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BY

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ABSTRACT

Introduction: Curcumin is a natural product derived from the turmeric rhizome (*Curcuma longa)* that possesses a variety of pharmacological properties including anti-inflammatory, anti-microbial and anti-cancer activities. It inhibited the inflammatory mediators' expression, suppressed the cell proliferation and reduced the percentage of invasion and migration in various cancer cell lines. In order to overcome the poor bioavailability of curcumin, several analogues have been synthesised. One of the curcumin analogues is known as 2,6-bis-(4-hydroxyl-3-methoxybenzylidine)cyclohexanone (BHMC). BHMC was synthesised from curcumin by removing the unstable β-diketone moiety and altering it into double bonds while maintaining the phenolic hydroxyl group. Curcumin has been proved to be pro-oxidant, antioxidant and natural chemoprotective agent that promotes ROS above the threshold level and causes cell death in malignant cells with minimal cytotoxicity effect to normal cells. Reactive oxygen species (ROS) is an unstable oxygen species that easily reacts with other molecules in cells. Increase of ROS level lead to the collapse of redox buffering system, elicited lipid peroxidation and disintegration of the mitochondrial membrane potential that eventually cause cell death in malignant cells. **Objective:** In this study, the aim is to determine the cytotoxic selective effect of BHMC and curcumin on human liver cancer, HepG2 and non-cancer human fibroblast, Hs27 cell lines through ROS pathways. **Methodology:** Cell viability of both cell lines treated with BHMC or curcumin were determined using MTT Assay. The accumulation of ROS level in HepG2 cells treated with BHMC was measured using DCFDA Assay. **Results and Discussion:** BHMC was observed to be approximately three times toxic towards HepG2 cell line compared to curcumin with IC_{50} of 16.00µM and 46.10µM respectively. However, BHMC was less toxic towards Hs27 cells with IC_{50} of more than 30µM. Thus, it is suggested that BHMC was cytotoxic selective towards normal cell lines. Further investigation shows that BHMC has a similar pattern in exerting the antioxidant effect as its parental compound. BHMC and curcumin act in dose dependent manner as high antioxidant activity is directly proportional to increase of concentration. **Conclusion:** BHMC has greater cytotoxic effect towards HepG2 cell line compared to normal Hs27 cell line. Although the exact mechanism is not fully elucidated, BHMC is suggested to trigger its cytotoxicity via ROS pathways.

Keywords: BHMC; Curcumin; HepG2; Hs27; ROS

KESAN SITOTOKSIK BHMC MELALUI LALUAN ROS TERHADAP GARISAN SEL HEPG2 DAN HS27

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ABSTRAK

Pengenalan: Curcumin adalah produk semula jadi yang berasal dari rizom kunyit (*Curcuma longa*) yang memiliki pelbagai aktiviti farmakologi termasuk aktiviti anti-inflamasi, anti-mikrob dan anti-kanser. Ia menghalang ekspresi perantara inflamasi, menghalang percambahan sel dan mengurangkan peratusan pencerobohan dan penghijrahan dalam pelbagai garisan sel kanser. Untuk mengatasi bioavailabiliti curcumin yang lemah, beberapa analog yang telah disintesis. Salah satu analog dikenali sebagai 2,6-bis-(4-hydroxyl-3-methoxybenzylidine)cyclohexanone (BHMC). BHMC disintesis dari curcumin dengan mengeluarkan β-diketone moiety yang tidak stabil dan mengubah kepada ikatan berganda sambil mengekalkan kumpulan hidroksil fenolik. Curcumin telah terbukti sebagai agen pro-oksidan, antioksidan dan kemoprotektif semula jadi yang menyebabkan ROS melebihi tahap ambang dan mendorong kepada kematian sel-sel malignan dengan kesan sitotoksisiti yang minimum kepada sel normal. Spesies Oksigen Reaktif (ROS) adalah spesies oksigen tidak stabil yang mudah bertindak balas dengan molekul lain dalam sel. Peningkatan tahap ROS menyebabkan keruntuhan sistem penampan redoks, menimbulkan lipid peroksidasi dan meluruhkan potensi membran mitokondria yang menyebabkan kematian sel terhadap sel-sel malignan. **Objektif:** Tujuan kajian ini adalah untuk mengetahui kesan sitotoksik BHMC dan curcumin pada garisan sel kanser hati manusia, HepG2 dan garisan sel fibroblas manusia bukan kanser, Hs27 melalui laluan ROS. **Metodologi:** Daya maju sel kedua-dua garisan sel yang dirawat dengan BHMC dan curcumin ditentukan dengan menggunakan Asai MTT. Pengumpulan tahap ROS dalam titisan sel HepG2 yang dirawat dengan BHMC diukur menggunakan Asai DCFDA. **Keputusan dan Perbincangan:** BHMC dilihat toksik tiga kali ganda terhadap garisan sel HepG2 berbanding curcumin dengan IC_{50} masing-masing 16.00µM dan 46.10µM. Walau bagaimanapun, BHMC kurang toksik terhadap sel Hs27 dengan IC_{50} lebih daripada 30µM. Oleh itu, dicadangkan bahawa BHMC bersifat toksik secara selektif terhadap garisan sel normal. Kajian lebih lanjut menunjukkan bahawa BHMC mempunyai corak yang serupa dalam memberi kesan antioksidan seperti sebatian asal. BHMC dan curcumin bertindak sebagai dos secara bersandar kerana aktiviti antioksidan yang tinggi berkadar langsung dengan peningkatan kepekatan. **Kesimpulan:** BHMC mempunyai kesan sitotoksik yang lebih besar terhadap garisan sel HepG2 berbanding dengan garisan sel normal Hs27. Walaupun mekanisme yang tepat tidak dijelaskan sepenuhnya, BHMC dicadangkan untuk mencetuskan sitotoksisitinya melalui laluan ROS.

Kata kunci: BHMC; Curcumin; HepG2; Hs27; ROS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GLOSSARY OF TERMS

AP-1	Activator protein 1 is a transcription factor that regulates gene expression in response to stimuli, including viral infections, stress, growth factors, cytokines, and bacterial
ATP	Adenosine triphosphate to provides energy in living cells
β-catenin	Catenin beta-1 involve in cells adhesion and gene transcription
BHMC	2,6-bis-(4-hydroxyl-3-methoxybenzylidine)cyclohexanone is analogue of curcumin
B-Raf	Proto-oncogene that related to serine/threonine kinase
CAT	Catalase breaks down hydrogen peroxide molecules into oxygen and water
Cdk	Cyclin-dependent kinases involve in regulating transcription, mRNA processing and cell cycle
c-Kit	Receptor of tyrosine kinase
COX-2	Cyclooxygenase is a type of pro-inflammatory proteins
CXCL	Chemokine (C-X-C motif) ligand is an inflammatory chemokine
CXCR	Chemokine receptors are integral membrane proteins that specifically bind and respond to chemokine
DCFDA	2',7'-dichlorofluorescence diacetate is a fluorescence dye that measure intracellular ROS activities
DHE	Dihydroethidium
DMEM	Dulbecco's Modified Eagle Medium is basal medium for cell growth

DMSO	Dimethyl sulfoxide that dissolve both polar and non-polar compounds
DNA	Deoxyribonucleic Acid that compose of two chains of polynucleotides that coil to one another that carry genetic informations
FLT-3	FMS-like receptor tyrosine kinase-3 is cytokine receptor that belongs to the receptor tyrosine kinase
G2/M	G2/M is DNA damage checkpoint
GSH	Glutathione is abundant low molecular weight thiol in cells
GSSC	Oxidised form of glutathione
GST	Glutathione-S-transferase is a detoxification enzyme in Phase II metabolising enzymes that protect cellular macromolecule from reactive molecules
HCC	Hepatocellular carcinoma is one type of primary liver cancer
HCV	Hepatitis C Virus that can cause liver infection, Hepatitis C
HBV	Hepatitis B Virus that can cause liver infection, Hepatitis B
HDI	Human Development Index is index of life expectancy, education, and per capita income indicators
HepG2	Human liver hepatocellular carcinoma
Hs27	Normal human fibroblast
IC_{50}	Concentration of compounds to produce cytotoxicity at 50% of population
IKK	IκB Kinase that involved in cellular response to inflammation

IL	Interleukin is inflammatory cytokines
iNOS	Inducibled nitric oxide synthase is a type of pro-inflammatory proteins
MAPK	Mitogen-activated protein kinase is serine/threonine-selective protein kinases directing cellular responses to stimuli including heat shock, proinflammatory cytokines, mitogens and osmotic stress.
MCP-1	Monocyte chemoattractant protein-1is type of inflammatory proteins
MMP-2	Matrix metallopeptidase 2 is a type of invasion-related proteins
MMP-9	Matrix metallopeptidase 9 is a type of invasion-related proteins
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide is used to measure metabolic activity of cells
NF-κB	Nuclear factor kappa B is transcription factors that regulate genes responsible for immune system
NO	Nitric Oxide is type of inflammatory mediators
PDGFR-β	Platelet-derived growth factor receptor beta
PGE_2	Prostaglandin E_2 is prostaglandin with oxytocic properties
PKD1	Protein Kinase D 1 is stress-activated serine/threonine kinase that regulate cell growth, angiogenesis and apoptosis.
PLGA	Poly(lactic-co-glycolic acid) is a copolymer of lactic acid and glycolic acid

