C-1) was indicated by DEPT 135. All these signals were characteristic of a quinic acid moiety. In addition, the ^{1}H NMR spectrum of constituent (**1**) showed the presence of para-substituted benzene ring proton signals [δ 7.48 (2H, d, J = 8.5 Hz, H-2', 6'), 6.81 (2H, d, J = 8.5 Hz, H-3', 5')] and olefinic double bond proton signals [δ 7.67 (1H, d, J = 16.0 Hz, H-7'), 6.38 (1H, d, J = 16.0 Hz, H-8')], where the coupling constant value of the olefinic double bond proton signals indicated *trans* configuration of the double bond. Therefore, constituent (**1**) was established as 5-*p*-*trans*-coumaroylquinic acid in which the NMR spectral data were consistent with the literature values (Lu *et al*., 2000). Constituent (**8**) showed one caffeoyl moiety with an ester carbonyl resonance at δ 168.7 (C-9') and a downfield shift of H-5 (δ 5.32, ddd, J = 8.9/8.9/4.4 Hz). By comparison to literature, constituent (**8**) was identified as chlorogenic acid (Lee *et al*., 2010). Electrospray ionisation-mass spectrometry (ESI-MS) further confirmed identity of constituents (**1**) ($C_{16}H_{18}O_8$) and (**8**) ($C_{16}H_{18}O_9$) with ion peak at *m/z* 337 $[M-H]^-$ and 353 $[M-H]^-$, respectively. The ^{1}H NMR spectrum of constituent (**2**) displayed a *para*-substituted phenyl ring containing two pairs of *ortho*-coupled proton at 7.87 (2H, d, J = 8.7 Hz, H-2, 6) and 6.81 (2H, d, J = 8.7 Hz, H-3, 5). Constituent (**2**) was identified as 4-

hydroxybenzoic acid by comparison with literature (Youn *et al*., 2010). Molecular formula of constituent (**2**) ($C_7H_6O_3$) was confirmed by negative ionization ESI-MS with strong ion peak at *m/z* 137 $[M-H]^-$. ^1H NMR and ^{13}C NMR spectra of constituents (**3**) and (**4**) were similar, except carbon signals in glucosyl moiety. Upfield shift of C-2', 4' and 6'; downfield shift of C-3' was displayed in constituent (**3**) compared to constituent (**4**). Constituent (**3**) was identified as rutin by comparison of NMR spectroscopic data reported in the literature (Kanada *et al*., 2012). Molecular weight of constituent (**3**) ($C_{27}H_{30}O_{16}$) was confirmed by ESI-MS with strong peak at *m/z* 609 $[M-H]^-$. ^1H NMR spectra of constituents (**4**) and (**6**) showed the signals of aromatic protons in AA'BB' system at δ 8.03 (2H, H-2', 6'), δ 6.87 (2H, H-3', 5') and 2H AX system at δ 6.17 (1H, d, J = 1.8 Hz, H-6), δ 6.36 (1H, d, J = 1.8 Hz, H-8). These proton signals suggested a kaempferol aglycone. Signals at δ 5.24/135.4 (H-1"/C-3) indicated the linkage between glucopyranose and aglycone at 3-hydroxyl. ^{13}C NMR spectrum of constituent (**6**) also showed signals of kaempferol aglycone and glucosyl moiety, therefore it was identified as kaempferol-3-*O*-glucoside (Feng *et al*., 2007). Constituent (**4**) displayed signals at 1.12/17.9 (H-6"'/C-6"') revealing the presence of methyl group of rhamnose unit whilst signals at δ 4.51/68.5 (H-1"'/C-6") indicated the linkage between rhamnopyranosyl and glucopyranose at 6"-hyroxyl. Constituent (**4**) was identified as kaempferol-3-*O*-rutinoside because of kaempferol aglycone, glucosyl moiety and rhamnosyl moiety that were observed in ^{13}C NMR spectrum (Feng *et al*., 2007). Identity of constituents (**4**) ($C_{27}H_{30}O_{15}$) and (**6**) ($C_{21}H_{20}O_{11}$) was further confirmed by ESI-MS with ion peak at *m/z* 593 $[M-H]^-$ and 447 $[M-H]^-$, respectively. In ^1H NMR spectrum of constituent (**5**), two groups of three aromatic protons were assigned to δ 7.06 (2H, br s, H-2',2"), δ 6.78 (2H, d, J = 8.1 Hz, H-5', 5"), δ 6.95 (2H, dd, J = 8.1/2.1 Hz H-6',6"). *Trans*-olefinic protons (H-7', 7" and H-8', 8") were showed by four doublets with J values of 16.0 Hz. These proton signals showed the presence of two caffeoyl moieties. The downfield shifts of H-

5 (m) and H-3 (m) at the region of δ 5.44 - 5.39 was due to esterification. Constituent (**5**) exhibited two ester carbonyl resonances (δ 168.9 for C-9', 168.4 for C-9") and a quinic acid moiety in ^{13}C NMR spectrum, which was identified as 3,5-dicaffeoylquinic acid by comparison with literature (Lee *et al*., 2010). ESI-MS also showed ion peak at *m/z* 515 $[M-H]^-$ which is consistent with molecular weight of constituent (**5**) ($C_{25}H_{24}O_{12}$). ^{1}H NMR spectrum of constituent (**7**) showed signals of a five carbon sugar unit (ribose) at δ 5.68 - 3.52 (H-1' to H-5') region. The presence of a carbon signal at δ 7.93 (H-8) suggested a nucleoside structure. Constituent (**7**) was confirmed to be guanosine by comparing with NMR spectral data reported in literature (Kim *et al*., 2003). Negative ionisation ESI-MS yielded strong ion peak at *m/z* 282 $[M-H]^-$ and confirmed the molecular weight of constituent (**7**) ($C_{10}H_{13}N_5O_5$). To our best knowledge, all constituents were isolated for the first time from *G. bicolor* leaves except constituent (**3**), rutin.

5-*p-trans*-coumaroylquinic acid (**1**): ^{1}H NMR (CD_3OD, 300 MHz) δ: 2.01-2.23 (4H, m, H-2, 6), 4.2 (1H, d, J = 3.1 Hz, H-3), 3.77 (1H, dd, J = 8.6/3.0 Hz, H-4), 5.33 (1H, m, H-5), 7.48 (2H, d, J = 8.5 Hz, H-2', 6'), 6.81 (2H, d, J = 8.5 Hz, H-3', 5'), 7.67 (1H, d, J = 16.0 Hz, H-7'), 6.38 (1H, d, J = 16.0 Hz, H-8'). ^{13}C NMR (CD_3OD, 75 MHz) δ: 76.1 (C-1), 38.2 (C-2), 72.0 (C-3), 71.2 (C-4), 73.4 (C-5), 38.7 (C-6), 177.1 (C-7), 127.2 (C-1'), 131.2 (C-2',6'), 116.8 (C-3',5'), 161.3 (C-4'), 146.7 (C-7'), 115.3 (C-8'), 168.6 (C-9'); ESI-MS *m/z* 337 $[M-H]^-$, 675 $[2M-H]^-$, 697 $[2M+Na-2H]^-$, 191 $[quinate]^-$ (NMR spectra shown in Appendix A1-A3; ESI-MS with the comparison between experimental and theoretical isotopic pattern are shown in Appendix I1 and I2).

4-hydroxybenzoic acid (**2**): ^{1}H NMR (CD_3OD, 300 MHz) δ: 7.87 (2H, d, J = 8.7 Hz, H-2, 6), 6.81 (2H, d, J = 8.7 Hz, H-3, 5). ^{13}C NMR (CD_3OD, 75 MHz) δ: 122.8 (C-1), 133.0 (C-2, C-6), 116.0 (C-3, C-5), 163.3 (C-4), 170.2 (C-7); ESI-MS *m/z* 137 $[M-H]^-$, 275 $[2M-H]^-$ (NMR spectra shown in Appendix B1 and B2; ESI-MS with the

comparison between experimental and theoretical isotopic pattern are shown in Appendix J1 and J2).

Rutin (**3**): ^{1}H NMR (CD_3OD, 300 MHz) δ: 6.20 (1H, d, J = 2.1 Hz, H-6), 6.39 (1H, d, J = 2.1 Hz, H-8), 7.66 (1H, d, J = 2.0 Hz, H-2'), 6.87 (1H, d, J = 8.4 Hz, H-5'), 7.61 (1H, dd, J = 8.6/2.0 Hz, H-6'), 5.10 (1H, d, J = 7.5 Hz, H-1"), 4.52 (1H, br s, H-1"'), 1.11 (3H, d, J = 6.0 Hz, H-6"'), 3.27-3.82 (m). ^{13}C NMR (CD_3OD, 75 MHz) δ: 158.5 (C-2), 135.6 (C-3), 179.4 (C-4), 162.9 (C-5), 99.9 (C-6), 166.0 (C-7), 94.9 (C-8), 159.3 (C-9), 105.6 (C-10), 123.1 (C-1'), 117.7 (C-2'), 145.8 (C-3'), 149.8 (C-4'), 116.0 (C-5'), 123.5 (C-6'), 104.7 (C-1"), 75.7 (C-2"), 78.1 (C-3"), 71.3 (C-4"), 77.2 (C-5"), 68.5 (C-6"), 102.4 (C-1"'), 72.1 (C-2"'), 72.2 (C-3"'), 73.9 (C-4"'), 69.7 (C-5"'), 17.9 (C-6"'); ESI-MS *m/z* 609 $[M-H]^-$ (NMR spectra shown in Appendix C1-C3; ESI-MS with the comparison between experimental and theoretical isotopic pattern are shown in Appendix K1 and K2).

Kaempferol-3-*O*-rutinoside (**4**): ^{1}H NMR (CD_3OD, 300 MHz) δ: 6.19 (1H, d, J = 1.8 Hz, H-6), 6.38 (1H, d, J = 1.8 Hz, H-8), 8.07 (2H, d, J = 8.7 Hz, H-2', 6'), 6.88 (2H, d, J = 8.7 Hz, H-3', 5'), 5.11 (1H, d, J = 7.5 Hz, H-1"), 4.51 (1H, br s, H-1"'), 1.12 (3H, d, J = 6.0 Hz, H-6"'), 3.25-3.82 (m). ^{13}C NMR (CD_3OD, 75 MHz) δ: 159.3 (C-2), 135.6 (C-3), 179.4 (C-4),163.0 (C-5), 100.1 (C-6), 166.4 (C-7), 95.0 (C-8), 158.6(C-9), 105.5 (C-10), 122.7 (C-1'), 132.4 (C-2', 6'), 116.1 (C-3', 5'), 161.6 (C-4'), 104.6 (C-1"), 75.7 (C-2"), 78.1 (C-3"), 72.3 (C-4"), 77.2 (C-5"), 68.5 (C-6"), 102.4 (C-1"'), 71.4 (C-2"'), 72.1 (C-3"'), 73.9 (C-4"'), 69.7 (C-5"'), 17.9 (C-6"'); ESI-MS *m/z* 593 $[M-H]^-$ (NMR spectra shown in Appendix D1-D3; ESI-MS with the comparison between experimental and theoretical isotopic pattern are shown in Appendix L1 and L2).

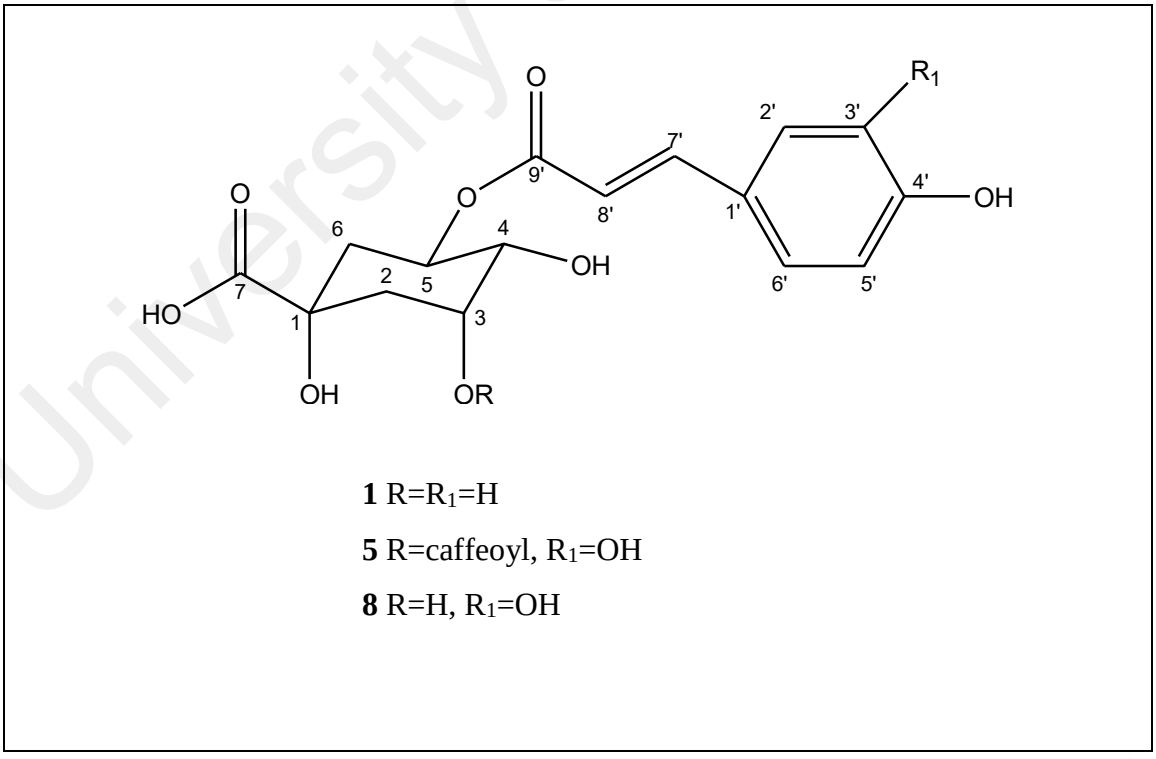
3,5-dicaffeoylquinic acid (**5**): ^{1}H NMR (CD_3OD, 300 MHz) δ: 2.34-2.14 (4H, m, H-2, 6), 5.44-5.39 (2H, m, H-3, 5), 3.99 (1H, dd, J = 7.8/3.0 Hz, H-4), 7.06 (2H, br s, H-2',2"), 6.78 (2H, d, J = 8.1 Hz, H-5', 5"), 6.95 (2H, dd, J = 8.1/2.1 Hz H-6',6"), 7.63

(1H, d, *J* = 16.0 Hz, H-7'), 7.58 (1H, d, *J* = 16.0 Hz, H-7"), 6.34 (1H, d, *J* = 16.0 Hz, H-8'), 6.28 (1H, d, *J* = 16.0 Hz, H-8"). ^{13}C NMR (CD_3OD, 75 MHz) *δ*: 74.7 (C-1), 37.6 (C-2), 72.5 (C-3), 70.6 (C-4), 71.9 (C-5), 35.9 (C-6), 177.3 (C-7), 127.8 (C-1'), 127.7 (C-1"), 116.4 (C-2',2"), 147.3 (C-3'), 147.1 (C-3"), 149.5 (C-4'), 149.4 (C-4"), 115.4 (C-5'), 115.2 (C-5"), 123.1 (C-6'), 123.0 (C-6"), 146.6 (C-7',7"), 115.1 (C-8'), 115.0 (C-8"), 168.9 (C-9'), 168.4 (C-9"); ESI-MS *m/z* 515 $[M-H]^-$, 537 $[M+Na-2H]^-$, 353 [caffeoylquinate]$^-$ (NMR spectra shown in Appendix E1-E3; ESI-MS with the comparison between experimental and theoretical isotopic pattern are shown in Appendix M1 and M2).

Kaempferol-3-*O*-glucoside (**6**): ^{1}H NMR (CD_3OD, 300 MHz) *δ*: 6.17 (1H, d, *J* = 1.8 Hz, H-6), 6.36 (1H, d, *J* = 1.8 Hz, H-8), 8.03 (2H, d, *J* = 8.3 Hz, H-2', 6'), 6.87 (2H, d, *J* = 8.3 Hz, H-3', 5'), 5.24 (1H, d, *J* = 6.1 Hz, H-1"), 3.71-3.19 (m). ^{13}C NMR (CD_3OD, 75 MHz) *δ*: 159.0 (C-2), 135.4 (C-3), 179.4 (C-4), 163.0 (C-5), 99.8 (C-6), 165.9 (C-7), 94.7 (C-8), 158.4 (C-9), 105.7 (C-10), 122.7 (C-1'), 132.1 (C-2', C-6'), 116.0 (C-3', C-5'), 161.5 (C-4'), 104.1 (C-1"), 75.7 (C-2"), 78.0 (C-3"), 71.3 (C-4"), 78.4 (C-5"), 62.6 (C-6"); ESI-MS *m/z* 447 $[M-H]^-$, 895 $[2M-H]^-$ (NMR spectra shown in Appendix F1-F3; ESI-MS with the comparison between experimental and theoretical isotopic pattern are shown in Appendix N1 and N2).

Guanosine (**7**): ^{1}H NMR (DMSO-d_6, 300 MHz) *δ*: 7.93 (1H, s, H-8), 5.68 (1H, d, *J* = 6.0 Hz, H-1'), 4.39 (1H, m, H-2'), 4.07 (1H, m, H-3'), 3.84 (1H, m, H-4'), 3.52 (2H, m, H-5').^{13}C NMR (DMSO-d_6, 75 MHz) *δ*: 151.6 (C-2), 153.9 (C-4), 116.9 (C-5), 157.1 (C-6), 135.8 (C-8), 86.5 (C-1'), 73.9 (C-2'), 70.6 (C-3'), 85.4 (C-4'), 61.6 (C-5'); ESI-MS *m/z* 282 $[M-H]^-$, 565 $[2M-H]^-$ (NMR spectra shown in Appendix G1-G3; ESI-MS with the comparison between experimental and theoretical isotopic pattern are shown in Appendix O1 and O2).

5-*O*-caffeoylquinic acid (Chlorogenic acid) (**8**): ^{1}H NMR (CD_3OD, 300 MHz) δ: 2.01-2.24 (4H, m, H-2, 6), 4.16 (1H, dt, *J* = 5.4/3.3 Hz, H-3), 3.71 (1H, dd, *J* = 8.4/3.1 Hz, H-4), 5.32 (1H, ddd, *J* = 8.9/8.9/4.4 Hz, H-5), 7.04 (1H, d, *J* = 2.0 Hz, H-2'), 6.77 (1H, d, *J* = 8.1 Hz, H-5'), 6.94 (1H, dd, *J* = 8.1/2.1 Hz, H-6'), 7.55 (1H, d, *J* = 15.9 Hz, H-7'), 6.26 (1H, d, *J* = 15.8 Hz, H-8'). ^{13}C NMR (CD_3OD, 75 MHz) δ: 76.1 (C-1), 38.1 (C-2), 71.3 (C-3), 73.4 (C-4), 71.9 (C-5), 38.8 (C-6), 177.1 (C-7), 127.7 (C-1'), 115.2 (C-2'), 146.7 (C-3'), 149.5 (C-4'), 116.4 (C-5'), 123.0 (C-6'), 147.1 (C-7'), 115.2 (C-8'), 168.7 (C-9'); ESI-MS *m/z* 353 $[M-H]^-$, 707 $[2M-H]^-$, 729 $[2M+Na-2H]^-$, 191 $[quinate]^-$ (NMR spectra shown in Appendix H1-H3; ESI-MS with the comparison between experimental and theoretical isotopic pattern are shown in Appendix P1 and P2).



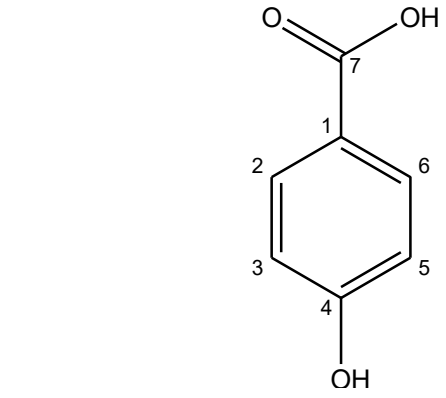
1 R=R_1=H

5 R=caffeoyl, R_1=OH

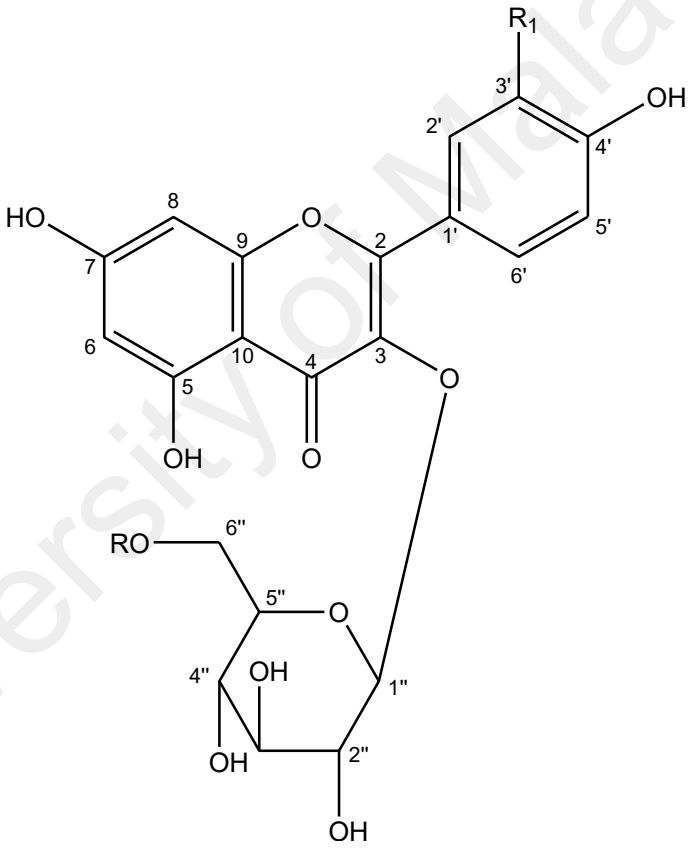
8 R=H, R_1=OH

Figure 4.12, continued

Figure 4.12, continued



2



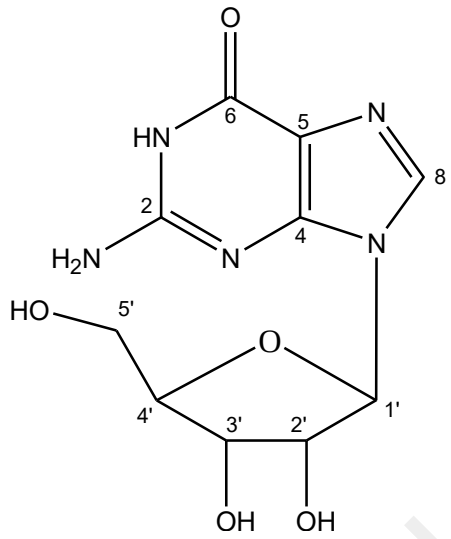
3 R=Rha, R_1=OH

4 R=Rha, R_1=H

6 R=R_1=H

Figure 4.12, continued

Figure 4.12, continued



7

Figure 4.12: Structures of isolated chemical constituents (1 - 8)

4.4.3 Cytotoxicity evaluation of isolated chemical constituents

MTT cytotoxicity assay was conducted to evaluate the cytotoxic effect of the isolated constituents against HCT 116 colon carcinoma and CCD-18Co normal colon cells. As shown in Table 4.4, all isolated chemical constituents did not show cytotoxic effect against HCT 116 cells at 72 hours incubation (with IC_{50} values > 100 µg/ml), except 3,5-dicaffeoylquinic acid (**5**, IC_{50} value of 79.7 ± 4.5 µg/ml), guanosine (**7**, IC_{50} value of 81 ± 6.6 µg/ml) and 5-*O*-caffeoylquinic acid (**8**, IC_{50} value of 79.3 ± 3.1 µg/ml).

Constituent with SI value more than 3 indicates high selectivity against cancerous cells compared to normal cells (Bézivin *et al.*, 2003). The cytotoxic effect of constituents (**5**), (**7**) and (**8**) against CCD-18Co normal colon cells were comparatively low with IC_{50} values of 350 ± 1.7, > 500 and 403.3 ± 9.5 µg/ml, respectively. Constituent (**7**) appeared to be highly selective against HCT 116 cells with SI value of 6.2, followed by constituent (**8**) with SI value of 5.1, and constituent (**5**) with SI value of 4.4. To our knowledge, the SI of constituents (**5**), (**7**) and (**8**) on HCT 116 and CCD-

18Co cells had not been reported. From SI point of view, constituents (**5**), (**7**) and (**8**) showed cytotoxicity against colon cancer cells while exerted minimal cytotoxicity against normal colon cells. The selective property of these three chemical constituents of *G. bicolor* could be useful against colon cancer.

Ethyl acetate extract of *G. bicolor* showed greater cytotoxic effect against HCT 116 cells compared to the isolated constituents with IC_{50} value of 29.2 µg/ml (Table 3.10). Constituents (**1**) **–** (**6**) isolated from ethyl acetate extract showed IC_{50} values more than 30 µg/ml, suggested that these constituents did not contribute to the cytotoxic effect of ethyl acetate extract individually. However, synergistic or potentiating interactions among these constituents and other unidentified active constituents (from fraction E9, Table 4.1) might be contributed to cytotoxic effect when present together in the extract. Studies by de Kok *et al*. (2008) also suggested that extract with mixture of constituents which showed higher anti-proliferation effect on cancerous cells than individual phenolic compound probably involved collective actions of multiple chemical constituents on the cells.

Although the other five chemical constituents (**1** – **4** and **6**) were not cytotoxic, they had other known biological activities. The 5-*p*-*trans*-coumaroylquinic acid (**1**) is a hydroxycinnamic acid derivative that formed from esterification of *p*-coumaric acid and a quinic acid. It could act as antioxidant especially towards hydroxyl radicals and protect HT-29 cells from oxidative stress (Ferguson *et al*., 2005; Serra *et al*., 2011).

The 4-hydroxybenzoic acid (**2**) is a phenolic acid which reported to possess antifungal, antibacterial activities and hypoglycemic effect on rats (Chong *et al*., 2009; Peungvicha *et al*., 1998). Pugazhendhi *et al*. (2005) showed the estrogenic effect of *p*-hydroxybenzoic acid which could increase the proliferation of human breast cancer cells (MCF7 and ZR-75-1).

Rutin (**3**) is a flavonol glycoside which rhamnose and glucose are linked to quercetin (also known as quercetin-3-*O*-rutinoside). Rutin (**3**) was reported to exhibit anti-inflammation activity by inhibiting the production of nitric oxide and the expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS) activated macrophages (Kazłowska *et al*., 2010). Besides that, it showed antioxidant activities in scavenging DPPH radicals and inhibiting lipid peroxidation. Its antioxidant activity was mainly contributed by the aglycone, quercetin (Chiang *et al*., 2004; Simić *et al*., 2007).

On the other hand, kaempferol-3-*O*-rutinoside (**4**) and kaempferol-3-*O*-glucoside (**6**) demonstrated lower antioxidant activity compared to kaempferol which is a potent antioxidant against superoxide, Fenton-mediated hydroxyl radicals and peroxynitrite (Fiorentino *et al*., 2007; Heijnen *et al*., 2001; Hou *et al*., 2004; Wang *et al*., 2006).

Table 4.4: Cytotoxic effect of isolated chemical constituents against HCT 116 and CCD- 18Co cells

Chemical constituents	IC_{50}[a] (µg/ml) (SI[b])	
	HCT 116	**CCD-18Co**
5-*p-trans*-Coumaroylquinic acid (**1**)	>100	ND
4-Hydroxybenzoic acid (**2**)	>100	ND
Rutin (**3**)	>100	ND
Kaempferol-3-*O*-rutinoside (**4**)	>100	ND
3,5-Dicaffeoylquinic acid (**5**)	79.7 ± 4.5 (4.4)	350 ± 1.7
Kaempferol-3-*O*-glucoside (**6**)	>100	ND
Guanosine (**7**)	81.0 ± 6.6 (>6.2)	>500
5-*O*-Caffeoylquinic acid (**8**)	79.3 ± 3.1 (5.1)	403.3 ± 9.5
Cis-platin[c]	2.9 ± 0.1 (>4.3)	>12.5

Data are presented as mean ± standard deviation from three independent experiments triplicate for each; [b]Selectivity index (SI); [c]Positive reference standard; ND = not determined.

4.5 Conclusion

In this work, eight chemical constituents were isolated from *G. bicolor* leaves for the first time, except rutin (**3**). The 3,5-dicaffeoylquinic acid (**5**), guanosine (**7**) and 5-*O*-caffeoylquinic acid (**8**) from *G.bicolor* leaves showed selective cytotoxic effect against HCT 116 cells by *in vitro* MTT assay. By consuming *G. bicolor*, these chemical constituents may have beneficial role in treatment of colon cancer.

CHAPTER 5: CAFFEOYLQUINIC ACIDS INDUCE CELL DEATH AND CELL CYCLE ARREST ON HCT 116 CELLS *VIA* FORMATION OF EXTRACELLULAR H_2O_2 AND OXIDATION PRODUCTS

5.1 Introduction

According to World Health Organisation's Globocan 2012 report (Ferlay *et al*., 2013), colorectal cancer (CRC) was the second most common cancer in Malaysia. Natural agents that can induce cell death and/or growth inhibition on early stage of neoplastic colonic epithelial cells in mucosa may prevent the development of CRC. Epidemiological studies showed that high intake of fruits and vegetables with abundant of (poly)phenolic compounds are linked to lower CRC incidence (Riboli & Norat, 2003).

Caffeoylquinic acids are phenolic acids that formed from esterification of caffeic acid(s) and a quinic acid. Mono-, di- and tri-caffeoylquinic acids are found in various plants such as apple, coffee, *Ilex paraguariensis* (mate tea), *Ipomoea batatas* (sweetpotato leaves), *Cynara scolymus* L. (artichoke) and herbs include *Chrysanthemum* spp., *Lonicera* spp. and *Arctium lappa* (Bravo *et al*., 2007; Clifford *et al*., 2007; Lin & Harnly, 2008; Luo *et al*., 2013). Caffeoylquinic acids demonstrate a wide range of bioactivities such as antioxidant, anti-inflammation, anti-carcinogenesis, anti-viral and cytotoxicity on human cancer cell lines including oral cancer cells (HSC-2, HSG), lung cancer cells (A549), breast cancer cells (MCF-7) and colon cancer cells (RKO, HT-29) (Burgos-Morón *et al*., 2012; Iwai *et al*., 2004; Jiang *et al*., 2000; McDougall *et al*., 1998; Morishita *et al*., 1997; Puangpraphant *et al*., 2011). Caffeoylquinic acids are widely accepted as antioxidants but could act as pro-oxidants under certain conditions. As reported by Jiang *et al*. (2000), 5-*O*-caffeoylquinic acid (CQ) at 2.5 mM could produce radicals which were detected by electron spin resonance (ESR) spectroscopy under alkaline condition. ESR method also showed that CQ

generated radicals in the presence of Cu^{2+} at concentrations of 1-10 mM (Iwasaki *et al*., 2011). The pro-oxidant activity of phenolic compound (particularly the one bearing catechol structure) is predicted to progress through the oxidation by oxygen to produce superoxide ($O_2^{\bullet-}$) and semiquinone radical. Subsequently, $O_2^{\bullet-}$ can be reduced by phenolic compound to hydrogen peroxide (H_2O_2). Additionally, $O_2^{\bullet-}$ can also dismutate spontaneously to H_2O_2 (Mochizuki *et al*., 2002). On the other hand, the OH groups of catechol structure of phenolic compound are contributing to antioxidant activity where electron or hydrogen atom can be transferred to free radicals (Hotta *et al*., 2001). Thus, caffeoylquinic acids which bear catechol structure(s) can have both anti- and pro-oxidant activity depending on the environmental condition.

The CQ and 3,5-dicaffeoylquinic acid (DCQ) which were isolated from *Gynura bicolor* exhibited cytotoxic effect on HCT 116 human colon cancer cells with IC_{50} of 79.7 and 79.3 µg/ml, respectively. The CQ and DCQ showed selectivity against colon cancer cells when compared with CCD-18Co normal colon cells with selective index of 4.4 and 5.1, respectively (Table 4.4).

In previous studies carried out by Jiang *et al*. (2000) and Burgos-Morón *et al*. (2012), selective cytotoxic effect of CQ was observed on human oral and lung cancer cells at concentrations of 0.5-1.4 mM compared to normal cells. These studies showed that catalase could protect the cancer cells from cell death effect induced by CQ. The results suggesting that the cell death effect of CQ involves H_2O_2. In the present study, the ability of CQ and DCQ to generate extracellular H_2O_2 and other oxidation products (green pigments) was demonstrated. The extracellular H_2O_2 and oxidation products of CQ and DCQ were playing the major role in causing cell death and cell cycle arrest on HCT 116 cells.

The specific objectives of the present study were as follows:

i. To investigate the cell death and cell cycle arrest effects of CQ and DCQ by flow cytometry

ii. To investigate the protective effects of antioxidants such as catalase, superoxide dismutase (SOD) and reduced glutathione (GSH) against the cell death and cell cycle arrest effects of CQ and DCQ

iii. To measure the extracellular H_2O_2 generated by CQ and DCQ

iv. To determine the level of intracellular reactive oxygen species (ROS) induced by CQ and DCQ

v. To determine the generation of oxidation products (green pigments) of CQ and DCQ in culture medium

5.2 Materials and methods

5.2.1 Chemicals and reagents

The CQ and DCQ were isolated from *G. bicolor* as described in section 4.3. McCoy's 5A medium, sodium bicarbonate, dimethyl sulfoxide (DMSO), catalase (catalogue No. C40), GSH, accutase, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), phosphate buffered saline (PBS), foetal bovine serum (FBS), penicillin/streptomycin (100X) and amphotericin B (250 µg/ml) were purchased from Sigma-Aldrich company. The SOD (catalogue No.190117) was from MP Biomedicals Company. Pierce ™ Quantitative Peroxide Assay kit was purchased from Thermo Scientific Company. FITC Annexin V Apoptosis Detection Kit I and CycleTEST™ PLUS DNA Reagent kit were purchased from BD Biosciences Company.

5.2.2 Cell line and stock solution of CQ and DCQ

HCT 116 cell line was purchased from American Type Culture Collection (ATCC). HCT 116 cell line was maintained in McCoy's 5A medium. Medium was supplemented with 10 % FBS, 1 % penicillin/streptomycin and 1 % amphotericin B. The cells were cultured in 5 % CO_2 at 37 ^oC in a CO_2 incubator. Stock solutions of CQ and DCQ were prepared in DMSO. The final concentration of DMSO in all the tested media were 0.5 %.

5.2.3 Annexin-V/PI flow cytometry

Firstly, HCT 116 cells (2 x 10^5 cells) were seeded in 60 mm culture dish. After 24 hours, medium was changed to fresh medium added with various tested samples. Depending on indicated treatment, single antioxidant or a combination of antioxidants was dissolved in medium. Appropriate medium was used to dilute the stock solution of CQ and DCQ to 0.5, 1 and 2 mg/ml. The final concentration of catalase, SOD and GSH was 200 units/ml, 30 units/ml and 4 mM, respectively. Appropriate medium added with DMSO (final concentration, 0.5 %) was used as untreated control. After 24 hours or 48 hours, cells were harvested and stained with FITC annexin-V and PI following the instruction of the FITC Annexin V Apoptosis Detection Kit I. Total of 10,000 cells were analysed by flow cytometer (BD FACSCanto II) using FACSDiva software.

5.2.4 Measurement of extracellular H_2O_2

The HCT 116 cells (2 x 10^5 cells) were seeded in 60 mm culture dish. After 24 hours, medium was changed to fresh medium added with various tested samples. Firstly, catalase or a combination of catalase and SOD was dissolved in medium. Appropriate medium was used to dilute the stock solution of CQ and DCQ to 1 and 2 mg/ml. The final concentration of catalase and SOD was 200 and 30 units/ml, respectively.

Appropriate medium added with DMSO (final concentration, 0.5 %) was used as untreated control. After 24 or 48 hours, medium was collected and centrifuged to remove dead cells. The concentration of H_2O_2 in the medium was measured according to Pierce ^{TM} Quantitative Peroxide Assay kits instruction. Tested medium added with catalase (final concentration, 1000 units/ml) was used as blank to remove absorbance due to sample in the medium. Each experiment was performed in triplicate.

