UNIVERSITI PUTRA MALAYSIA

CYTOTOXIC EFFECT OF BHMC ON HEPG2 CELL LINES VIA TRYPAN BLUE EXCLUSION ASSAY

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ABSTRACT

Cytotoxic Effect of BHMC on HepG2 Cell Lines via Trypan Blue Exclusion Assay

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Introduction: Hepatocellular carcinoma (HCC) is one of the primary liver cancers and a leading cause of death due to cancer worldwide. It is often associated with risk factors such as viruses and excessive alcohol consumption. Several bioactive compounds have been explored to target a possible therapeutic approach in cancer treatment and one of it is curcumin. Curcumin, a bioactive compound derived from the rhizomes part of *Curcuma longa* (turmeric) is known to possess various pharmacological properties in cancer treatment including anti- proliferative, anti-inflammatory and chemotherapeutic. It has been proven that curcumin exhibit anti-cancer effects by inducing cell death and inhibit cell proliferation. Apoptosis is induced by curcumin through exerting direct cytotoxic effect that eventually leads to reduction in cell viability. However, due to its low bioavailability, 2, 6-bis-(4-hydroxyl-3-methoxybenzylidine) cyclohexanone (BHMC), an analogue of curcumin is synthesised to enhance the anti-cancer properties by removing unstable β-diketone moiety from the structure and modify it into conjugated double bond. BHMC is believed to be more selective in terms of cytotoxicity. **Objective:** This study aims to determine and compare the cytotoxic effect of BHMC and curcumin on the viability of HepG2 and Hs27 cells. **Methodology:** Percentage of HepG2 and Hs27 cells viability following treatment of BHMC and curcumin were determined by Trypan Blue Exclusion Assay. **Results:** Treatment with BHMC at 24 hours significantly reduced HepG2 cells at 30-60%. A remarkable reduction at 70-80% of cell populations were observed after 48 hours treatment with lower concentration compared to curcumin. On the contrary, BHMC only reduced 20-30% of Hs27 cells at 24 hours and 40-60% at 48 hours. Meanwhile, curcumin reduced 40-60% of HepG2 cells for 24 and 48 hours but only reduced 30% of Hs27 cells at similar concentration and timepoints. **Discussion:** Although BHMC and curcumin reduced the percentage of HepG2 cell viability in concentration and time dependent manner, anti-proliferative effect exerted by BHMC was greater compared to curcumin. BHMC also showed a cytotoxic selective effect on normal Hs27 cells especially at 48 hours with less cell death at lower concentration compared to its effect on HepG2 cells. The cyto-selective effect on Hs27 was observed to be similar with its parental compound, curcumin. **Conclusion:** BHMC mediate greater cytotoxic effect in HepG2 by reducing the cell viability and modulate higher percentage of cell death at lower concentration compared to curcumin. BHMC also possessed a cytotoxic selective effect towards Hs27 cells due to less cell death observed at similar concentrations.

Keywords: BHMC, curcumin, HepG2, Hs27

ABSTRAK

Kesan Sitotoksik BHMC Terhadap Garisan Sel HepG2 dan Hs27 Melalui Asai Trypan Blue Exclusion

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Pengenalan: Karsinoma hepatoselular (HCC) adalah antara kanser hati utama dan merupakan penyebab utama kematian akibat kanser seluruh dunia. Faktor-faktor risiko seperti virus dan penyalahgunaan akohol sering dikaitkan dengan HCC. Beberapa sebatian bioaktif telah diterokai untuk mensasarkan kemungkinan pendekatan terapi dalam rawatan kanser. Kurkumin, sebatian bioaktif berasal dari bahagian rizom *Curcuma longa* (kunyit) diketahui memiliki pelbagai sifat farmakologi dalam rawatan kanser termasuk anti- proliferatif, anti-radang dan kemoterapi. Ia telah terbukti bahawa kurkumin mempamerkan kesan anti-kanser dengan mendorong kematian sel dan menghalang percambahan sel. Apoptosis didorong oleh curcumin melalui kesan sitotoksik yang akhirnya membawa kepada pengurangan kebolehhidupan sel. Walau bagaimanapun, disebabkan oleh bioketersediaan yang rendah, 2, 6-bis-(4-hydroxyl-3-methoxybenzylidine) sikloheksanon (BHMC), analog kurkumin disintesis untuk meningkatkan sifat-sifat anti-kanser dengan mengeluarkan struktur yang tidak stabil iaitu moieti β-diketon dari struktur dan mengubahnya menjadi ikatan ganda dua terkonjugat. **Objektif:** Kajian ini bertujuan untuk menentukan dan membandingkan kesan sitotoksik kurkumin dan BHMC mengenai daya maju sel HepG2 dan Hs27. **Metodologi:** Peratusan daya maju sel HepG2 dan Hs27 berikutan rawatan curcumin dan BHMC ditentukan oleh ujian Pengecualian Trypan Blue. **Keputusan:** Rawatan dengan BHMC pada 24 jam mengurangkan sel HepG2 secara signifikan pada 30-60%. Pengurangan ketara pada 70-80% populasi sel juga diperhatikan pada 48 jam dengan kepekatan rawatan yang lebih rendah berbanding kurkumin. Sebaliknya, BHMC hanya mengurangkan 20-30% sel Hs27 pada 24 jam dan 40-60% pada 48 jam. Sementara itu, kurkumin mengurangkan 40-60% sel HepG2 selama 24 dan 48 jam tetapi hanya mengurangkan 30% sel Hs27 pada kepekatan dan titik waktu yang sama. **Perbincangan:** Walaupun BHMC dan kurkumin mengurangkan peratusan daya maju sel HepG2 pada kepekatan dan masa bersandar, kesan anti-proliferatif yang ditunjukkan oleh BHMC lebih besar berbanding kurkumin. BHMC juga menunjukkan kesan selektif sitotoksik pada sel normal Hs27 terutama pada 48 jam dengan kematian sel yang lebih sedikit pada kepekatan yang lebih rendah berbanding kesannya pada sel HepG2. Kesan sito-selektif pada Hs27 diperhatikan serupa dengan sebatian induknya, curcumin. **Kesimpulan:** BHMC menunjukkan kesan sitotoksik yang lebih besar dalam kedua-dua garisan sel dengan mengurangkan daya maju sel dan memodulasi peratusan kematian sel yang lebih tinggi pada kepekatan yang lebih rendah berbanding dengan kurkumin. BHMC juga mempunyai kesan sitotoksik memilih terhadap sel Hs27 kerana kurang kematian sel diperhatikan pada kepekatan yang sama.