Raf-1	Proto-oncogene serine/threonine protein kinase that also known as proto-oncogene c-RAF or simply c-Raf or even Raf-1
ROS	Reactive oxygen species is chemical reactive species that contain oxygen such as hydrogen peroxide
STAT-3	Signal transducer and activator of transcription 3
TNF	Tumour Necrosis factor is pro-inflammatory cytokines
VEGFR	Vascular Endothelial Growth Factor Receptors (VEGFRs) are tyrosine kinase receptors responsible for binding with VEGF

CHAPTER ONE

INTRODUCTION

1.1 Background

Cancer is defined as a large group of diseases characterised by the abnormal growth of cells which is malignant that will invade the surrounding tissues and metastasise to other organs (World Health Organization, 2019). According to study conducted by Heron (2019), cancer is the second leading cause of death worldwide in 2018. In a study conducted by GLOBOCAN (2018), it stated that 18,078,957 new cases of cancer and 9,555,027 deaths due to cancer in 2018. Liver cancer has also been reported to contribute to 841,080 of cancer incidences and 781,631 of mortality. In Malaysia, liver cancer contribute to 4.4% of incidences from 43,837 new cases of cancer and 7.3% of mortality from 26,395 deaths due to cancer (GLOBOCAN, 2018). The incidence rates of liver cancer is higher in male that mainly living in lower Human Development Index (HDI) such as northern and western Africa and eastern and South East Asia compared to female (Bray et al., 2018).

Primary liver cancer is the malignant tumor that starts from liver. There are few types of primary liver cancer including hepatocellular carcinoma (HCC) (75%-85%), intrahepatic cholangiocarcinoma (10%-15%) and other rare types (5%-10%) such as fibrolamellar HCC, angiosarcoma and hepatoblastoma (Bray et al., 2018). HCC is one of neoplastic liver disease that originated from hepatocytes at hepatic parenchymal mainly function in metabolism of cholesterol, glucose and glycogen, urea, detoxification and blood clotting (Vera-Ramirez et al., 2013). The development of HCC can be due to infections such as Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) or risk factors including alcoholic and non-alcoholic cirrhosis, obesity

and iron overload or dietary hepatocarcinogens such as aflatoxins and nitrosamines (S. Darvesh, B. Aggarwal, & Bishayee, 2011). People that abuse alcohol have 22% increase risk if drink alcohol more than six times per day, people that infected with HBV have 10%-25% of lifetime risks, people that infected with HCV have 20% to develop cirrhosis which will develop HCC due to its almost exclusively in cirrhosis liver (Balogh et al., 2016). Treatments for HCC are by surgery and liver transplantation but it is limited to 20% of patients due to several factors such as multifocality and size of tumour which effective at early stage but it's usually diagnose at advance stage of HCC (Balogh et al., 2016).

One of the alternative ways is to find the new treatment of liver cancer, which is by treating with bioactive compound, curcumin. Curcumin is a natural occurring polyphenol that derived from rhizome of turmeric or *curcuma longa*. It was firstly identified by Lampe and Milobezka in 1910 and possess variety of pharmacological properties such as anti-inflammatory activities and anti-cancer activities (Jurenka & Ascp, 2009). Curcumin has also been used as the treatment for inflammation in the long history of Ayurvedic Medicine. The anti-inflammatory activity of curcumin is triggered via suppressing the NF-κB activation that cause the downregulation of iNOS, COX-2 and inflammatory cytokines (Jurenka & Ascp, 2009). Chronic inflammation can lead to the development of cancer due to DNA damage. Curcumin also has shown to have anti-proliferation activity on cancer cells by suppressing the IκB Kinase (IKKs) that cause inhibition of NF-κB activation (Hartojo et al., 2010). Curcumin also has anti-metastatic activity by reduce the expressions of MMP-2, MMP-9, Snail and Twist in breast cancer cells and squamous cell carcinoma that lead to decrease in percentage of invasions and migrations (A. Y. L. Lee et al., 2015; Razak et al., 2017). It is also has been found that inflammation can promote tumour proliferation and MMP-9

expression that allow metastasise of cancer by forming invadopodia (Harun et al., 2018). Curcumin have pro-oxidant, antioxidant and chemoprotective properties that can target malignant cells selectively (Liu et al., 2016). In the same study, it stated that high level of ROS can cause the cancer cell undergo apoptosis while mild level of ROS can promote cell proliferation. According to Atsumi, Tonosaki and Fujisawa (2006), curcumin is pro-oxidant because it can generate ROS to induce the cell death of cancer cell. Curcumin also can scavenging the ROS by induce the phase II metabolism enzymes such as Glutathione S Transferase (GST) to neutralise ROS derived from chemical carcinogen as its antioxidant properties (Iqbal et al., 2003). According to Martin-Cordero et al., (2012), cancer cells that consistently under oxidative stress are more vulnerable to ROS-induced cytotoxicity compared to normal cells. However, curcumin has limitation in the poor bioavailability despite all the therapeutic effects it has shown (Anand et al., 2007). It has low water solubility, low chemical stability, low cellular uptake and low availability in cytoplasm (Tomeh, Hadianamrei, & Zhao, 2019). It is suggested due to the lack of hydroxyl group, unstable β-diketone structure and hydrophobicity that affect the bioavailability of curcumin.

Many approaches has been done to improve the bioavailability of curcumin such as nanoformulations, combination with adjuvants, phospholipid complex and structural modification (Gupta, Patchva, & Aggarwal, 2013). Several analogues of curcumin were also synthesised to overcome the limitation and improve the effect. One of the analogue is known as 2,6-bis-(4-hydroxyl-3-methoxybenzylidine)cyclohexanone (BHMC). It was synthesised at Institute of Bioscience, UPM based on the structure of curcumin by removing the unstable β-diketone and modifying it into double bond while preserving the hydroxyl group (Tham et al., 2010). In order to improve the therapeutic effects of curcumin, stable

cyclohexanone has been added to substitute β-diketone moiety, since aldo-ketoreductase will rapidly metabolize in the liver (Y. Z. Lee et al., 2012). BHMC possesses variety of pharmacological properties such as anti-metastatic activity as its parent compound, analgesic activity and anti-inflammatory activity by inhibition of cyclooxygenase and lipoxygenase (Harun et al., 2018; Razak et al., 2017).

1.2 Objectives

1.2.1 General Objective

To determine the cytotoxic effect of BHMC and curcumin on human liver cancer cells, HepG2 and non-cancerous human fibroblast, Hs27 through ROS induction.

1.2.2 Specific Objective

- To compare the effect of BHMC and curcumin on cell viability in both cell lines.
- To measure the level of ROS upon the treatment of BHMC and curcumin in both cell lines.

1.3 Hypothesis

Since BHMC is an analogue of curcumin that exhibit pro-oxidant and antioxidant properties, it is hypothesised that BHMC has greater cytotoxic effect on HepG2 cells which may be exerted through the induction of ROS level above the threshold compared to curcumin.

CHAPTER TWO

LITERATURE REVIEW

2.1 Curcumin

2.1.1 Introduction to Curcumin

Curcumin or diferuloylmethane is one of the phytochemicals that can be found from rhizome of *Curcuma longa* (turmeric) belong to Zingiberaceace family that said to have beneficial effects in term of pharmacology aspect such as anti-cancer, anti-microbial, anti-inflammatory and anti-oxidant properties (Syed Alwi, Zahari, Haron, & Alexander, 2019). Curcumin is one of polyphenol compound that presence in turmeric about 60-780%, while other polyphenol compound can be found in turmeric are demethoxycurcumin (15-30%) and bisdemethoxycurcumin (2-6%) (Rohman, 2012). According to Anirudhan & Binusreejayan (2016), Curcumin has the ability to inhibit cancer cells due to anti-oxidant and anti-inflammatory properties. It is said that has been used by Indian race as treatment for sinusitis and rheumatism but also been used by other people as treatment for anorexia, biliary and hepatic disorders (Hu et al., 2015). Phytochemicals or Polyphenolic compound can be found naturally in the plant as the secondary metabolite that has preventive properties and can target specific pathway which is good in fighting cancer (Syed Alwi et al., 2019).

2.1.2 Anti-cancer properties of Curcumin

Anti-tumour properties of Curcumin has potential effects on various of cancer cells such as colon, lung, prostate, breast and pancreatic cancers while it is also exhibits the potential to reverse the multidrug resistance in cancer cells (Hu et al., 2015).