5.2.5 Measurement of intracellular ROS level

HCT 116 cells (2×10^5 cells) were seeded in 60 mm culture dish. After 24 hours, medium was changed to fresh medium added with 2 mg/ml of CQ or DCQ. After 24 hours or 48 hours, cells were harvested and washed with PBS. A volume of 500 µl PBS containing 10 µM of DCF-DA was used to resuspend the cells. After 30 minutes of incubation in dark, 10,000 of cells were analysed by flow cytometer (BD FACSCanto II) using FACSDiva software. Cells treated with 500 µM of H_2O_2 for 20 minutes were used as a positive control.

5.2.6 Measurement of green pigment

Firsty, catalase or a combination of catalase and SOD or GSH was dissolved in medium. Appropriate medium was used to dilute the stock solution of CQ and DCQ to 1 and 2 mg/ml. The final concentration of catalase, SOD and GSH was 200 units/ml, 30 units/ml and 4 mM, respectively. The tested medium without the presence of HCT 116 cells was incubated for 0, 24 and 48 hours, under 5 % CO_2 at 37 ^oC. The green pigment in the medium was measured at 680 nm using microplate reader (Thermo Scientific Multiskan GO). Each experiment was performed in triplicate.

5.2.7 Cell cycle arrest flow cytometry

Firstly, HCT 116 cells (2 x 10^5 cells) were seeded in 60 mm culture dish. After 24 hours, medium was changed to fresh medium added with various tested samples. Catalase or GSH was dissolved in medium first. Appropriate medium was used to dilute the stock solution of CQ and DCQ to 0.5, 1 and 2 mg/ml. The final concentration of catalase and GSH was 200 units/ml and 4 mM, respectively. Appropriate medium added with DMSO (final concentration, 0.5 %) was used as untreated control. After 24 or 48 hours, cells were harvested and stained with PI following the instruction of the CycleTEST^{TM} PLUS DNA Reagent kit. Total of 30,000 cells were analysed by flow cytometer (BD FACSCanto II) using FACSDiva and ModFit softwares.

5.2.8 Statistical analysis

Annexin-V/PI and cell cycle arrest flow cytometry data were analysed using Student's *t*-test, with *P* values < 0.05 being regarded as significant difference.

5.3 Results

5.3.1 CQ and DCQ induce cell death effects on HCT 116 cells

At the beginning of study, investigation was carried out to determine the cell death effects of CQ and DCQ by using annexin-V/PI flow cytometry. Annexin-V/PI flow cytometry provides more information such as the event of early, late apoptosis and necrosis compared to MTT assay. At 24 hours treatment, CQ induced minimal cell death effect on HCT 116 cells (Figure 5.1). However, 12.7 % increase in late apoptotic/necrotic cells was observed when HCT 116 cells were exposed to 2 mg/ml of CQ for 48 hours (Figure 5.1). As shown in Figure 5.2, the percentage of late apoptotic/necrotic cells increased in concentration- and time-dependent manner when HCT 116 cells were treated with DCQ. HCT 116 cells treated with 2 mg/ml of DCQ for

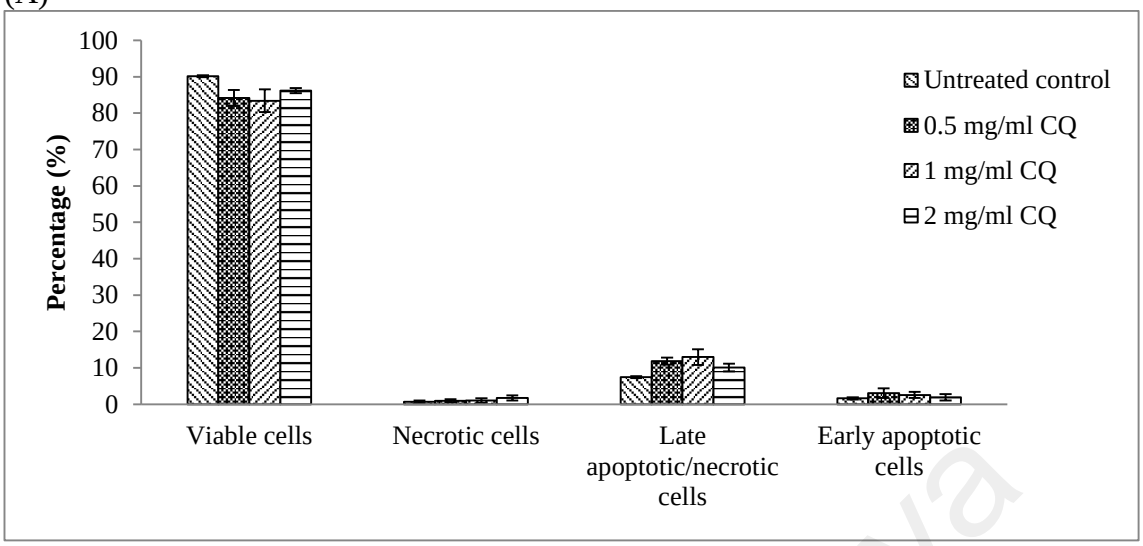
48 hours showed substantial increase of 76 % in late apoptotic/necrotic cells compared to untreated control.

5.3.2 Impact of catalase / catalase with SOD on cell death effects of CQ and DCQ

Investigation was then carried out to determine whether the cell death effects of CQ and DCQ were due to pro-oxidant activity by using cell impermeable antioxidants such as catalase and SOD. The SOD is an antioxidant enzyme which converts $O_2^{\bullet-}$ to H_2O_2, in turn, catalase catalyses the conversion of H_2O_2 to water and oxygen. The addition of catalase (200 units/ml) could increase the percentage of viable cells by 4.7% compared to HCT 116 cells treated with 2 mg/ml of CQ alone (Figure 5.3). As shown in Figure 5.4, the co-treatment of catalase and 2 mg/ml of DCQ could reduce the percentage of late apoptotic/necrotic cells and increase the percentage of early apoptotic cells by 55.8 % and 10.8 % respectively, compared to cells treated with 2 mg/ml of DCQ only. These results indicate that catalase can reduce and delay the cell death effects of CQ and DCQ suggesting the involvement of H_2O_2.

Besides H_2O_2, pro-oxidant activity of CQ and DCQ is likely to generate $O_2^{\bullet-}$ and may play a role in the cell death effects. As reported by Li *et al*. (2010), pro-oxidant activity of green tea epigallocatechin-3-gallate (EGCG) was shown to cause cytotoxicity and such effects can be inhibited by the combination of catalase and SOD. However, the percentage of viables cells did not increase when catalase was used together with SOD (30 units/ml) compared to treatments added with catalase alone (Figure 5.3 and 5.4). Thus, H_2O_2 appears to play a more critical role than $O_2^{\bullet-}$ in contributing to the cell death effects of CQ and DCQ.

(A)



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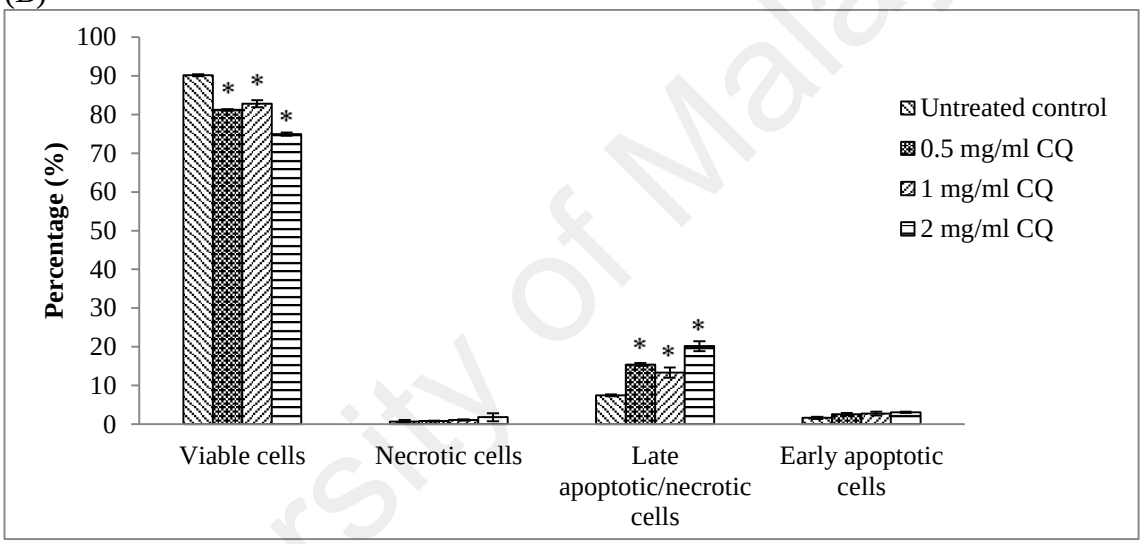
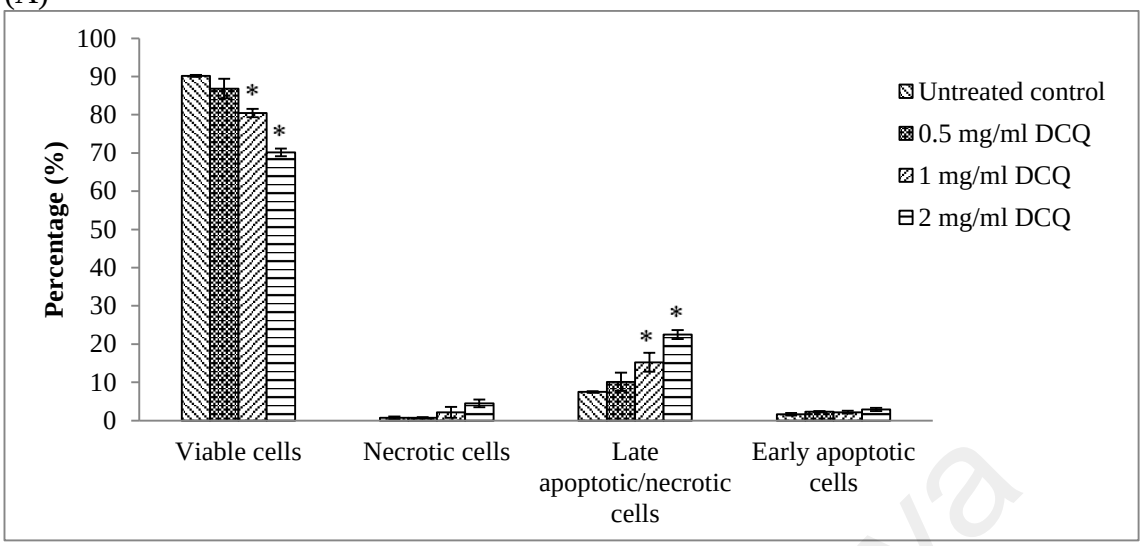


Figure 5.1: Cell death induction of CQ

(A) HCT 116 cells were treated with CQ for 24 hours. (B) HCT 116 cells were treated with CQ for 48 hours. Cell death was assessed by annexin-V/PI flow cytometry. Results are mean ± SD, n = 3. (*$p < 0.05$, in comparison with untreated control)

(A)



(B)

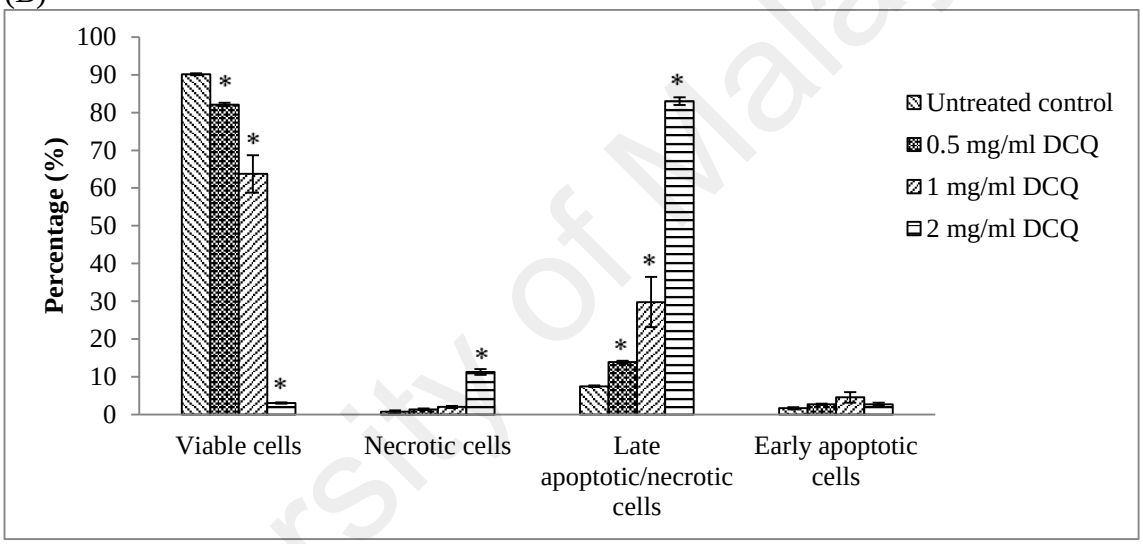


Figure 5.2: Concentration and time dependent cell death induction of DCQ

(A) HCT 116 cells were treated with DCQ for 24 hours. (B) HCT 116 cells were treated with DCQ for 48 hours. Cell death was assessed by annexin-V/PI flow cytometry. Results are mean ± SD, n = 3. (*$p < 0.05$, in comparison with untreated control)

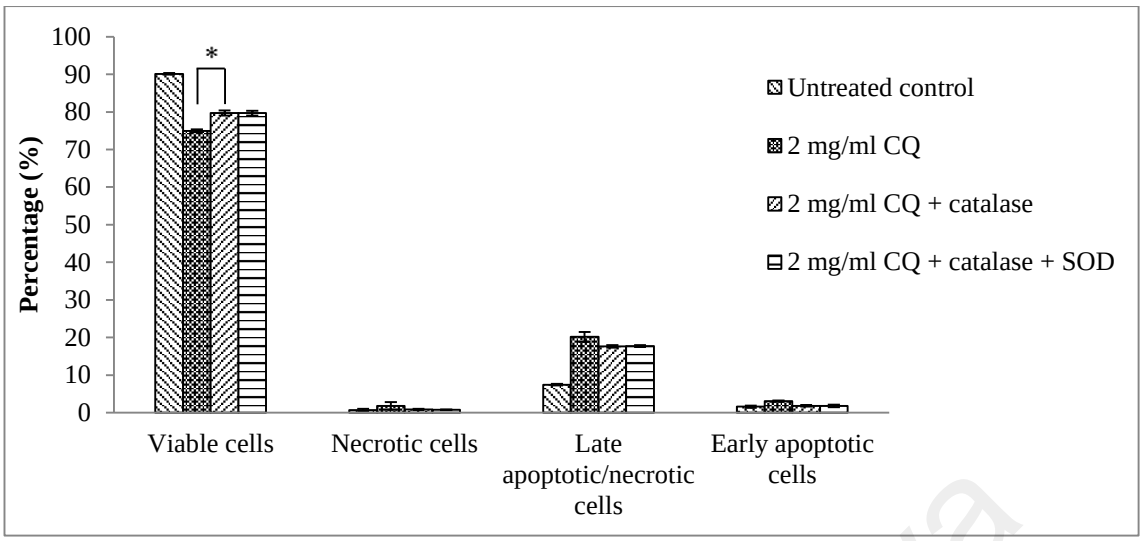


Figure 5.3: Protective effect of catalase against CQ cell death induction

HCT 116 cells were treated with CQ in the absence or presence of catalase / catalase with SOD for 48 hours. The final concentration of catalase and SOD was 200 units/ml and 30 units/ml, respectively. Cell death was assessed by annexin-V/PI flow cytometry. Results are mean ± SD, n = 3. (*$p < 0.05$, Student's *t*-test)

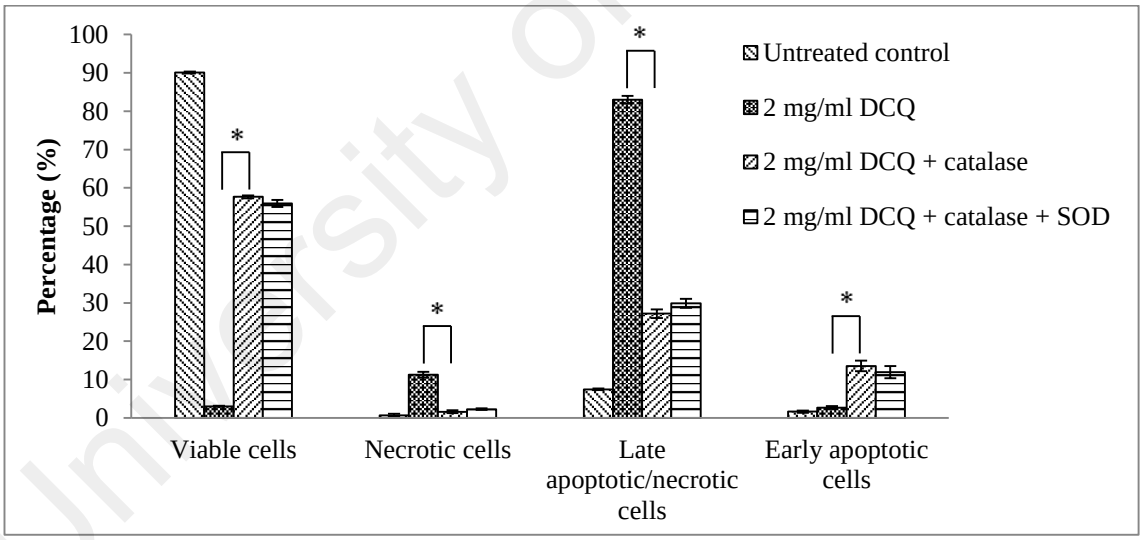


Figure 5.4: Protective effect of catalase against DCQ cell death induction

HCT 116 cells were treated with DCQ in the absence or presence of catalase / catalase with SOD for 48 hours. The final concentration of catalase and SOD was 200 units/ml and 30 units/ml, respectively. Cell death was assessed by annexin-V/PI flow cytometry. Results are mean ± SD, n = 3. (*$p < 0.05$, Student's *t*-test)

5.3.3 Generation of extracellular H_2O_2 by CQ and DCQ in HCT 116 cell culture medium

In this study, the HCT 116 cell culture medium was collected in order to determine the presence of extracellular H_2O_2 produced by CQ and DCQ. Substantial amount of H_2O_2 was detected in HCT 116 cell culture medium added with CQ or DCQ (Figure 5.5). The DCQ induced generation of H_2O_2 in concentration- and time-dependent manner while CQ only showed concentration-dependent manner. After 24 hours treatment, 2 mg/ml of CQ and DCQ generated 24.5 and 29.1 µM of H_2O_2, respectively. After 48 hours treatment, 2 mg/ml of DCQ could induce the generation of 98.7 µM of H_2O_2 while 2 mg/ml of CQ could only generate 24.8 µM of H_2O_2. In untreated control and media added with catalase showed no detectable level of H_2O_2.

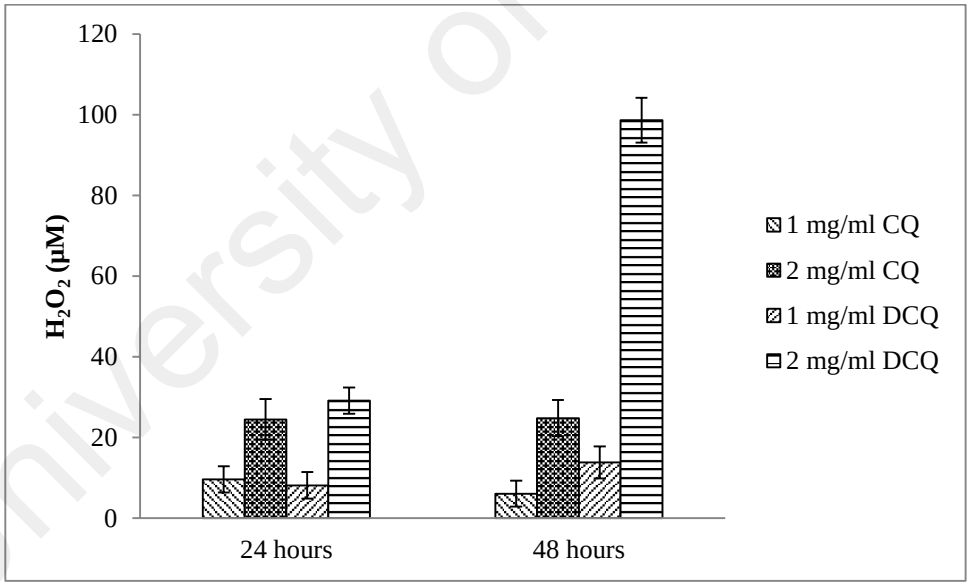


Figure 5.5: Extracellular concentration of H_2O_2 produced by CQ and DCQ
Following the treatments of CQ and DCQ in HCT 116 cell culture, the medium was collected to measure the concentration of H_2O_2. H_2O_2 was not detected in untreated control and medium added with catalase or catalase + SOD. The final concentration of catalase and SOD was 200 units/ml and 30 units/ml, respectively. Results are mean ± SD, n = 3.

5.3.4 Intracellular ROS level

In present study, the ability of CQ and DCQ to increase the intracellular ROS level in HCT 116 cells was investigated by using DCF-DA. Some studies used DCF-DA to determine the level of intracellular H_2O_2 but DCF-DA reacts indirectly with H_2O_2. It cannot be used to measure intracellular H_2O_2 accurately (Halliwell & Whiteman, 2004). Therefore, DCF-DA is a probe used to detect general oxidative stress or ROS within the cells. DCF-DA is cell permeable and will be deacetylated by esterases to dichlorofluorescin (DCFH) once enter the cell. DCFH can be oxidised to fluorescent dichlorofluorescein (DCF) by various radicals, peroxidases and heme proteins (Kalyanaraman *et al*., 2012).

In untreated control, most of the cells (99.6 %) were within R1 which is the low ROS level region (Figure 5.6). In positive control, cells were treated with 500 µM of H_2O_2 and the percentage of cells in R2 (high ROS or DCF positive region) was increased by 12.4 % compared to untreated control. In the treatments of CQ and DCQ, the cells did not show apparent sign of increase of intracellular ROS as most of the treated cells present in R1 region. The intracellular ROS of HCT 116 cells treated with 2 mg/ml of DCQ for 48 hours was not determined because of low number of live cells for the experiment.

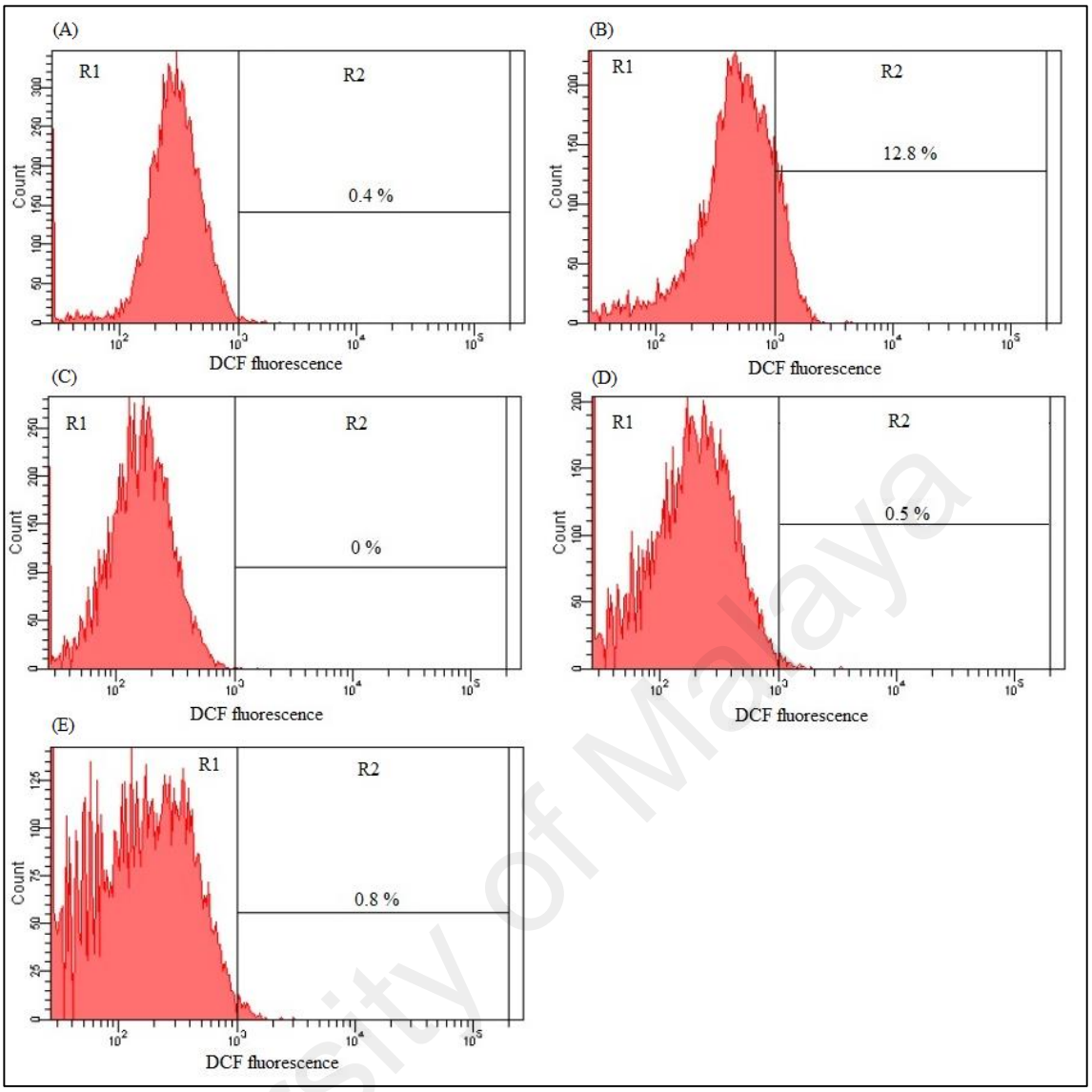


Figure 5.6: Intracellular ROS level of HCT 116 cells treated with CQ and DCQ (A) Cells were treated with 0.5 % of DMSO as untreated control. (B) Cells were treated with 500 µM of H_2O_2 for 20 minutes as positive control. (C and D) Cells were treated with 2 mg/ml of CQ for 24 and 48 hours, respectively. (E) Cells were treated with 2 mg/ml of DCQ for 24 hours.

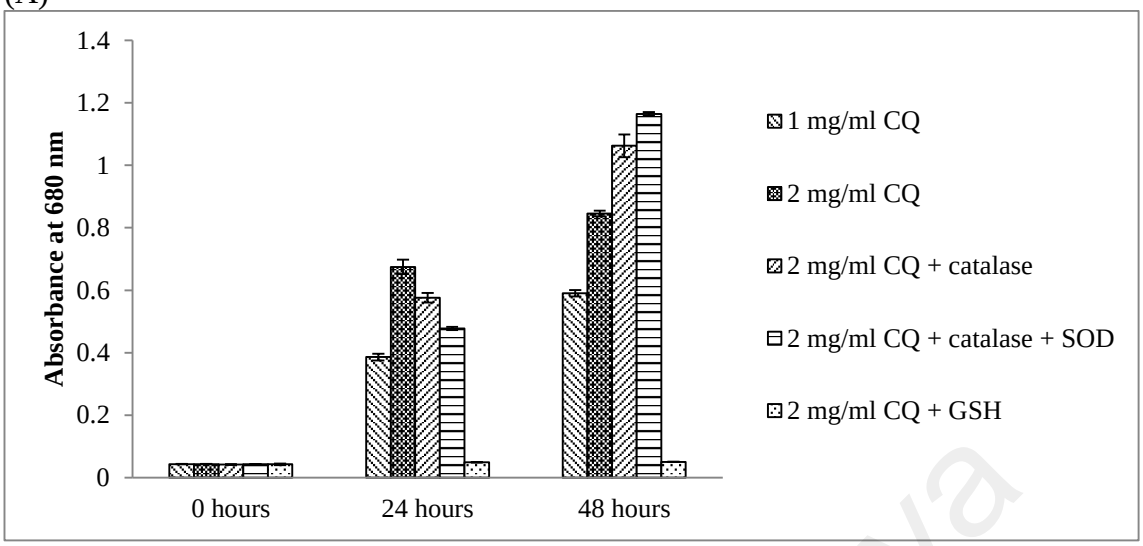
5.3.5 Generation of green pigments by CQ and DCQ

During the experiments, a colour change from pinkish to greenish in medium containing CQ or DCQ was observed, suggesting chemical reactions were occurred in the medium. As reported by Yabuta *et al.* (2001) and Namiki *et al*. (2001), CQ can react with various free amino acids to form green pigments (quinone products) in alkaline condition. The green pigments have maximal absorbance at around 680 nm. Therefore, absorbance value at 680 nm of the medium added with CQ or DCQ was measured in the

present study. The absorbance value of the medium was increased in concentration- and time-dependent manner (Figure 5.7). The results suggest that similar green pigments which reported by Yabuta *et al*. (2001) and Namiki *et al*. (2001) are formed in the medium. The DCQ showed higher rates of green pigments formation compared to CQ. In the presence of catalase / catalase with SOD, the rates of green pigments formation had decreased at 24 hours treatments. It seems that $O_2^{\bullet -}$ and H_2O_2 can influence the rates of initial green pigments formation. However, the formation rates of green pigments increased after 48 hours. This effect is remained to be further investigated.

GSH is a tripeptide which can donate electron to free radicals and react with another oxidised GSH to form glutathione disulfide (GSSG). GSH is also known to be effective in trapping reactive chemical intermediates such as quinones, thus, GSH was used to inhibit the formation of green pigments (presumably quinone products) in this study. As shown in Figure 5.7, the addition of GSH (4 mM) could completely inhibit the formation of green pigments after 24 and 48 hours treatments.

(A)



(B)

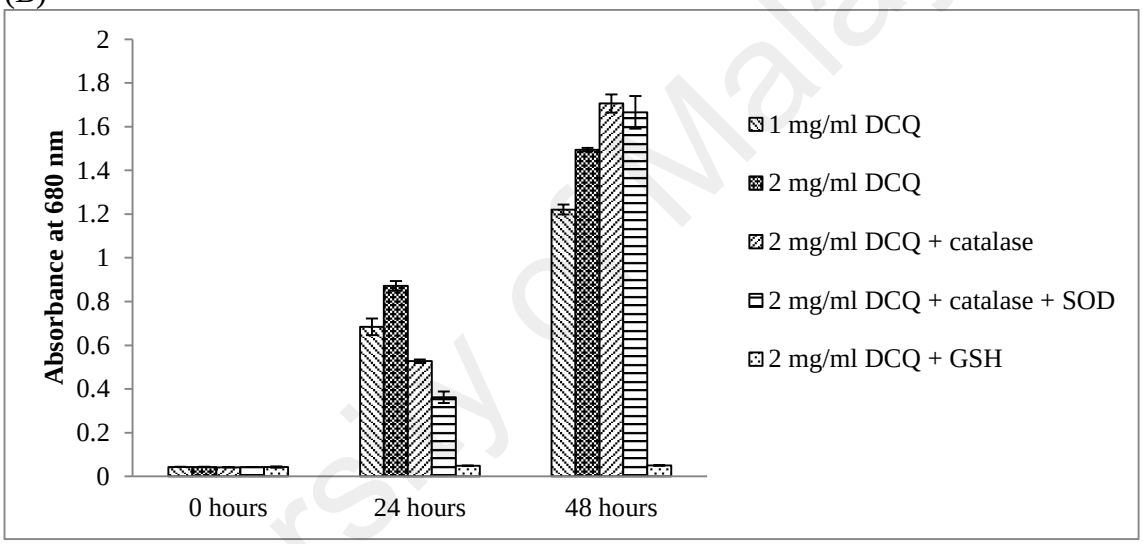


Figure 5.7: Green pigment formation in medium

(A) Following 0, 24 and 48 hours, the medium added with CQ was measured at 680 nm. (B) Following 0, 24 and 48 hours, the medium added with DCQ was measured at 680 nm. The final concentration of catalase, SOD and GSH was 200 units/ml, 30 units/ml and 4 mM, respectively. Results are mean ± SD, n = 3.

5.3.6 GSH protected HCT 116 cells from CQ and DCQ cell death effects

As mentioned above, CQ and DCQ could undergo oxidation to produce green pigments in the medium. The green pigments probably involve in the cell death effects of CQ and DCQ. Thus, the ability of GSH to prevent the cell death effects of the compounds was investigated as GSH could inhibit the formation of green pigments completely. The addition of GSH inhibited the cell death effect induced by DCQ in a

greater extent compared to catalase (compare Figure 5.4 and 5.8). The results showed GSH could increase the percentage of viable cells by 20 % more than catalase, thus proven that the extracellular H_2O_2 and green pigments are involved in cell death effect of DCQ. In contrast, the protective effect of catalase and GSH against CQ cell death effect was similar (compare Figure 5.3 and 5.8). Apparently, the cells can be protected from cell death by removing the extracellular H_2O_2 even in the presence of green pigments. The results suggesting that 2 mg/ml of CQ (48 hours) did not generate toxic concentration of quinone products but generated toxic levels of H_2O_2. Therefore, the cell death of HCT 116 cells induced by 2 mg/ml of CQ at 48 hours is mainly caused by the extracellular H_2O_2.

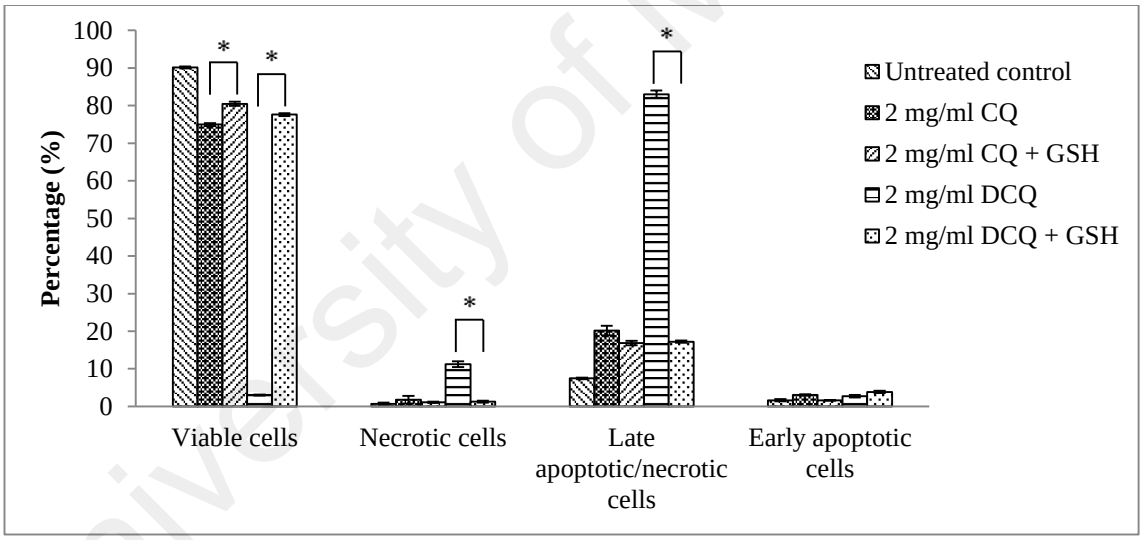


Figure 5.8: Protective effect of GSH against CQ and DCQ cell death induction
HCT 116 cells were treated with CQ or DCQ in the absence or presence of GSH for 48 hours. The final concentration of GSH was 4 mM. Cell death was assessed by annexin-V/PI flow cytometry. Results are mean ± SD, n = 3. (*$p < 0.05$, Student's *t*-test)

5.3.7 Impact of catalase and GSH on cell cycle arrest effects of CQ and DCQ

The investigation of cell cycle arrest effects of CQ and DCQ was included in the present study because little is known on this. Figure 5.9 and 5.10 showed CQ and DCQ could induce HCT 116 cells arrested at S phase. At 1 and 2 mg/ml of CQ after 48 hours treatments, the percentage of cells at S phase increased by 5.1 and 13.2 % respectively,

compared to untreated control. At 1 and 2 mg/ml of DCQ after 24 hours treatments, the percentage of cells at S phase increased by 8.0 and 8.2 %, respectively, compared to untreated control. As described above, catalase and GSH provide protection against cell death effects of CQ and DCQ. Therefore, the impact of catalase and GSH on cell cycle arrest effects of CQ and DCQ was also investigated in this study,

As shown in Figure 5.11, the S phase arrest was shifted to G0/G1 phase arrest in co-treatment of CQ and catalase; while co-treatment with GSH could completely protect HCT 116 cells from cell cycle arrest. It seems that in the absence of H_2O_2, CQ is still able to induce HCT 116 cells to arrest at G0/G1 phase and such effects is most likely due to the green pigments. The results indicated that both H_2O_2 and green pigments take part in cell cycle arrest effects of CQ. In DCQ treatments, addition of catalase could slightly prevent the HCT 116 cells from arrested at S phase while GSH could completely protect the HCT 116 cells from S phase arrest (Figure 5.12). The observations on the treatment of 1 mg/ml of DCQ at 24 hours, indicated that green pigments play the main role in contributing to the S phase arrest effect of DCQ.