Kata Kunci: BHMC, kurkumin, HepG2, Hs27

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AIDS	Acquired immunodeficiency syndrome which is an infection caused by Human Immunodeficiency Virus (HIV)
Akt	Ak strain transforming is a group of protein kinase which is important in cell proliferation and metabolism
Bcl-2	B-cell lymphoma 2 is a protein that regulate cell death
Bcl-xL	B-cell lymphoma-extra large is one of anti-apoptotic protein that is located in mitochondria
Bcl-xs	Gene product of Bcl-X gene which carries a pro-apoptotic function
BHMC	2,6-bis-4-(hydroxyl-3-methoxy-benzylidine) cyclohexanone is a derivative of curcumin
ß-catenin	Protein found in cells and tissues of the body and involved in the cell-cell adhesion process
CAM	Cell adhesion molecules which is located on the surface of the cells and involved in cell adhesion process
CCI	Chronic constriction injury
COX-2	Cyclooxygenase 2 are enzymes that are involved in the production of prostaglandins that causes inflammation
Cyclin D1	Protein that regulates the cell cycle
DMEM	Dulbecco's Modified Eagle Medium is used for the growth of

	mammalian cells
DMSO	Dimethyl sulfoxide is a chemical that is used as solvent
DR4	Death receptor 4 that is located on surface of cells which bind another proteins triggering apoptosis
DR5	Death receptor 5 that is located on surface of cells which bind another proteins triggering apoptosis
EMT	Epithelial-mesenchymal transition occurs in tumour cells causing increase in malignancy and invasiveness
ER	Estrogen receptor is a type of transcription factor that regulates transcription process of genes
Fas	A membrane protein of the death receptor that is involved in the apoptosis
G0	Resting phase in cell cycle
G2	A phase in cell cycle where cells undergone a rapid growth
GSH	Glutathione is an anti-oxidant produced in the body to neutralize free radicals
G6PD	G6PD deficiency is an inherited disorder characterized by deficient in enzyme glucose-6-phosphate dehydrogenase (G6PD)
HA	Hyaluronic acid which is naturally present on the connective tissue
HBV	Hepatitis B Virus which caused Hepatitis B liver infections

HCV	Hepatitis C Virus which caused Hepatitis C liver infections
HCC	Hepatocellular carcinoma which is the common malignancy of liver cancer
HepG2	Human hepatocellular carcinoma cells
Hs27	Normal human fibroblast cells
HL-60	Human promyleocytic leukemia cells
ICC	Intrahepatic cholangiocarcinoma is the second most common malignancy of liver cancer
IC_{50}	Concentration of drug that reduced 50% the population
IL	Interleukins are group of cytokines that act as signal in immunes system
IKßα	Belongs to group of cellular proteins that involved in the inhibition of NF-κB signalling pathway
IKKß	Enzyme complex which is involved in the cellular response to inflammation
iNOS	Inducible nitric oxide synthase is a gene present on chromosome 17 and stimulate production of nitric oxide
JAK-STAT	Janus Kinase and Signal Transducer and Activator Transcription is a pathway which regulates embryonic development
JAK2	Janus Kinase 2 gene encodes for protein that regulates cell growth and proliferation

J5	Human hepatocellular carcinoma cells
LPS	Lipopolysaccharide which is a component of the bacterial cell membrane
MAPK	Mitogen-activated protein kinase is a pathway that integrate signals and produce response in cell proliferation and apoptosis
MCP-1	Monocyte chemoattractant protein-1 is the chemokines involved in the migration of monocytes
MCF-7	Human estrogen receptor positive breast cancer cell lines
MDA-MB-231	Human estrogen negative breast cancer cell lines
miRNA	A class of non-coding RNAs involved in the gene expression
MMP-9	Matrix metalloproteinase 9 which is highly expressed in inflammation and certain cancers
NAC	N-acetyl cysteine is an anti-oxidant involved in cancer prevention
NFκß	Protein involved in the transcription of DNA and regulation of genes
NO	Nitric oxide is a molecule produced by the body and cause vasodilation
PBS	Phosphate Buffered Saline used as buffer for washing steps
p53	A tumour suppressor gene
p38	Mitogen-activated protein kinase that produced pro-

	inflammatory cytokines
p65	A transcription factor involved in the nuclear transcription
PI3K	A group of protein kinase located on plasma membrane and involved in cellular functions
ROS	Reactive Oxygen Species is a reactive chemicals derived from the oxygen
RAW 264.7	Monocyte cell line
RFA	Radiofrequency ablation which is a minimally invasive techniques used in the treatment of cancer
STAT3	Member of STAT protein family that control cell growth and proliferation
SOD	Superoxide dismutase is an anti-oxidant that protects the body from oxidative stress
SI	Selectivity Index is an indicator used to determine the degree of selectivity of a compound
Smad2	Protein involved in the transcription process
TNBC	Triple negative breast cancer refers to cancer cells that do not have an estrogen or progesterone receptor
TGF-ß	Cytokines involved in the immune system
TGF-ß1	Cytokines that are highly expressed in various diseases
UV	Ultraviolet is a form of electromagnetic radiation

Wnt	Signalling pathway involve in gene transcription
VEGF	Protein that promotes formation of new blood vessels
3T3	Normal mouse fibroblast cells
4T1	Mice breast cancer cells

CHAPTER 1

INTRODUCTION

1.1 Background

Cancer refers to a broad range of diseases that may originate practically in any organ or tissue of the body when abnormal cells grow uncontrollably, invade the body's adjacent areas, and metastasized to other parts of the organs (World Health Organisation, 2021). Cancer can begin almost anywhere in the trillions of cells that comprise the human body. A quarter of cancer cases worldwide reported were caused by infections in developing countries. World Health Organisation (WHO) also implied that global cancer rates are expected to rise by 2020 (Eaton, 2003).

In conjunction with GLOBOCAN (2020), a total of 19, 292, 789 new cancer incidences and 9, 958, 133 deaths due to cancer globally were reported in 2020 and Asia recorded 49.3% and 58.3% of the total incidence and mortality rate respectively. Liver cancer also contributed 4.7% of the cancer incidences reported and 8.3% or a total of 830,180 deaths worldwide. It was demonstrated that liver cancer attributes 4.4% or 1,929 of new cancer incidences in Malaysia (Cancer Country Profile, 2020). In Malaysia, liver cancer ranked eighth as common cancer that occurred for both gender and ranked as fifth common cancer among males (Mohamed et al., 2018). Although there are advancements in both diagnosis and treatment, cancer is still the major burden of disease worldwide with rapid increasing in incidence and death (Balogh et al., 2016).

Liver cancer is a type of tumour that occur in the cell of the liver. It comprises of primary tumours which are hepatocellular carcinoma (HCC) which is the common malignancy, intrahepatic cholangiocarcinoma (ICC) and rare tumours,

which are fibrolamellar carcinoma and hepatoblastoma (Sia et al., 2017). Primary liver cancer is one of the leading cause of mortality due to cancer globally and still poses significant threat to the public health system. Increase in the prevalence of liver cancer is due to several risk factors, including infections caused by viruses, nutritional status, genetics and autoimmune diseases and toxin such as excessive alcohol consumption (Ferenci et al., 2010). Besides, most incidences of HCC is also attributed to Hepatitis B and Hepatitis C viruses infection and other risk factors such as liver cirrhosis from heavy drinking as well as exposure to dietary carcinogens (Alwi et al., 2019). HCC itself contributes to majority of primary liver cancer cases, with over 800, 000 new cases every year. The occurrence is most remarkable in sub-Saharan Africa and Asia where the cases are attributable to the fact that there is a very high incidence of Hepatitis B (HBV) infection (Sia et al., 2017).

Most cases of HCC occurs along with chronic liver disease, therefore therapy and control of its complications is mandatory. In several regions of Africa and Asia, HCC is the most common malignant disease due to failure in identifying individuals presented with Hepatitis B and C viruses. Besides, lacked of effective treatment after the diagnosis of HBV infection also accounts for approximately 60% and 23% of overall liver cancer in developing and developed nations respectively (Colagrande et al., 2016).