Curcumin, the isolated yellow pigment shows that it is a DNA-damaging agent that can exhibit the properties of supressing cell proliferation, induced apoptosis and cell arrest at the G2/M phase by regulating NF-κB and protein kinase B (Akt) (Syed Alwi et al., 2019; Wang, Cheng, Luo, & Zhuang, 2008). According to Moses, Garcia-Bloj, Harvey, & Blancafort (2018), there are 10 hallmarks of cancer consists of sustain proliferative signals, evade growth suppressor, avoid immune systems, switch on replicative immortality, tumour-promoting inflammation, activate invasion and metastasize, induce angiogenesis, mutation and genome instability, escape from apoptosis and deregulate cellular energetics. In anti-cancer aspect, Curcumin shows that it can overcome some of the hallmarks of cancer such as promote apoptosis, inhibit metastasize, angiogenesis and cancer formation in various cancer cells (Wang et al., 2008). Curcumin's anti-inflammatory activity is activated by suppressing the NF-kB activation, which triggers downregulation of iNOS, COX-2 and inflammatory cytokines that can lead to the development of cancer due to DNA damage (Jurenka & Ascp, 2009). Curcumin has also been shown to have anti-proliferation activity on cancer cells by suppressing the IκB Kinase (IKKs) that induces NF-κB activation inhibition (Hartojo et al., 2010). Curcumin also has anti-metastatic activity in breast cancer cells and squamous cell carcinoma by reducing the expressions of MMP-2, MMP-9, Snail and Twist that result in the decrease in percentage of invasions and migrations (A. Y. L. Lee et al., 2015; Razak et al., 2017). Inflammation has also been found to promote tumour proliferation and the expression of MMP-9 that allow formation of invadopodia for cancer to metastasise (Harun et al., 2018). According to Tomeh, Hadianamrei and Zhao (2019), curcumin can suppress proliferation and promote apoptosis in prostate cancer by activate protein kinase D1 (PKD1) that cause oncogenic signals such as β-catenin and MAPK. In the same study, similar cell lines,

curcumin suppress expression of CXCL 1 and 2 which in inflammatory cytokines that lead to inhibition of chemotactic receptors CXCR4, a metastasise promoting gene.

2.1.3 Derivative of Curcumin

In many studies, curcumin has been shows to exert various pharmacological properties and has been classifies as GRAS (Generally Recognised as Safe) from The United States Food and Drug Administration (FDA) (Gupta et al., 2013). However, there is limitation of curcumin which is poor bioavailability. This due to the factors of low water solubility, low chemical stability, low cellular uptake and low availability in cytoplasm (Tomeh et al., 2019). In a study conducted by Hewlings & Kalman (2017), poor bioavailability of curcumin also due to the poor absorption, rapid metabolism and rapid elimination after administered that cause the dosage of curcumin to be administered increase and cause curcumin to exert its toxic or side effect. There are many approaches has been done to improve the bioavailability of curcumin such as nanoformulations, combination with adjuvants, liposomes, phospholipid complex and structural modification (Gupta et al., 2013). In the same study, the use of piperine as adjuvant results in increase of bioavailability of curcumin up to 2000%. Curcumin loaded with liposomes shows the reduced interleukin-6 (IL-6) production by macrophage and twenty-fold cytotoxic activity exert on hepatocellular carcinoma, lung carcinoma, colorectal carcinoma and cervical carcinoma (Tomeh et al., 2019). Other approach has been done which is drug delivery systems including polymeric nanoparticles, liposomes, nanogels, peptide and protein formulations and cyclodextrin complexes (Tomeh et al., 2019). In the same study, nanoformulations of curcumin with synthetic polymer poly(D, L-lactic-co-glycolic acid) (PLGA) can inhibits the expression mRNAs that code for inflammatory cytokines such as CXCR3 and

CXCL10, and promote anti-inflammatory cytokines such as interleukin-10 (IL-10) at 15-fold lower concentration compared to curcumin at the brain. For nanogels approach, curcumin-loaded hybrid nanogels shows significant difference by inhibiting cell proliferation compared to cells treated by curcumin (Tomeh et al., 2019).

2.2 BHMC

2,6-bis(4-Hydroxy-3-Methoxybenzylidene) Cyclohexane (BHMC) is a curcumin analogue that being produce in order to improve the bioavailability of curcumin and maintain the therapeutic effects of curcumin (Y. Z. Lee et al., 2012). BHMC is synthesise by removing the unstable β-diketone moiety, modifying into conjugated double bonds and remain the phenolic OH functional group of curcumin (Tham et al., 2015). In order to enhance the therapeutic effects of curcumin, stable cyclohexanone was added to replace β-diketone moiety due to it can rapidly metabolise in liver by aldo-ketoreductase (Y. Z. Lee et al., 2012; Syed Alwi et al., 2019).

According to Lee et al. (2012), BHMC was studied in inflammation, hyperalgesia, sepsis and murine breast cancer. In the study, Ming-Tatt et al. (2012) suggested that inducible nitric oxide synthase isoform (iNOS), TNF-α, IL-6 and monocyte chemotactic protein (MCP)-1 was involved in mechanism of pain. BHMC can inhibit the production NO, cytokines and other inflammatory mediator by inhibiting the cyclooxygenase and lipoxygenase activity that will disturb the signal transduction as involved in pain mechanism (Ming-Tatt et al., 2012). In addition, Tham et al. (2011) stated that BHMC is more selective in reduce the synthesis of inflammatory mediators such as MCP-1, iNOS, IL-10, IL-6, and TNF-α while production of prostaglandin E_2 (PGE_2), IL-1β and IL-8 are not affected compared to

curcumin. BHMC shows to inhibit the hyperalgesic activity induced at opioid receptor by sciatic nerve ligation (Ming-tatt et al., 2013)

The MAPK activation can be inhibited when treated with BHMC by losing of AP-1-DNA binding due potent effects on p38 activity by binding to ATP pocket on p38 that will block the synthesis of several proinflammatory mediators (Tham et al., 2011). A study conducted by Tham et al. (2015) stated that BHMC suppressed transendothelial leukocyte migration and endothelial permeability by reduce the MCP-1 synthesis and modulate the adhesion molecule expression of endothelial cell in order to block the specific event of leukocytes recruitment at the site of inflammation.

In septic host, immune suppression will be developed after the activation of p38 signalling pathway (Tham et al., 2011). According to Tham et al. (2011), administration of BHMC shows the inhibition of p38 that increase the survival rates by reduce the mortality due to severe systemic inflammatory syndrome. In comparison to curcumin, BHMC is more potent and superior in reduce mortality due to lethal sepsis from severe systemic inflammatory syndrome (Y. Z. Lee et al., 2012; Tham et al., 2011). However, Tham et al. (2011) suggested that BHMC has small range in margin of safety which show toxicity in severe renal and liver damage of septic host.

According to Harun et al. (2018), cancer has the ability to undergo abnormal cell division to proliferate and invade neighbouring tissues which will metastasise to other organs. In the same study, Harun et al. (2018) suggested that the percentage of migration and invasion of breast cancer cells has been decreased after treated with BHMC and Curcumin especially on breast cancer cells and oral Squamous Cell Carcinoma can avert the invasion process. In another study by Razak et al. (2017) stated that BHMC possessed the anti-metastasise properties as its precursor, Curcumin

that suppressed the inflammatory mediators and MMP9 which shows that BHMC better in anti-metastasise properties.

2.3 Reactive Oxygen Species (ROS)

2.3.1 Roles of ROS in Cancer Therapy

Reactive Oxygen Species (ROS) is oxygen containing chemical reactive species that also been called as reactive oxygen metabolites (ROMs), reactive oxygen intermediates (ROIs) and oxygen radicals (Y. R. Li & Trush, 2016). ROS consist of oxygen molecule which is unstable and easily react with other molecule in cells cause to damage to DNA, RNA and protein, protein cross-linking and apoptosis via ROS build up (Uy, McGlashan, & Shaikh, 2011). Free radicals is defined as any chemical species with one or more unpaired electrons that are capable of independent existence via occupying an atomic or molecular orbital by itself (Y. R. Li & Trush, 2016). ROS that containing unpaired electrons called free radicals such as superoxide (O_2·-) and hydroxyl radicals (HO·), and ROS that not contain unpaired electrons called non radicals molecules such as hydrogen peroxide (H_2O_2) which can be found produced in mitochondria electron transport chain and endoplasmic reticulum (Y. R. Li & Trush, 2016; Liu et al., 2016). According to Liu et al. (2016), higher level of ROS can induce apoptosis in cancer cells while mild increase of ROS level can enhance cell proliferation. It is shows that the chemopreventive properties by targeting high level of ROS in cancer cells that can cause the cell death while nearby normal cells are unaffected with the ROS toxicity (Liu et al., 2016).