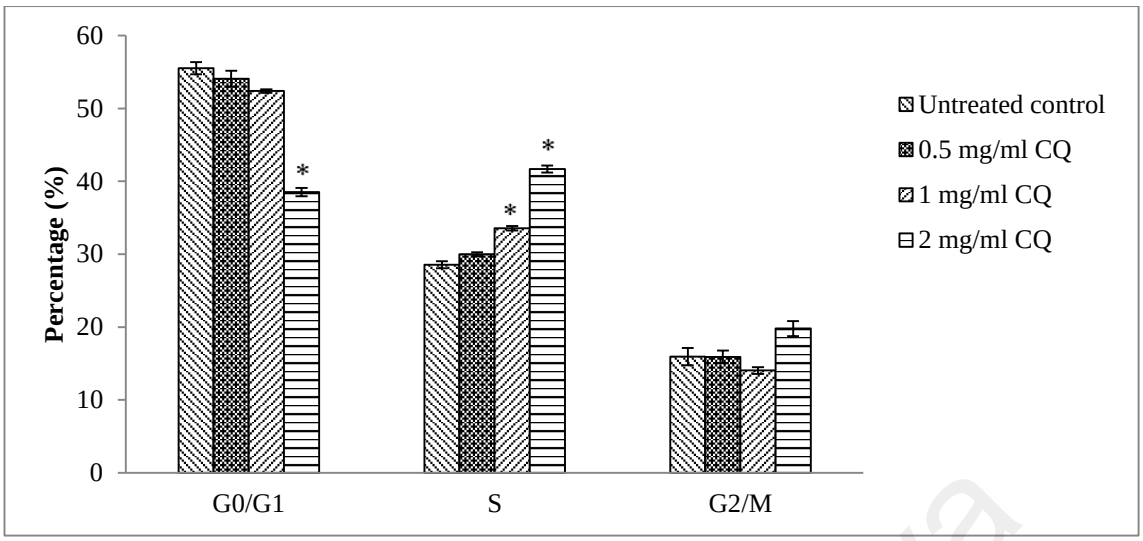


Figure 5.9: Cell cycle arrest effect of CQ

HCT 116 cells were treated with CQ for 48 hours and cell cycle distribution was assessed as described in material and methods. Results are mean ± SD, n = 3. (*p < 0.05, in comparison with untreated control)

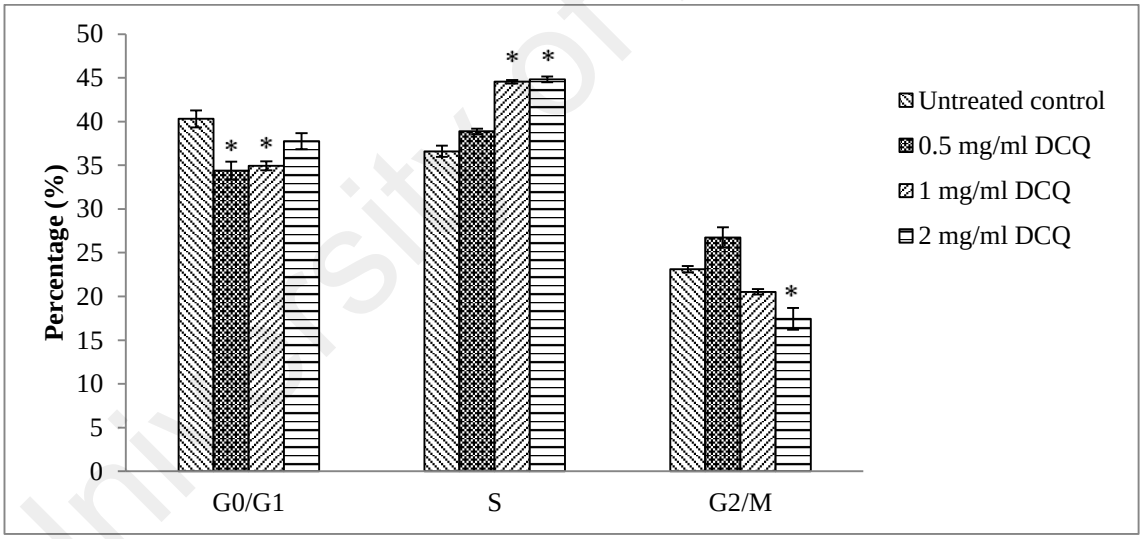


Figure 5.10: Cell cycle arrest effect of DCQ

HCT 116 cells were treated with DCQ for 24 hours and cell cycle distribution was assessed as described in material and methods. Results are mean ± SD, n = 3. (*p < 0.05, in comparison with untreated control)

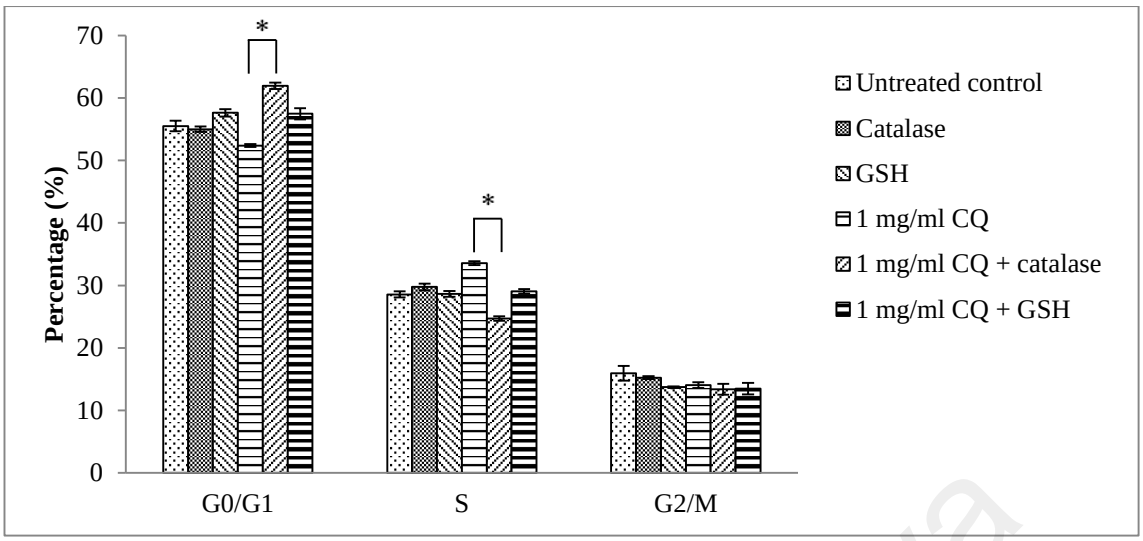


Figure 5.11: Protective effect of catalase and GSH against CQ cell cycle arrest
HCT 116 cells were treated with CQ in the absence or presence of catalase / GSH for 48 hours. The final concentration of catalase and GSH was 200 units/ml and 4 mM, respectively. Results are mean ± SD, n = 3. (*$p < 0.05$, Student's *t*-test)

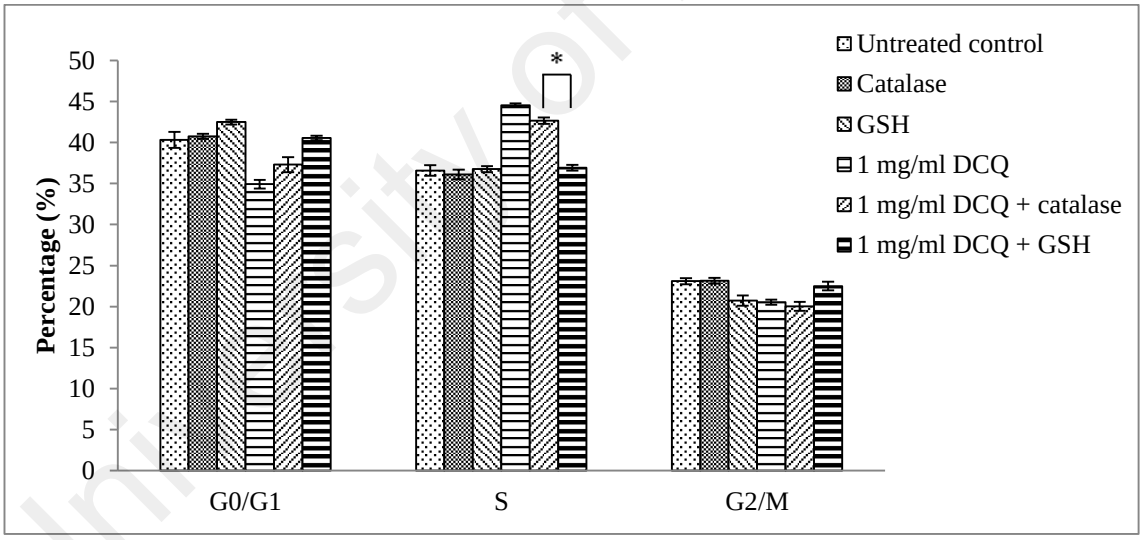


Figure 5.12: Protective effect of catalase and GSH against DCQ cell cycle arrest
HCT 116 cells were treated with DCQ in the absence or presence of catalase / GSH for 24 hours. The final concentration of catalase and GSH was 200 units/ml and 4 mM, respectively. Results are mean ± SD, n = 3. (*$p < 0.05$, Student's *t*-test)

5.4 Discussion

Previous studies by Burgos-Morón *et al*. (2012) and Jiang *et al*. (2000) observed that catalase could protect cancer cells (K562, A549, HSC-2, HSG) from the cytotoxic effect of CQ. The other study by Rakshit *et al*. (2010) reported PEG-catalase and *N*-

acetylcysteine could give similar protective effect against CQ cytotoxicity. These studies suggest that cytotoxicity of CQ involves oxidative stress which is induced by H_2O_2, but it is unclear whether intracellular H_2O_2 or extracellular H_2O_2 causes the cytotoxicity. In the present study, the increase of intracellular ROS level was not observed but the level of extracellular H_2O_2 was increased in the treatments of CQ. The cell death effect of CQ on HCT 116 cells is mainly depending on extracellular H_2O_2. Similarly, the increase of extracellular H_2O_2 level was detected but not intracellular ROS when HCT 116 cells were treated with DCQ.

The results in present study are in agreement with the report by Hu *et al*. (2011) which showed that intracellular ROS was not increased in HT-29 cells treated with methyl 3,5-dicaffeoylquinic acid. As shown in this study, the cell death effect of DCQ on HCT 116 cells is partly contributed by extracellular H_2O_2. Puangpraphant *et al*. (2011) observed that low concentration (100 µM) of 3,4- and 3,5-dicaffeoylquinic acid mixture could induce apoptosis in RKO and HT-29 cells, most probably *via* formation of extracellular H_2O_2. On the other hand, the high sensitivity of RKO and HT-29 cells towards H_2O_2 may explain the ability of dicaffeoylquinic acid mixture to induce apoptosis at much lower concentration compared to present study.

Beside cell death effects, CQ and DCQ also induce cell cycle arrest on HCT 116 cells which partly contributed by extracellular H_2O_2. Part of the cell cycle arrest effects could be contributed by the quinones that involved in the formation of green pigments as catalase could not completely protect HCT 116 cells from cell cycle arrest effects. Quinones may reduce the availability of free amino acids (Yabuta *et al*., 2001) and covalently bind to thiol groups of cell surface receptors (Thornton *et al*., 1995). Study demonstrated that quinones (oxidation products) of EGCG can covalently bind to epidermal growth factor receptors (Hou *et al*., 2005). The inactivation of receptors might occur and interfere with the cell signalling to induce the cell cycle arrest effects

on HCT 116 cells. GSH can provide protection against cell death and cell cycle arrest effects of CQ and DCQ. Most likely, GSH protected the HCT 116 cells from quinones directly and H_2O_2 indirectly. GSH can react with oxidised caffeoylquinic acids (quinones) to form various GSH-conjugated products and protected HCT 116 cells (Xie *et al*., 2012). GSH probably did not directly scavenge H_2O_2 to protect HCT 116 cells because of low reaction rates (Winterbourn & Metodiewa, 1999) but may reduce the quantity of H_2O_2 resulted from the inhibition of oxidative green pigments formation.

Both CQ and DCQ were showed to oxidise in the cell culture medium with the generation of H_2O_2 and green pigments in section 5.3.3 and 5.3.5. CQ and DCQ showed good scavenging activity against various free radicals except H_2O_2 (Iwai *et al*., 2004; Kono *et al*., 1997; Sroka & Cisowski, 2003) which may explain the accumulation of H_2O_2 in culture medium. DCQ exhibited higher oxidation rates as determined by the production of H_2O_2 and green pigments (Figure 5.5 and 5.7). The difference between these compounds is the number of catechol moiety, which DCQ has additional one catechol moiety compared to CQ. The catechol moiety is prone to oxidation and it is important in contributing to antioxidant activity. Some other phenolic compounds had been found to be unstable in culture media and prone to oxidation and generation of H_2O_2, semiquinone radicals and quinones (Babich *et al*., 2011; Long *et al*., 2010).

Previous MTT assay showed that CQ and DCQ induced cytotoxic effect on HCT 116 cells at lower concentration with IC_{50} values of 79.7 and 79.3 µg/ml, respectively (Table 4.4). In the present annexin-V/PI flow cytometry experiments, higher concentrations of CQ and DCQ were needed to show the cell death effects. The differences may due to the number of cells and exposure time used in the experiments. In MTT assay, 3 X 10^3 cells were used for seeding and treatments were done for 72 hours. While, 2 X 10^5 cells were used for seeding and treatments were done for 48 hours in annexin-V/PI flow cytometry. Gülden *et al*. (2010) reported that cytotoxicity of

H_2O_2 was cell concentration- and time-dependent mainly because high cell number could eliminate extracellular H_2O_2 at higher rate compared to low cell number.

Bioavailability of caffeoylquinic acids is frequently found to be very low in plasma because of rapid metabolism and limited absorption (Erk *et al*., 2012; Stalmach *et al*., 2014). Studies demonstrated that about 64 % of the ingested mono-caffeoylquinic acid and 57 % of the ingested di-caffeoylquinic acid could reach colon before absorption (Erk *et al*., 2012; Stalmach *et al*., 2010). Thus, colon epithelial cells may expose to high concentration of intact caffeoylquinic acid molecules. Under normal physiological condition, oxidation of caffeoylquinic acids might be limited because of colonic hypoxia environment. However, at the site of increased inflammation that usually associated with CRC (McLean *et al*., 2011; Roncucci *et al*., 2008), oxidation of CQ and DCQ may take place and lead to the generation of semiquinone radicals, quinones and ROS because of enhanced secreted myeloperoxidase from inflammatory cells (O'Brien, 2000, Xie *et al*., 2012). According to the results in present study, these oxidation products of caffeoylquinic acid can exert its cell death and cell cycle arrest effects on colon cancer cells. Previous study by Moridani *et al*. (2001) suggested metabolic conversion of CQ to cytotoxic semiquinone radical and quinone could occur intracellularly in the presence of peroxidase or tyrosinase or cytochrome P450. It is important to note that cell permeability of caffeoylquinic acids is rather limited (Farrell *et al*., 2011). Thus, intracellular oxidation of caffeoylquinic acids might not be the major event.

As shown in Table 4.4, CQ and DCQ demonstrated selective cytotoxicity against colon cancer cells. Cancer cells that are frequently under oxidative stress may be more sensitive to H_2O_2 challenge compared to normal cells. Thus, this could be the reason for the selectivity of both caffeoylquinic acids against HCT 116 carcinoma cells compared to normal CCD-18Co cells. Galati and O'Brien (2004) suggested that

antioxidant activity of phenolic compounds contributed to chemoprevention while their pro-oxidant activity contributed to anticancer.

5.5 Conclusion

High concentrations of CQ and DCQ could induce cell death and cell cycle arrest on HCT 116 cells. The oxidation of CQ and DCQ resulted in the generation of extracellular H_2O_2 and quinone products which contributed to the cell death and cell cycle arrest effects. Thus, high concentration of dietary CQ and DCQ may play a role in inhibiting the growth of cancer cells in colon. Further study is warranted to investigate *in vivo* generation of H_2O_2 and quinone products from CQ or DCQ especially at the site of inflammation.

CHAPTER 6: ANTI-PROLIFERATION EFFECT OF GUANOSINE ON HCT 116 CELLS

6.1 Introduction

Nucleosides such as adenosine and guanosine (structures shown in Figure 6.1) are formed when ribose links to adenine and guanine, respectively. These molecules are crucial for deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) synthesis, cellular energy (ATP) and cell signalling (ATP, GTP). Nucleosides have been implicated as neurotransmitters in nervous system, especially adenosine. Adenosine has been found to influence the functions of neural and glial cells such as proliferation, differentiation and survival through P1 receptors with four subtypes (A_1, A_{2A}, A_{2B}, A_3) (Burnstock *et al*., 2011). On the other hand, guanosine has also been shown to exert similar effects but the specific mechanisms or receptors are remained to be fully elucidated (Rathbone *et al*., 1999).

Studies reported that extracellular guanosine was one of the factors that could stimulate the proliferation of astrocytes, rat brain microglia and human brain capillary endothelial (Kim *et al*., 1991; Rathbone *et al*., 1992a & 1992b). Moreover, guanosine can elicit differentiation (neurite outgrowth) of rat adrenal pheochromocytoma cells (PC-12), human neuroblastoma cells (SH-SY5Y) and melanoma cells (B16F10) to neuron-like cells (Gysbers & Rathbone, 1996; Guarnieri *et al*., 2009; Naliwaiko *et al*., 2008). These cellular effects of extracellular guanosine are associated with the increase of intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Bau *et al*., 2005). These cAMP and cGMP can act as secondary messengers that regulate intracellular signalling pathways. In contrast, literatures have shown that guanosine elicit anti-proliferation and cytotoxic effects on various cancer cell lines including leukemic T cells (Jurkat, MOLT 4, KM-3), B cells

(GM 558, MGL 8), lymphoma cells (Raji), glioblastoma cells (U87), B16F10 and SH-SY5Y cells (Batiuk *et al*., 2001; Garozzo *et al*., 2010; Guarnieri *et al*., 2009; Sidi & Mitchell, 1984; van der Kraan *et al*., 1988). It is suggested that guanosine is converted to guanine and ribose phosphate by purine nucleotide phosphorylase (PNP). In turn, guanine can be salvaged to GMP by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) with the consumption of 5-phosphoribosyl-pyrophosphate (PRPP). Subsequently, GMP can be converted to GTP with the consumption of ATPs. The intracellular GTP level is increased while intracellular ATP level is decreased when cells are exposed to guanosine (Batiuk *et al*., 2001; Garozzo *et al*., 2010). The anti-proliferation effect of guanosine can be reversed by addition of adenosine which can be salvaged to restore the adenine nucleotide levels (Batiuk *et al*., 2001; Garozzo *et al*., 2010). The disruption of the balance between adenine nucleotide and guanine nucleotide pools is the mechanisms linked to the anti-proliferation effect of guanosine. However, the mechanisms are not completely understood in particular the potential disruption of cell signalling pathways. In present study, the focus was on the changes of signalling pathways in HCT 116 cells after guanosine exposure.

As shown in Table 4.4, guanosine showed selective cytotoxicity against HCT 116 cells with IC_{50} value of 81 µg/ml compared to normal colon CCD-18Co cells. The result demonstrated that guanosine showed high selective index (> 6.2). To our knowledge, the anti-proliferation effect of guanosine on HCT 116 cells has not been reported.

The specific objectives of the present study were as follows:

i. To investigate the cell death effect of guanosine on HCT 116 cells

ii. To investigate the cell cycle arrest effect of guanosine on HCT 116 cells

iii. To determine the levels of signalling- and cell cycle- related proteins associated with cell cycle arrest effect of guanosine

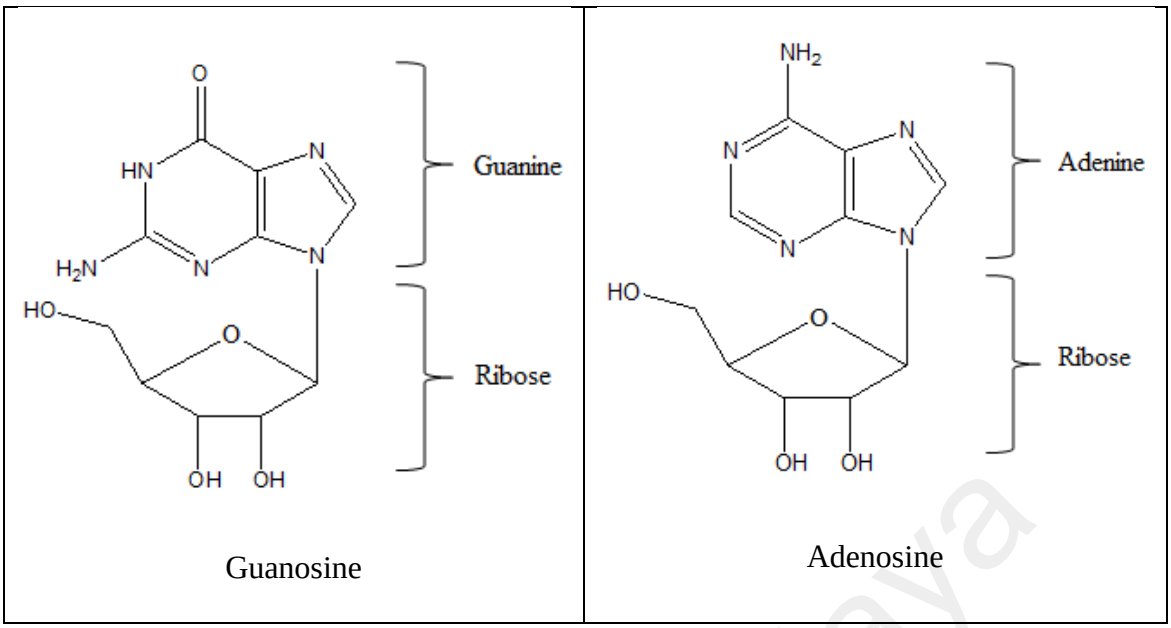


Figure 6.1: Structures of nucleosides

6.2 Materials and methods

6.2.1 Chemicals and antibodies

Guanosine was isolated from *G. bicolor* as described in section 4.3. Glycerol, Triton X-100, EDTA-2Na, phenylmethanesulfonyl fluoride (PMSF), mercaptoethanol and D-ribose were purchased from Sigma-Aldrich. FITC Annexin V Apoptosis Detection Kit I and CycleTEST^{TM} PLUS DNA Reagent kit were purchased from BD Biosciences Company. Antibodies for AKT (4691), P-AKT (4060), ERK1/2 (4695), P-ERK1/2 (4370), S6 (2217), P-S6 (4858), AMPK (5832), P-AMPK (2535), p38 (8690), P-p38 (4511), JNK (9258), P-JNK (4668), p21 (2947), p27 (3686), cyclin D1 (2978) and β-actin (8457) were purchased from Cell Signaling Technology. Prestained protein marker, biotinylated protein ladder, glycine, Tris-base, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), nonfat dry milk were purchased from Cell Signaling Technology. Dithiothreitol (DTT), ammonium persulfate (APS), 30 % acrylamide:bis solution (29:1), tetramethylethylenediamine (TEMED), tween 20, bromophenol blue were purchased from Bio Rad. Goat anti-rabbit horseradish peroxidase conjugated antibody, RIPA buffer, Pierce™ BCA Protein Assay Kit, Halt™ Protease and

Phosphatase Inhibitor Cocktail, SuperSignal West Pico chemiluminescent substrate, nitrocellulose membrane and blot paper were purchased from Thermo Scientific.

6.2.2 Cell culture and guanosine stock

All the experiments, HCT 116 cells were cultured in McCoy's 5A medium supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin and 1 % amphotericin B. The cells were cultured in 5 % CO_2 at 37 ^{o}C in a CO_2 incubator. Stock solution of guanosine was prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO in all the experiments was 0.5 %.

6.2.3 Annexin-V/PI flow cytometry

Firstly, HCT 116 cells (2 x 10^5 cells) were seeded in 60 mm culture dish. After 24 hours, HCT 116 cells were treated with various concentration of guanosine. HCT 116 cells treated with 0.5 % DMSO was used as untreated control. After 72 hours, cells were harvested and stained with FITC annexin-V and PI following the instruction of the FITC Annexin V Apoptosis Detection Kit I. Total of 10,000 cells were analysed by flow cytometer (BD FACSCanto II) using FACSDiva software.

6.2.4 Cell cycle arrest flow cytometry

Firstly, HCT 116 cells (2 x 10^5 cells) were seeded in 60 mm culture dish. After 24 hours, medium was changed to fresh medium added with various tested samples. Depending on indicated treatment, D-ribose was dissolved in medium first. Appropriate medium was used to dilute the stock solution of guanosine to 300 µg/ml. The final concentrations of D-ribose were 1, 2 and 4 mM. Appropriate medium added with DMSO (final concentration, 0.5 %) was used as untreated control. After 24, 48 and 72 hours, cells were harvested and stained with PI following the instruction of the

CycleTEST^{TM} PLUS DNA Reagent kit. Total of 30,000 cells were analysed by flow cytometer (BD FACSCanto II) using FACSDiva and ModFit softwares.

6.2.5 Microscopic observation

HCT 116 cells treated with guanosine were observed for morphological changes under inverted light microscope (Leica).

6.2.6 Western blot

At the end of the experiments, HCT 116 cells on the 60 mm culture dish were washed twice with cold PBS. 100 µl of cold RIPA buffer (Halt™ Protease and Phosphatase Inhibitor Cocktail added) or lysis buffer (25 mM Tris-HCl pH7.5, 420 mM NaCl, 0.2 mM EDTA-2Na, 10 % glycerol, 0.5 mM DTT, 0.5 mM PMSF, 0.5 % Triton X-100, Halt™ Protease and Phosphatase Inhibitor Cocktail) was added onto the cells and rapidly scrapped and collected into microcentrifuge tube. The microcentrifuge tube with RIPA buffer was incubated on ice for 15 minutes. Then, sample was sonicated for 3 seconds followed by 30 seconds incubation on ice. The same method was repeated twice. If lysis buffer was used, the incubation time was 30 minutes with vortex at every 10 minutes. The sample was centrifuged at 14000 X g at 4 ^{o}C for 10 minutes. The supernatant was collected for protein quantification with Pierce™ BCA Protein Assay Kit. After protein quantification, the aliquot samples were stored at -80 ^{o}C. Mini PROTEAN tetra cell (Bio Rad) was used to run SDS-PAGE, short and spacer glass plates were cleaned with 70 % ethanol. The plates were clamped on the casting frame and checked for leakage with deionised water. 10 % (w/v) of APS was prepared freshly. Then, 10% of resolving gel solution (1.0 mm thickness) was prepared and loaded into the gel cassette. Deionised water was used to cover the surface of resolving gel solution. After 1 hour, deionised water was removed. 4 % of stacking gel solution was loaded and

comb was included; the stacking gel would be ready in 1 hour. Equal amount of proteins were mixed with 5 X sample buffer (Appendix Q) and heated for 5 minutes at 95 ^{o}C then keep on ice before loading. The gel cassette was fixed to electrode assembly and loaded with running buffer (Appendix Q). The comb was removed and protein samples were loaded into wells. The gel cassette with electrode assembly was put into tank and loaded with running buffer. The gel electrophoresis was run at 180V for about 45 minutes. The gel was removed and transferred into transfer buffer for 15 minutes. Nitrocellulose membrane, blot papers and fiber pads were soaked in transfer buffer (Appendix Q) for 15 minutes as well. Mini Trans-Blot (Bio Rad) was used for western blotting. Gel holder cassette was used to sandwich the nitrocellulose membrane with gel along with blot papers and fiber pads. A roller was used to remove any air bubbles. The gel holder cassette was put into the electrode module and then placed into the tank. The tank was loaded with transfer buffer and run at 70V for 1.5 hours under cooling condition. The membrane was transferred into 25 ml of tris buffered saline (TBS) buffer and rocked for 5 minutes at room temperature. After washing, the membrane was soaked in 25 ml of blocking buffer (Appendix Q) and rocked for 1 hour at room temperature. The membrane was washed with 15 ml of tris buffered saline with tween-20 (TBST) buffer for 5 minutes. The membrane was added with primary antibody solution (Appendix Q) and rocked for 16 hours at 4 ^{o}C. The membrane was washed with 15 ml of TBST buffer (Appendix Q) for 3 times, each time 5 minutes at room temperature. The membrane was added with horseradish peroxidase conjugated antibody solution and rocked for 1 hour at room temperature. Then, membrane was washed with 15 ml of TBST buffer for 3 times, each time 5 minutes. The membrane was added with SuperSignal West Pico chemiluminescent substrate and viewed with ChemiDoc (Bio Rad). The data were analysed by Image Lab software.

6.2.7 Statistical analysis

Annexin-V/PI and cell cycle flow cytometry data was analysed using Student's *t*-test, with *P* values < 0.05 being regarded as significant difference.

6.3 Results and discussion

6.3.1 Cell death induction of guanosine on HCT 116 cells

The cell death effect of guanosine on HCT 116 cells was investigated by annexin-V/PI flow cytometry to confirm the cytotoxic effect of guanosine observed in the MTT assay (Table 4.4). To my surprise, guanosine can only induce notable cell death effect on HCT 116 cells at high concentrations (500 µg/ml) and long incubation times (72 hours). At 72 hours treatment, 500 µg/ml of guanosine caused a marked decrease in the percentage of viable cells to 71.8 % compared to untreated control which showed 90.1 % of viable cells (Figure 6.2). In comparison with untreated control, the percentage of late apoptotic/necrotic cells increased by 12.7 % while percentage of early apoptotic cells increased by 3.9 %.

Previous MTT assay showed that guanosine induced cytotoxic effect on HCT 116 cells at low concentration with IC_{50} values of 81 µg/ml (Table 4.4). In the present annexin-V/PI flow cytometry experiment, higher concentration of guanosine is required to show the cell death effect. The differences may due to the number of cells used in the experiments. In MTT assay, 3×10^3 cells were used for seeding while 2×10^5 cells were used for seeding in annexin-V/PI flow cytometry. The results demonstrate that the cytotoxicity of guanosine on HCT 116 cells may be cell number dependent.

Batiuk *et al*. (2001) demonstrated that guanosine could induce necrotic and apoptotic cell death on Jurkat cells but at low concentration (142 µg/ml) and short incubation (24 hours). Batiuk *et al*. (2001) also reported that guanosine induced accumulation of GDP and GTP along with the depletion of ATP and

deoxyribonucleotide (dGTP and dATP). The deoxyribonucleotides are important for the DNA synthesis in cell cycle while ATP is source of energy for survival and growth of cells. The study suggested that guanosine could deplete these vital nucleotides to cause Jurkat cells undergo apoptosis and necrosis. In agreement, Sidi and Mitchell (1984) demonstrated that guanosine could induce accumulation of GTP in GM 558 cells with growth inhibitory effect. In present study, depletion of ATP by guanosine could take place in HCT 116 cells to cause cell death effect.

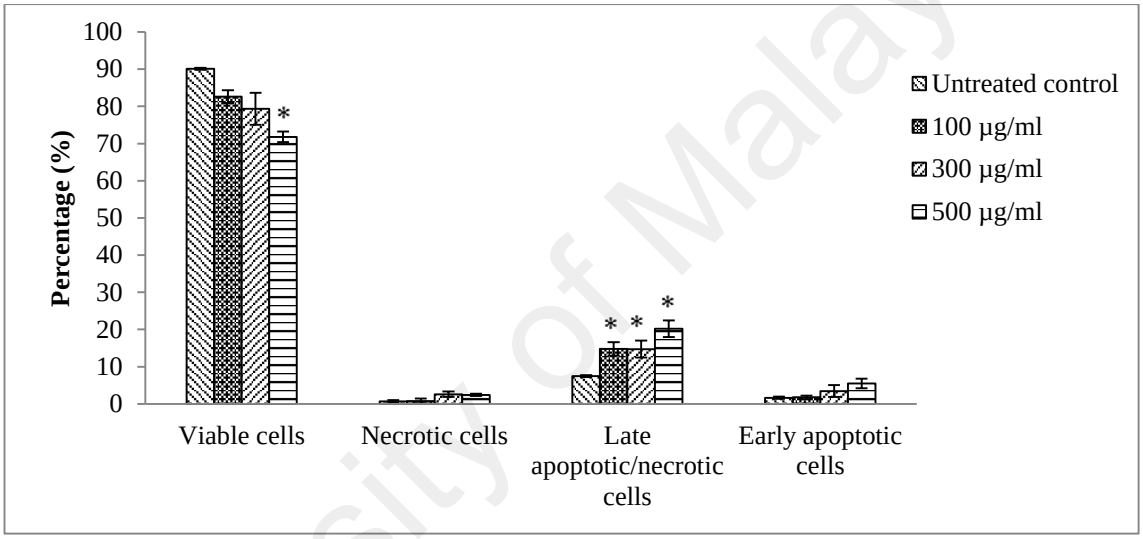


Figure 6.2: Cell death effect of guanosine on HCT 116 cells

HCT 116 cells were treated with guanosine for 72 hours and analysed by Annexin-V/PI flow cytometry. Results are mean ± SD, n = 3. (*p < 0.05, in comparison with untreated control)

6.3.2 Cell cycle arrest effect of guanosine

As observed above, the cell death response of HCT 116 cells to guanosine was not drastic. However, guanosine could have an impact on the cell cycle distribution of HCT 116 cells. At 24 hours treatment, guanosine (100, 300 and 500 µg/ml) could increase the proportion of cells in G0/G1 phase (Figure 6.3). The cell cycle distribution of HCT 116 cells was shifted when treated with 300 and 500 µg/ml of guanosine for 48 and 72 hours but not 100 µg/ml of guanosine. The results suggesting that low concentration of guanosine can only induce cell cycle arrest effect for short periods (24

hours). In contrast, high concentration of guanosine can interfere with cell cycle process for longer periods (48 and 72 hours). Studies by Guarnieri *et al*. (2009) and Moosavi *et al*. (2006) demonstrated that guanosine could induce cell cycle arrest on SH-SY5Y and K562 cells in S phase. Guarnieri *et al*. (2009) reported that upregulation of cyclin E2/cdk2 and downregulation of cyclin B1/B2 was responsible for cell cycle arrest effect of guanosine as determined by microarray gene expression system. Studies had shown that cell cycle arrest effect of guanosine was accompanied by differentiation effect (Guarnieri *et al*. 2009; Naliwaiko *et al*., 2008). However, in present study, the differentiation of HCT 116 cells into neurite-like cells was not observed when treated with guanosine (Figure 6.4). The differentiation effect of guanosine might be cell type dependent.