Sorafenib, one of the widely used standard treatments in the advanced stage of HCC is limited in its efficacy as it is only able to extend the survival of patients by only several months (Grazie et al., 2017). Other treatment of HCC includes liver resection and surgery. However, in patients that are presented with chronic liver disease, surgery is known to increase the risk of liver failure, especially after repeated resection. While surgical resection can be one of the curative choices, it is

also limited due to several reasons including high blood loss complication rates and associated with morbidity and mortality (Daher et al., 2018). In the medical and pharmaceutical fields, worldwide disease research needs to be strengthened, mainly focusing on helping the areas where the available resources are limited (Ferenci et al., 2010). Treatment of metastatic tumours involved chemotherapy; however, it is associated with severe side effects such as bone marrow depletion, neurotoxicity, gastrointestinal reactions, and damaged liver and kidney. Anti-cancer chemotherapy induces long-term toxicity and other side effects that can cause discomfort to the patient (Nurgali et al., 2018). Besides, chemotherapy is capable of inducing multidrug resistance that can cause treatment failure following the recurrence of the cancer (Feng et al., 2017).

Resistance to therapy is a significant constraint for effective curative treatment of human cancers at a disseminated level. Besides that, frequent occurrence of drug resistance reflects the mutation in the genetic composition of tumour cells resulting to adaptation to treatment of chemotherapy or the blockage of survival pathways by newly targeted drugs (Heffeter et al., 2008). Likewise, authors also described that the genetic instability and heterogeneity of the tumour always cause cells to establish drug resistance, develop mutations in the drug target or activation of other signalling pathways that will eventually resulting in acquired drug resistance and a poor tumour prognosis.

Therefore, extensive studies are conducted to develop alternatives for cancer treatment. Phytochemicals are the secondary metabolites with defensive or preventive properties and naturally present in plants and most of them are chemotherapeutic agents. These natural compounds have been demonstrated to exhibit various biological properties which target particular pathways and enzymes

and combat carcinogenesis efficiently (Alwi et al., 2019). Many studies have focused on bioactive compounds synthesised from plants to develop novel anti-cancer therapy, especially curcumin and BHMC. In the treatment of HCC, multiple treatment protocols are used, but they are often related to numerous side effects. Thus, a study by Abdel-Hamid et al. (2018), reported that natural ingredients are useful in the treatment as well as prevention of HCC.

Many studies have been conducted on the immunomodulatory activities of Curcuma *longa* and its main component, curcumin, concerning to all of the Curcuma species explored (Yuandani et al., 2021). Curcumin, one of the polyphenolic compounds are extensively studied for the treatment of cancer, in particular HCC. It has been shown that turmeric curcuminoids and their derivatives have numerous biological and pharmacological properties, which include anti-oxidant, anti-inflammatory, anti-cancer, anti-microbial, neuro-protective, cardio-protective and radio-protective (Amalraj et al., 2017). The history of curcumin isolation started about two centuries ago when two researchers discovered a 'yellow coloured substance' from rhizomes of Curcuma *longa* (Amalraj et al., 2017). Despite the aforementioned bioactive compound's efficacy, it possesses one major challenge as a potential drug candidate, which is poor bioavailability. Curcumin's limited bioavailability appears to be due to poor absorption, rapid metabolism, and systemic clearance (Gupta et al., 2013).

2, 6-bis-(4-hydroxyl-3-methoxybenzylidine) cyclohexanone, also known as BHMC are curcumin's derivatives, which is believed to exert excellent anti-cancer properties than curcumin. BHMC has been synthesised by substituting a monocyclic ketone of the curcumin diketone moiety (Razak et al., 2017). Nonetheless, many

studies have not been done to explore the potential anti-cancer effect in the derivatives of curcumin on liver cancer cells.

1.2 Objectives

1.2.1 General Objective

To determine the cytotoxic effect of BHMC and curcumin on human liver cancer cells (HepG2) and human fibroblast (Hs27).

1.2.2 Specific Objectives

- To measure and compare the cytotoxic effect of BHMC and curcumin on human liver cancer, HepG2.
- To determine the cytotoxic selective effect of BHMC and curcumin towards Hs27 cells.

1.3 Hypothesis

It is hypothesised that BHMC mediates greater cytotoxic effect on HepG2 cells at lower concentration and exert its cytotoxic selective effect by causing lower cell death upon treatment in normal Hs27 cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Curcumin

2.1.1 Introduction to curcumin

Curcumin, the primary constituent of *Curcuma longa* (turmeric) is a natural polyphenol found in the rhizome of turmeric. It is also known as diferuloylmethane has anti-oxidant, anti-microbial, and anti-inflammatory properties making it one of widely used medicinal herb in Asian countries (Hewlings & Kalman, 2017). Curcuma, which has been used as Ayurvedic medicine, is a potent active component for the treatment of various diseases, such as asthma, liver disease, and anorexia (Qadir et al., 2016). Furthermore, it is also one of the bioactive compounds derived from turmeric which exhibit pharmacological effects on many diseases, including inflammatory conditions (Aggarwal et al., 2019).

Curcumin has been shown to exhibit anti-inflammatory, anti-oxidant, and anti-carcinogenic in several studies (Qadir et al., 2016). Moreover, it is also showed that curcumin exhibit chemo-preventive, radio-sensitization and chemo-sensitization (Aggarwal et al., 2017). Several biological activities are also reported in this study where one of them is neuro-protective effects in Alzheimer's and Parkinson's disease. Moreover, curcumin was reported to inhibit a spectrum of cancers from development, progression, and metastases. The negative control of different factors particularly growth factors, cytokines, protein kinases and oncogenic proteins are mainly used to mediate these anti-cancer actions (Shanmugam et al., 2015).

2.1.2 Anti-cancer properties of curcumin

Most beneficial properties of curcumin that have been extensively studied are anti-cancer properties. Curcumin is a regulator at various levels of cancer development stages including replication, metastases, angiogenesis, and apoptosis (Mbese et al., 2019). Curcumin, a natural chemotherapeutic agent for cancer, can affect several signalling molecules such as cell survival protein, inflammatory molecules and many more due to the chemical structure and function (Liu et al., 2016). The study on the anti-cancer properties was done through several mechanisms by reducing tumour proliferation and growth, invasion, metastasis, neo-angiogenesis and chemo resistivity (Wang et al., 2020). They described that tumour proliferation is regulated by curcumin through multiple signalling pathways particularly human trophoblastic surface antigen (Trop2), in tumour progression. In the same study, Wang et al. (2020) described that curcumin prevents proliferation of cancer cells through various signalling pathways such as CyclinD1-PI3K-Akt, JAK2-STAT3 and Wnt-β-catenin signal pathways.

Inhibition of angiogenesis by curcumin was modulated in a study by AS and RA (2008), where curcumin suppressed vascular endothelial growth factor (VEGF) and other angiogenic factors mainly essential fibroblast and epidermal growth factor. It is also revealed that this compound could inhibit the expression of genes that are important for angiogenesis.