2.3.2 Roles of ROS in Curcumin

According to Cao et al. (2015), the study state that curcumin has been shown to cause arrest of cell cycle and lead to apoptosis in different tumour cell types. In vitro study, curcumin can upregulate Cdk inhibitors and p53 while downregulate Cyclin D1, Cdk-1, cdc2, NF-κB which are molecular target that affected and lead to cell cycle arrest and apoptosis (Montopoli, Ragazzi, Froldi, & Caparrotta, 2009). Treatment of curcumin cause the cell cycle arrest at G2/M phase that involve the regulator protein in the transition from G2 phase to M phase which are cyclin B1 and cyclin D1 (Cao et al., 2015; Lavin, Gatei, Chen, Kijas, & Kozlov, 2010). In gastric carcinoma AGS cells, the cell cycle was arrested at G2/M phase via reduction of cyclin D1 and promotion of cyclin B1 in dose-dependent manner when treated with curcumin (Cao et al., 2015). In a study conducted by Y. Zhu and S. Bu (2017), curcumin has the synergetic effect if combined with gemcitabine that will lead to total cell cycle arrest at S phase and G2/M phase. Curcumin possesses pro-oxidant, antioxidant and chemoprotective properties which can selectively target malignant cells (Liu et al., 2016). In the same study stated that high ROS levels can induce apoptosis of cancer cells while low ROS levels can promote cell proliferation. According to Atsumi, Tonosaki and Fujisawa (2006), curcumin is pro-oxidant as it can produce ROS to cause cancer cell death. Treatment of curcumin on HepG2 cause the increase level of ROS especially hydroxyl radicals (HO·) after confirmed with catalase (CAT) and H_2O_2 – scavenging enzyme and dihydroethidium (DHE) (Liu et al., 2016). In cells, Glutathione (GSH) is low-molecular weight thiol that most abundant and responsible in maintaining redox buffering system (Liu et al., 2016). Curcumin can also scavenge the ROS by triggering the enzymes in phase II metabolism such as Glutathione S Transferase (GST) to neutralise chemical carcinogen-derived ROS as its antioxidant properties (Iqbal et al.,

2003). The ratios of GSH/GSSG are used to estimate the redox buffering system in cells and it shows the sudden decrease of GSH/GSSC ratio after treated with curcumin (Liu et al., 2016). It indicates that curcumin enhance the ROS level and cause redox buffering system in cells to collapse. Increase of ROS level will cause the changes in mitochondrial membrane potential by induce lipid peroxidation that lead to leakage of cytochrome C and activate the intracellular pathway of apoptosis (Liu et al., 2016). According to Martin-Cordero et al., (2012), cancer cells that are continuously under oxidative stress are more vulnerable to the cytotoxicity caused by ROS compared to normal cells. Curcumin promote apoptosis by induce ROS generation, disturb mitochondria membrane potential and reduce glutathione in MDA-MB-231 cell line (Tomeh et al., 2019).

2.4 Liver Cancer

2.4.1 Epidemiology of Hepatocellular Carcinoma

Liver cancer is one of second leading cancer which frequent in men as second most common cancer while in women as sixth common cancer that is lethal (Hu et al., 2015). Hepatocellular Carcinoma (HCC) is one of the most threatening type of cancer under Liver Cancer which eventually has cause 781, 631 mortality cases in 2018 (Bray et al., 2018). Liver cancer contribute 841,080 new cases and 781,631 deaths from 18,078,957 cancer incidence and 9,555,027 mortality in the world (GLOBOCAN, 2018). In the same study, in Malaysia, liver cancer has been reported about 4.4% of incidences from 43,837 new cases of cancer and 7.3% of mortality from 26,395 deaths due to cancer.

According to Hu et al. (2015), HCC has the characteristic of high metastasise activity among other liver cancer such as Liver Angiosarcoma, Cholangiocarcinoma and Hepatoblastoma that cause HCC to have poor prognosis. As stated in a study by Yousef, Alsaab, Sau, & Iyer (2018), all of liver cancer types shows that HCC has contribute about 75-90% aside from other type of liver cancers. There are few types of primary liver cancer that starts at the liver region such as hepatocellular carcinoma (HCC) (75%-85%), intrahepatic cholangiocarcinoma (10%-15%) and other types (5%-10%) (Bray et al., 2018). Other types of liver cancer including fibrolamellar HCC, angiosarcoma and hepatoblastoma (Bray et al., 2018).

2.4.2 Risk Factors and Signs and Symptoms of Hepatocellular Carcinoma

The risk factor of HCC are iron overload, obesity, alcoholic and non-alcoholic cirrhosis, type 2 diabetes mellitus and ingestion of dietary carcinogens (Kuo & Lin, 2003; Vera-Ramirez et al., 2013; Yousef et al., 2018). According to Ni et al. (2019), HCC are frequently cause by the infection of Hepatitis B and Hepatitis C due to virus. According to a study conducted by (Mejia & Pasko, 2020), it stated that Hepatitis C Virus (HCV) is the leading cause of liver cirrhosis and hepatocellular carcinoma development. In a study by Qiu et al. (2014), it stated that HepG2 is liver cancer with negative Hepatitis B Virus (HBV) infection while Hep 3B is liver cancer associate with positive HBV infection that cause it to be tumourigenic. Both of the cells line was taken from different person that shows different in gene expressions, drug effects and drug responses in signalling pathway (Qiu et al., 2014). The development of cirrhosis and liver cancer increase by 20% of HCV, 10-25% of HBV and 22% of alcohol consumption (Balogh et al., 2016).

In a study conducted by (C. Li et al., 2012), the signs and symptoms of hepatocellular carcinoma including fever and chills, tenderness in the right-upper-quadrant abdomen and abdominal discomfort, jaundice, nausea, vomiting, and diarrhoea. In other study by (Mejia & Pasko, 2020) stated that signs and symptoms including anorexia, weight loss, palpable mass on the abdomen, variceal bleeding, coagulopathy and encephalopathy. In the same study suggested that HCC can cause the rupture of intratumor and capsular that cause bleeding and manifest the abdominal pain and haemorrhagic shock. It is also been found that liver cancer patient will have the presence of hepatomegaly and ascites (Al-Sarraf, Go, Kithier, & Vaitkevicius, 1974).

2.4.3 Staging System and Treatment of Hepatocellular Carcinoma

In liver cancer prognosis, there are few staging systems has been used including Barcelona Clinic Liver Cancer (BCLC), Tumour, Node, and Metastasis (TNM), "GRoupe d'Etude et de Traitement du Carcinoma Hépatocellulaire" (GRETCH), Japan Integrated Staging (JIS), Cancer of Liver Italian Program (CLIP) and Chinese University Prognostic Index (CUPI) (Messaoudi et al., 2019). New staging system being used commonly are BCLC and Hong Kong Liver Cancer (HKLC) that provide specific treatment on each stages (Wallace et al., 2018). In early detection and development of HCC, BCLC is known as the best staging system that has been validated by cohort studies (Pons, Varela, & Llovet, 2005). According to Selçuk (2017), it stated that components in BCLC staging system based on the number, volume and invasiveness of tumour, performance status, Child-Pugh score, spread to extrahepatic region and Okuda stage. BCLC classification based on the liver function and tumour characteristics including size and spreading of tumour, number of hepatic

nodule, functional index, Child-Pugh score, pressure of portal vein and level of bilirubin (Messaoudi et al., 2019). There are 5 stages of liver cancer in BCLC staging system which are Stage 0, Stage A, Stage B, Stage C and Stage D depending on the criteria and characteristics mention above. According to Bruix, Reig and Sherman (2016), it is suggested that Stage 0 and Stage A in BCLC indicate the patient with solitary lesion and nodule less than 3cm with functional liver. In Stage 0 and A, the curative treatment are resections, liver transplantation and ablation with the survival rates from 60% to 80% in 5 years (Selçuk, 2017). Treatments for HCC are by surgery and liver transplantation but it is limited to 20% of patients due to several factors such as multifocality and size of tumour which effective at early stage but it's usually diagnose at advance stage of HCC (Balogh et al., 2016). In Stage B, patient with intermediate HCC presented with large and multifocal tumour in absence of invasion to vascular and region beyond the liver (Bruix et al., 2016). The treatment suggested is locoregional therapies and transarterial chemoembolization (TACE) (Pons et al., 2005). In Stage C with advance HCC that macrovascular invasion and metastasise beyond the liver can be treated with sorafenib therapies (Selçuk, 2017). It is tyrosine kinase inhibitors that can prolong the survival of HCC patient (Bruix et al., 2016). In Stage D or end stage liver cancer usually patient receive supportive and symptomatic care (Selçuk, 2017).

Treatment for HCC patient are chemotherapy and liver transplantation but a few of new drugs were studied and produced due to the difficulties in finding suitable donor and common drug show non-specific activity when administered (Anirudhan & Binusreejayan, 2016). According to a study conducted by Y. J. Zhu, Zheng, Wang and Chen (2017), sorafenib is a FDA-approved drug used to treat renal cell carcinoma and advanced HCC that result in the extension of survival period of patient for 3-5 months.

In a study conducted by Bruix, Reig and Sherman (2016), sorafenib is a known multikinase inhibitors that promote apoptosis, inhibits cancer cell proliferation and angiogenesis of tumour that result in reducing 30% risk of death in advanced HCC. It can inhibits NF-κB and promote STAT-3-mudolated resistance that cause the increase of anticancer drugs efficacy (Chen et al., 2017). In the same study, it is suggested that sorafenib can become resistance in the treatment of HCC due to long exposure in the body as the cancer progress. Inhibition of Raf-1, B-Raf and Kinases in various signalling pathways lead to the inhibition of tumour cell proliferation (Y. J. Zhu et al., 2017). It can target specific kinases such as hepatocyte factor receptor (c-Kit), platelet-derived growth factor receptor (PDGFR-β), Fms-like tyrosine kinase (FLT-3), vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-3 to inhibit the progression or development of angiogenesis in HCC (Y. J. Zhu et al., 2017).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 BHMC and Curcumin

2,6-bis-(4-hydroxyl-3-methoxybenzylidine)cyclohexanone (BHMC) was kindly supply by Associate Professor Dr. Tham Chau Ling from the Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM). BHMC was synthesised chemically from curcumin at the Natural Products Laboratory, Institute of Bioscience, UPM (Tham et al., 2010). Curcumin was purchased from Nacalai Tesque. Dimethyl Sulfoxide (DMSO) from ATCC was used to dissolve both compound to final stock solution of 50mM and diluted according to appropriate concentration used for assays. Due to DMSO toxicity toward cells, final concentration of DMSO was maintained below 0.1% in all assay.