(A)

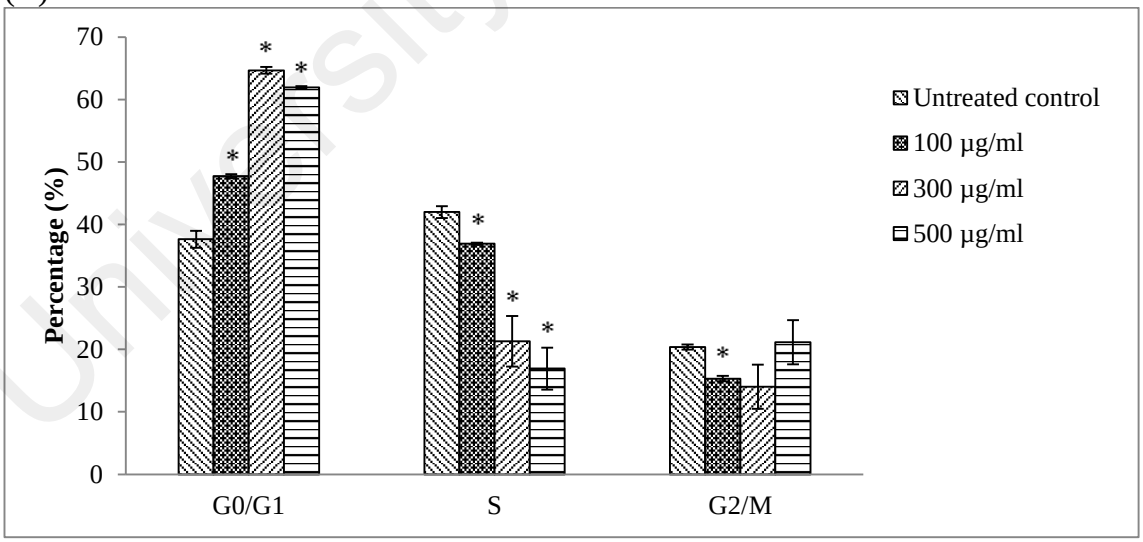
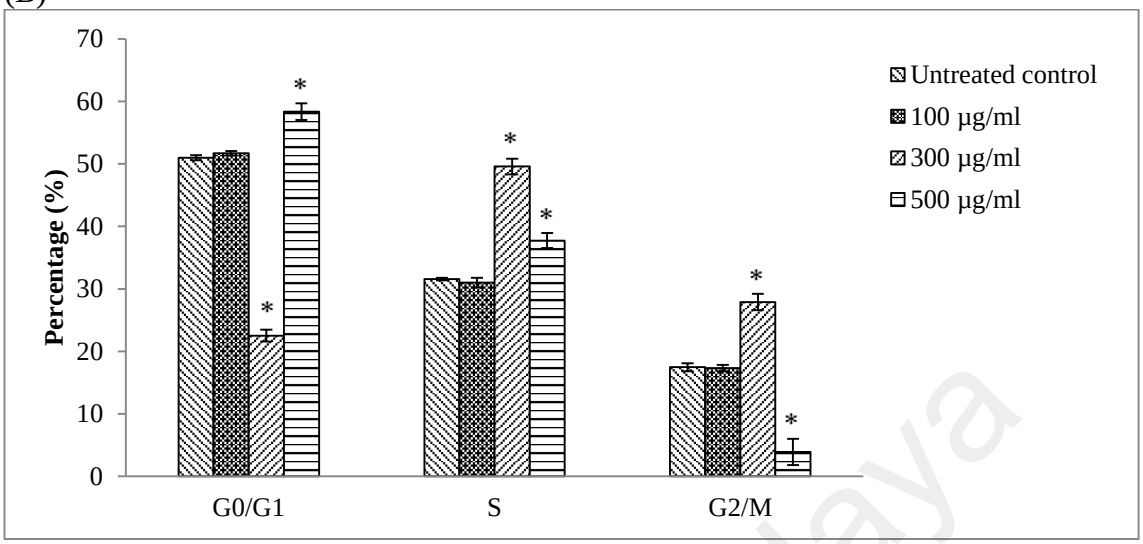


Figure 6.3, continued

Figure 6.3, continued

(B)



(C)

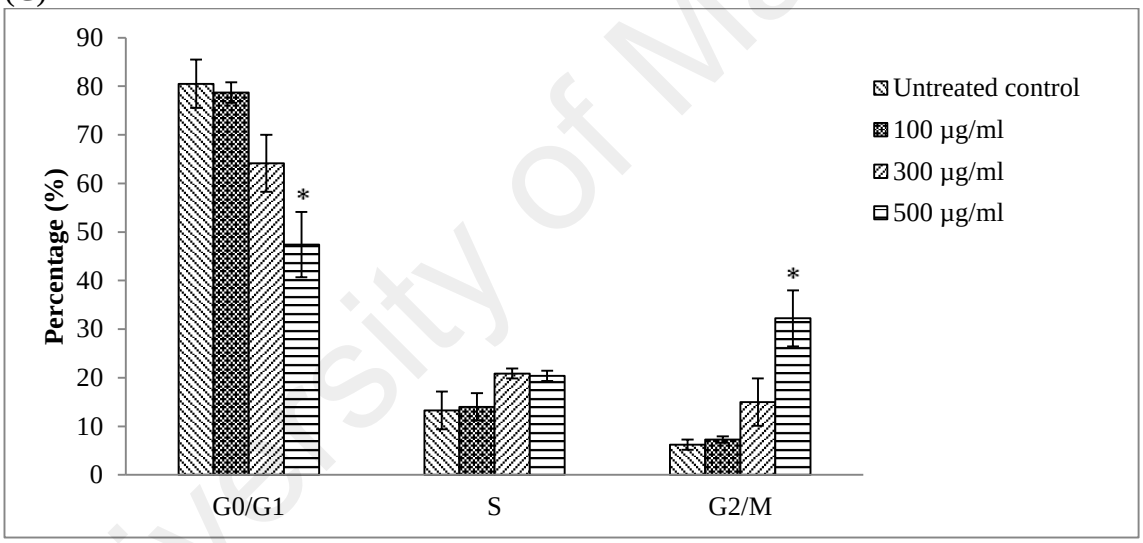


Figure 6.3: Cell cycle arrest effect of guanosine

(A) HCT 116 cells were treated with guanosine for 24 hours. (B) HCT 116 cells were treated with guanosine for 48 hours. (C) HCT 116 cells were treated with guanosine for 72 hours. Treated cells were stained with PI and analysed by flow cytometry. Results are mean ± SD, n = 3. (*$p < 0.05$, in comparison with untreated control)

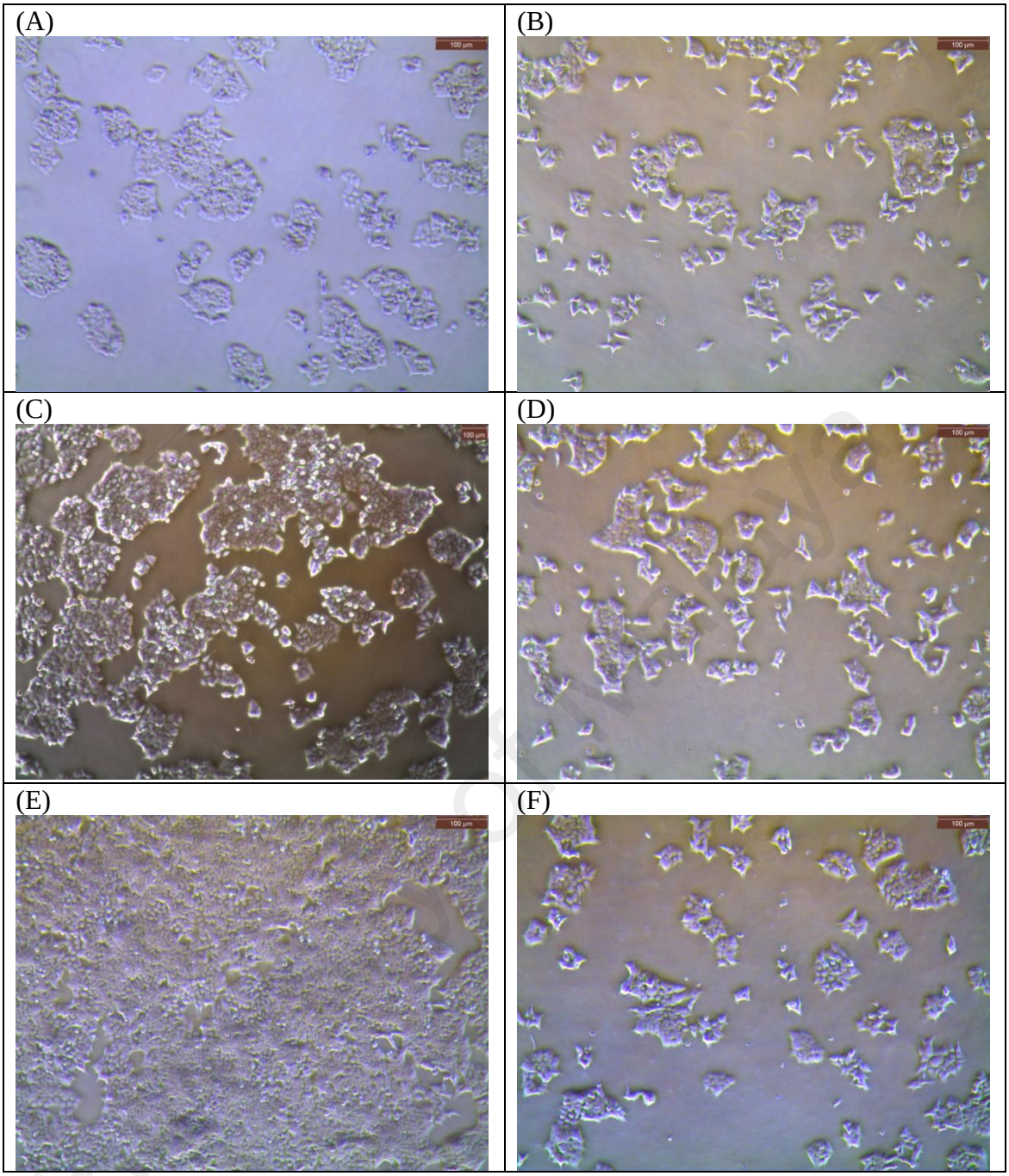


Figure 6.4: Morphological observation of guanosine treated HCT 116 cells
(A) Untreated control 24 hours. (B) Cells treated with 300 µg/ml of guanosine for 24 hours. (C) Untreated control 48 hours. (D) Cells treated with 300 µg/ml of guanosine for 48 hours. (E) Untreated control 72 hours. (F) Cells treated with 300 µg/ml of guanosine for 72 hours. (All are under 50x magnification).

6.3.3 Investigation of impact of D-ribose in preventing cell cycle arrest induced by guanosine

As discussed in Introduction, the anti-proliferative effect of guanosine is linked to the depletion of ATP. It was hypothesised that addition of D-ribose may help to

stimulate the production of ATP through pentose phosphate pathway and purine *de novo* synthesis pathway (Salerno *et al*., 1999). D-ribose can be converted to ribose-5-phosphate by ribokinase to stimulate the production of glyceraldehyde-3-phosphate (G3P) and PRPP in pentose phosphate pathway and purine *de novo* synthesis pathway, respectively (Figure 6.5).

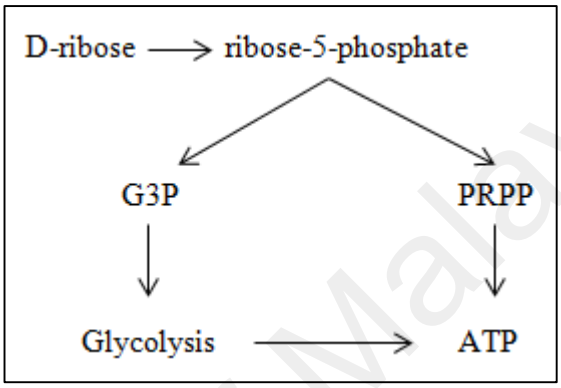


Figure 6.5: General scheme showing the stimulation of ATP production by D-ribose

In the present study, D-ribose had been added along with guanosine to investigate the effect on cell cycle of HCT 116 cells. The results failed to show any protective effect of D-ribose against cell cycle arrest effect of guanosine (Figure 6.6). The possible explanation is that the cells could be running low in adenine base not the ribose when exposed to guanosine. In order to restore the off balanced adenine nucleotides pool, feeding the cells with sources of adenine base such adenine and adenosine would be more effective.

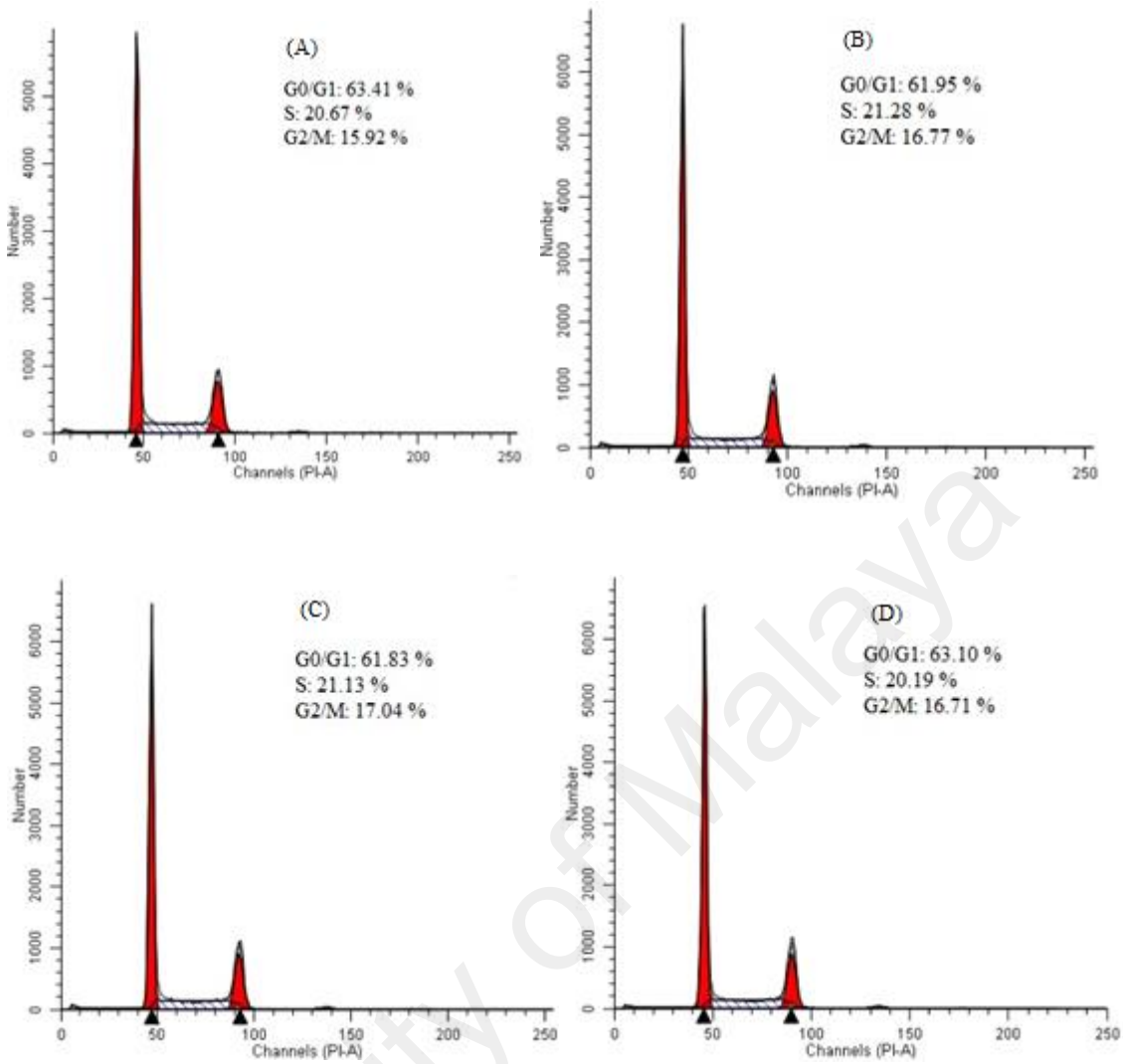


Figure 6.6: Cell cycle of HCT 116 cells treated with 300 µg/ml of guanosine in the presence or absence of D-ribose at 24 hours

(A) 300 µg/ml of guanosine. (B) 300 µg/ml of guanosine with 1 mM of D-ribose. (C) 300 µg/ml of guanosine with 2 mM of D-ribose. (D) 300 µg/ml of guanosine with 4 mM of D-ribose. Treated cells were stained with PI and analysed by flow cytometry. The results are the representative of duplicate experiments.

6.3.4 Guanosine activates ERK1/2, p38, JNK, AKT and S6 phosphorylation but decreases the phosphorylation of AMPK

The effect of guanosine on selected proteins that related to cell signalling pathways was investigated to provide additional mechanisms of action of guanosine in anti-proliferation. The mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that regulate various cellular activities such as proliferation, survival, differentiation and migration. The MAPK family is consisting of extracellular signal-

regulated kinases (ERKs), p38s and c-JUN N-terminal kinases (JNKs) which transduce the signals into gene transcription in response to cellular environment (Berridge, 2012). In general, ERKs are usually activated by growth stimuli while p38s and JNKs are activated by stress related stimuli. The results showed that guanosine (300 μg/ml) increased the phosphorylated ERK1/2, p38 and JNK in the treatments of 24 hours (Figure 6.7). The elevated phosphorylated ERK1/2 was persisting to 48 hours treatment but not p38. In 48 hours treatment, phosphorylated JNK was slightly increased compared to untreated control. As shown in untreated controls at 24 and 48 hours, HCT 116 cells need lower level of phosphorylated ERK1/2 for proliferation. These results suggesting that over activation of the ERK1/2 is inhibiting the proliferation of HCT 116 cells rather than promoting.

The anti-proliferation effects of guanosine could be associated with metabolic stresses caused by the disruption of ATP balance. In this study, two metabolisms related pathways, mammalian target of rapamycin 1 (mTOR1) and AMP-activated protein kinase (AMPK) pathways were investigated. The mTOR1 is a serine/threonine kinase [downstream of protein kinase B (AKT)] that promotes cell growth by activating anabolic activities especially protein synthesis. This is done through activation of ribosomal S6 kinase 1 (S6K1) and inhibition of eukaryotic initiation factor 4E binding protein 1 (4E-BP1) by phosphorylation (Laplante & Sabatini, 2009). The resulting activated S6K1 can phosphorylate ribosomal protein S6 (Meyuhas & Dreazen, 2009). Generally, phosphorylation of S6 is a good indicator of S6K1 activity. Western blot analysis revealed that guanosine increased the phosphorylated AKT in 24 hours treatment (Figure 6.7). Furthermore, the increased of phosphorylated S6 was observed in 24 and 48 hours of guanosine treatments. The phosphorylated AKT can promote the activation of mTOR1 by phosphorylating tuberous sclerosis complex 2 (TSC2) and proline-rich AKT substrate of 40 kDa (PRAS40) (Manning & Cantley 2007). Therefore,

the results suggesting that mTOR1 activity of HCT 116 cells was not interrupted by the treatments of guanosine. AMPK is a sensor can be activated by low intracellular ATP level to regulate metabolisms. Once activated, it can promote glycolysis and fatty acid oxidation to generate ATP while inhibit anabolisms such as protein, fatty acid and cholesterol synthesis (Towler & Hardie, 2007; Hardie, 2011). The results showed that the levels of phosphorylated AMPK were decreased by guanosine treatments at 24 hours and sustained until 48 hours (Figure 6.7). Most of the cancer cells have high activity of glycolysis which is well known as the Warburg effect. Glycolysis is not only supplying ATP but also intermediates for citric acid cycle which is important for energy, lipid and amino acid synthesis. Based on the decrease of phosphorylated AMPK, the catabolisms (glycolysis, fatty acid oxidation) of HCT 116 cells may be downregulated when exposed to guanosine.

6.3.5 Guanosine decreases the level of cyclin D1

The investigation was carried out to determine cell cycle related proteins that were involved in the cell cycle arrest effect induced by guanosine. Study showed that p38 could mediate the upregulation of p16 expression and lead to cell cycle arrest (Thornton & Rincon, 2009). But p16 should not be involved because HCT 116 cells lack of p16 (Okamoto *et al*., 1994). The activation of p53 by p38 and JNK may take place and induce the expression of p21 to cause cell cycle arrest on HCT 116 cells (Wagner & Nebreda, 2009). In addition, sustained activation of ERK and p38 has been shown to upregulate the expression of p21 and induce cell cycle arrest (Park, 2014; Todd *et al*., 2004). However, the results of 24 and 48 hours treatments of guanosine did not drastically increase the levels of p21 and p27 in HCT 116 cells (Figure 6.8). Therefore, the cell cycle arrest effects observed in guanosine treated HCT 116 cells were not associated with p21 and p27. Study showed that the expression of cyclin D1

could be downregulated by ERK and p38 (Densham *et al*., 2008). Study also had shown that p38 could phosphorylate to inhibit cyclin D1 and mediate the inhibition of cyclin D1 gene expression (Thornton & Rincon, 2009). The western blot analysis revealed that guanosine (300 µg/ml) treatments decreased cyclin D1 levels at 24 and 48 hours treatments (Figure 6.8). The results showed that elevated phosphorylated ERK1/2 and p38 may involve in the downregulation of cyclin D1 to interfere with the cell cycle transition.

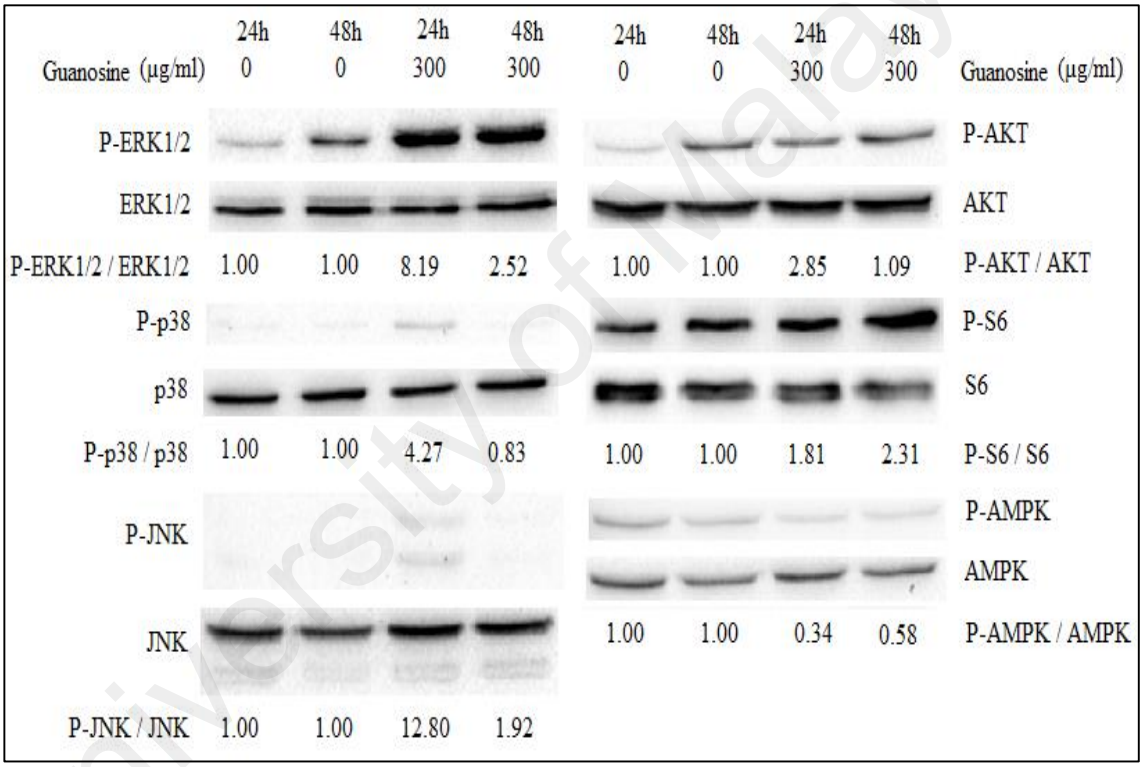


Figure 6.7: Effect of guanosine on ERK1/2, p38, JNK, AKT, S6 and AMPK activation

HCT 116 cells were treated with guanosine for 24 hours and 48 hours. After treatments, cell lysates were analysed by western blot. ERK1/2, p38, JNK, AKT, S6, AMPK as loading controls. Band intensities are shown as fold changes compared to untreated controls after normalisation.

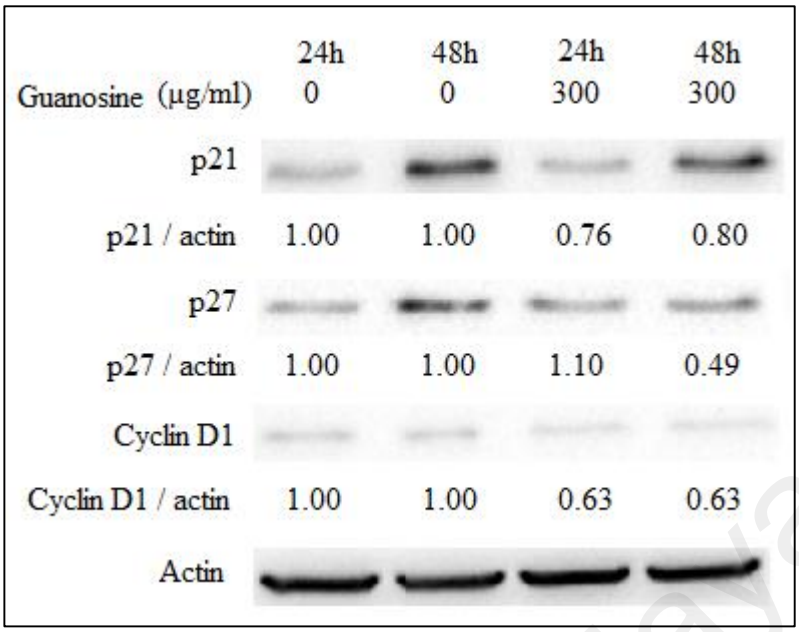


Figure 6.8: Effect of guanosine on levels of p21, p27 and cyclin D1

HCT 116 cells were treated with guanosine for 24 hours and 48 hours. After treatments, cell lysates were analysed by western blot. Actin as loading control. Band intensities are shown as fold changes compared to untreated controls after normalisation to actin expression.

6.4 Conclusion

The anti-proliferation effect of guanosine on HCT 116 cells was mainly due to cell cycle arrest. The increased activation of ERK1/2, p38 and JNK were observed in HCT 116 cells treated with guanosine. The increased activation of ERK1/2 and p38 pathways may lead to downregulation of cyclin D1 which contributed to cell cycle arrest effect. The decreased activation of AMPK was also associated with the anti-proliferation effect of guanosine. The results suggest that guanosine can induce cell cycle arrest through the activation of ERK1/2, p38 and JNK pathways along with attenuation of AMPK pathway.

CHAPTER 7: CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

The ethyl acetate extract of *G. bicolor* with highest total phenolic content among the extracts showed higher activity in DPPH radicals scavenging, metal chelating and antioxidant activity against β-carotene bleaching. The ethyl acetate extract of *G. bicolor* also exhibited cytotoxic activity against human cancer cell lines and induced apoptotic and necrotic cell death, especially towards the HCT 116 and HCT-15 colon cancer cells. The acute oral toxicity indicated that *G. bicolor* has negligible level of toxicity when administered orally and regarded as safe.

Overall, methanol extract of *G. procumbens* demonstrated better DPPH radical scavenging and antioxidant activity against β-carotene bleaching while hexane extract showed better metal chelating activity. Ethyl acetate extract of *G. procumbens* exhibited moderate cytotoxicity against cancer cells while low cytotoxicity against normal cells. The acute oral toxicity test indicated that *G. procumbens* has negligible level of oral toxicity. Taken together, the results indicated the potential benefits of *G. bicolor* and *G. procumbens* in prevention and treatment of colon cancer.

In this study, six chemical constituents, 5-*p*-*trans*-coumaroylquinic acid (**1**), 4-hydroxybenzoic acid (**2**), rutin (**3**), kaempferol-3-*O*-rutinoside (**4**), 3,5-dicaffeoylquinic acid (**5**) and kaempferol-3-*O*-glucoside (**6**) were isolated from ethyl acetate extract of *G. bicolor*. Guanosine (**7**) and 5-*O*-caffeoylquinic acid (**8**) were successfully isolated from water extract. All chemical constituents were isolated from *G. bicolor* leaves for the first time, except rutin (**3**). The 3,5-dicaffeoylquinic acid (**5**), guanosine (**7**) and 5-*O*-caffeoylquinic acid (**8**) showed selective cytotoxic effect against HCT 116 cells by *in vitro* MTT assay.

Further investigation demonstrated that 3,5-dicaffeoylquinic acid (**5**) and 5-*O*-caffeoylquinic acid (**8**) could undergo oxidation in culture medium to generate H_2O_2 and green pigments (presumably quinones). Extracellular H_2O_2 and quinones were shown to induce cell death and cell cycle arrest in HCT 116 cells. High concentration of 3,5-dicaffeoylquinic acid (**5**) and 5-*O*-caffeoylquinic acid (**8**) may be able to inhibit the growth of cancer cells in colon when high level of H_2O_2 and quinones were formed as a result of oxidation. Further study is warranted to investigate *in vivo* generation of H_2O_2 and quinone products from both compounds especially at the site of inflammation. The study can include four animal groups, 1) untreated non-induced group, 2) untreated induced inflammation by using dextran sodium sulfate (DSS), 3) non-induced group oral administered with 3,5-dicaffeoylquinic acid (**5**) / 5-*O*-caffeoylquinic acid (**8**), 4) induced inflammation group oral administered with 3,5-dicaffeoylquinic acid (**5**) / 5-*O*-caffeoylquinic acid (**8**). The colonic semisolid and fluid may be collected to determine the level of H_2O_2 (FOX assay) and GSH-conjugated oxidation products (LC-MS).

The guanosine (**7**) inhibits the proliferation of HCT 116 cells through cell cycle arrest rather than cell death induction. The increased activation of ERK1/2, p38 and JNK pathways were found to associate with the cell cycle arrest effect of guanosine (**7**). Whilst, the decreased activation of AMPK was also observed which could lead to downregulation of catabolic activities of HCT 116 cells. In addition, the level of cyclin D1 was found to decrease when HCT 116 cells were exposed to guanosine (**7**). In order to further confirm that guanosine (**7**) interferes with cell cycle progression, the expression of CDK4/6 is suggested to be investigated in future study.

By consuming *G. bicolor*, these chemical constituents may have beneficial role in treatment of colon cancer. In conclusion, the findings in the present study support the common belief that ethnopharmacological selection of *G. bicolor* and *G. procumbens* is a useful criterion in drug discovery. As suggestions, further studies on the mutagenic

and toxicity effect over a longer period of time involving detection of effects on vital organ functions should be carried out to ensure that the plants are safe for human consumption.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of publications

Teoh, W. Y., Sim, K. S., Richardson, J. S. M., Abdul Wahab, N., & Hoe, S. Z. (2013). Antioxidant capacity, cytotoxicity and acute oral toxicity of *Gynura bicolor*. *Evidence Based Complementary and Alternative Medicine,* article ID 958407.

Teoh, W. Y., Tan, H. P., Ling, S. K., Abdul Wahab, N., & Sim, K. S. (2016). Phytochemical investigation of *Gynura bicolor* leaves and cytotoxicity evaluation of the chemical constituents against HCT 116 cells. *Natural Product Research, 30*: 448-451.

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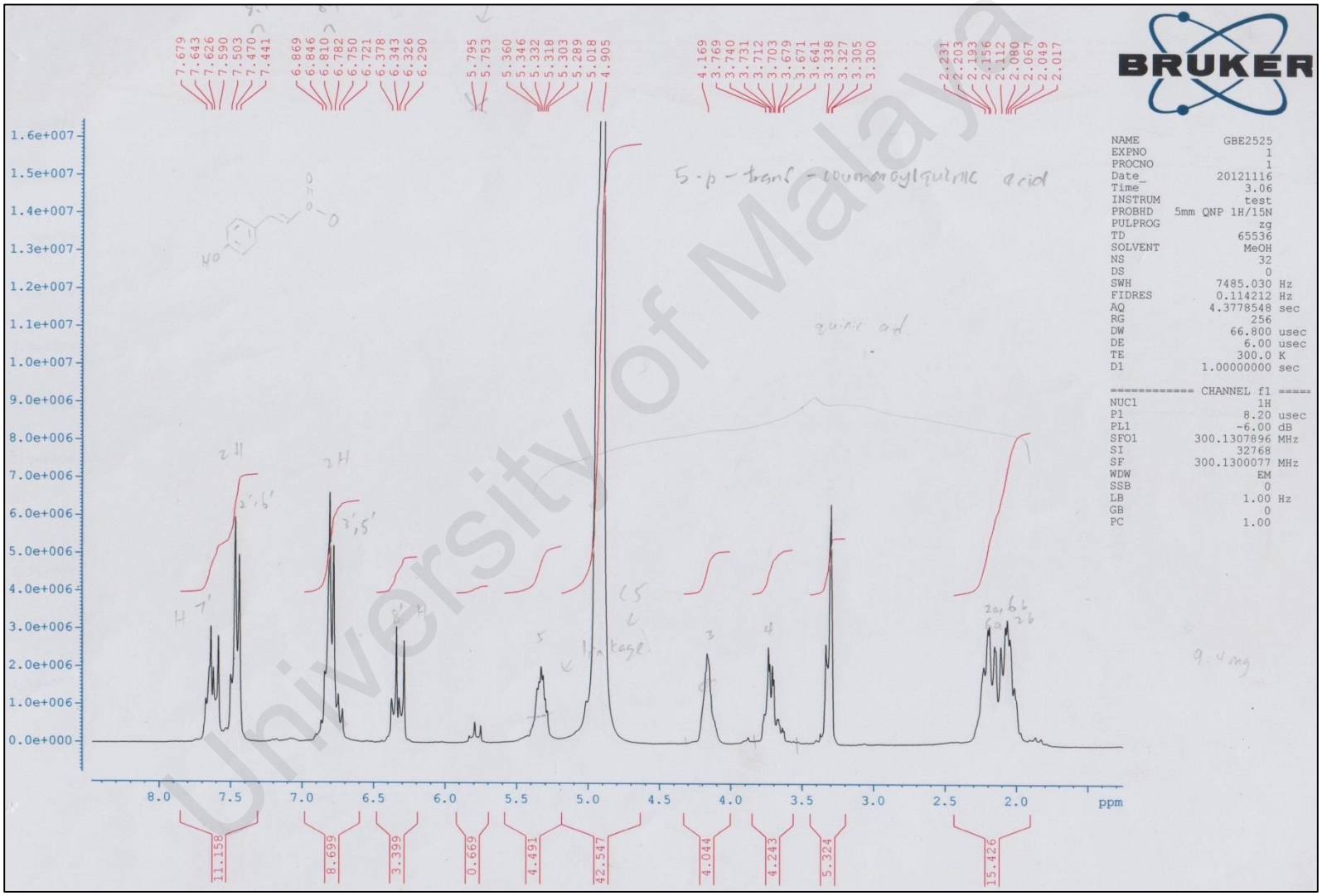
List of presentations

Teoh, W. Y., Abdul Wahab, N., & Sim, K. S. Cytotoxic activities of selected *Gynura* species in Malaysia. 16th Biological Sciences graduate Congress (BSGC), 12-13th Dec 2011, National University of Singapore, Singapore.