Persistent and chronic inflammation are one of the hallmark in the development of cancer. A few mechanisms on how curcumin down-regulates the pro-inflammatory signalling pathways are explained and described in a few studies. Tham et al. (2010) reported that curcumin had been identified to exhibit various molecular targets. Curcumin reduces the expression of various transcription factors

and inflammatory mediators such as signal transducers, activators of transcription protein (STAT), chemokines, monocyte chemotactic protein (MCP)-1, interleukins (IL) and cyclooxygenase (COX-2) (Tham et al., 2011). Due to its favourable effects on diseases with chronic inflammation, curcumin has been one of the widely cited natural molecule worldwide (Tham et al., 2015). Besides, Sandur et al. (2007) suggested that structure of bis-alpha, beta-unsaturated ß-diketone, two methoxy and phenolic hydroxyl groups as well as two double conjugated bonds play parts in anti-inflammatory activities of curcumin.

One of curcumin's major anti-cancer benefits is causing cell death to impede tumour development. The cell death caused by curcumin (apoptosis) is governed by three processes that affect anti-apoptotic proteins such Bcl-xl and Bcl-xs, which influence the production of cytochrome c and Reactive Oxygen Species (ROS) and inducing cell cycle arrest via caspases pathway alteration (Feng et al., 2017). Apart from anti-proliferation and anti-inflammation, curcumin also possesses anti-oxidant properties. The study by Hewlings and Kalman (2017) reported that curcumin could increase the anti-oxidants activities of superoxide dismutase in the serum. Consequently, curcumin is also capable of modulating Glutathione (GSH), catalase and SOD enzyme in neutralizing the free radicals.

The addition of N-acetyl-L-cysteine (NAC) and glutathione decreased the cytotoxicity of curcumin and Reactive Oxygen Species (ROS) generation, indicating a possible connection between cytotoxicity and ROS. In the findings, Fujisawa et al. reported that the addition of NAC or glutathione significantly reduced ROS production by curcumin (Fujisawa et al., 2004). Apart from that, some studies have proven that curcumin also exhibits anti-oxidant properties by efficiently scavenge the ROS and inhibit lipid peroxidation (Barzegar & Moosavi-Movahedi, 2011).

2.1.3 Induction of cell death by curcumin

Cell death or apoptosis is mediated through two major pathways, which are intrinsic and extrinsic. In a review by Tomeh et al. (2019), they reported that the intrinsic pathway functions by activating the mitochondrial membrane to suppress the production of anti-apoptotic protein, Bcl-2 and Bcl-xL. Subsequently, the extrinsic apoptotic pathway increases cell death receptors and activates apoptosis-related tumour necrosis factors (Tuorkey, 2014). Curcumin contributes to the modulation of DR 4 and DR 5 death receptor expression (Ashour et al., 2014; Lee et al., 2012). This suggested that curcumin induces mitochondrial apoptosis of tumour cells, DNA self-repair dysfunctionality and endoplasmic reticulum stress.

Besides that, curcumin activates caspase-3, releases cytochrome c, and inhibits Bcl-2, causing apoptosis in several tumour cells including advanced HL-60 and U937 (Rao et al., 2011). Curcumin inhibits EMT tumour cells, preventing tumour cell invasion and metastases by lowering TGF-β expression and inactivating the downstream signalling pathways. Besides that, curcumin can reduce the TGF-β1 expression levels in liver cancer, inhibit Smad2 phosphorylation and nuclear translocation, decrease snail expression levels and Smad2's unique binding site to the snail promoter (Wang et al., 2020). Choudhuri et al. (2005) described that since curcumin predominantly induces apoptosis in cells with high proliferative rate, the apoptosis in tumour cells is even higher than usual. The findings has shown that the anti-cancer dosage of curcumin inhibit non-cancerous cells in the G0 phase, however they do not necessarily trigger apoptosis.

Furthermore, cell death-induced by curcumin are reported in other cancer cell lines. Curcumin facilitates apoptosis in melanoma cells by activating caspases via

Fas-receptor assembly, whereby it inhibits the NFκB pathway and represses the XIAP apoptotic inhibitor (Bush et al., 2001). Apart from this, curcumin also regulates the expression of several genes such as Bcl-2, Bcl-xL, cyclooxygenase 2 (COX-2) and matrix metalloproteinase (MMP)-9, thus facilitating cell cycle arrest along with suppression proliferative activities, triggering the apoptosis (Hahn et al., 2018; W. H. Lee et al., 2014). Moreover, curcumin also induces apoptosis via activation of p53 tumour suppressor gene, leading to cell death in the G2 stage of cell cycle (Sa & Das, 2008). Another study also reported that effect of curcumin on inducing tumour cell apoptosis is mediated via inhibition of IKKβ kinase, which results in decreased phosphorylation of the NF-κB inhibitor and retention of the cytoplasm (Duarte et al., 2010). Although the modulatory action of curcumin on several cellular targets related to numerous diseases are shown effective, one of the significant drawbacks of curcumin is the bioavailability (Tham et al., 2010).

2.1.4 Limitations of curcumin

A variety of pharmacological effects have been modulated in curcumin such as antioxidant, anti-microbial, anti-inflammatory and anti-cancer effects. In addition, several cell signalling pathways have also been shown to control by curcumin. However, curcumin has several limitations despite its effectiveness including poor bioavailability, poor water solubility and rapid metabolism and elimination in the systemic (Feng et al., 2017). Razak et al. (2017) also described the limitations of curcumin which are classified as “pans-assay interference compounds” including instability of the compound, poor solubility and selectivity and multiple modes of interference, especially in the in vitro assays.

Curcumin had been demonstrated to modulate therapeutic effects in many forms of cancer, including lung, cervix, prostate, breast, liver cancers and osteosarcoma. However, due to its poor solubility and low bioavailability, curcumin's in vivo activities are restricted (Feng et al., 2017). The study on the pharmacokinetics of curcumin demonstrated that curcumin undergoes reduction and later hepatic conjugation upon being administered orally (Sandur et al., 2007). In improving its bioavailability, the use and the synthesis of curcumin analogues also play a significant role (Aggarwal et al., 2017).

Tham et al. (2010) illustrated in their study that the synthesis of curcumin analogues is a strategy to circumvent the limitation of curcumin's bioavailability. This is because curcumin is quickly metabolised by aldo-keto reductase enzyme in the liver due to the presence of the β-diketone moiety, thereby reducing curcumin's possible therapeutic effect on various forms of the disease. Thus, many studies have focused on curcumin's synthetic derivative, which exhibits the same activities as curcumin but with longer bioavailability. In addition, Mbese et al. (2019) explained that altering the chemical structure of curcumin helps improve its physiochemical and pharmacokinetic properties, not only the pharmacological activity of the drug molecule and the binding of the drug receptor.

On the other hand, curcumin is reported to be an unstable molecule due to its sensitivity towards oxygen, alkaline pH and radiation with ultraviolet (UV) light (Metzler et al., 2013). The biological action of curcumin is strongly linked to its structural constituent. By developing various changes to the structure of curcumin as elucidated in several studies, researchers are expecting to produce enhanced biological activity of curcumin's derivatives, some of which focus on the advantages of the additional biological effects (Anand et al., 2007).