3.1.2 Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM), Penicillin Streptomycin Mixed Solution, 0.25% Trypsin-EDTA with Phenol Red, Dimethyl Sulfoxide (DMSO), Trypan Blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Nacalai Tesque (Kyoto, Japan). Fetal Bovine Serum (FBS) was purchased from Tico Europe (Amstelveen, Netherlands). Phosphate Buffer Saline (PBS) tablets and Absolute Ethanol denatured (99.6%) were purchased from Fisher Scientific (Massachusetts, USA), 2',7'-dichlorofluorescence diacetate (DCFDA) was purchased from ChemCruz (Huissen, Netherlands), Hydrogen Peroxide (H_2O_2) was purchased from HmbG Chemicals (Kuala Lumpur, Malaysia).

3.1.3 Cell Lines

Human hepatocellular carcinoma, HepG2 and normal human fibroblast, Hs27 were purchased from American Type Culture Collection (ATCC) (Virginia, USA). HepG2 was obtained from 15 years adolescent male Caucasian while Hs27 was obtained from skin of newborn and both cell lines are adherent cells. According to ATCC, HepG2 has the sub-cultivation ratio of 1:4 to 1:6 while for Hs27 has the sub-cultivation ration of 1:2 to 1:4 with medium renewal of both cell lines are required twice per week. HepG2 has doubling time of 48 hours while Hs27 has doubling time of 36 hours. The HepG2 and Hs27 cell lines were cultured in DMEM that contains 10% FBS and 1% Penicillin Streptomycin. Both cell lines were incubated at 37˚C in a 5% CO_2 incubator.

3.1.4 Instruments

The instrument used in cell culture including micropipettes (0.5 to 10µL, 10 to 20µL, 100 to 200µL and 100 to 1000µL) and multichannel pipette (30 to 300µL) from Eppendorf Research Plus (Hamburg, Germany), Pipet-aid from Thermo Scientific (Massachusetts, USA), 5ml, 10ml and 25ml serological pipette from Lab Serv (Longford, Ireland), 96-well flat bottom clear plate, 96-well flat bottom black plate, 6-well plate, 15ml and 50ml centrifuge tubes from Fisher Scientific (Massachusetts, USA), cryovial, 1.5ml and 2ml microcentrifuge from Nest (Jiangsu, China), microplate reader, Infinite F50 and fluorescence microplate reader, Infinite 200 Pro from Tecan (Männedorf, Switzerland), Biohazard Safety Cabinet (BSC) class II from ESCO (Changi, Singapore), water bath, WNB14 from Memmert (Schwabach,

Germany), centrifuge Rotofix 32 from Jora-aki Technology Sdn. Bhd (Cheras, Selangor), CO_2 incubator, Galaxy 170R from Eppendorf AG (Petaling Jaya, Selangor), biomedical freezer (-30˚C), MDF-U333 and Ultra-low temperature freezer, MDF-U73V VIP Series from Sanyo (Osaka, Japan) and Olympus CK40 Inverted Phase Contrast Microscope (Tokyo, Japan). These instruments were provided by laboratories in Faculty of Medicine and Health Sciences, UPM.

3.2 Cell Culture

3.2.1 Thawing Frozen Cell

Frozen cells in cryovial tube was thawed using water bath at the temperature of 37˚C for a minute. Then, 70% ethanol was used to wipe the cryovial tube before transferred into BSC class II. The thawed cells were then transferred into 15mL centrifuge tube that containing pre-warmed complete growth media. The tube containing cell suspension was centrifuged at 15000 rpm for 5 minutes. Then, the supernatant was discarded and the pellet was resuspended with 3mL complete growth media in centrifuge tube. The cells were transferred into T25 flask and placed into 5% CO_2 incubator at 37˚C.

3.2.2 Sub-culturing of Adherent Cells

The cells were split into new flask when the confluency reach 80-90%. Old media was removed, and the cells were washed thrice with 2mL of PBS. Cells were harvested by adding 1mL of trypsin/EDTA into the flask and incubated for 5-7 minutes at 37˚C. The side of the flask was gently tapped to aid in the cell's detachment. The cells were resuspended with 2mL complete growth media and transferred into 15mL

centrifuge tube for centrifugation purposes at 15,000 rpm for 5 minutes. Supernatant was discarded and the pellet was resuspended in 1mL complete growth media. The cells were transferred with appropriate ratio into T25 culture flask containing complete growth media and incubated at 37˚C 5% CO_2.

3.2.3 Cell Counting

The cells were split into new flask when the confluency reach 80-90% by discarded the old media from the culture flask. The cells were washed thrice with 2mL of PBS using serological pipette. The monolayer cells were trypsinised by adding 1mL of trypsin/EDTA and incubated for 5-7 minutes in CO_2 incubator at 37˚C. Cells were examined under fluorescence microscope to ensure the cell's detachment. The cells were resuspended in 2mL complete growth media and transferred into 15mL centrifuge tube and centrifuged at 15000 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended with 1mL complete growth media. Cell counting was done using haemocytometer. Approximately, 10µL of cell suspension mixed with trypan blue filled the chamber of haemocytometer and observed under the inverted microscope. The number of viable cells was calculated to get the average cell count from 4 quadrants, multiplying by 2 to the power of dilution factor, multiplying by 10000 and get the final value of counted cells in the number of viable cells/mL of cell suspension.

3.2.4 Cell Seeding

The cell count of cell suspension was obtained using haemocytometer. The amount of cell suspension and complete growth media that needed for new density of

cell suspension was calculated using $M_1V_1=M_2V_2$ formula. The new density of cell suspension was transferred into sterile petri dish. Approximately, 100µL and 2ml of new density cell suspension was transferred into 96-well and 6-well plates respectively. The plates were incubated in 5% CO_2 incubator at 37˚C overnight before the treatment for the assay.

3.2.5 Preparation of stock and working solutions of curcumin, BHMC, hydrogen peroxide and DMSO

BHMC powder was dissolved in DMSO to produce 15mM and 50mM BHMC as stock solution. To prepare working solution, stock solution was diluted using complete growth media. The working concentrations for MTT assay were 50µM, 25µM, 12.5µM, 6.25µM, 3.125µM, 1.563µM and 0.78µM. Using $M_1V_1=M_2V_2$ formulation, 50mM BHMC was diluted in complete growth media to final concentration of 50µM BHMC and the subsequent concentration of working solutions were prepared via serial dilution. From MTT assay, the concentration that has been chosen for DCFDA assay were 5µM, 10µM and 15µM.

Similarly, curcumin powder was dissolved in DMSO to produce 50mM curcumin as stock solution. To prepare the working solution, stock solution was further diluted using growth media to required concentrations for MTT assay.

Meanwhile, hydrogen peroxide (H_2O_2) was dissolved in PBS to produce 0.01M H_2O_2 as stock solution. Using $M_1V_1=M_2V_2$ formulation, 0.01M H_2O_2 was diluted in complete growth media to final concentration of working solution 100µM curcumin that act as positive control in DCFDA assay. DMSO acts as negative control and the final concentration was maintained below 0.1% in all assay.

3.3 Growth Inhibition Assay - MTT Assay

MTT assay was used to determine the cytotoxicity activity of BHMC and curcumin. It was performed by using method of Liu et al. (2016) with some modification. MTT or 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide was metabolised by mitochondria dehydrogenase that is specifically present in viable cells. The cells were harvested with trypsin-EDTA after reach 70-80% of confluency. Initially, 100µl of HepG2 and Hs27 cells suspension of 4 X 10^5 (cells/mL) were seeded in triplicate of 96-well plate. The cells were incubated overnight at 37ºC with 5% CO_2 and treated with various concentrations of curcumin and BHMC (0, 0.78, 1.563, 3.125, 6.25, 12.5, 25 and 50µM). Cisplatin and DMSO acts as positive control and negative control respectively. The treated cells were then incubated again at 37ºC with humidifies 5% CO_2 for 24 hours. After incubation time, 20µl of 5mg/ml of MTT Solution that was dissolved in 1x PBS then added into each well and the plate was incubated at 37ºC for 4 hours. After 4 hours, microplate reader was used to measure the insoluble formazan precipitate in the plate at absorbance of 570nm with reference wavelength of 630nm. A graph of cell viability (%) against compound concentration was plotted to determine IC_{50} from the dose-dependent graph. The percentage of cell viability was calculated by using formula below:

$$Cell\ Viability\ (\%) = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ negative\ control\ cells}\ X\ 100\%$$