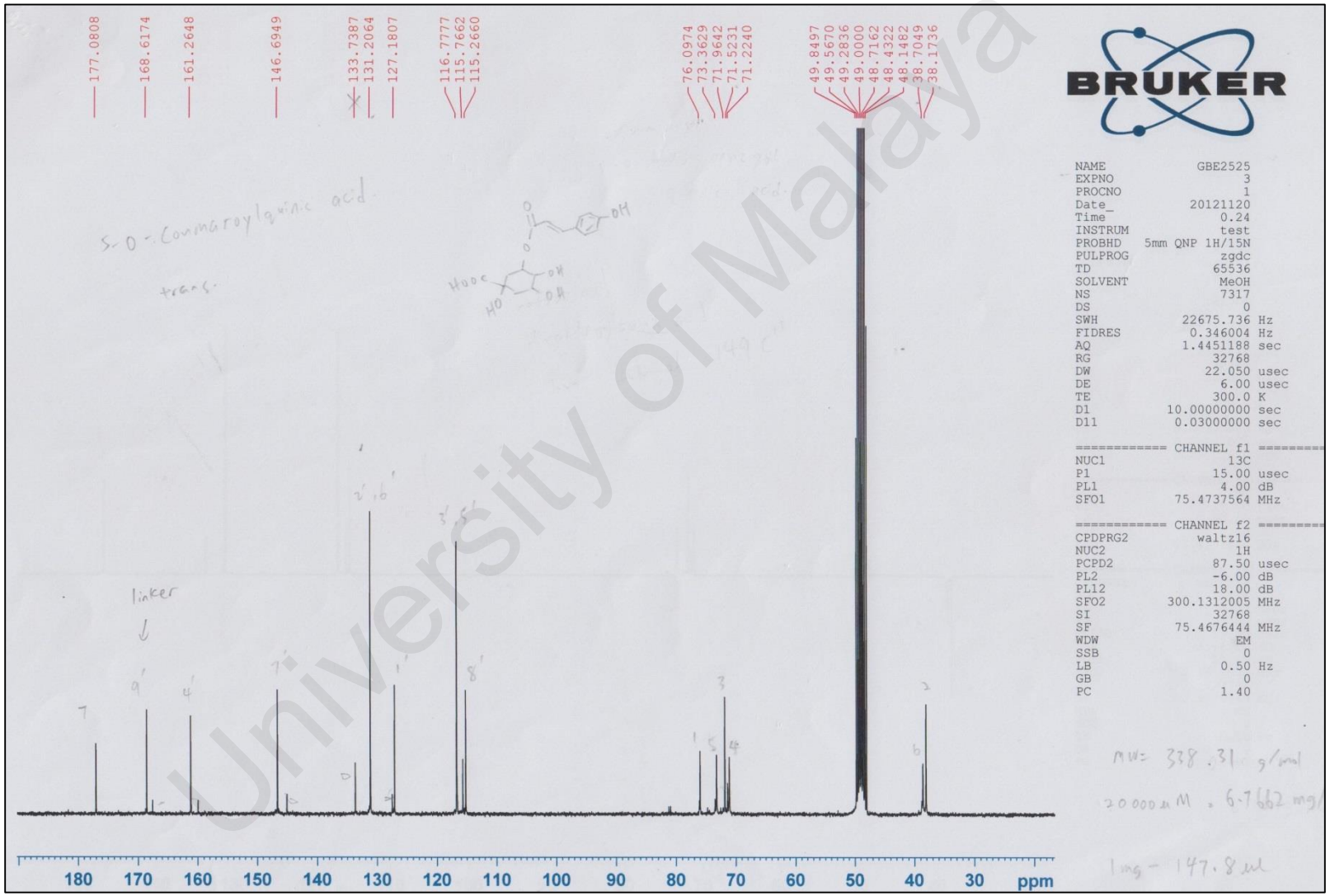
Teoh, W. Y., Abdul Wahab, N., & Sim, K. S. Extracellular oxidation products of 3,5-dicaffeoylquinic acid induce cell death and cell cycle arrest on HCT 116 cells. International Conference on Antioxidants and Degenerative Diseases (ICADD), 3-4th June 2015, Kuala Lumpur, Malaysia.

Teoh, W. Y., Abdul Wahab, N., & Sim, K. S. Extracellular oxidation products of 3,5-dicaffeoylquinic acid induce cell death and cell cycle arrest on HCT 116 cells. Annual Colloquium on Drug Development from Natural Products (CENAR), 12th Aug 2015, University of Malaya, Malaysia.

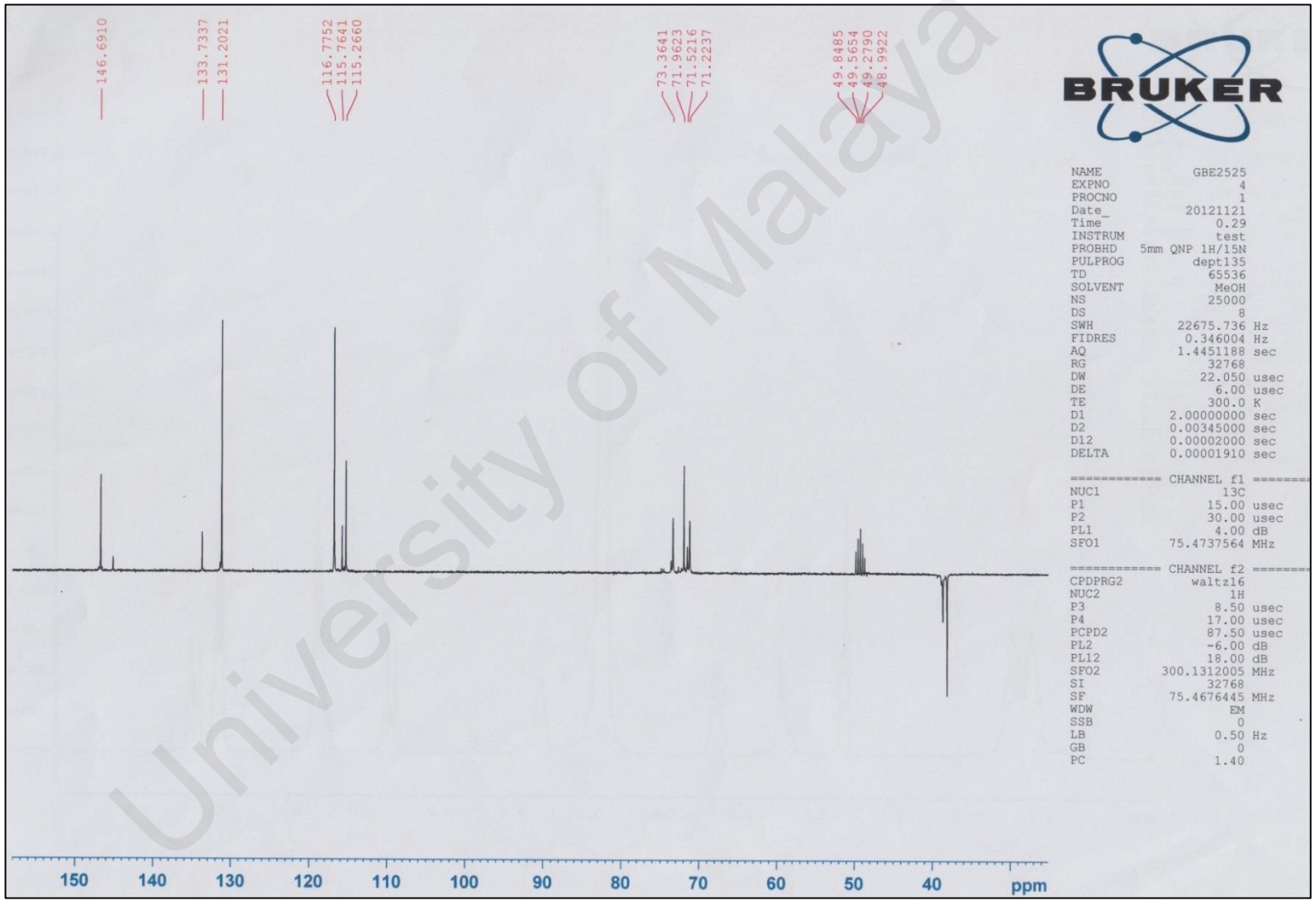
Teoh, W. Y., Abdul Wahab, N., & Sim, K. S. Antiproliferative effect of *Gynura bicolor* on human colon carcinoma HCT 116 cells. 2nd International Conference of Traditional and Complementary Medicine on Health (ICTCMH), 24-27th Oct 2015, GIS NTU Convention Center, Taipei, Taiwan.



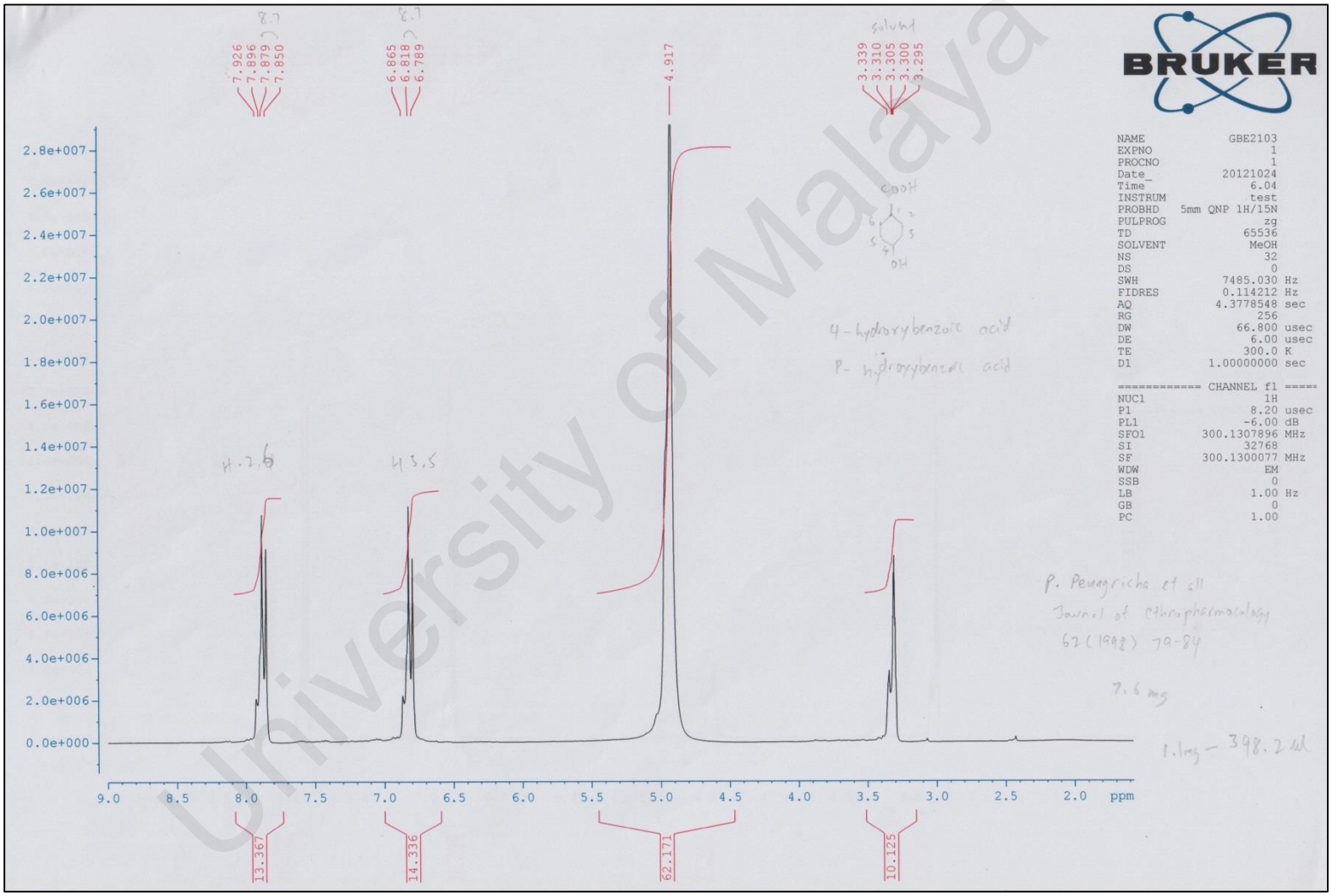
Appendix A1: 1H NMR spectrum of 5-*p-trans*-coumaroylquinic acid (1)



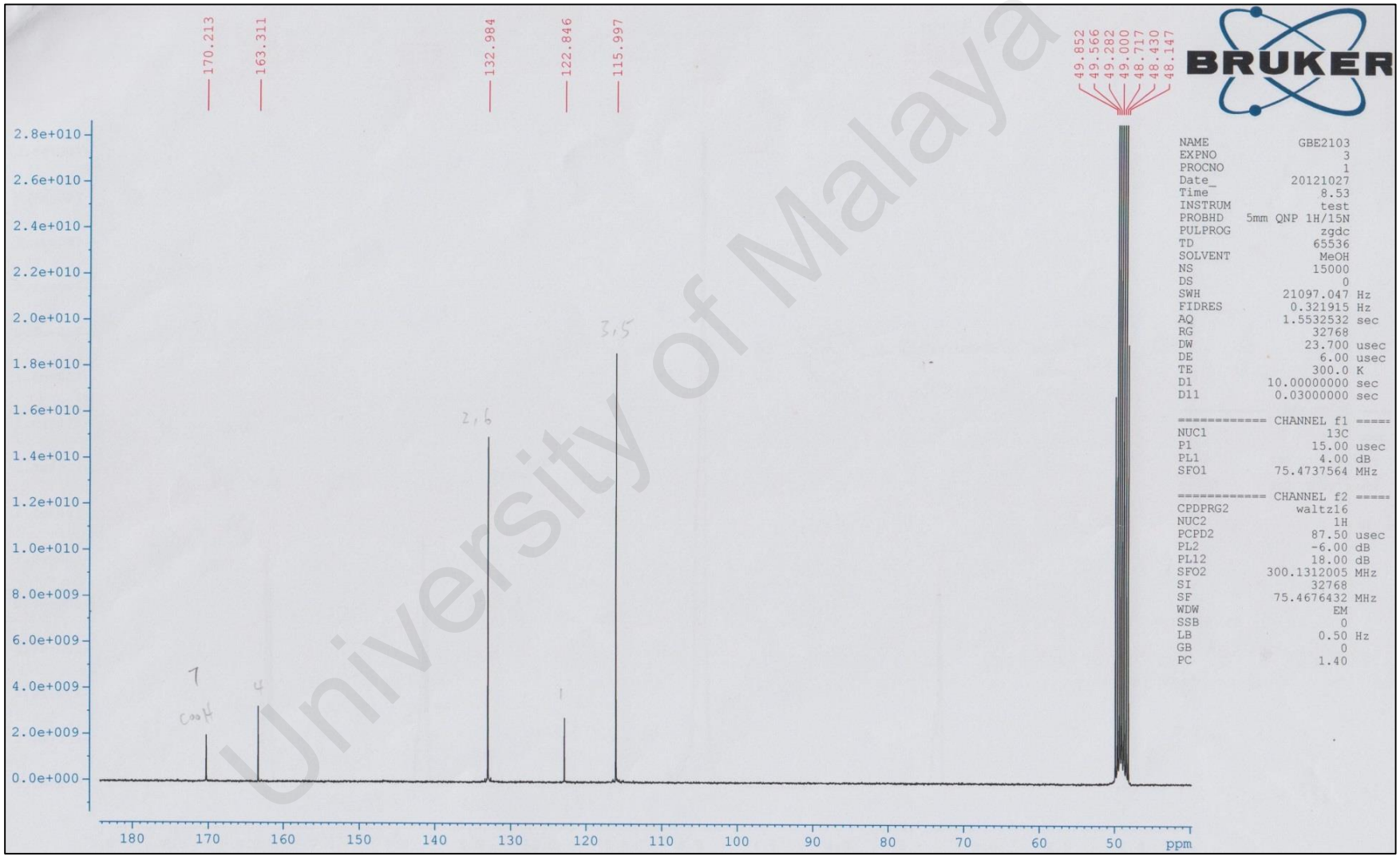
Appendix A2: 13C NMR spectrum of 5-*p*-*trans*-coumaroylquinic acid (1)



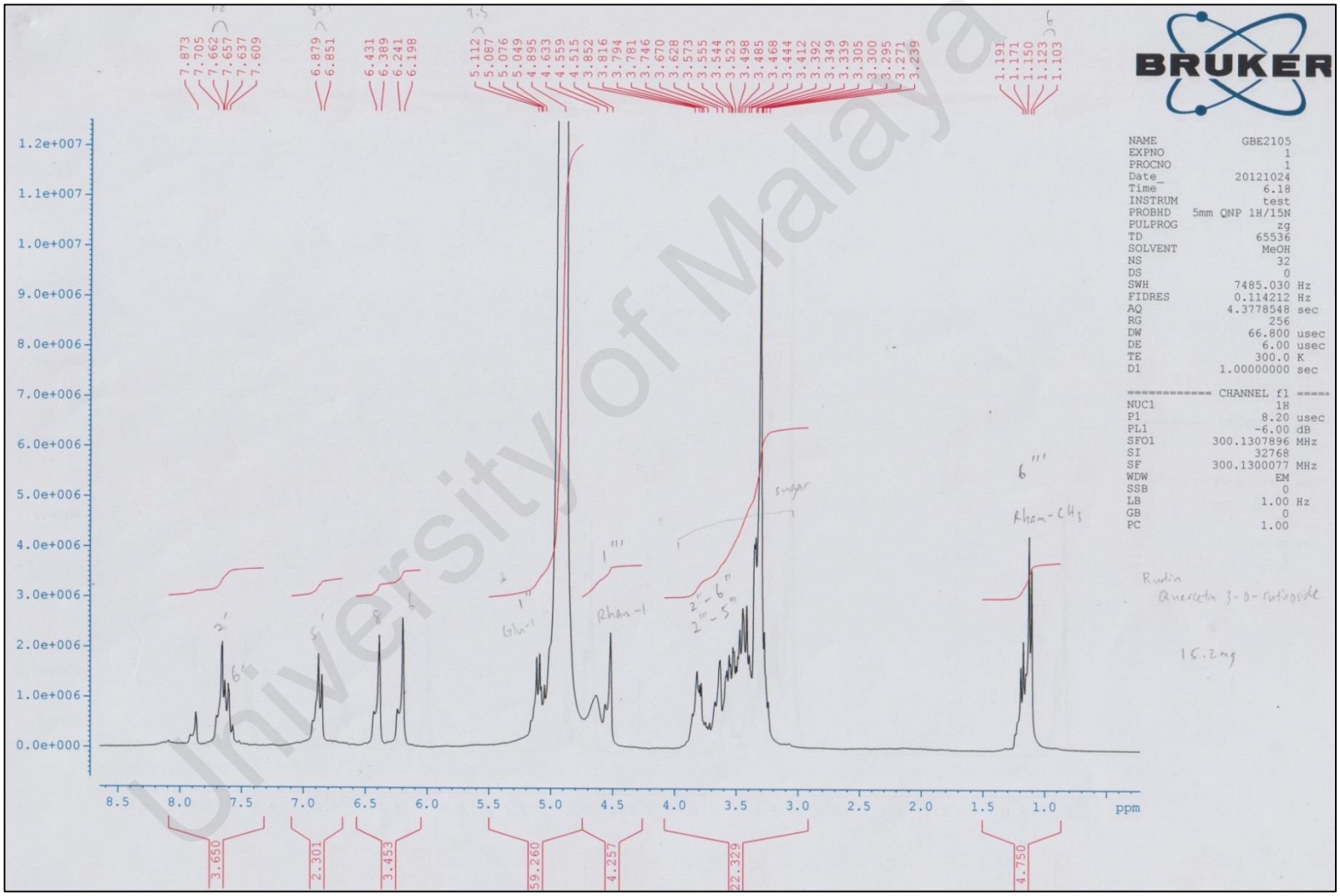
Appendix A3: DEPT 135 NMR spectrum of 5-*p-trans*-coumaroylquinic acid (1)



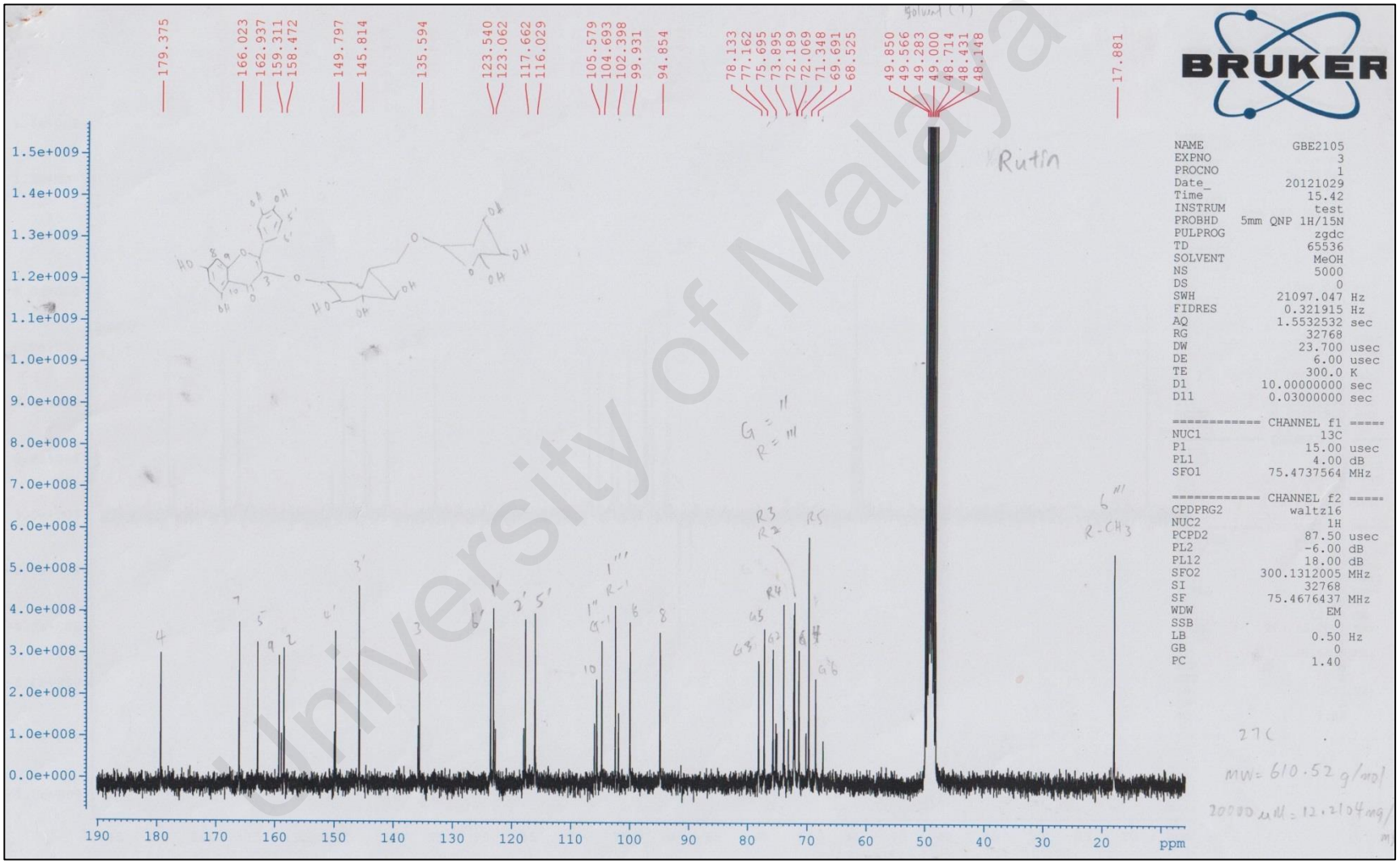
Appendix B1: 1H NMR spectrum of 4-hydroxybenzoic acid (2)



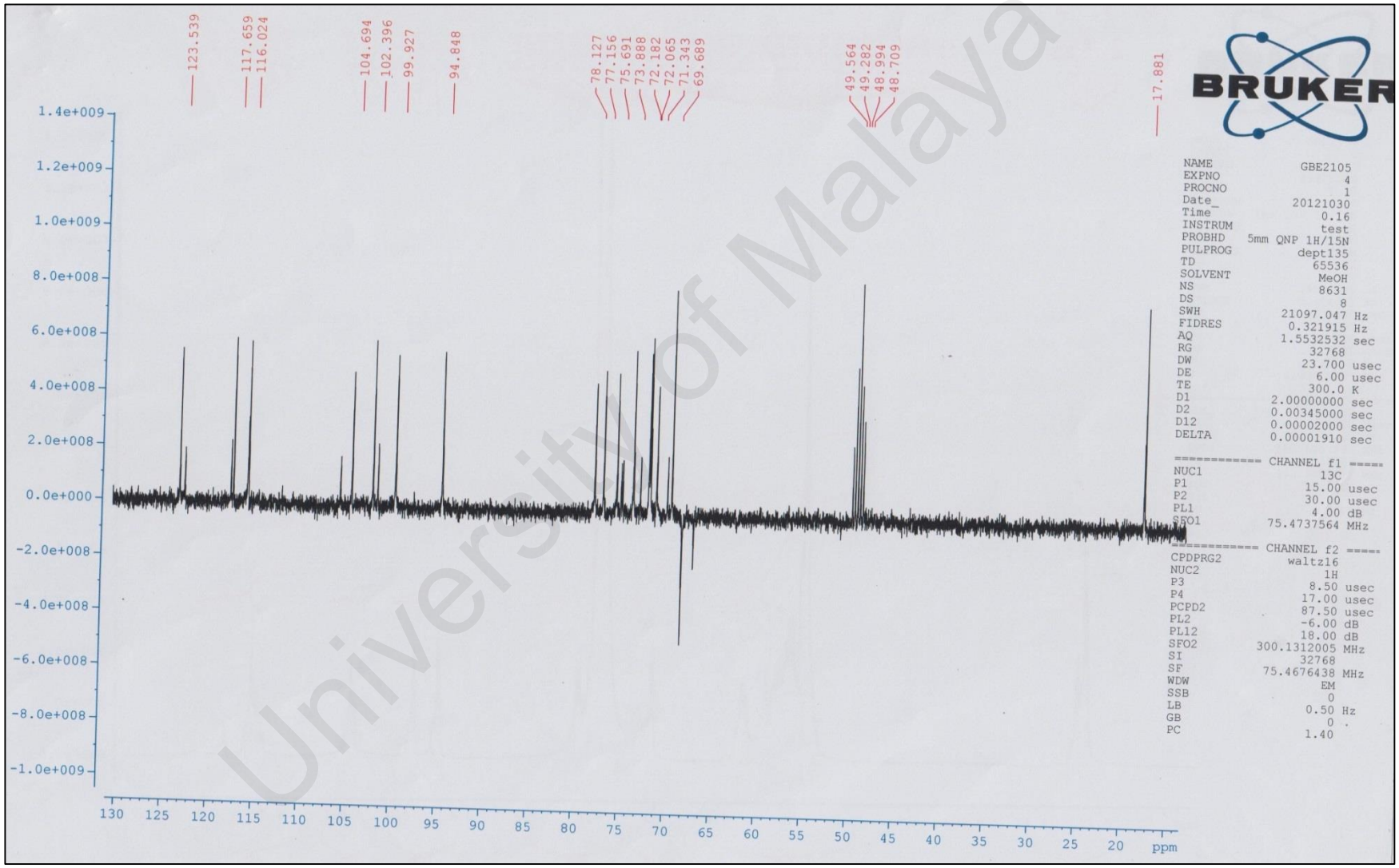
Appendix B2: 13C NMR spectrum of 4-hydroxybenzoic acid (2)



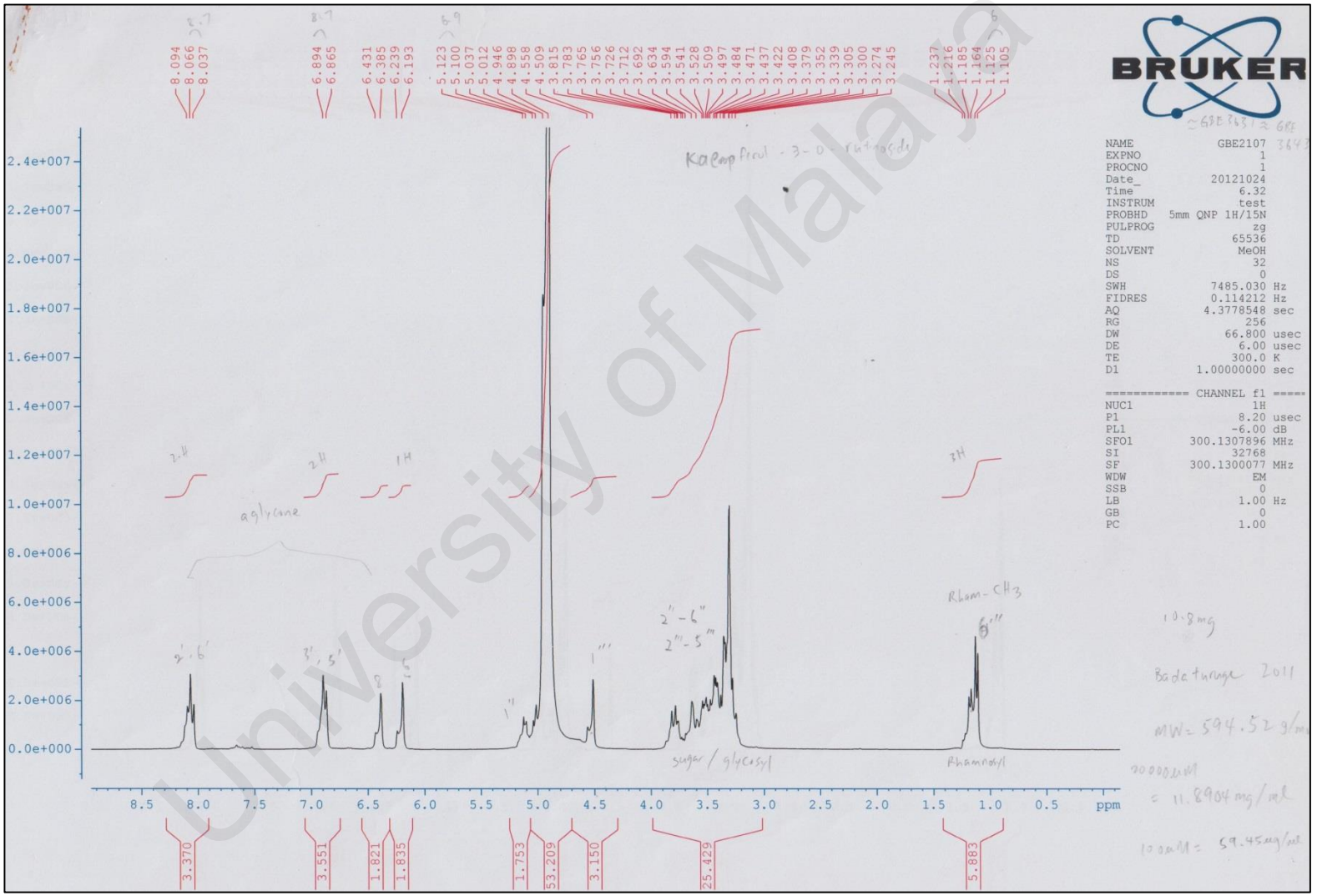
Appendix C1: 1H NMR spectrum of rutin (3)



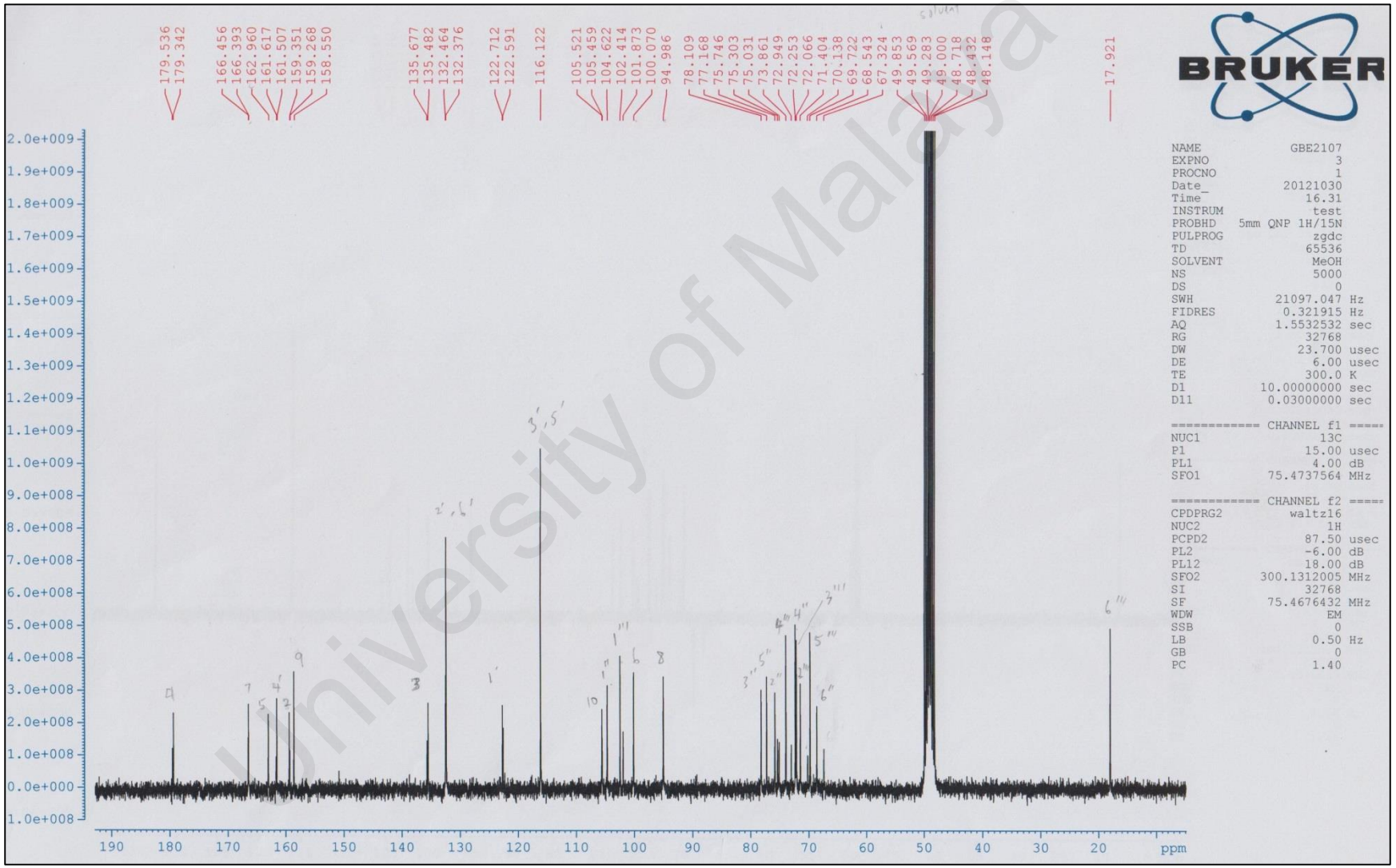
Appendix C2: 13C NMR spectrum of rutin (3)



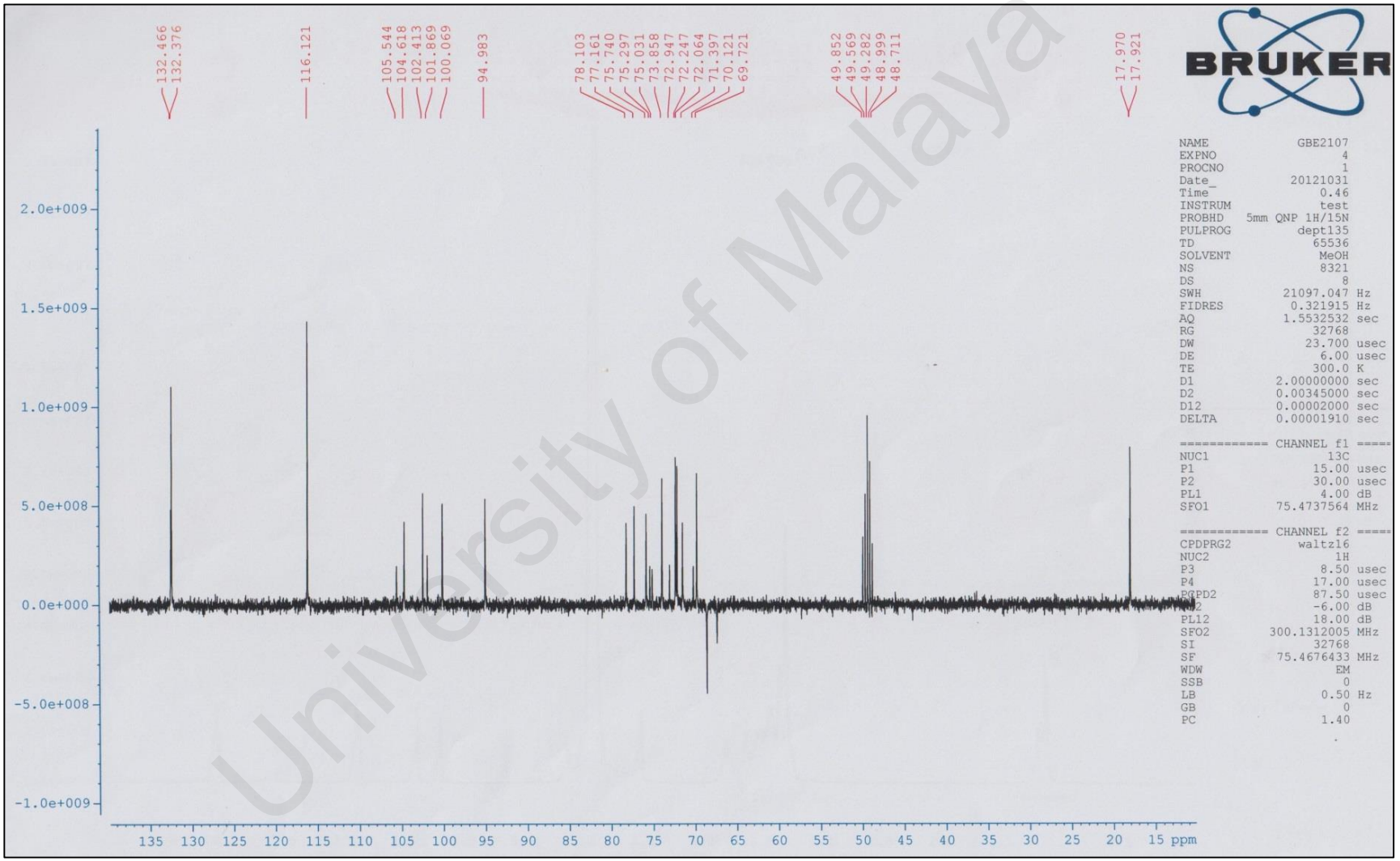
Appendix C3: DEPT 135 NMR spectrum of rutin (3)