2.2 BHMC

A number of synthetic derivative of curcumin are synthesised to enhance the anti-tumour effects and improve bioavailability of curcumin while maintaining the same safety profile as the parental compound, curcumin (Alwi et al., 2019). 2,6-bis-(4-hydroxyl-3-methoxybenzylidine) cyclohexanone (BHMC) is an analogue of curcuminoid that was developed to increase curcumin bioavailability while maintaining its therapeutic impact (Tham et al., 2010). It was synthesised based on curcumin structure by removing the unstable β-diketone structure and transforming it into conjugated double bonds as well as retaining the phenolic OH group that is crucial to the anti-inflammatory properties of curcumin (Tham et al., 2015).

Previous study by Tham et al. (2010; 2011) reported that BHMC significantly suppressed nitric oxide production (NO), TNF-a, MCP-1, interleukin (IL) 6 and 10 through a major pro-inflammatory signalling pathway inhibition. On the other hand, BHMC also reduces vascular inflammation by downregulating the synthesis of chemokine, CAM expression and endothelial migration.

According to Tham et al. (2010), JAK-STAT pathways play a crucial role in inflammation, regulating a certain amount of cytokines and growth factors. The findings explained that curcumin inhibits almost various pro-inflammatory mediators while BHMC exhibits selective inhibition of pro-inflammatory mediators. Besides, the generation of BHMC by modifying the original structure of the parent compound has induced a degree of selectivity in its target molecules.

Tham et al. (2011) also reported that BHMC mildly inhibits the IκBα phosphorylation induced by LPS while inhibiting the nuclear translocation of NF-κB p65. These finding further underpin and show a similar pattern to the blockage in the phosphorylation of IκBα. It serves as protein's suppressors to activate NF-κB dimers

(Viatour et al., 2005). The findings demonstrated that BHMC does not trigger the phosphorylation of MAPK as it fails to inhibit p38 phosphorylation. This finding is in contrast with curcumin that down-regulates the phosphorylation of MAPKs. Furthermore, the results showed potent inhibition of p38 by BHMC which directly acts on kinase.

Furthermore, BHMC also mediates cell death and reduce cell proliferation in cancer cells implicating an effective anti-cancer properties. Cytotoxic effect of BHMC was determined and evaluated on several cancer cell lines including liver and breast cancer (Alwi et al., 2019; Razak et al., 2017). The studies demonstrated that BHMC modulate higher percentage of cell deaths but at three folds lower concentration compared to curcumin. Findings from these studies indicated that BHMC exhibit enhanced anti-proliferative activities compared to the parent compound and suggested that the activities shown is mainly contributes due the change in structure and presence of functional groups.

Harun et al. (2018) highlighted on the anti-tumour properties of BHMC towards the invasion of breast cancer, MDA-MB-231 cells. The invasion of tumour cells are characterised by the formation of invadopodia, and thus this study reported that BHMC significantly reduced the number of cells which formed invadopodia following the treatment of BHMC at various concentrations. Besides, BHMC also down regulate the key players involved in the invasion and metastasis stage such as matrix metalloproteinase protein (MMP-9).

2.3 Liver Cancer

2.3.1 Hepatocellular carcinoma

Liver cancer is the fourth most prevalent cause of cancer-related mortality in the world and ranked sixth in incidents in which hepatocellular carcinoma is the most common type of liver cancer (Marrero, 2005). According to Center and Jemal (2011), 75% to 90% of primary liver cancer cases are hepatocellular carcinomas (HCC), malignant tumours that originate in the liver parenchymal cells. Another type of primary hepatic cancer is intrahepatic cholangiocarcinoma (ICC), a tumour that develop in the bile ducts. Hepatocellular carcinoma is a common cancer, which requires a multidisciplinary approach for prevention, screening, diagnosis and treatment. In addition, the variety of clinical circumstances in which HCC might express itself may reflect the disease's underlying pathophysiological complexity (Clark et al., 2015).

2.3.2 Risk Factors of Liver Cancer

Liver cancer particularly hepatocellular carcinoma, is associated with several risk factors. Chronic Hepatitis B and C, alcoholism, metabolic liver disease especially non-alcoholic fatty liver disease and exposure to food toxins such as aflatoxins and aristolochic acid are the risk factors for HCC (Yang et al., 2019). In the same study, the author also highlighted that all of these risk variables are theoretically avoidable, demonstrating the great potential for in reducing the worldwide burden of HCC. Aside from that, the most significant risk factors for HCC include infection with the Hepatitis B and C virus, which integrate their genomes into the DNA of the host, creating alterations (Chen et al., 2021). Most patients who have HCC appear with advanced illness, and even with a number of

effective treatments like surgery, liver transplantation and palliative clinical care, including radiofrequency ablation (RFA), survival rates remain low (Mohamed et al., 2018).

2.3.3 Current treatment of HCC and challenges

Treatment of HCC relies on the tumour's stage, the patient's health status and liver function capacity (Wang et al., 2015). Concurrently, according to Anwanwan et al. (2020), the treatment selection also relies on the features of the tumour, the severity of the associated liver dysfunction, age, other co-morbidities and the availability of local medical resources. It is well proven that sorafenib is used to target the tyrosine kinase enzyme in the advanced stage of HCC (Llovet et al., 2008). The only approved systemic therapy for HCC is sorafenib and used to treat individuals with asymptomatic HCC who have regained liver capacity and are not the candidates for potentially curative treatments such as transplantation or surgical resection (Wang et al., 2015).

Furthermore, sorafenib is the only approved multi-kinase inhibitor for HCC therapy (Li et al., 2015). However, following partial hepatectomy for HCC, controversy surrounds its usage as adjuvant treatment (Chedid et al., 2017). Thus, new medications such as lenvatinib as the first line of drug and second line of drugs including regorafenib, cabozanib and ramucirumab have been shown to enhance clinical outcomes though overall median survival remains about one year (Llovet et al., 2018). In the context of widespread of HCC, therapy often remains a difficult issue due to a higher rate of late detection and a scarcity of therapeutic alternatives for advanced stage (Grazie et al., 2017).

CHAPTER 3
METHODOLOGY

3.1 Materials

3.1.1 BHMC and curcumin

Curcumin powder was purchased from Nacalai Tesque. 2,6-bis-(4-hyroxyl-3-methoxybenzylidine)cyclohexane (BHMC) was generously supplied by Associate Professor Dr. Tham Chau Ling from Department of Biomedical Sciences, Faculty of Medicine and Health Sciences. Dimethyl Sulfoxide (DMSO), which was used as solvent in this research was purchased from ATCC. Both compounds were dissolved in DMSO from ATCC to a final stock solution of 50mM and diluted to the required concentration for assays. The final DMSO concentration in all assays was fixed at concentrations below 0.1% due to the toxicity to the cells.

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), and Trypan Blue were purchased from Nacalai Tesque (Kyoto, Japan). Phosphate Buffer Saline (PBS) in tablet form and Absolute Ethanol denatured (99.6%) were purchased from Fisher Scientific (Massachusetts, USA). Meanwhile, Fetal Bovine Serum was purchased from Tico Europe (Amstelveen, Netherlands).