3.4 DCFDA Assay

The intracellular ROS was measured using cellular ROS detection assay kit with fluorescent probes (2',7'-dichlorofluorescence diacetate) (DCFDA). DCFDA

assay was performed by using method recommended by Alexander et al. (2019) with some modification. The cells were observed under inverted microscope to ensure the confluency has reach 70-80%. Firstly, 100µL of HepG2 and Hs27 were seeded at cell density of 4 X 10^5 cells/mL into required well in 96-well black plate. The plate was incubated at 37˚C overnight in 5% CO_2 incubator for the attachment of cells to the well. Working solution of BHMC, curcumin, DMEM, H_2O_2 and 0.1% DMSO were prepared on the sterile petri dish. The old media was removed, and the cells attached in the well were treated with 200µL of BHMC (5µM, 10µM and 15µM), curcumin (25µM and 50µM), 100µM H_2O_2 and 0.1% DMSO. The well with DCFDA solution only acts as blank, 0.1% DMSO acts as negative control and H_2O_2 acts as positive control. The plate was wrapped with aluminium foil then incubated at 37˚C in 5%CO_2 incubator for desired incubation timeframe (18 and 24 hours). The DCFDA solution was dissolved in DMSO to produce 25mM DCFDA as stock solution. Then, 25µM DCFDA solution was prepared by diluting 25mM DCFDA in PBS. After incubation time, the plate was washed once with 100µL of PBS and 100µL of 25µM DCFDA solution were added into each well of 96-well black plate. The plate was wrapped with aluminium foil then incubated for an hour at 37˚C in 5%CO_2 incubator. After incubation time, the plate was read on fluorescent microplate reader with RFU (excitation/emission) at 495nm/529nm. A graph of RFU (495nm/529nm) against compound concentration was plotted.

CHAPTER FOUR

RESULTS

4.1 Cytotoxic effect of BHMC and Curcumin on HepG2 and Hs27 cell lines by using MTT Assay

The cytotoxic effect of BHMC was compared with its parental compound, curcumin on HepG2 and Hs27 cell lines by MTT Assay. The cell viability of both cell lines was shown in figure 1 to 2 after treated with BHMC and Curcumin at several concentrations (0.78, 1.563, 3.125, 6.25, 12.5, 25 and 50µM) for 24 hours. The data obtained for both compounds was used to determine the IC_{50} values (Table 1). Data are presented as mean ± S.E.M. and represent of three independent experiments. From Table 1, data obtained shows there is significant difference of IC_{50} value of BHMC compared to curcumin in each cell line. There is also significant difference of IC_{50} value of BHMC in HepG2 cell line compared to Hs27 cell line. In comparison to curcumin, BHMC was found more toxic towards HepG2 and Hs27 cell lines with lower IC_{50} value at incubation time of 24 hours. It is shown that BHMC and curcumin selectively toxic toward HepG2 cell line compared to Hs27 cell line with lower IC_{50} value at incubation time of 24 hours. The IC_{50} value of BHMC were approximately 3 to 5 folds lower than curcumin on HepG2 cell line. Hence, BHMC was found to be more potent and selectively cytotoxic toward HepG2 compared to curcumin.

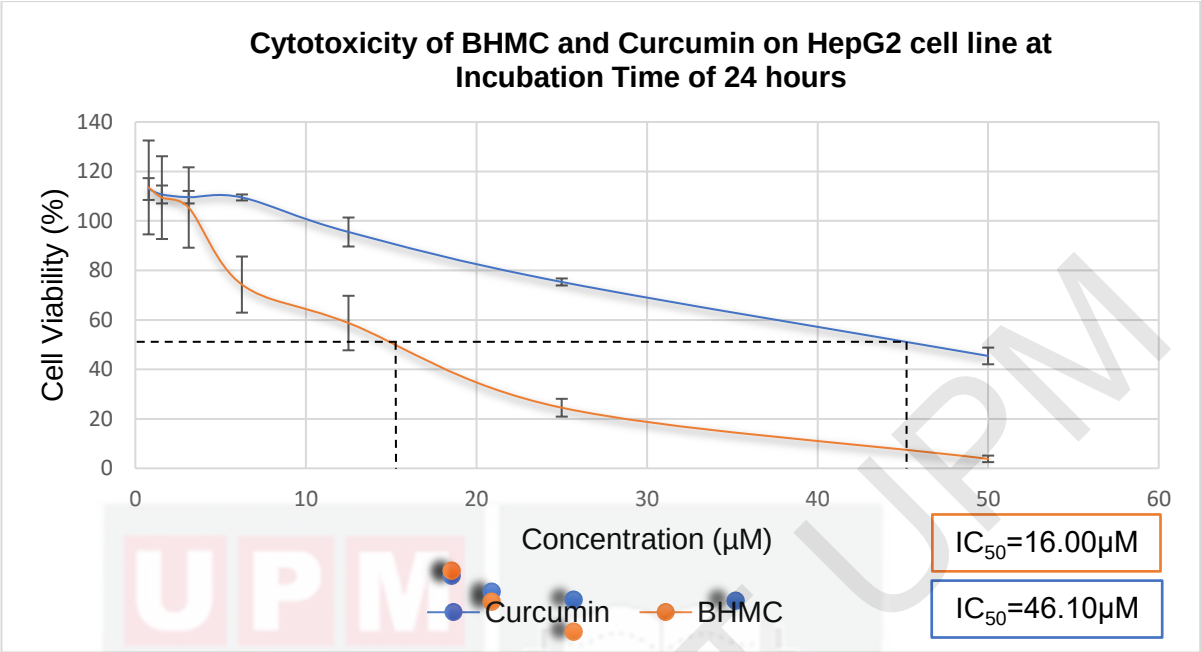

Figure 1: Percentage of cell viability of HepG2 cell line treated with BHMC and Curcumin at incubation time of 24 hours using MTT Assay. Data are presented as mean ± S.E.M. and represent of three independent experiments. The IC_{50} of BHMC and Curcumin in Hs27 cell line are 16µM and 46.1µM respectively as determined by using extrapolation of the curve.

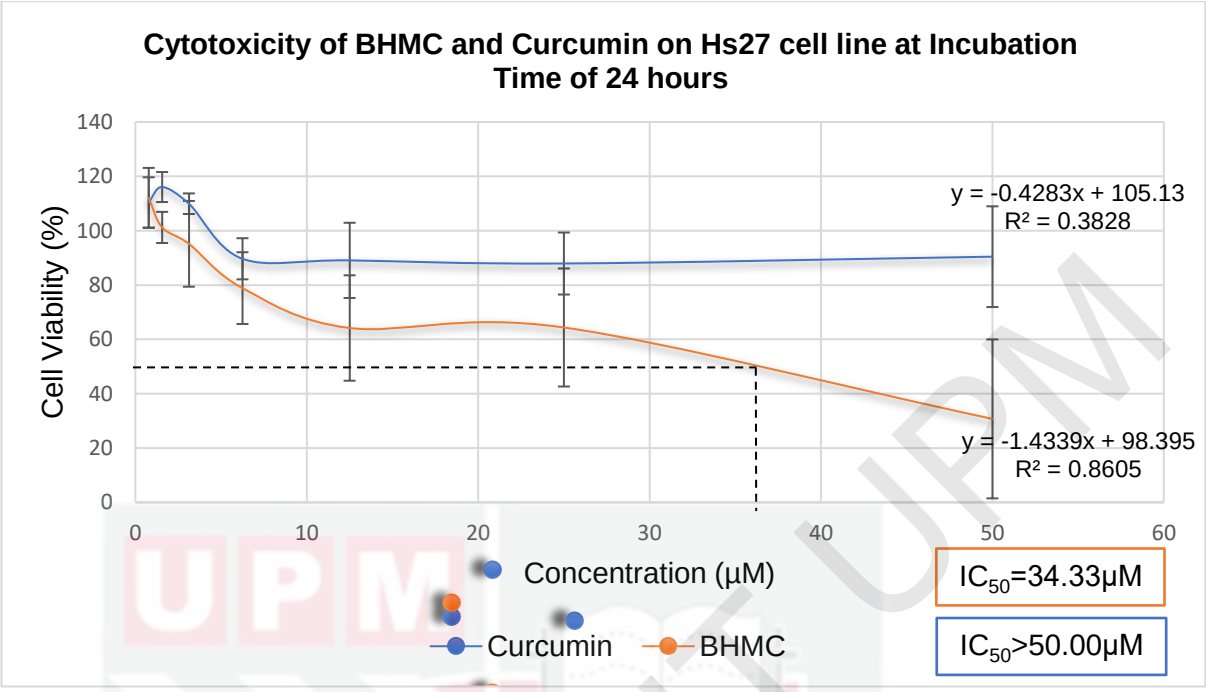

Figure 2: Percentage of cell viability of Hs27 cell line treated with BHMC and Curcumin at incubation time of 24 hours using by MTT Assay. Data are presented as mean ± S.E.M. and represent of three independent experiments. The IC_{50} of BHMC and Curcumin in Hs27 cell line are 34.33µM and more than 50µM respectively as determined by using extrapolation of the curve.