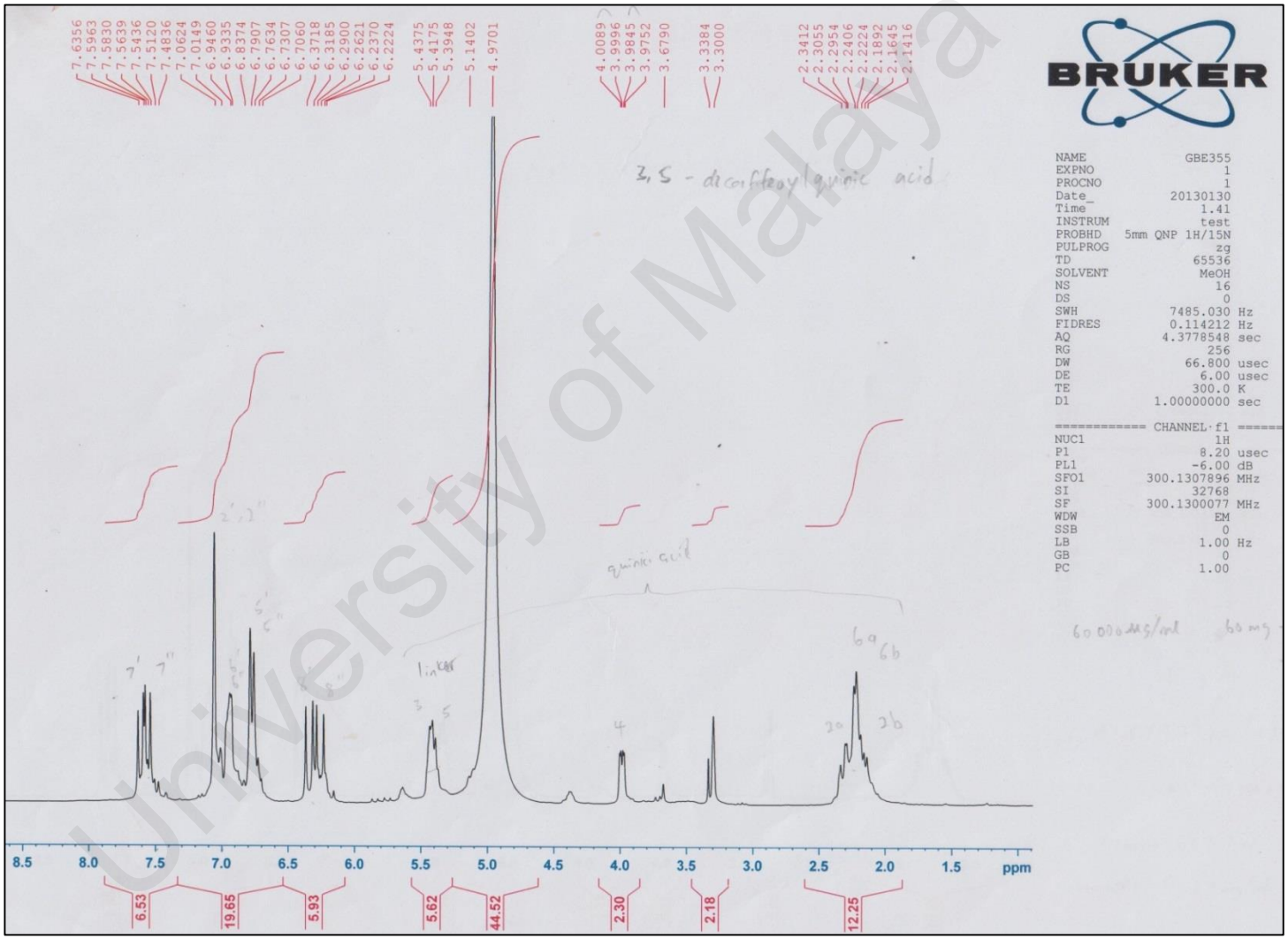
Appendix D1: 1H NMR spectrum of kaempferol-3-*O*-rutinoside (4)



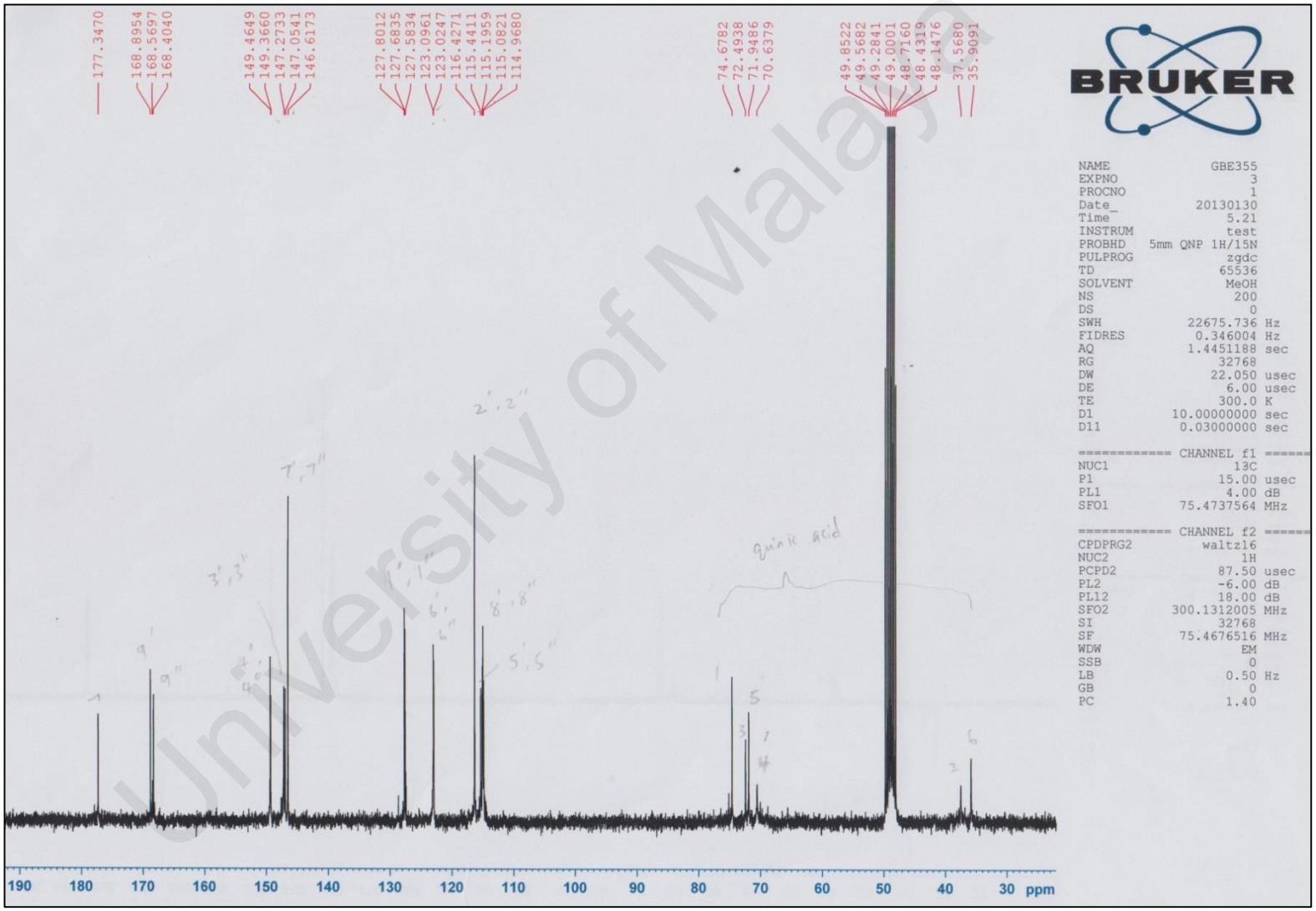
Appendix D2: 13C NMR spectrum of kaempferol-3-*O*-rutinoside (4)



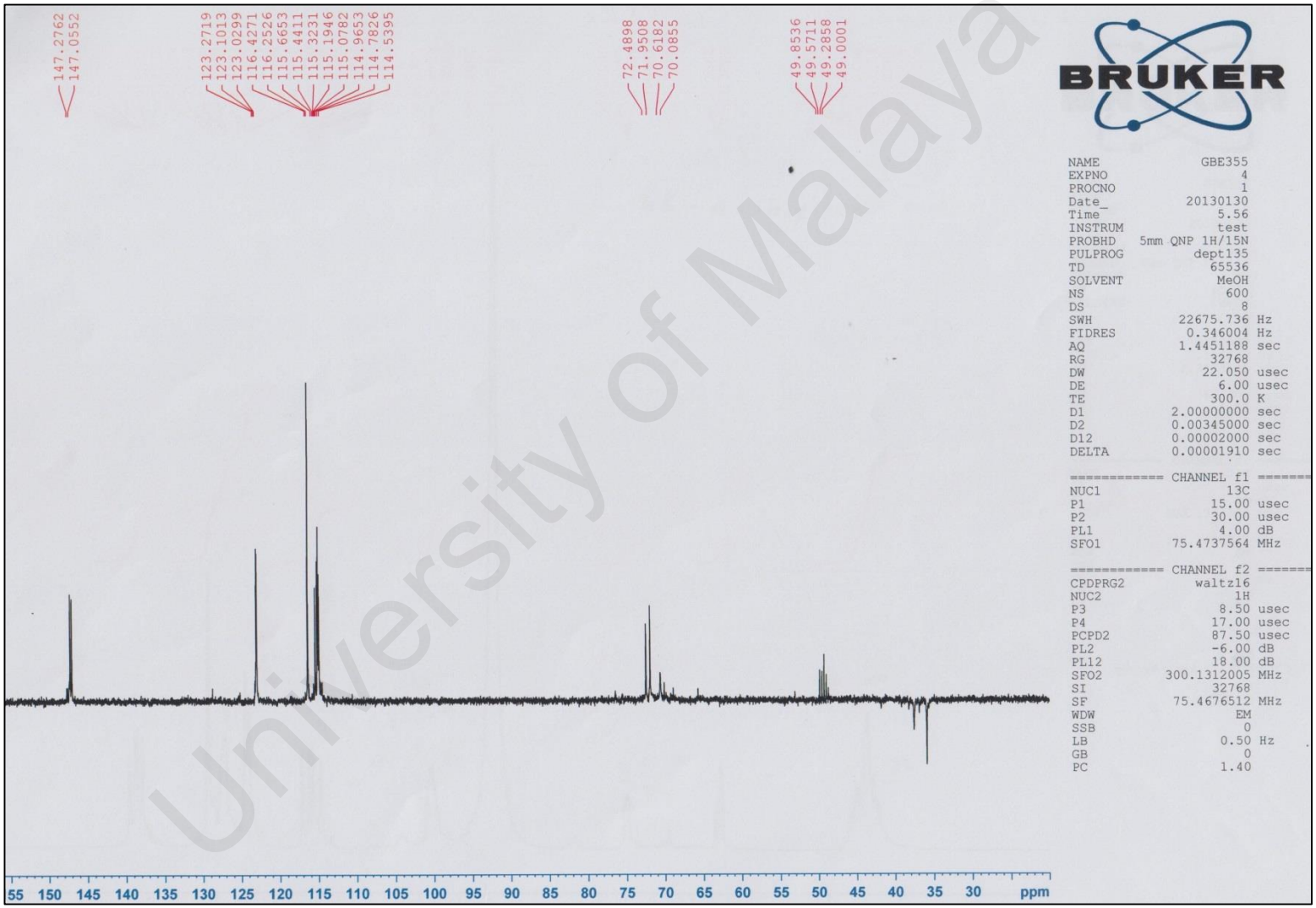
Appendix D3: DEPT 135 NMR spectrum of kaempferol-3-*O*-rutinoside (4)



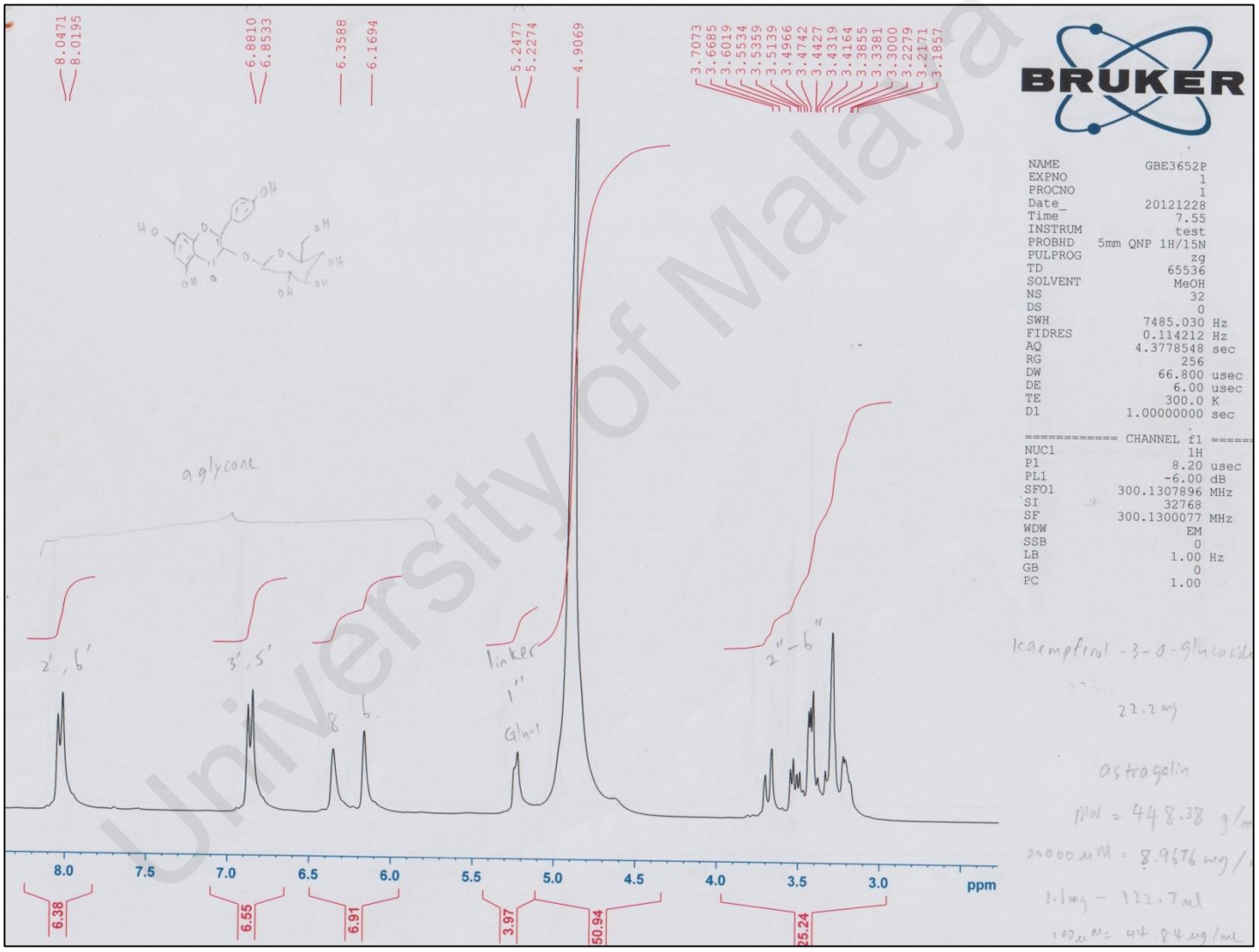
Appendix E1: 1H NMR spectrum of 3,5-dicaffeoylquinic acid (5)



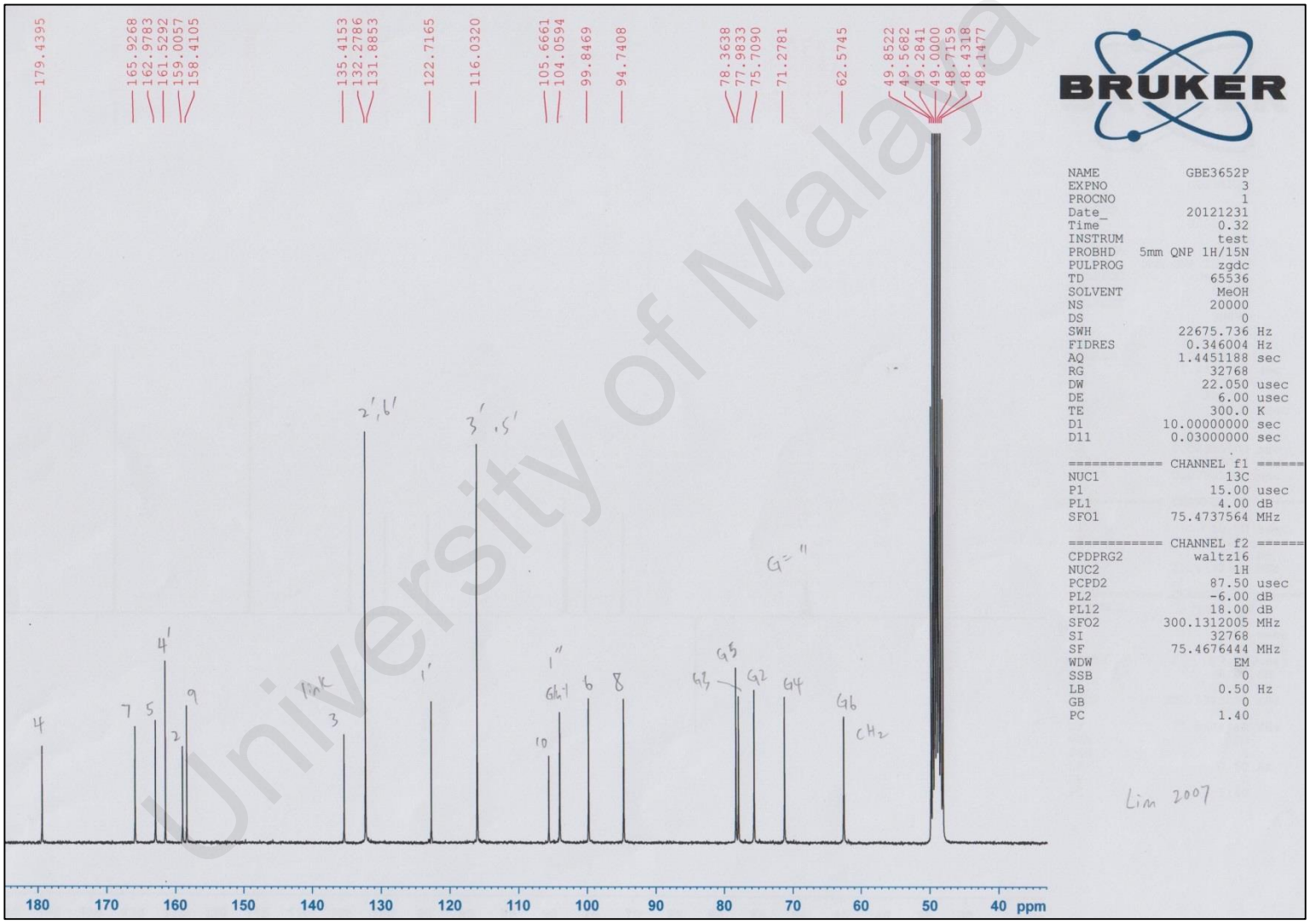
Appendix E2: 13C NMR spectrum of 3,5-dicaffeoylquinic acid (5)



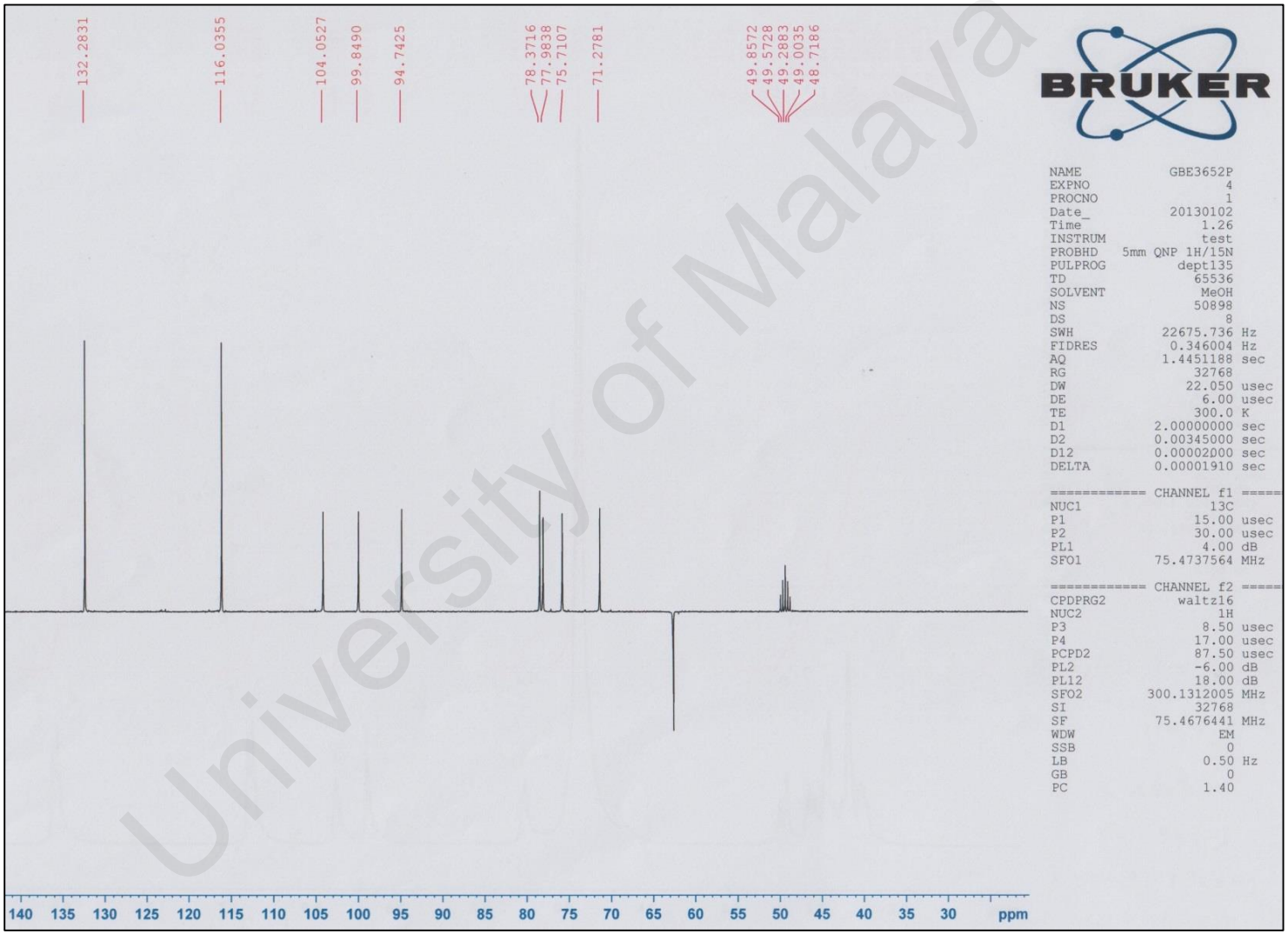
Appendix E3: DEPT 135 NMR spectrum of 3,5-dicaffeoylquinic acid (5)



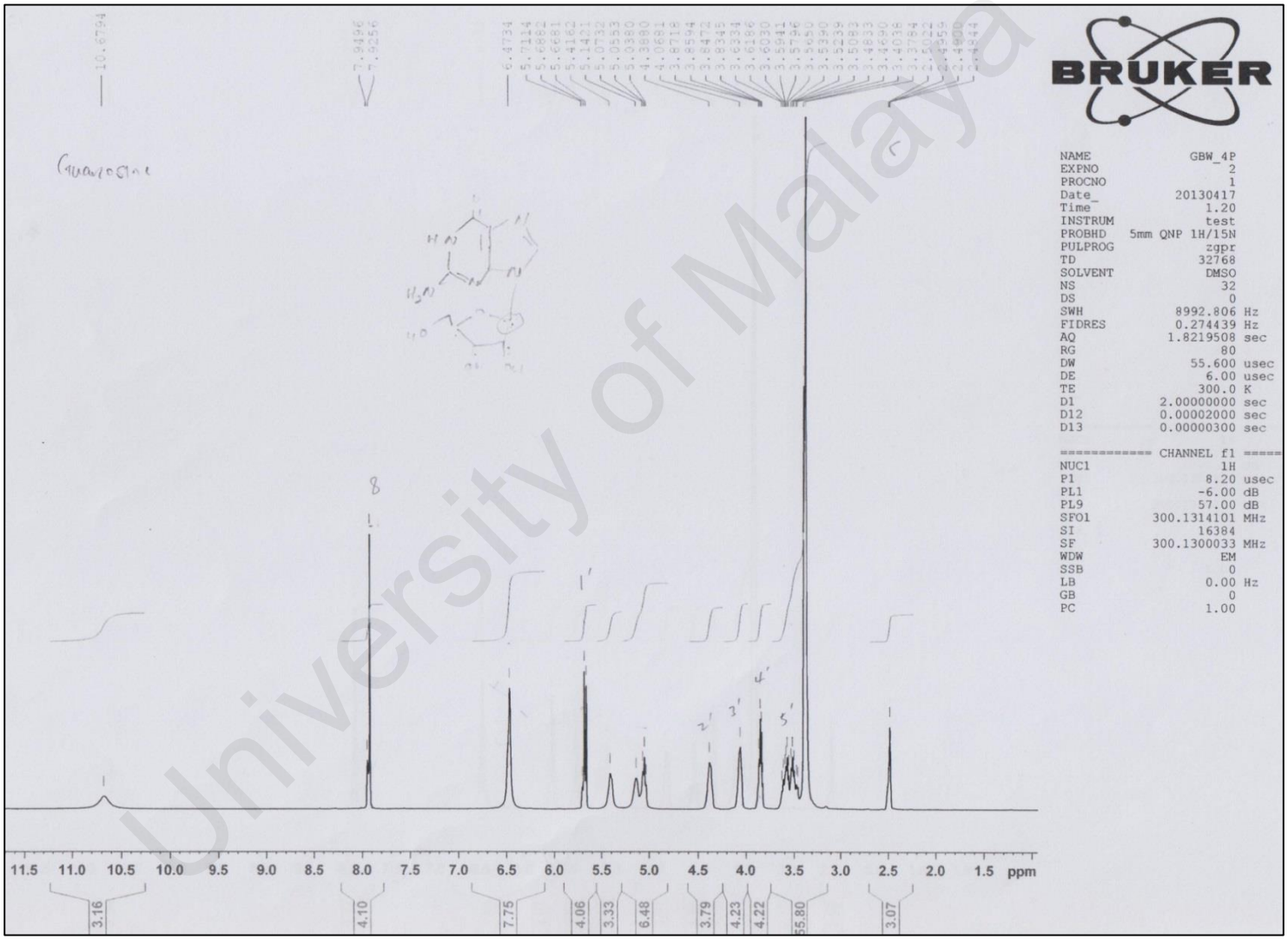
Appendix F1: 1H NMR spectrum of kaempferol-3-*O*-glucoside (6)



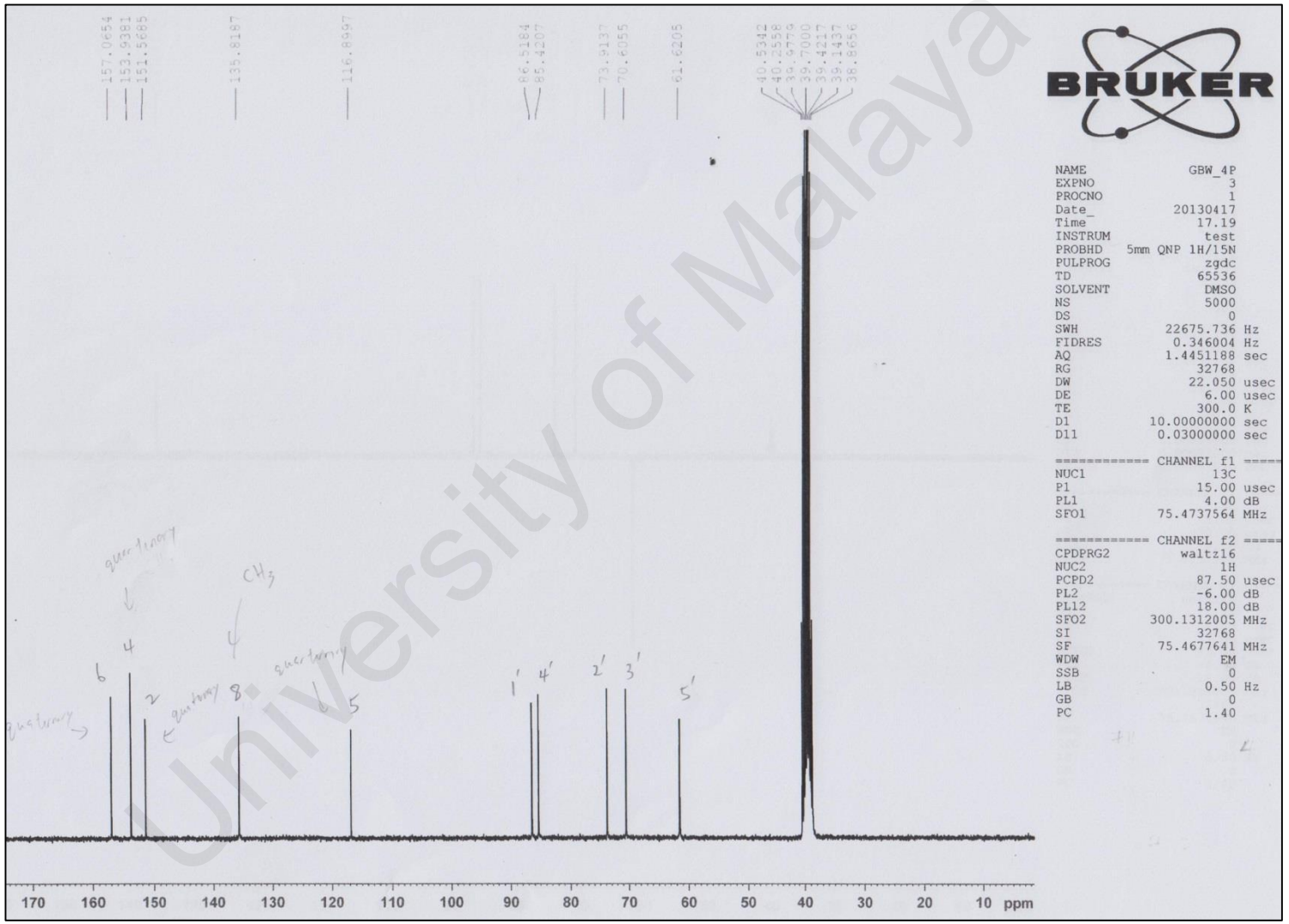
Appendix F2: 13C NMR spectrum of kaempferol-3-*O*-glucoside (6)



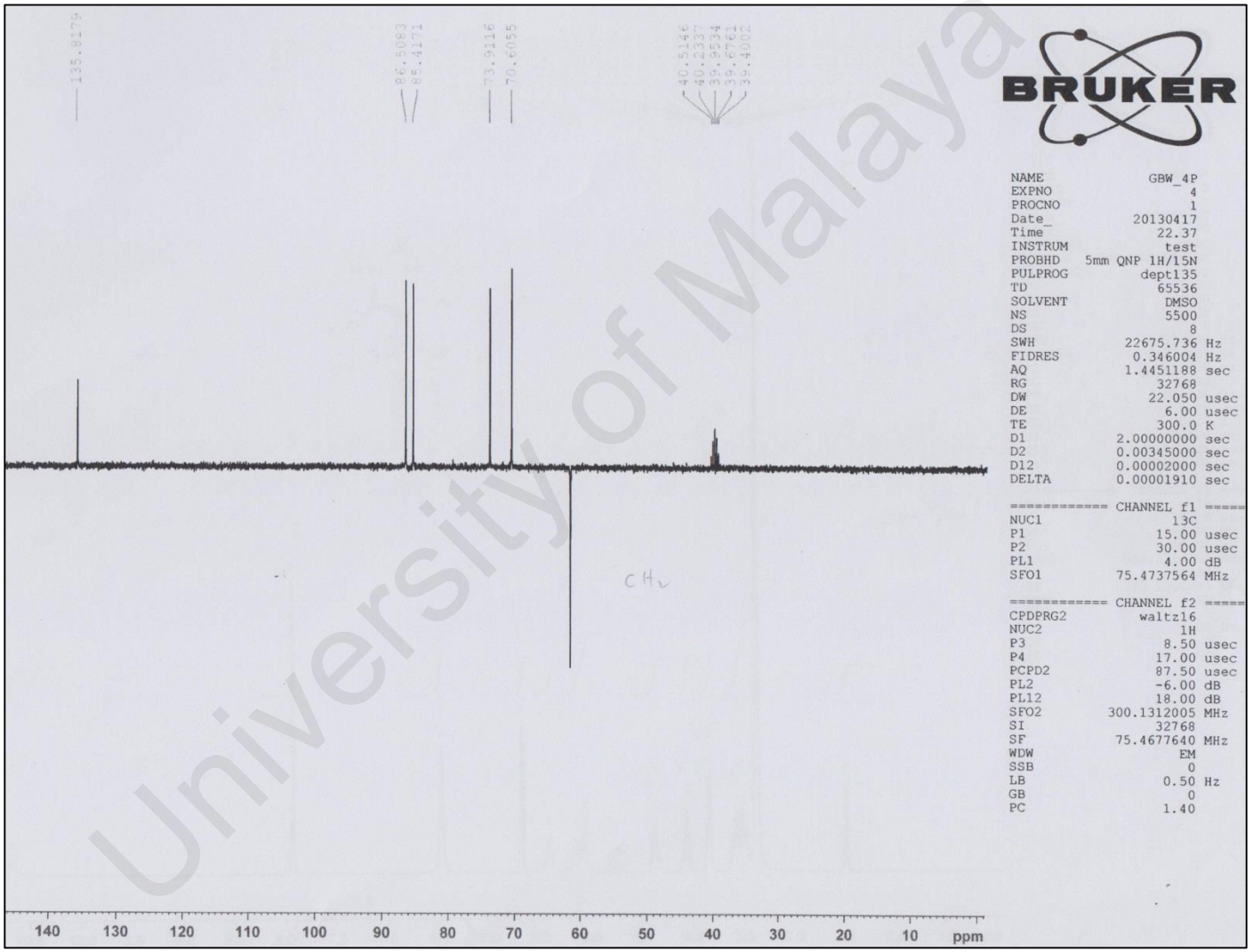
Appendix F3: DEPT 135 NMR spectrum of kaempferol-3-*O*-glucoside (6)