3.1.3 Cell lines

Human hepatocellular carcinoma, HepG2 and human fibroblast, Hs27 were purchased from American Type Culture Collection (ATCC). The cells were seeded, cultured in a complete DMEM medium, and incubated in a 5% incubator at 37ºC. According to ATCC, HepG2 cells originated from the liver and are obtained from a 15-year-old male with hepatocellular carcinoma disease and have epithelial-like morphology and adherent cells when cultured. HepG2 is studied widely due to its benefits in terms of availability and human protein secretions. It is also encapsulated in alginate and has proliferated with strong cell-to-cell interaction and cell activity to form compact cell spheroids (Baquerizo et al., 2015). Concurrently, Hs27 was obtained from foreskin of normal new born and possessed phenotype of G6PD type A. Hs27 has a doubling time of 36 hours while HepG2 has a doubling time of 48 hours. Both cell lines were cultured in DMEM with 1% Penicillin Streptomycin and 10% FBS. The cells were incubated in a 5% CO_2 incubator at 37˚C.

3.1.4 Instruments

The instruments used in this project are haemocytometer, micropipettes of (0.5-10µL, 10-20µL, 100-200µL and 100-1000µL), 10ml and 25ml serological pipette from Lab Serv (Longford, Ireland), pipet-aid from Thermo Scientific (Massachusetts, USA), 96-well flat bottom clear plate, 6-well plate, 15ml and 50 ml centrifuge tubes from Fisher Scientific (Massachusetts, USA), cryovial, 1.5 ml micro centrifuge tube from Nest (Jiangsu, China), microplate reader 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). All these instruments were provided by laboratories in Faculty of Medicine and Health Sciences, UPM.

3.2 Cell Culture

3.2.1 Thawing of frozen cells

The frozen cells in the cryovial tube were thawed by transferring the cells from -80 ºC freezer into -30 ºC, then are put into a 37 ºC water bath for few minutes. The cells were then transferred and mixed into a 15mL centrifuge tube containing pre-warmed complete growth media. The mixture was centrifuged at 15000 rpm for 5 minutes. The supernatant was discarded, and the retrieved cell pellet was re-suspended together with 3mL complete growth media. Finally, the cells were transferred into a T25 flask and placed into a 5% CO_2 incubator at 37ºC.

3.2.2 Sub-culturing adherent cells

Sub-culturing of cells is done when the confluency of cells reached 70-80%. The old media was removed and was washed thrice with 1mL PBS. 1 mL of 0.25% Trypsin was added to the cells and were incubated for 5 minutes in 5% CO_2 incubator at 37 ºC. Consequently, the cells were observed using an inverted microscope, and the flask was gently tap to facilitate the detachment of cells from the flask. Next, 2mL of complete growth media with a ratio of 2:1 to trypsin was added to the flask. The cell suspension was then added into 15 mL centrifuge tube and was spined at 15000 rpm for 5 minutes for Hs27 cell lines while 12000 rpm for 5 minutes for HepG2 cell lines. The supernatant was then discarded and the cell pellet was resuspended with 1 mL of complete growth media. Next, cell suspension with appropriate ratio was transferred into a new T25 culture flask that contained

complete growth media and was incubated for 5 minutes in 5% CO_2 incubator at 37 ^{o}C.

3.2.3 Cell counting

The old media is removed when the cells confluency reached 70-80%. The cells were then washed trice with 1 mL PBS to remove the remaining old media and protein bound to the cells. Next, 1mL of 0.25% trypsin was added to detach the cells and was incubated for 5 minutes in 5% CO_2 incubator at 37 ºC. The cells' detachment was observed under an inverted microscope. 2 mL of complete growth media with a ratio of 2:1 to trypsin was then added to the flask. The cell suspension was transferred to 15 mL centrifuge tube and spin at 15000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended with 1 mL complete growth media. Three dots of 100µL trypan blue dye were transferred onto a parafilm using a micropipette for cell counting. Next, 10µL of cell suspension was pipetted onto the first dot of trypan blue dye. The dye and cell suspension was resuspended and 10µL was transferred to the next dot of trypan blue. The procedure was repeated until it reached the last dot. Lastly, the chamber of haemocytometer was filled with 10µL of cell suspension together with trypan blue. Haemocytometer was placed under an inverted microscope and observed by focusing on four quadrants of sixteen corner squares with 10X objective lens. Live cells which do not take up the dye were counted.

3.2.4 Cell Seeding

The amount of cell suspension (number of viable cells/mL) and complete growth media needed for new density of cell suspension using the formula of M1V1=M2V2. The new density of cell suspension was transferred into a sterile petri dish. Then, 100µL and 2mL of new cell suspension were transferred into 96-well plate and 6-well plates consecutively. The plates were incubated in 5% CO_2 incubator at 37 ºC overnight.

3.2.5 Preparation of working solution

BHMC and curcumin powder were dissolved in DMSO to produce stock solution of 20mM and 50mM, respectively. It was then further diluted to produce working solutions with concentrations of 10µM, 15µM and 20µM for BHMC whereas for curcumin, the working solutions with concentrations of 25µM and 50µM were prepared. The stock solution of positive control, cisplatin was also diluted to produce a final working solution of 100µM. 0.1% DMSO was used as a negative control, hence 100% of DMSO was diluted in complete growth media to generate the final working solution.

3.3 Trypan Blue Exclusion Assay

Trypan blue exclusion test is used to evaluate the viability of cells. It carries the concept that living cells which have intact cell membranes prevent the passage of dyes such as trypan blue, eosin, or propidium while dead cells which do not have intact membranes will allow the passage of dye into the cells (Strober, 2015). The assay was performed consequently after MTT assay to confirm the percentage of viable cells obtained from MTT assay. This assay was performed according to Alwi et al. (2019) with some improvisation. First, HepG2 cells with a density of 1.5x 10^5

cells/ml were seeded in each well of a 6-well plate. The cells were then incubated for 24 hours to allow cell attachment.

Subsequently, cells were treated with 15 µM of cisplatin for positive control, 0.1% DMSO for negative control, 25 and 50 µM for curcumin and three concentrations of BHMC which are 10, 15 and 20µM. Cells were then incubated for three different time point (24, 48 and 72 hours). Following incubation period, growth media contained treatments were discarded and the cells from each well were harvested using trypsin. The harvested cells were incubated for 10 minutes and centrifuged at 12000 rpm for 5 minutes. Following that, the supernatant was discarded and cell pellet was resuspended with 1ml of complete media. Accordingly, 10µL of trypan blue dye solution was mixed and diluted with an equivalent amount of cell suspension. Cell suspension was pipetted onto a haemocytometer and observed under an inverted microscope. The number of viable and dead cells were counted using a digital counter and tabulated. The following formulas were used to determine the number of viable and dead cells. This experiment was performed in triplicate.

$$Number\ of\ counted\ cells = \frac{n}{4}\ X\ 2^{df}\ X\ 10^4$$

$$Percentage\ of\ viable\ cells = \frac{number\ of\ viable\ cells\ counted}{total\ number\ of\ cells\ counted}\ X\ 100\%$$

3.4 Statistical Analysis

Data were analysed by using software packaged Prism GraphPad Prism version 8 and IBM SPSS Statistics version 24. Error bars (±) represent the standard error mean (S.E.M) for each data set. The data for the two groups were compared and analysed using Independent Samples T-Test. Comparisons and analysis between more than two groups of data were carried out using Two-way analysis of variance (ANOVA) followed by Dunnett post hoc test. The probability of p-value < 0.05 was statistically significant.