Table 1: Cytotoxicity of BHMC and Curcumin on HepG2 and Hs27 cell lines reflected by IC_{50} values at incubation time of 24 hours by using MTT Assay.

Incubation Time (Hours)	IC_{50} ± S.E.M. (µM)			
	HepG2		Hs27	
	BHMC	Curcumin	BHMC	Curcumin
24	$16.00 \pm 2.22^{a, c}$	$46.10 \pm 0.26^{b, c}$	$38.30 \pm 2.83^{a, d}$	$>50 \pm 0.00^{b, d}$

Data are presented as mean ± S.E.M. and represent of three independent experiments. [a,b]Different superscript letters indicate significant differences ($p<0.05$) compared within different compounds from same cell lines at incubation time of 24 hours by using independent-samples T test. [c,d]Different superscript letters indicate significant differences ($p<0.05$) compared within same compounds from different cell lines at incubation time of 24 hours by using independent-samples T test.

4.2 Effect of BHMC and Curcumin on ROS Level in HepG2 Cell Lines

The effect of BHMC on ROS level in HepG2 cell line was determined by using DCFDA Assay. Relative Fluorescent Unit (RFU) represents the fluorescent released from DCF due to oxidation of DCFDA detected by fluorescent microplate reader with excitation/emission at 495nm/529nm that reflect the ROS generation level in HepG2 cell line. The Relative Fluorescent Unit (RFU) detected in HepG2 after treated with BHMC and curcumin on different concentration (5, 10 and 15µM) and (25 and 50µM) respectively with incubation times of 18 and 24 hours as shown in Figure 3 and 4. Data are presented as mean ± S.E.M. and represent of three independent experiments. Cells treated with 0.1% DMSO and 100µM Hydrogen Peroxide acts as negative and positive controls respectively. Data obtained shows that BHMC acts as antioxidant properties followed its parental compound, curcumin. The finding shows that accumulation of ROS level acts in time dependent manner after treated with both compounds. Data obtained also shows a significant decrease of relative fluorescence units was detected in HepG2 cell line incubated with 50µM of curcumin. Hence, it is suggested that BHMC triggered cell death via ROS pathways.

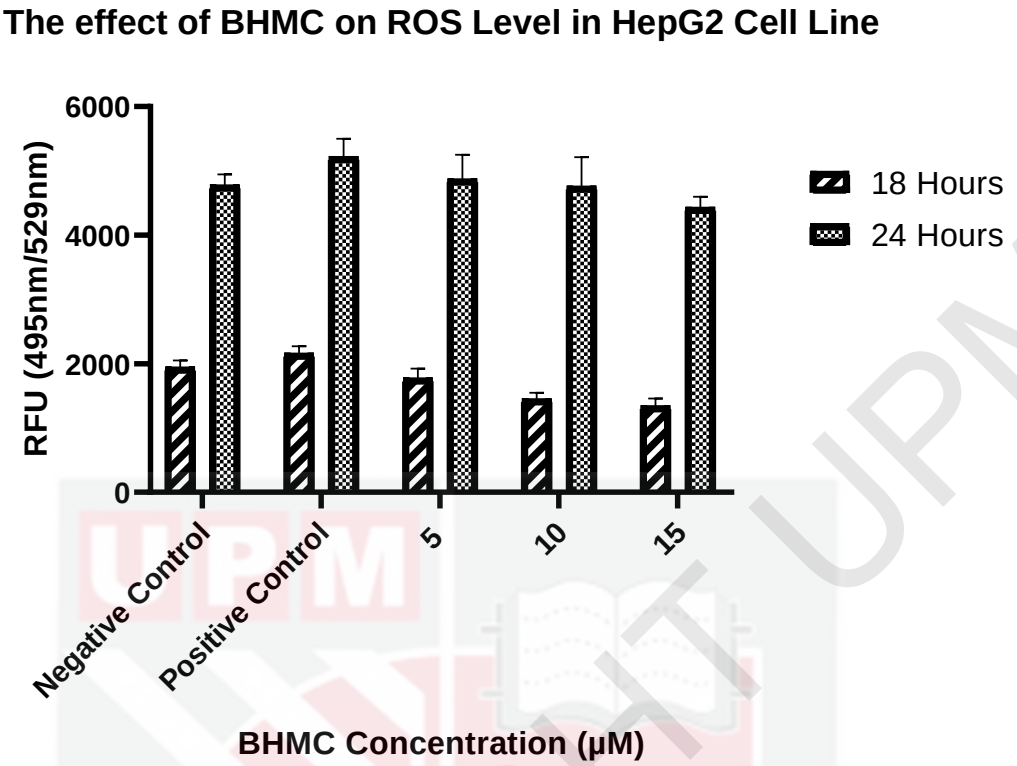

Figure 3: The effect of BHMC on ROS level in HepG2 cell line was determined by using DCFDA Assay. Data are presented as mean ± S.E.M. and represent of three independent experiments.

The effect of Curcumin on ROS Level in HepG2 Cell Line

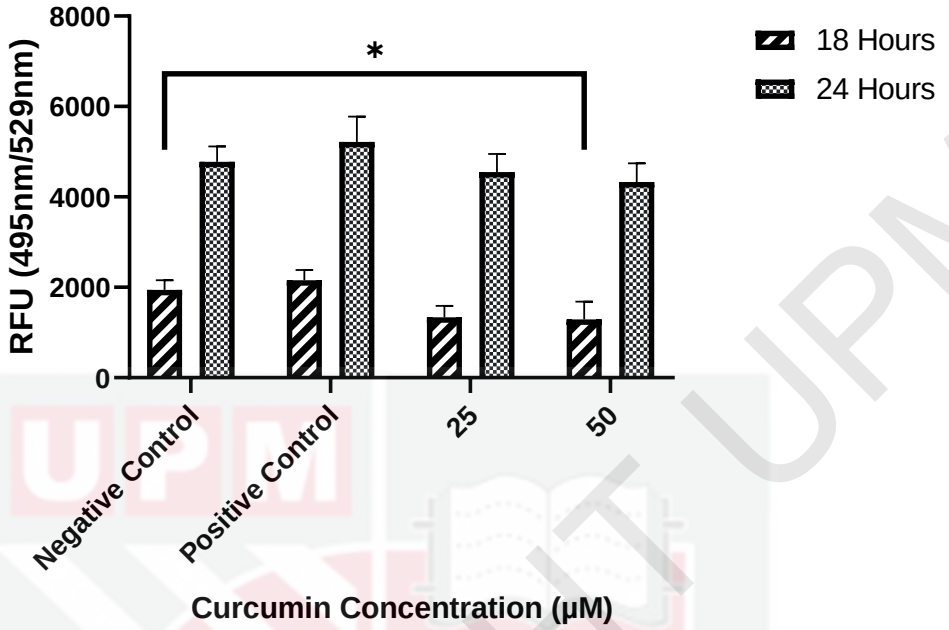

Figure 4: The effect of BHMC on ROS level in HepG2 cell line was determined by using DCFDA Assay. Data are presented as mean ± S.E.M. and represent of three independent experiments. *Statistically significant differences are indicated ($p < 0.05$) compared with the negative control group for 18 hours using One-way ANOVA followed by Dunnett's Multiple Comparison.

CHAPTER FIVE

DISCUSSION

Curcumin is one of compounds that possess a variety of pharmacological properties including anti-inflammation, anti-cancer and anti-nociceptive activities in *in vitro* and *in vivo* studies. However, preclinical and clinical studies shows the limitation of curcumin which is poor bioavailability that cause by the lack of hydroxyl group, hydrophobicity and unstable β-diketone moiety structure of curcumin (Tomeh et al., 2019). Numerous approach has been done to enhance therapeutic effects of curcumin by improving its bioavailability such as nanoformulations, phospholipid complexes, combination with adjuvants and structural modification (Gupta et al., 2013). Hence, several of analogues were produced, one of the analogues is known as 2,6-bis-(4-hydroxyl-3-methoxybenzylidine)cyclohexanone, BHMC was synthesised by eliminating the unstable β-diketone moiety and modifying it into bis enone system (double bond) while preserving the hydroxyl group (Tham et al., 2010).

In previous study, BHMC shows to have anti-inflammatory activity by suppressing the gene that code for iNOS expression and inhibits secretion of MCP-1 and IL-10. It's also exhibits its anti-inflammatory activity via inhibition of p38 activity in MAP kinase signalling pathway that reduce the mortality of sepsis-induced mice (Tham et al., 2011). BHMC has anti-proliferation activity similar to its parental compound by delayed the tumour development in 4T1 breast cancer cell line as lower number of mitotic cells was observed in tumour (Razak et al., 2017). BHMC also shows significant suppression of MMP-9 expression compared to curcumin that shows its anti-metastasise activity. In MDA-MB-231 cell line, number of cell forming invadopodia was significantly reduce upon the treatment of BHMC that result in decrease percentage of invasion and migration of cancer cell (Harun et al., 2018). The

inhibition of invadopodia formation due to the suppression of MT1-MMP, β-PIX and MMP-9 expression which is invasion-related proteins as BHMC interrupt the cytoskeleton. BHMC exerts anti-cancer properties in same mechanism as its parental compound, curcumin as stated in studies above.