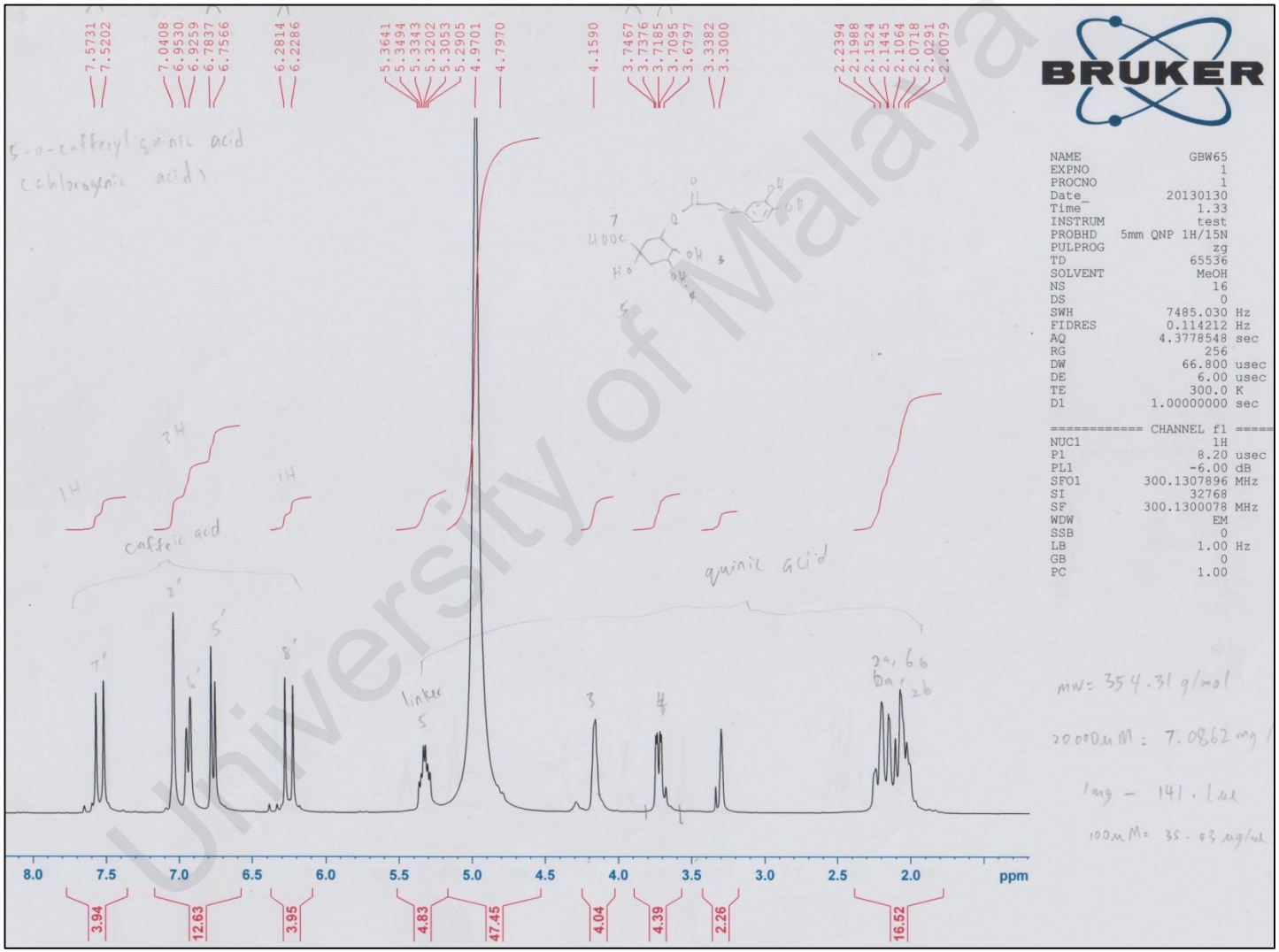
Appendix G1: 1H NMR spectrum of guanosine (7)



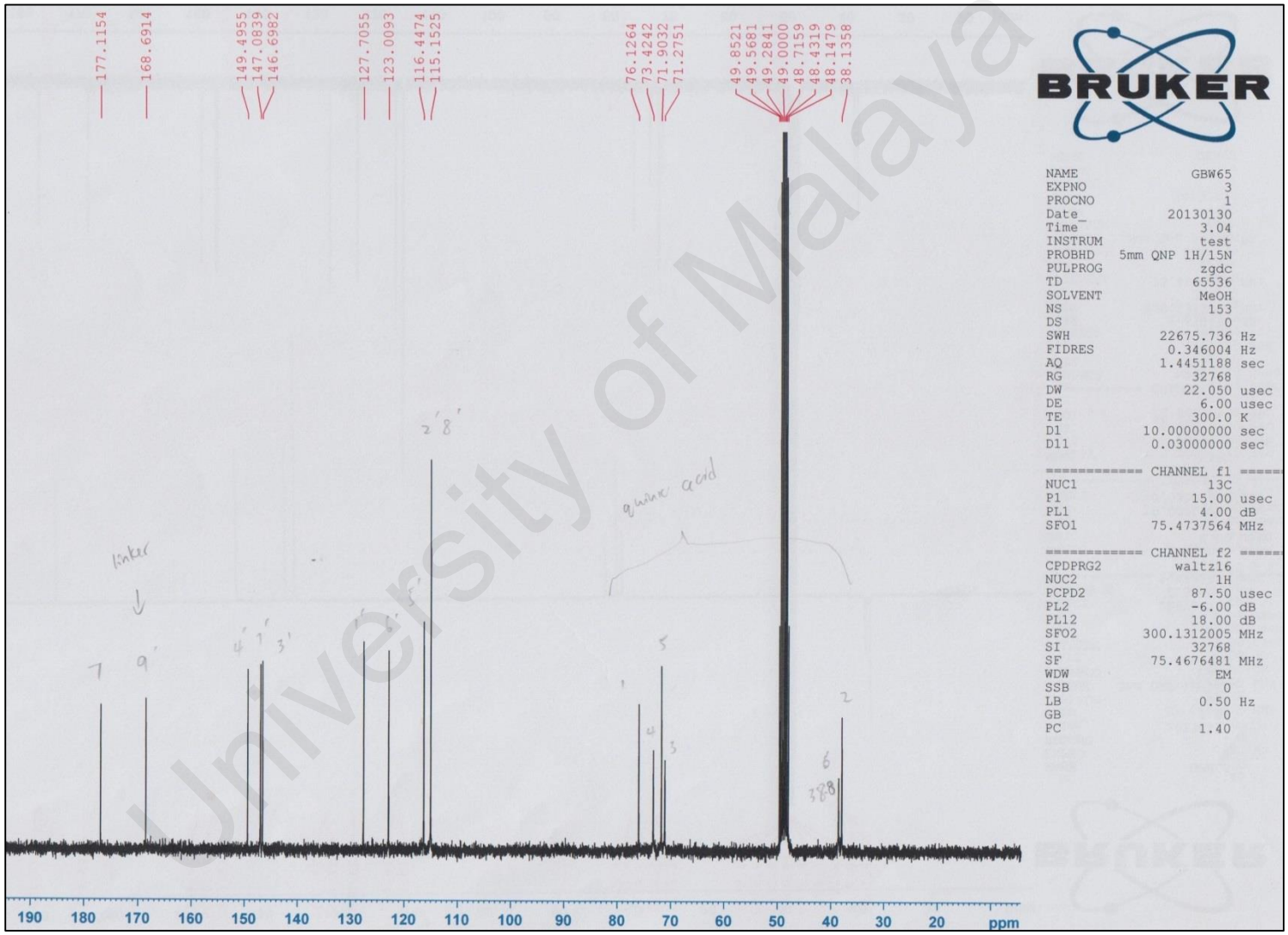
Appendix G2: 13C NMR spectrum of guanosine (7)



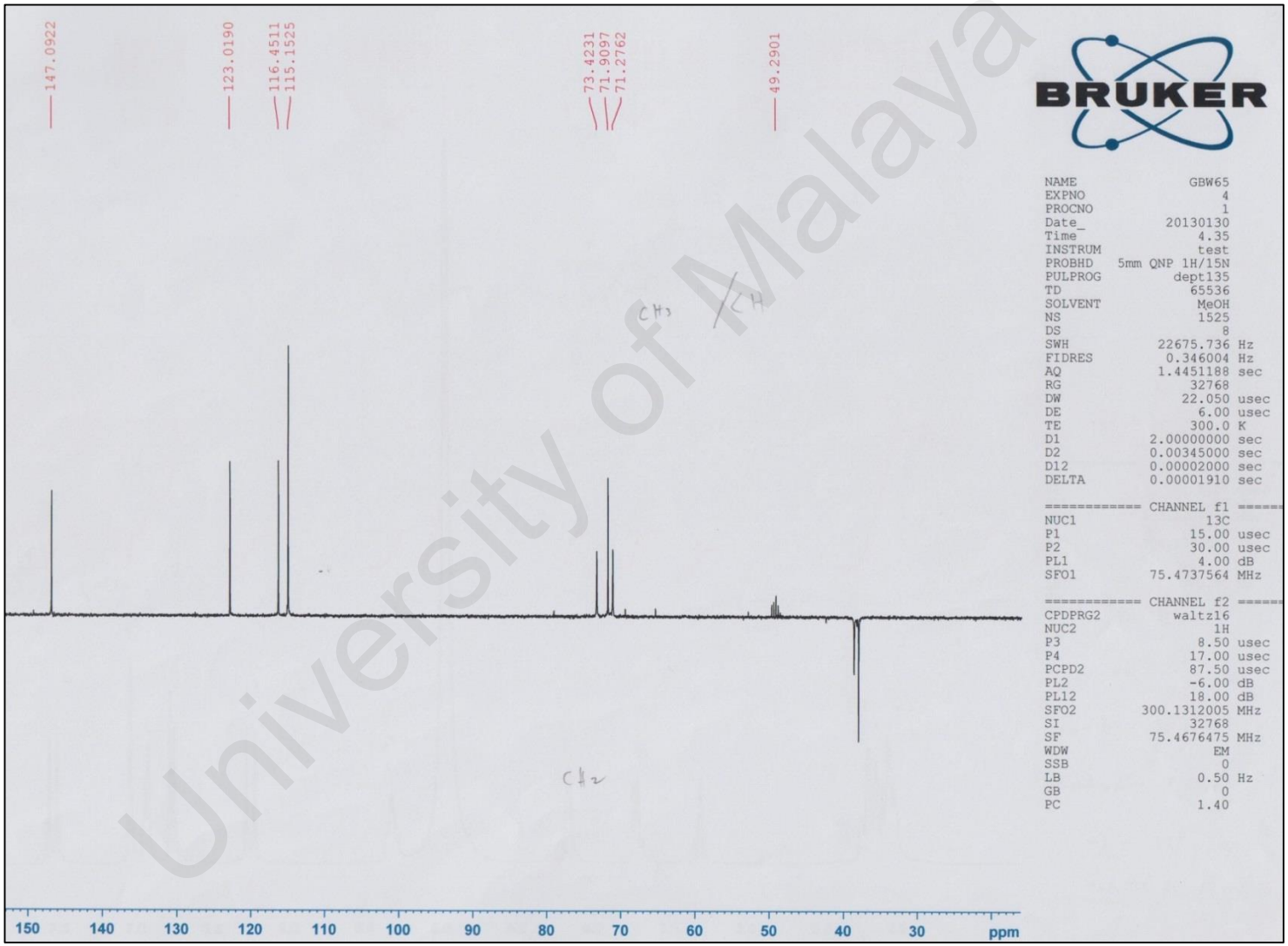
Appendix G3: DEPT 135 NMR spectrum of guanosine (7)



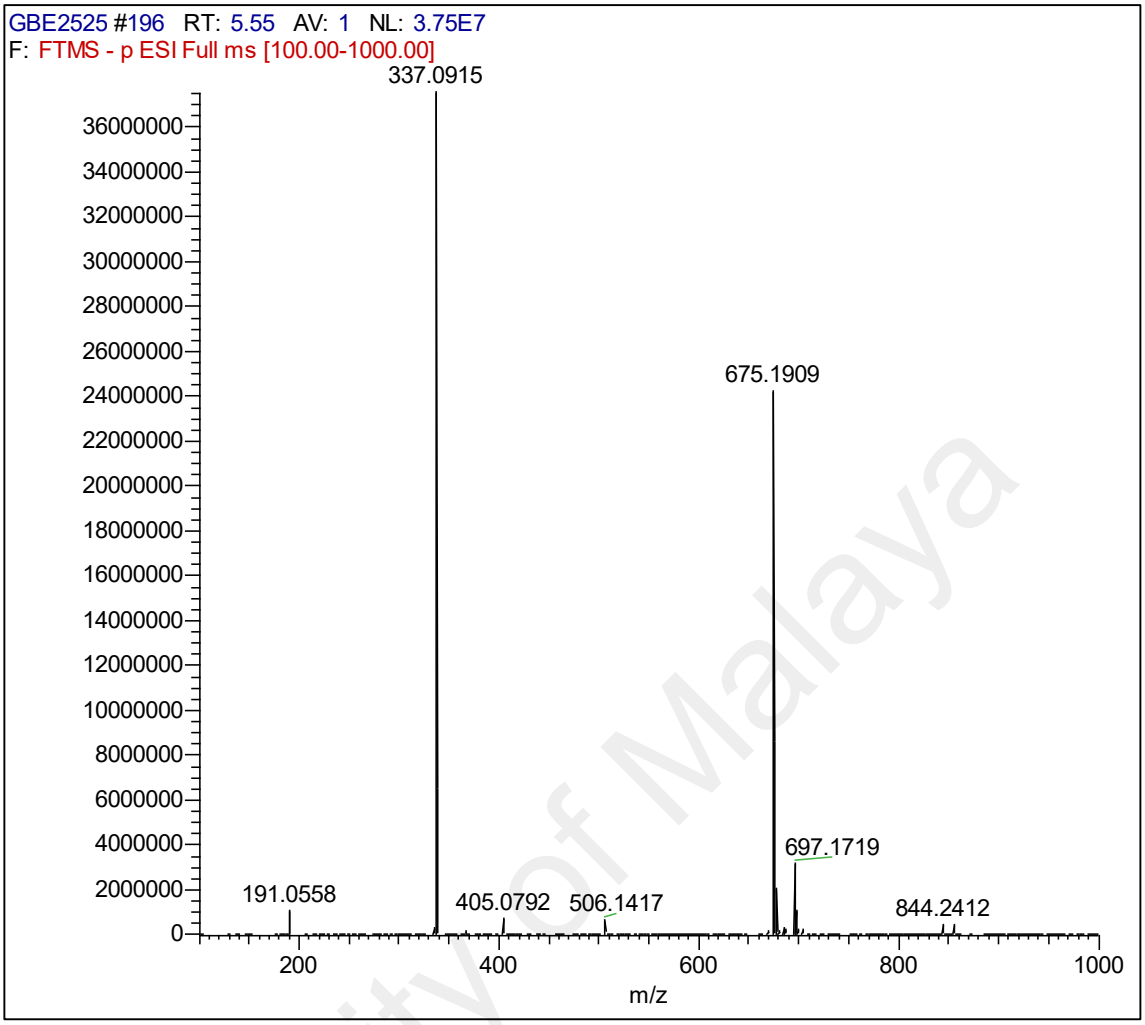
Appendix H1: 1H NMR spectrum of 5-*O*-caffeoylquinic acid (8)



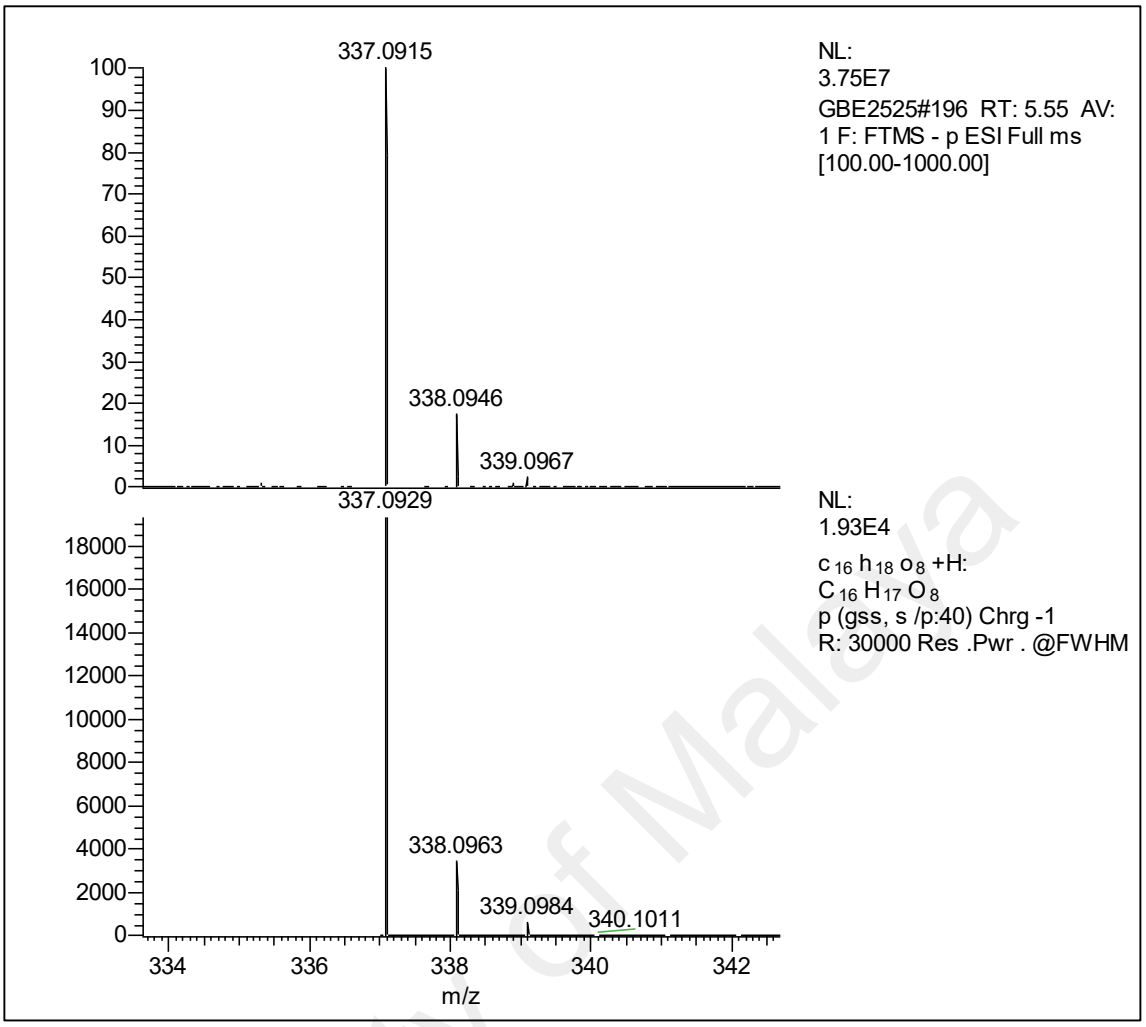
Appendix H2: 13C NMR spectrum of 5-*O*-caffeoylquinic acid (8)



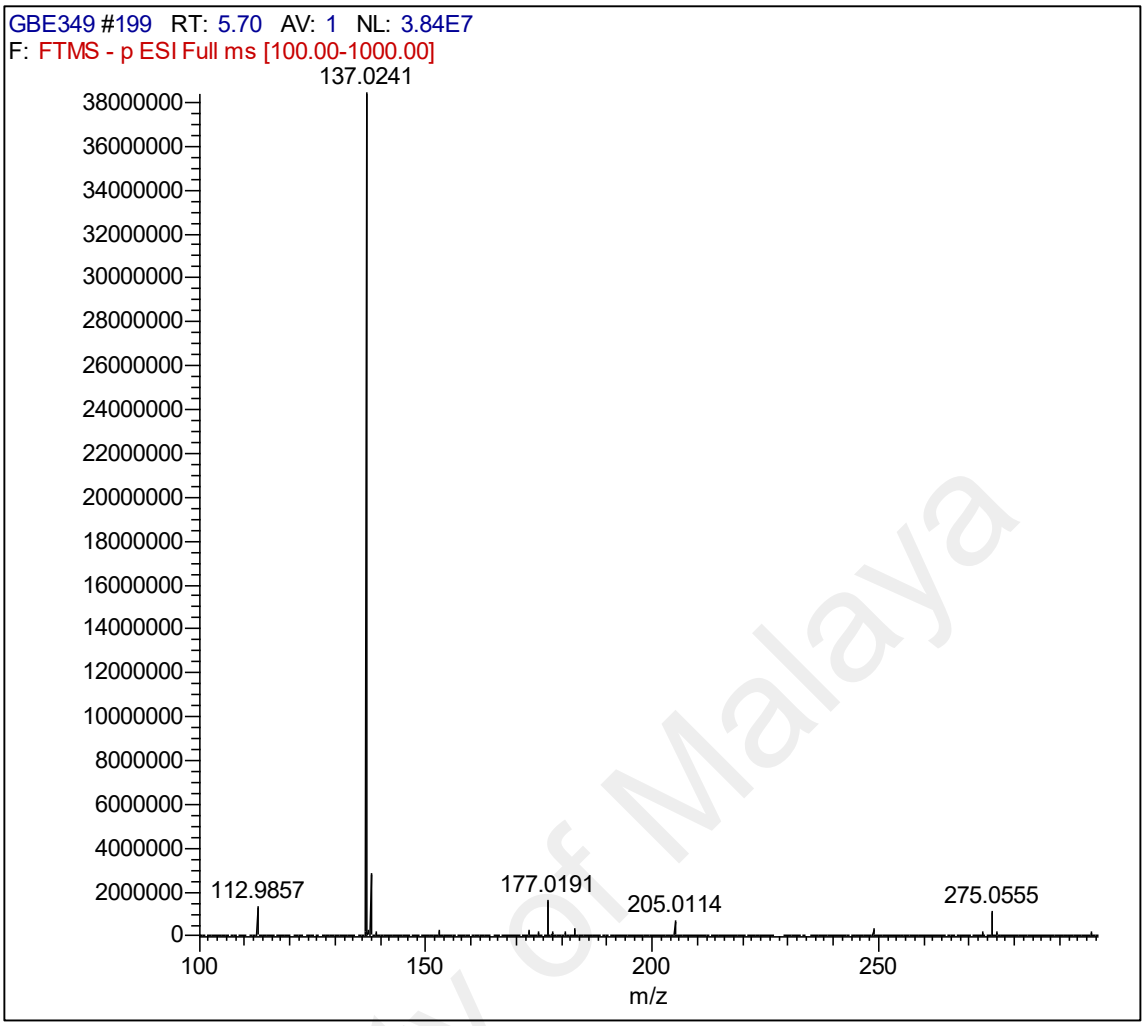
Appendix H3: DEPT 135 NMR spectrum of 5-*O*-caffeoylquinic acid (8)



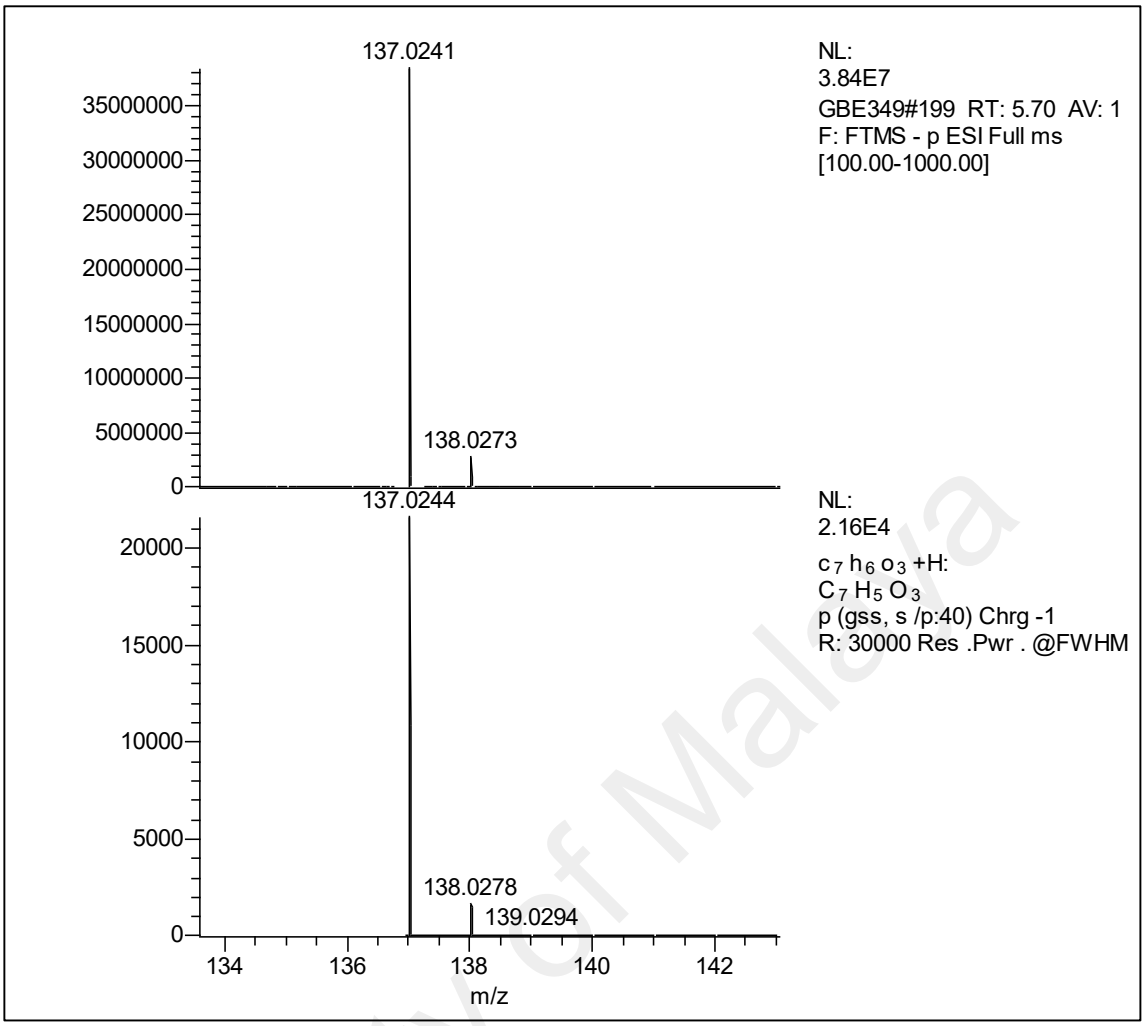
Appendix I1: ESI-MS of 5-*p*-*trans*-coumaroylquinic acid (1)



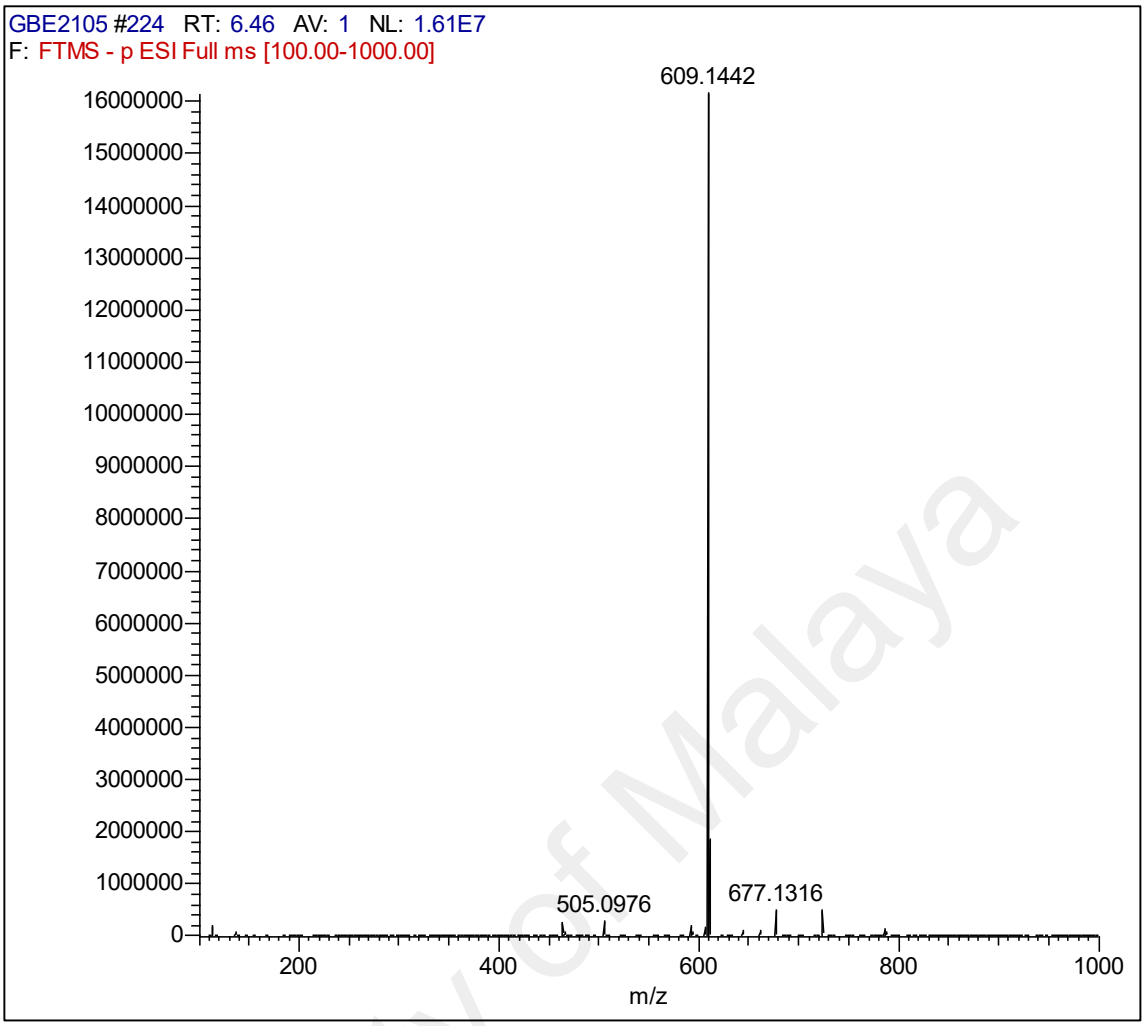
Appendix I2: Isotopic pattern of 5-*p*-*trans*-coumaroylquinic acid (1)



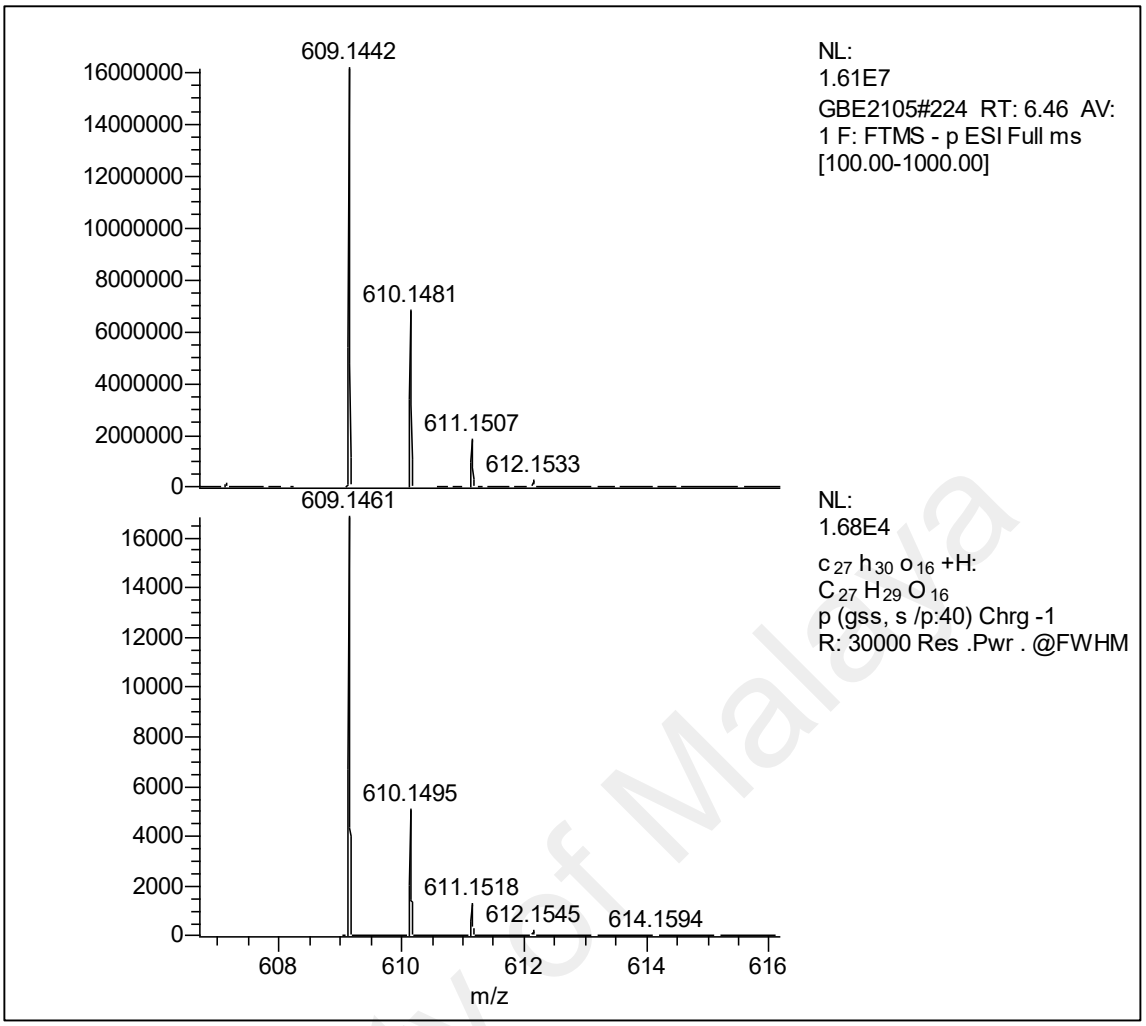
Appendix J1: ESI-MS of 4-hydroxybenzoic acid (2)



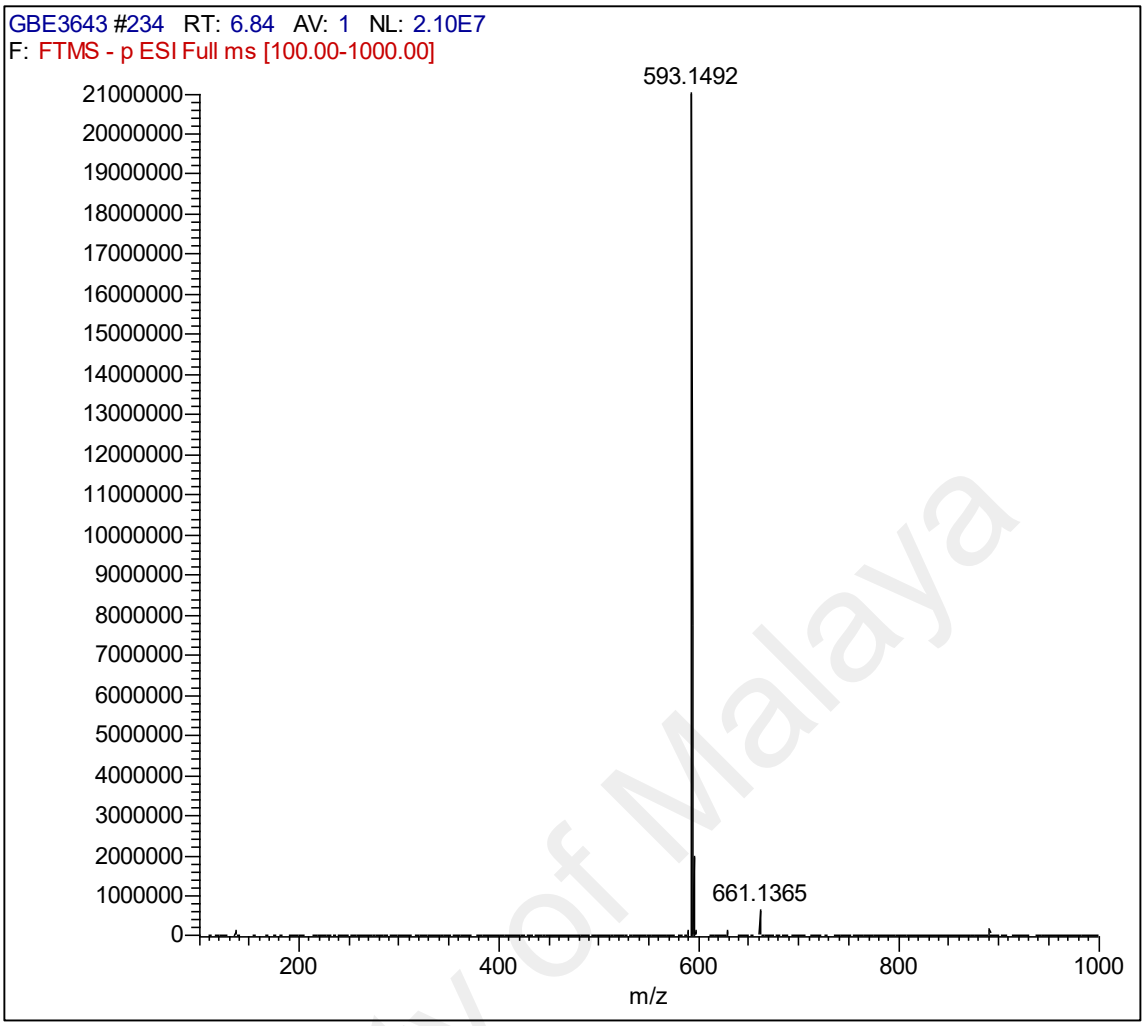
Appendix J2: Isotopic pattern of 4-hydroxybenzoic acid (2)