CHAPTER 4

RESULTS

4.1 Cytotoxic effect of BHMC on cell viability of HepG2 and Hs27 determined by Trypan Blue Exclusion Assay

The cytotoxic effect of BHMC on HepG2 and Hs27 cell lines were determined via Trypan Blue Exclusion Assay. Percentage of cell viability was illustrated in Figures 1-2 following incubation with different concentration of BHMC (10, 15 and 20µM). The concentrations were selected based on the IC_{50} values obtained from MTT assay in the previous study. Normal cell lines were included to compare and establish the cytotoxic selective effect. Figure 1-2 demonstrated that the cell viability of HepG2 decreased in a time and concentration-dependent manner ($p < 0.01$). At 24 hours timepoint, BHMC reduced HepG2 cells population by 30-60% and a remarkable reduction of 70-80% cell population was observed at longer exposure of 48 hours as shown in Figure 1. On contrary, BHMC only reduced 20-30% of normal Hs27 cells population after 24 hours treatment and a lower cell death of 40-60% was recorded after 48 hours treatment, as shown in Figure 2. In addition, the percentage of cell viability of normal Hs27 cells does not show a significant reduction and cell death. Therefore, selected concentrations of BHMC were observed to be very potent towards HepG2 but exert minimal toxicity towards Hs27.

As shown in Table 1, there is a statistically significant difference in the cell viability after treatment of BHMC between the two cell lines. From the table, BHMC exhibited time and dose-dependent anti-proliferative activities in HepG2 and Hs27 cells. However, anti-proliferative activities of BHMC at 24 hours are less evident in

which only 15 and 20uM of BHMC is significant at 24 hours timepoint. The anti-proliferative activities of BHMC are more significant at 48 hours, where it reduced more than 50% cell viability of HepG2 compared to Hs27. This indicates that at prolonged exposure of the treatment, more cell death are observed. Besides, lower cell death were also observed on Hs27 cells, indicating a potential cytoselective effect of BHMC.

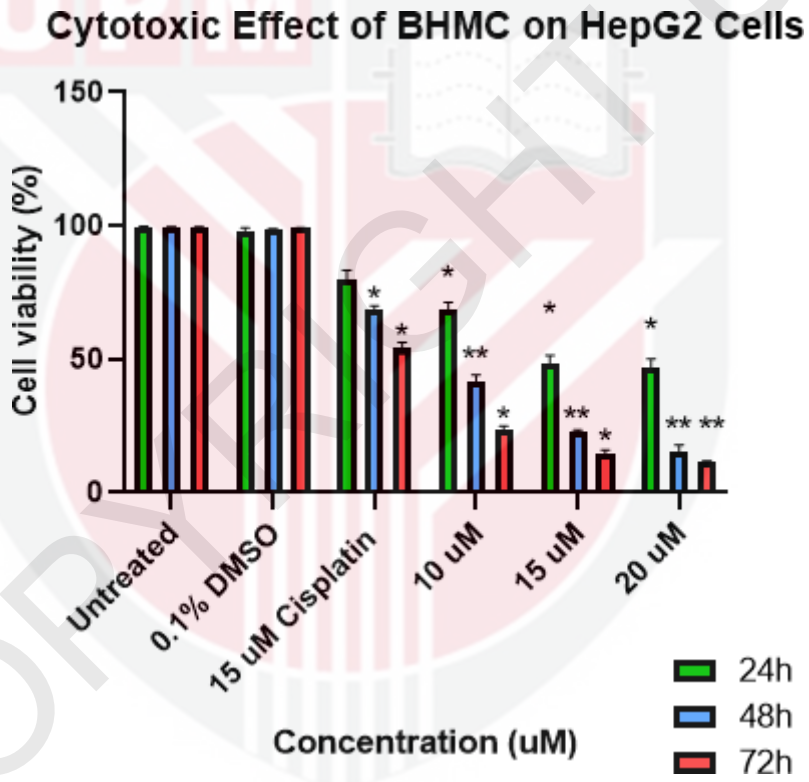

Figure 1: Effect of BHMC on the cell viability of HepG2 at different incubation time (24, 48 and 72 hours) as determined by Trypan Blue Exclusion Assay. Data is presented as mean ± S.E.M and represent of three independent experiments. Statistically significant different were indicated with (*p< 0.05, **p< 0.01) compared with the normal group using Two-way ANOVA followed by Dunnett's post hoc test.

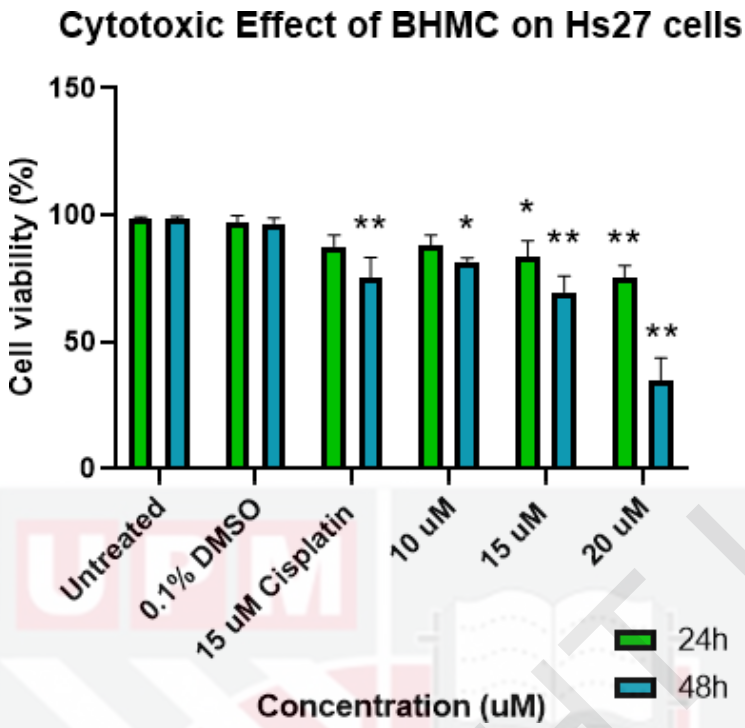

Figure 2: Effect of BHMC on the cell viability of Hs27 cells at different incubation time (24 and 48 hours) as determined by Trypan Blue Exclusion Assay. Data is presented as mean ± S.E.M and represent of three independent experiments. Statiscally significant different were indicated with (*p< 0.05, **p <0.01) compared with the normal group using Two-way ANOVA followed by Dunnett's post hoc test.

Table 4.1: Cytotoxic effect of BHMC on the cell viability of HepG2 and Hs27 at different time points as determined by Trypan Blue Exclusion Assay.