In this study, the cytotoxic effect of BHMC and curcumin were compared on HepG2 and Hs27 cell lines. BHMC exhibits its cytotoxic effect against curcumin at lower IC_{50} value in HepG2 cell line. In MDA-MB-231 breast cancer cell line, BHMC is 3-4 times more active than curcumin (Zamrus et al., 2018). The results show that BHMC exerts its cytotoxicity at IC_{50} value of 16µM while curcumin at IC_{50} value of 46.1µM in HepG2 cell line at incubation time of 24 hours. In Hs27 cell line, it's shows that IC_{50} value of BHMC at 34.33µM while it is suggested that IC_{50} value of curcumin more than 50µM. It's shows there is significant difference of IC_{50} value of BHMC compared to curcumin in each cell line. Thus, BHMC exerts 3 times more toxic toward HepG2 cell line as it is more active than curcumin. There is also significant difference of IC_{50} value of BHMC in HepG2 cell line compared to Hs27 cell line. BHMC exerts higher IC50 value at 34.33µM in Hs27 cell line while IC50 value in HepG2 cell line is 16µM. In this study, BHMC shows to be more cytotoxic compared to curcumin in HepG2 cell lines. This is due the structural modification by removing the unstable β-diketone of curcumin into more stable α, β-unsaturated bis-enone system that is crucial to improve its biological effect including anti-cancer as in bis-chalcones and chalcones (Razak et al., 2017). The presence of β-diketone moiety and active methylene group cause rapid metabolism of curcumin by aldo-ketoreductase in liver that result in poor bioavailability of curcumin (K. H. Lee et al., 2012). The cytotoxic effect of both compounds act in dose-dependent manner. Thus, it is suggested that BHMC has greater cytotoxicity than curcumin and selectively cytotoxic toward HepG2 cell line.

In previous study, it's shows that BHMC and curcumin treatment in HepG2 and 3T3 cell lines act in time-dependent manner which exert their cytotoxicity with lower IC_{50} values at incubation time of 72 hours compared to 24 and 48 hours. Hence, cytotoxic effect of BHMC and curcumin on HepG2 and Hs27 cell lines should be evaluated and verify if both compounds act in time-dependent manner in both cell lines.

Curcumin have antioxidant, pro-oxidant and chemotherapeutic properties that induce apoptosis through ROS pathways in cancer cells (Liu et al., 2016). Increase of ROS level can lead to two situations which are high level of ROS induce the cell death while mild level of ROS induces the cell proliferation. Curcumin exhibits pro-oxidant in leukemic cell (HL-60) by increase the free radical production and reduce the level of glutathione (Feng et al., 2017). Accumulation of ROS can trigger protein misfolding and Endoplasmic Reticulum stress-induced apoptosis. ROS can regulate several signalling pathways including IKK/NF-κB, MAPK/Erk1/2 and PI3K/Akt (Liou & Storz, 2010). One of curcumin analogue, L48H37 increase the ROS level, activate ER stress pathway and modulate p-STAT pathway that lead to the reduce growth of lung cancer.

Cancer cells have high level of ROS which is detrimental to the cells (Liou & Storz, 2010). Maintaining a reduction-oxidation (redox) balance by removing excessive ROS in cancer cells is crucial in the progression of the cancer and prevention of apoptosis as it can activate factors that associate with chemoresistance mechanism such as AP-1, NF-κB and HIF-1α (Aggarwal et al., 2019). There are two major antioxidant system regulate to maintain the redox balance in cells which are thioredoxin and glutathione systems (Dai et al., 2015). In normal cell, antioxidant agent prevents the oxidative damage and apoptosis by induction of cytoprotective

enzyme or scavenging ROS. Antioxidant agents can prevent the activation of chemoresistance pathways mediated by oxidative stress in cancer cells.

Nfr2 is transcription factors that act as master regulator of cellular antioxidant response to the oxidative stress (Vomund, Schäfer, Parnham, Brüne, & Von Knethen, 2017). In this study, BHMC shows to follow its parental compound which exhibits antioxidant properties. In comparison to the negative control, curcumin shows significant reduction of ROS level at 50µM in HepG2 cell line. Curcumin activate the Nrf2 regulation in order to protect the normal cell by scavenging ROS (Negrette-Guzmán et al., 2017). In malignant cells, Nrf2 were highly upregulated that function as cellular antioxidant response to the stress. As cancer cells inherits the high ROS level, a mild transient oxidative stress exerted by antioxidant agents such as curcumin can contribute to the increase of ROS level above the threshold in malignant cells. It is suggested that increase level of ROS not necessarily cause the cell death but the molecules derived from low or high ROS level can lead to the cell death (Larasati, Yoneda-kato, Nakamae, & Yokoyama, 2018). In comparison to normal cells, cancer cells are more vulnerable towards cytotoxicity induced by ROS as it's consistently under oxidative stress (Martin-Cordero et al., 2012). It is suggested that reactive molecules that derived from ROS can cause the cell death such as reactive carbonyls and reactive aldehydes (Larasati et al., 2018). Reactive carbonyls such as alkenals is strong electrophiles that cause the Michael addition to the proteins, lipids and DNA as nucleophilic groups that arise due to oxidative stress via lipid peroxidation and glycoxidation (Ellis, 2007). Direct interaction of reactive aldehydes exerts cytotoxicity that cause membrane transporter, microtubules, signalling proteins, transcription factors and enzymes to loss their function. It's also induced apoptosis by underlying mechanism that activate caspases. As BHMC work in similar pathway with curcumin,

it is suggested that BHMC exhibit its cytotoxicity and cause the cell death in low or high level of ROS via ROS-derived reactive molecules.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

BHMC has greater cytotoxic effect towards HepG2 cell line compared to normal Hs27 cell line. BHM also shows to has antioxidant properties as its parental compound, curcumin. Low or high level of ROS can induce cell death due to several reactive molecules derived from ROS. Although exact mechanism is not fully elucidated, BHMC is suggested to trigger its cytotoxicity that induce apoptosis via ROS pathways and selectively cytotoxic toward liver cancer cells.

6.2 Recommendations

In this study, BHMC and curcumin demonstrated cytotoxic effect toward non-cancerous human fibroblast. However, there is necessarily to evaluate the cytotoxicity of both compounds on other normal cell lines such as normal human hepatocytes, THLE-3 and normal mouse hepatocytes, AML-12 (American Type Culture Collection (ATCC), 2016b, 2016a). It is recommended to determine the mode of cell death via morphology analysis with staining to see the presence of apoptosis hallmarks. Then, it is crucial to proceed with the Annexin V-FITC Staining to determine the effect of BHMC on mode of cell death in HepG2 cell line. Next, the effect of BHMC on glutathione metabolism should be determined using Total Glutathione Assay and Oxidised Glutathione Assay. Glutathione (GSH) is the most abundant thiol with low molecular weight in cells that play important roles in balancing intracellular redox status. To see the effect of BHMC can cause imbalance of cellular redox homeostasis, the ratio of GSH and Oxidised Glutathione (GSSG) can be measured. Lastly, the

underlying mechanism of BHMC triggering apoptosis via intracellular ROS pathways can be determined by evaluating the expression of proteins involved in ROS pathways via ELISA or western blot.

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APPENDICES

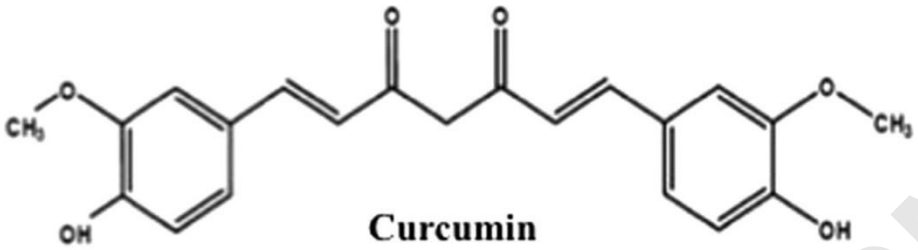

Figure 5: Chemical structure of curcumin

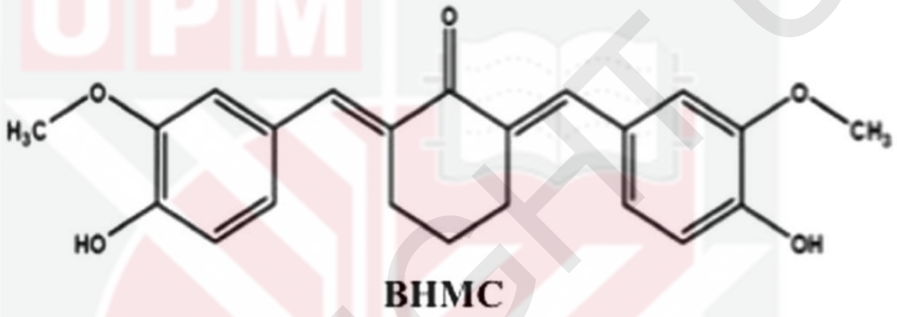

Figure 6: Chemical structure of 2,6-bis-4-(hydroxyl-3-methoxybenzilidine)-cyclohexanone, BHMC