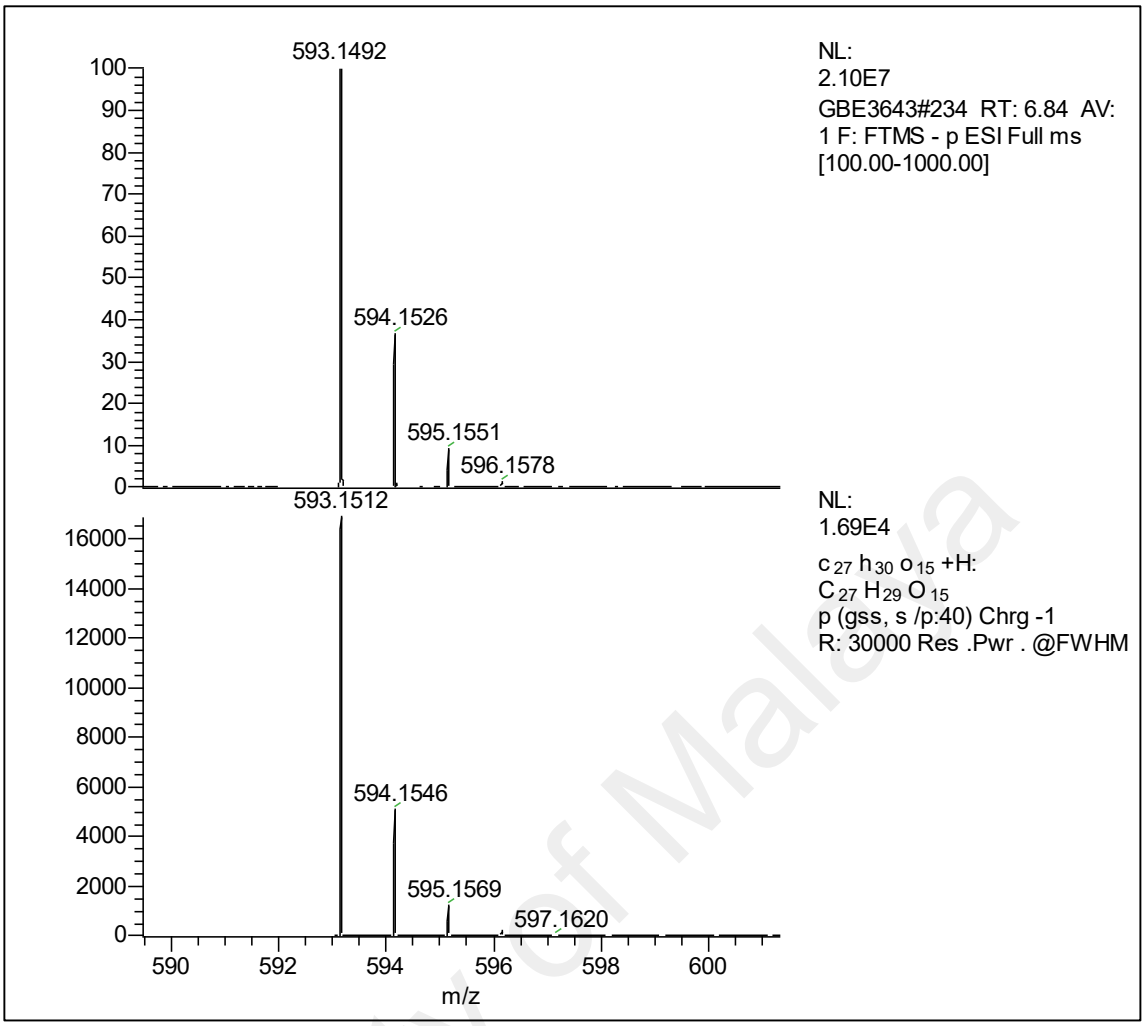
Appendix K1: ESI-MS of rutin (3)



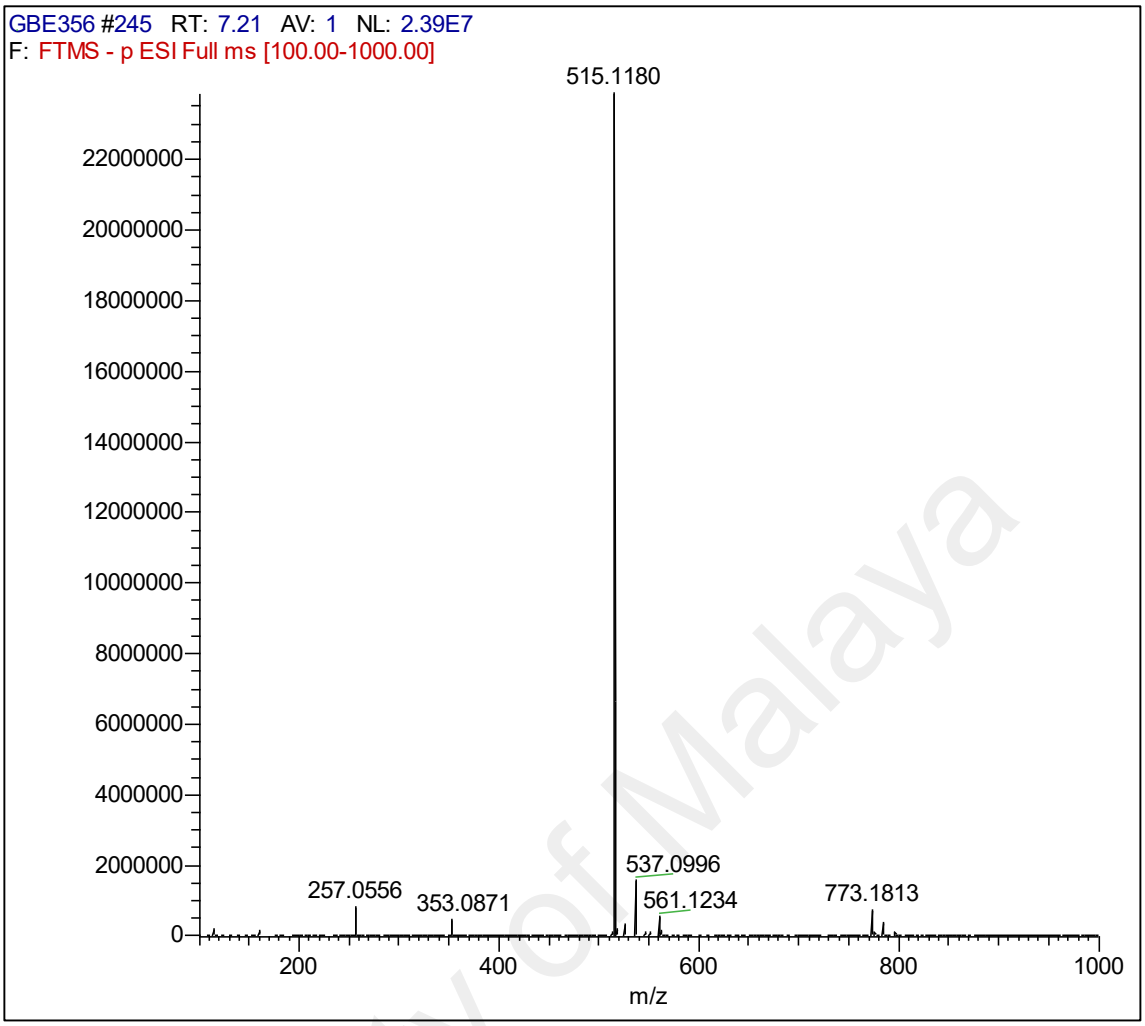
Appendix K2: Isotopic pattern of rutin (3)



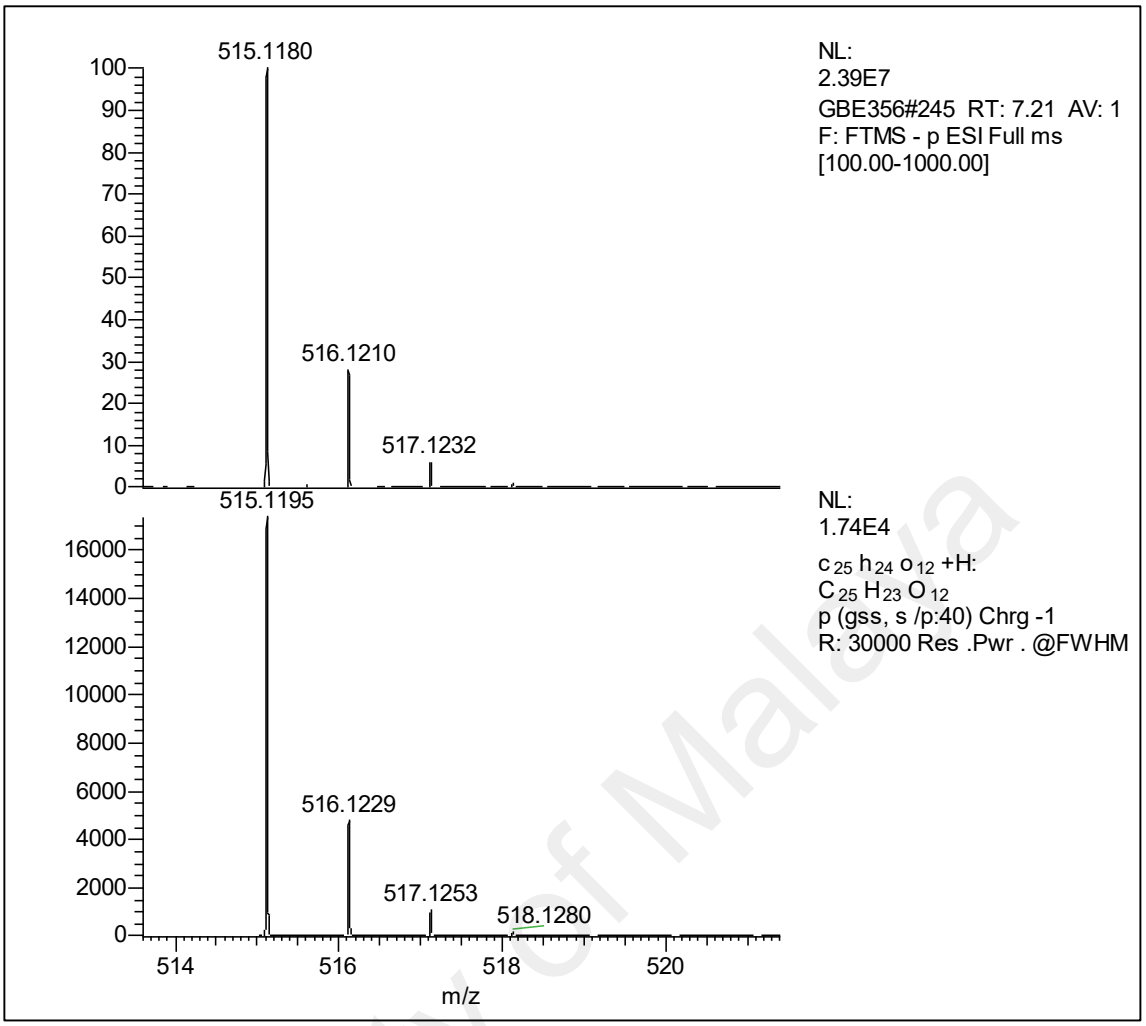
Appendix L1: ESI-MS of kaempferol-3-*O*-rutinoside (4)



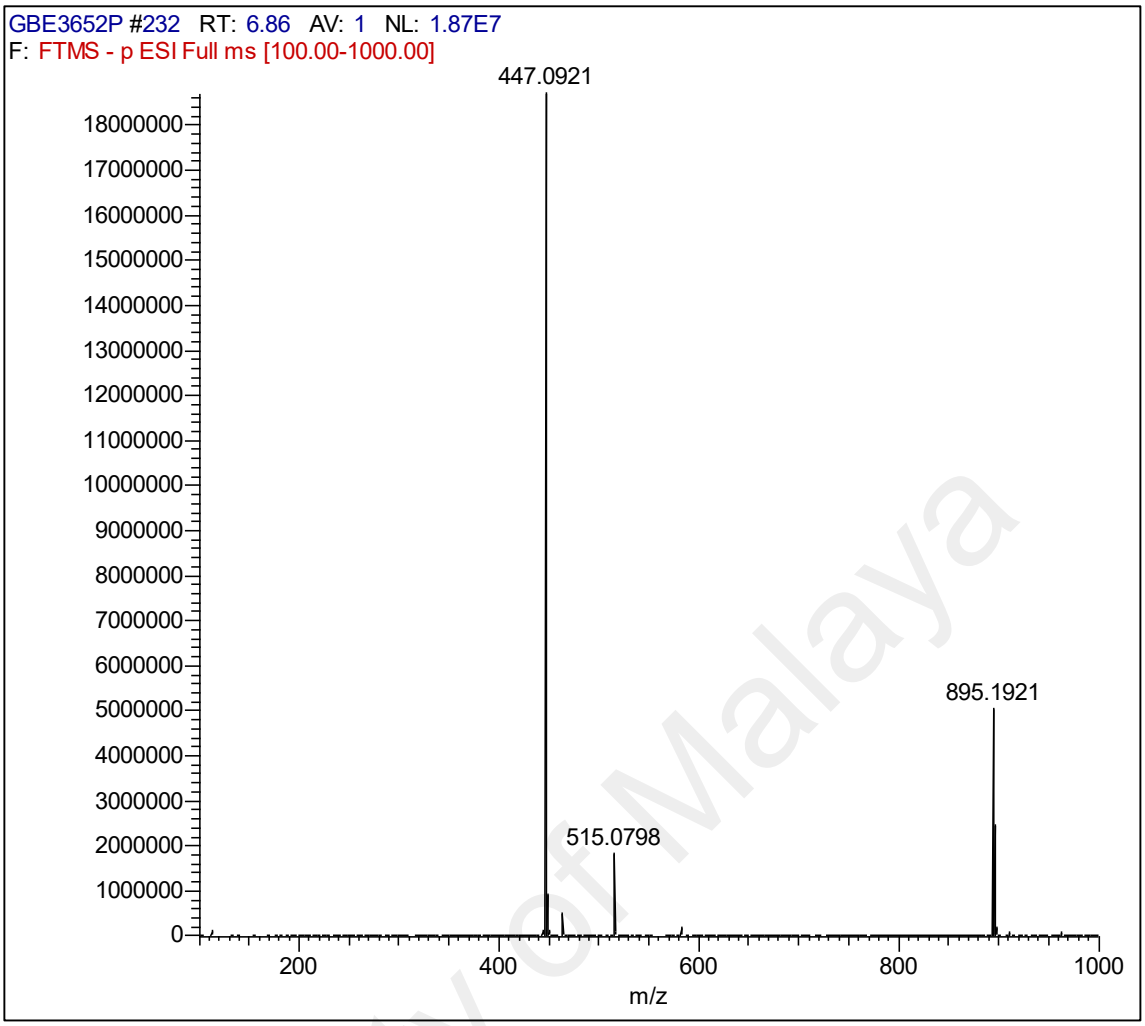
Appendix L2: Isotopic pattern of kaempferol-3-*O*-rutinoside (4)



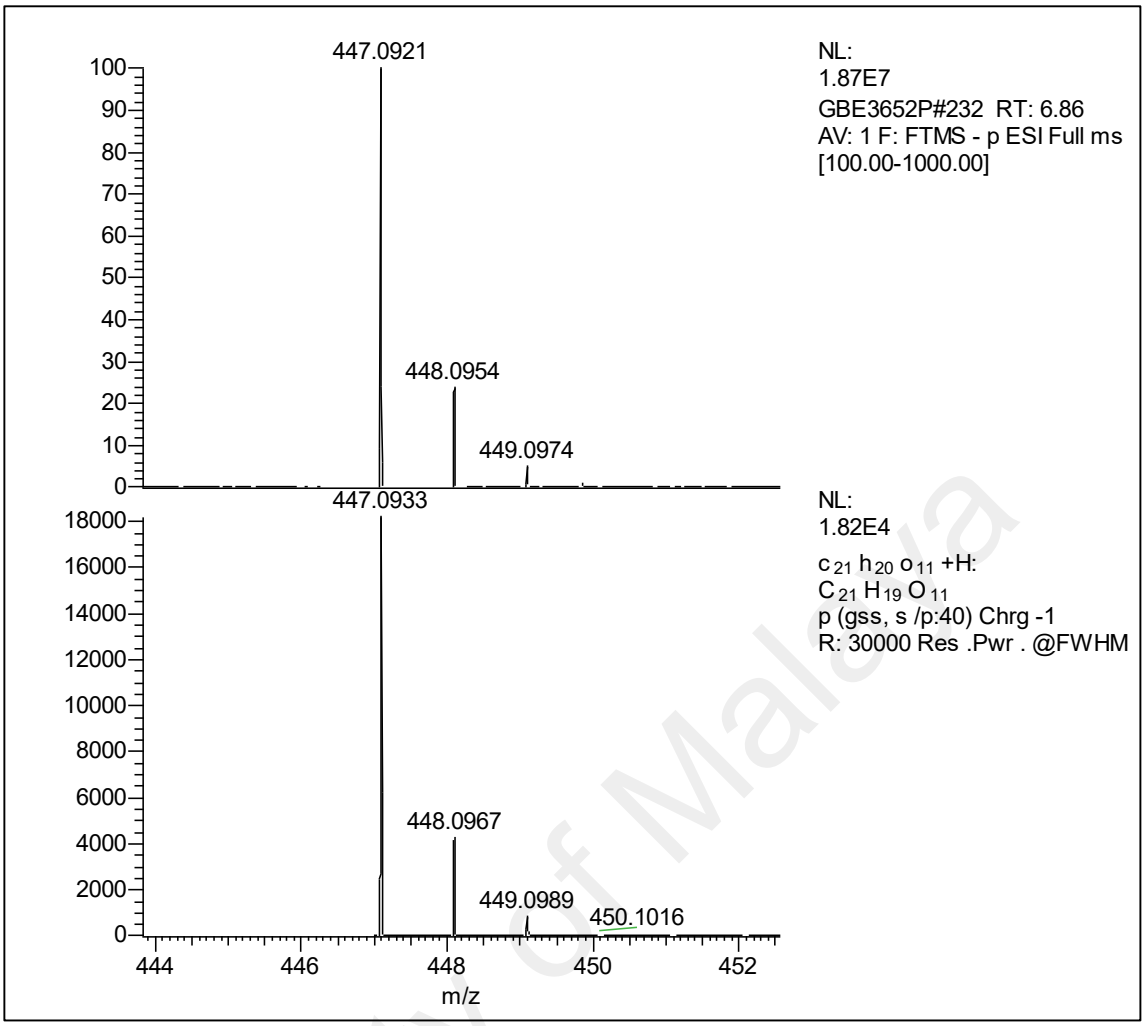
Appendix M1: ESI-MS of 3,5-dicaffeoylquinic acid (5)



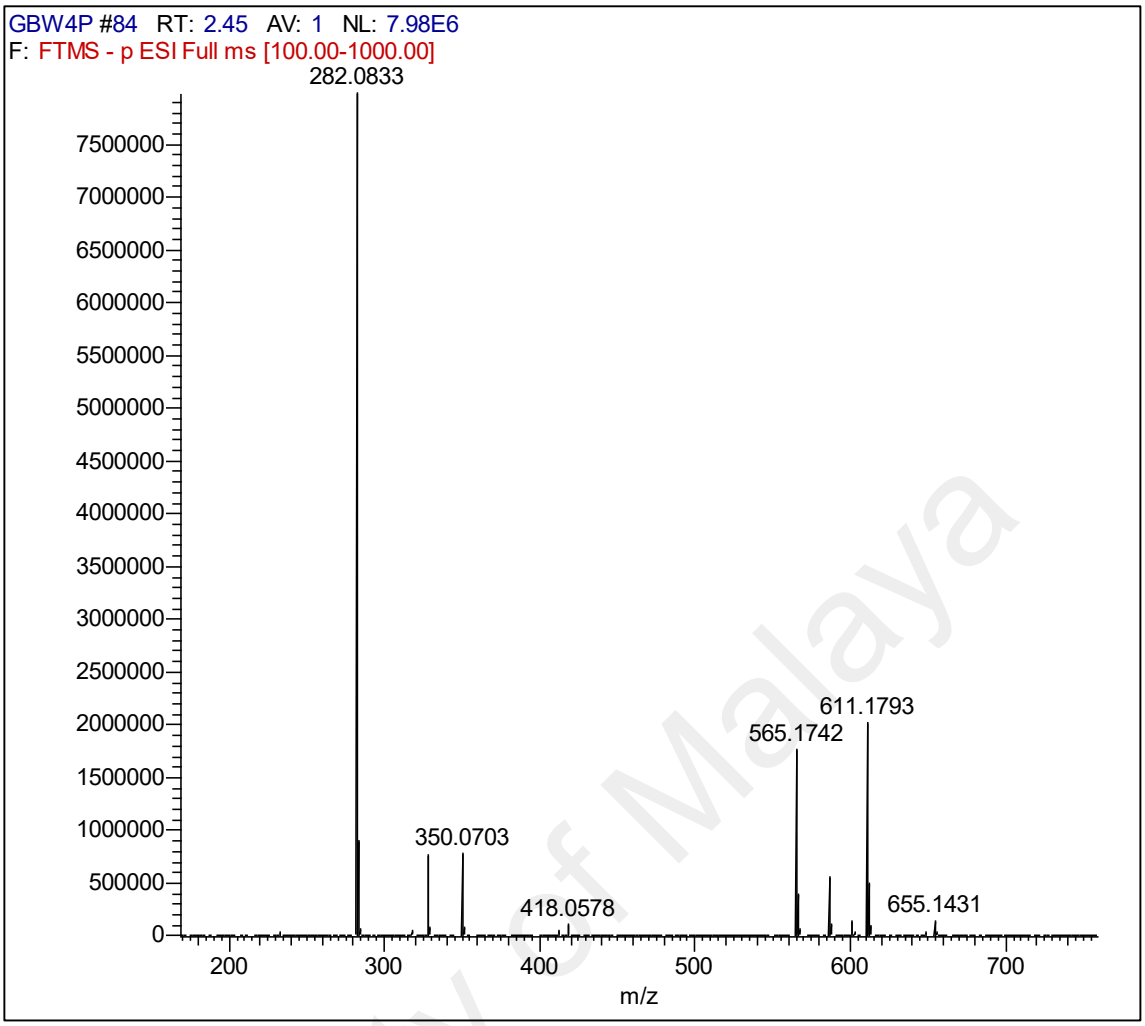
Appendix M2: Isotopic pattern of 3,5-dicaffeoylquinic acid (5)



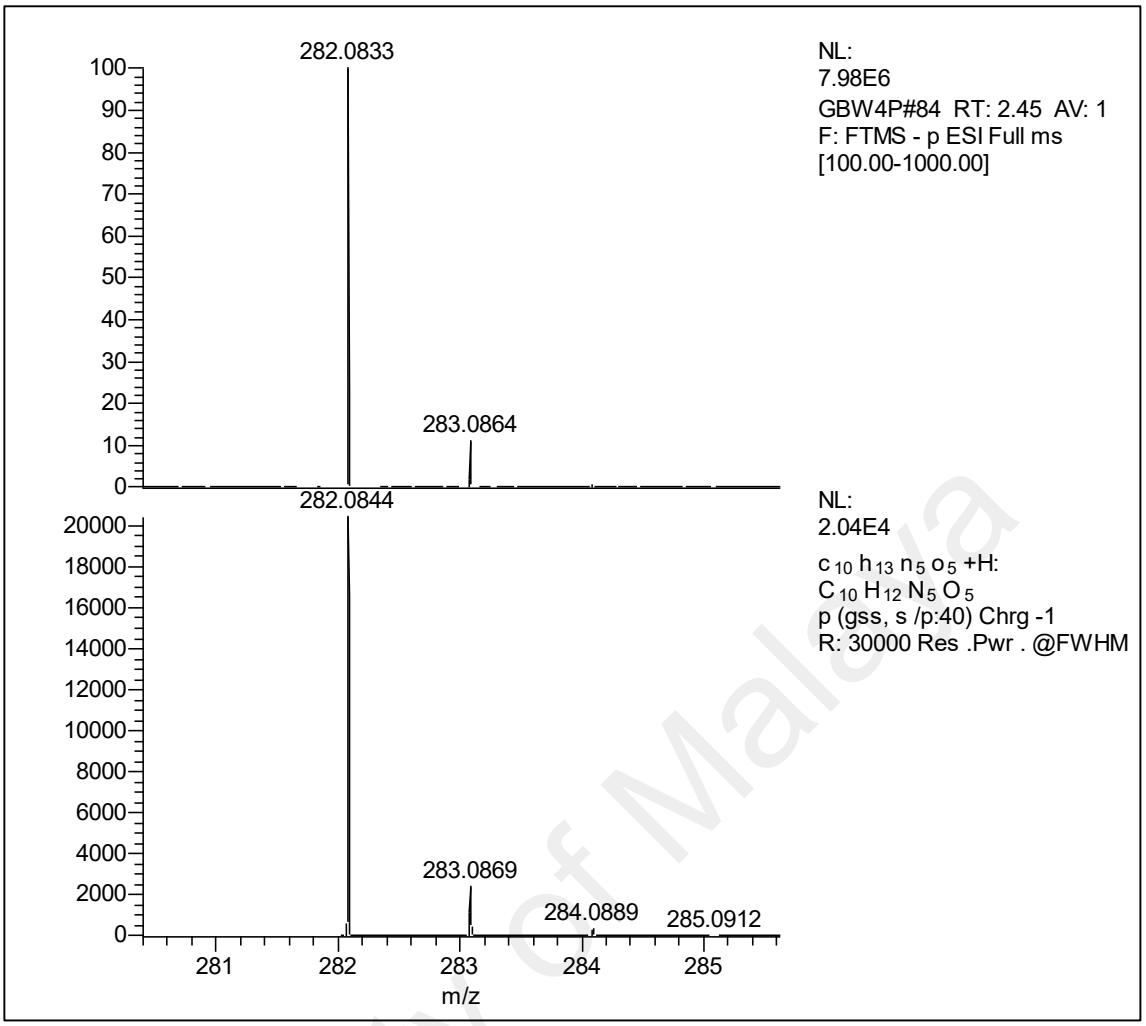
Appendix N1: ESI-MS of kaempferol-3-*O*-glucoside (6)



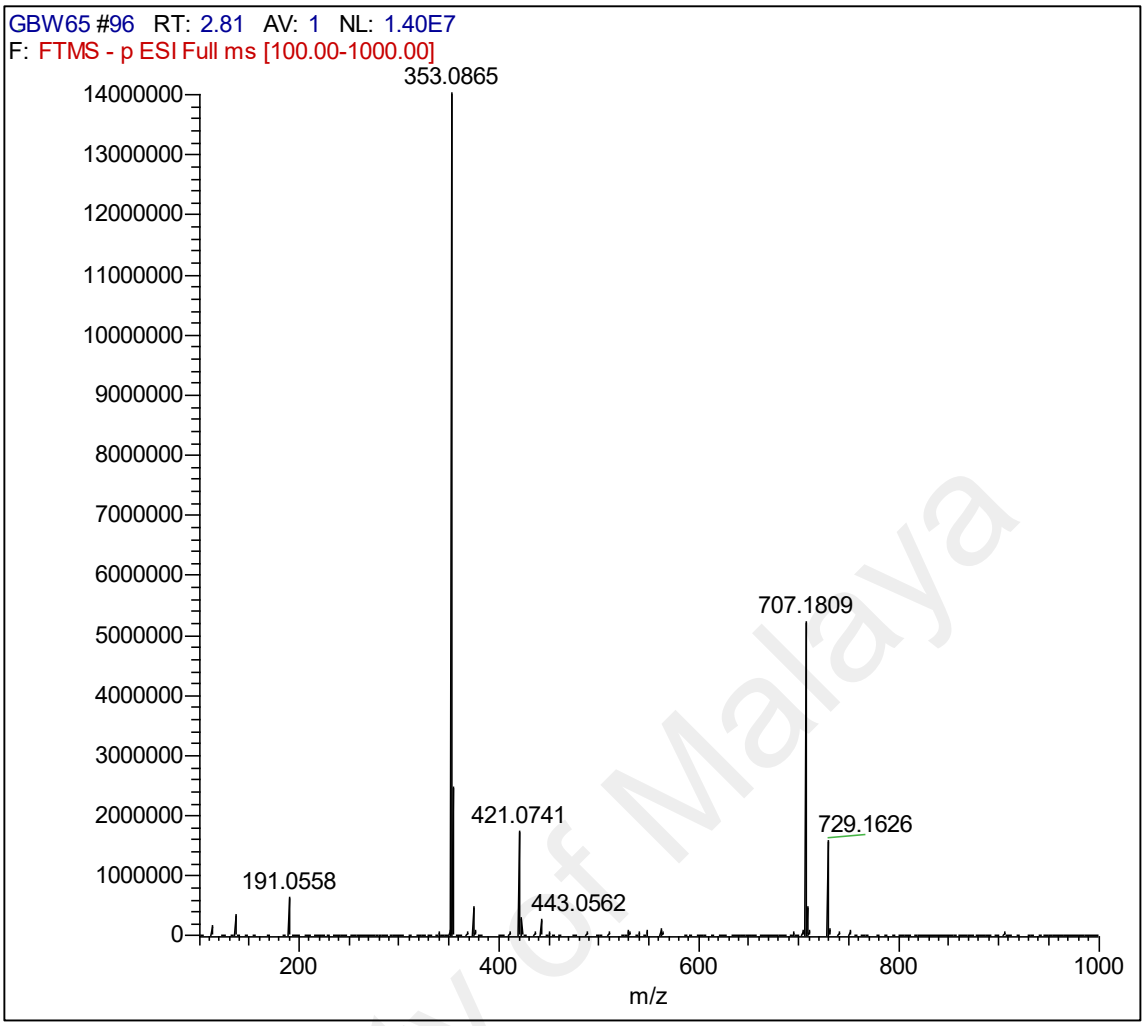
Appendix N2: Isotopic pattern of kaempferol-3-*O*-glucoside (6)



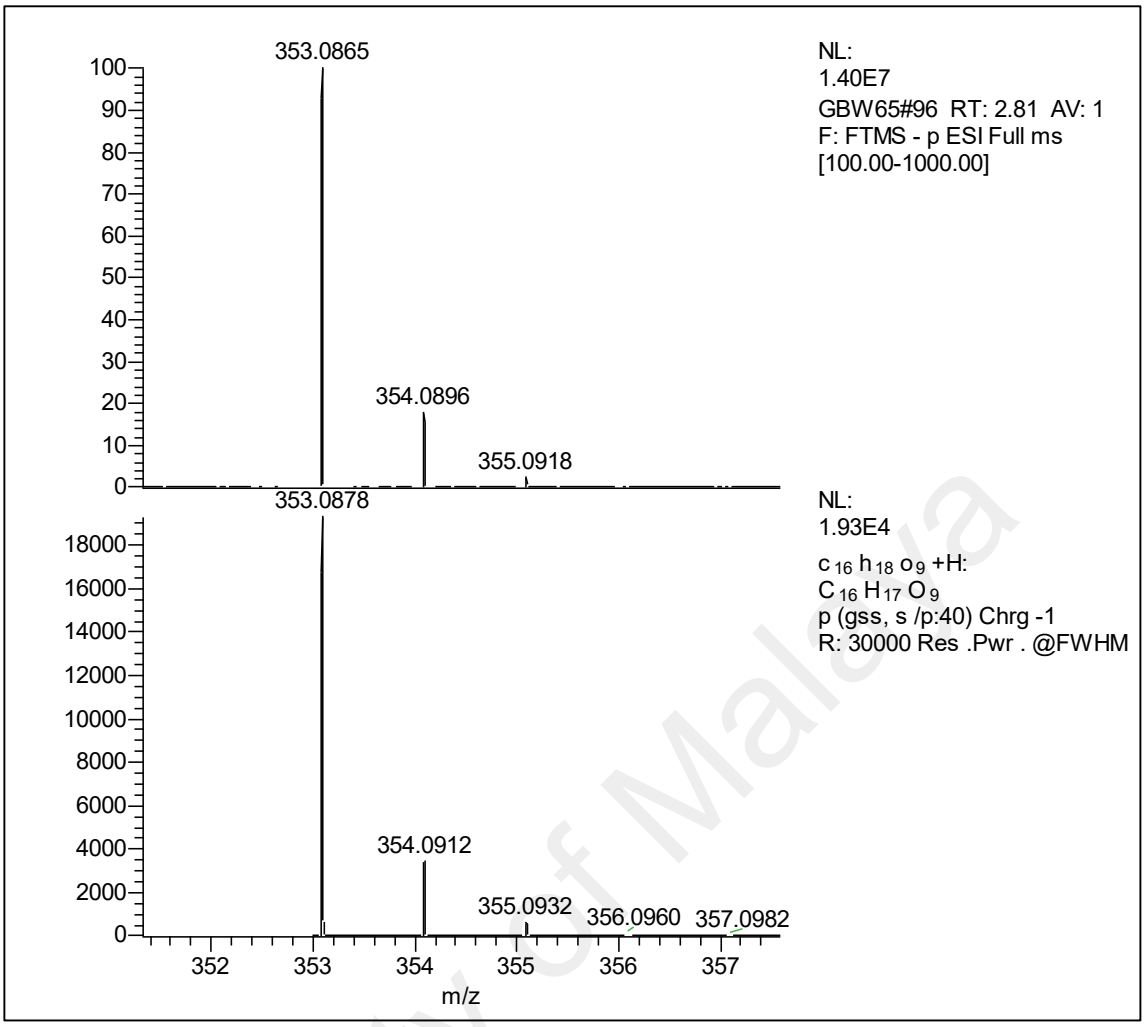
Appendix O1: ESI-MS of guanosine (7)



Appendix O2: Isotopic pattern of guanosine (7)



Appendix P1: ESI-MS of 5-*O*-caffeoylquinic acid (8)



Appendix P2: Isotopic pattern of 5-*O*-caffeoylquinic acid (8)

Appendix Q

Western blot preparations:

1. Preparation of 10 % resolving gel

	Volume (µl)
Deionized water	4700
30% acrylamide/bis (29:1)	4000
1.5M Tris-HCl, pH 8.8	3000
10% SDS	120
10% APS	120
TEMED	12

2. Preparation of 4 % stacking gel

	Volume (µl)
Deionized water	3600
30% acrylamide/bis (29:1)	800
0.5M Tris-HCl, pH 6.8	1500
10% SDS	60
10% APS	60
TEMED	6

3. Preparation of sample buffer (5X)

	Volume (ml)
0.5M Tris-HCl, pH6.8	3.5
Glycerol	4.0
10 % SDS	2.0
0.5 % bromophenol blue	0.25

Add 10 µl of mercaptoethanol to 190 µl of sample buffer (5X) and use immediately

4. Preparation of running buffer

Tris-base	3.03 g
Glycine	14.4 g
SDS	1.0 g
Deionized water	1 L

5. Preparation of transfer buffer

Tris-base	3.03 g
Glycine	14.4 g
Methanol	200 ml
Deionized water	Top up to 1 L

6. Preparation of Tris buffered saline with Tween-20 (TBST)

NaCl	8.006 g
Tris-base	2.422 g
Tween-20	1 ml
Deionized water	Top up to 1 L after pH 7.6

7. Preparation of Tris buffered saline (TBS)

NaCl	8.006 g
Tris-base	2.422 g
Deionized water	Top up to 1 L after pH 7.6

8. Preparation of blocking buffer

TBST	60 ml
Nonfat dry milk	3.0 g

9. Preparation of primary antibody solution

TBST	20 ml
BSA	1.0 g
Antibody	Accordingly

10. Preparation of horseradish peroxidase conjugated antibody solution

TBST	20 ml
Nonfat dry milk	1.0 g
Antibody	Accordingly