Timepoint	BHMC concentration	Cell Lines	
		HepG2	Hs27
24 Hours	Untreated	99.63 ± 0.23	99.63 ± 0.42
	0.1% DMSO	98.20 ± 0.99	97.37 ± 1.39
	15 µM cisplatin	88.36 ± 4.32	87.66 ± 2.56
	10 µM	73.82 ± 2.94[c]	88.14 ± 2.24[d]
	15 µM	59.85 ± 2.07[a,c]	83.70 ± 3.62[b,d]
	20 µM	53.20 ± 4.17[c]	75.00 ± 2.88[d]
48 Hours	Untreated	99.39 ± 0.27	99.63 ± 0.55
	0.1% DMSO	98.47 ± 0.47	96.68 ± 1.26
	15 µM cisplatin	81.66 ± 4.82	75.00 ± 4.80
	10 µM	41.36 ± 2.65[a,c]	81.66 ± 0.85[b,d]
	15 µM	22.59 ± 0.85[a,c]	69.64 ± 3.71[b,d]
	20 µM	15.60 ± 2.26[a,c]	35.00 ± 5.00[b,d]

Data are presented as mean ± S.E.M and represent three independent experiments. [a,b] Different superscript letters indicate significant difference at ($p < 0.05$) compared between cell lines at 24 and 48 hours using Independent-Samples T-test. [c,d] Different superscript letters indicate significant difference at ($p < 0.05$) compared between time points using Two Way ANOVA

4.2 Cytotoxic effect of Curcumin on cell viability of HepG2 and Hs27 determined by Trypan Blue Exclusion Assay

Similarly, cytotoxic effect of curcumin on HepG2 and Hs27 cell lines were determined via Trypan Blue Exclusion Assay. Percentage of cell viability were illustrated in Figure 3-4 following incubation with different concentrations of curcumin (25 and 50µM) at different time points (24, 48 and 72 hours). Concentrations were selected based on the IC_{50} values obtained from MTT assay in the previous study. As shown in Figure 3, curcumin reduced HepG2 cells population by 30-40% after 24 hours treatment at selected concentrations and more than 50% reduction observed after 48 hours treatment, respectively. Lower percentage of cell death ranging 30-40% was observed in Hs27 treated with curcumin after 24 and 48 hours treatments. The plotted graph depicted a constant trend on cell viability percentage, thus illustrating the cytotoxic selective effect of curcumin on the normal Hs27 cells.

As illustrated in Table 2, there is a statistically significant difference in cell viability of HepG2 and Hs27 after treatment with curcumin. At 24 hours, only concentration of 50uM is statistically significant between HepG2 and Hs27. This indicated that curcumin causes lower cell death as compared to BHMC at 24 hours and only minimal toxicity were observed in Hs27 cells at both concentrations. On the other hand, it was observed that the cytotoxic effect of curcumin is significant for 25 and 50uM concentrations at 48 hours timepoint. Therefore, at prolonged exposure of curcumin, more cell death in cancer HepG2 cells were observed. Besides, lower cell death were also observed on Hs27 cells, indicating a cyto-selective effect of curcumin.

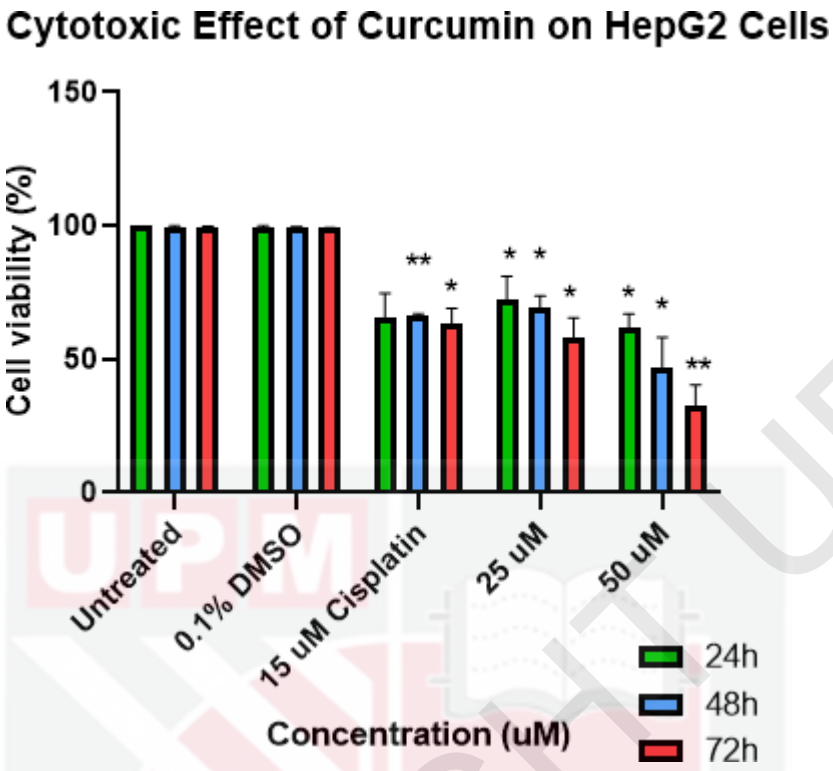

Figure 3: Effect of curcumin on the cell viability of HepG2 at different incubation time (24, 48 and 72 hours) as determined by Trypan Blue Exclusion Assay. Data are presented as mean ± S.E.M and represent of three independent experiments. Statistically significant different were indicated with (*p< 0.05, **p< 0.01) compared with the normal group using Two-way ANOVA followed by Dunnett's post hoc test.

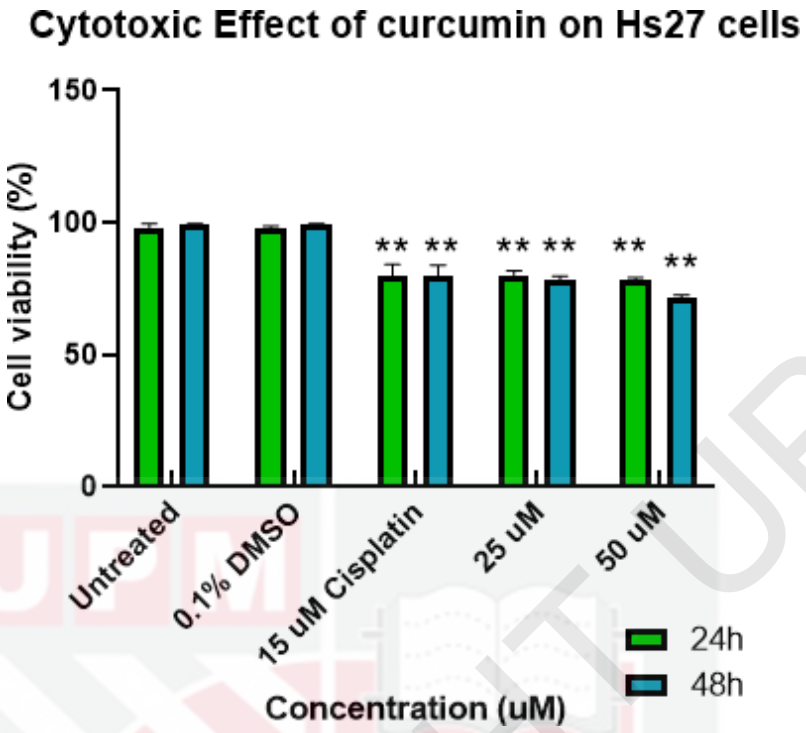

Figure 4: Effect of curcumin on the viability of Hs27 cells at different incubation time (24 and 48 hours) as determined by Trypan Blue Exclusion Assay. Data is presented as mean ± S.E.M and represent of three independent experiments. Statistically significant different were indicated with (**p <0.01) compared with the normal group using Two-way ANOVA followed by Dunnett's post hoc test.

Table 4.2: Cytotoxic effect of curcumin on the cell viability of HepG2 and Hs27 at different time points as determined by Trypan Blue Exclusion Assay.

Timepoint	Curcumin concentration	Cell Lines	
		HepG2	Hs27
24 Hours	Untreated	99.63 ± 0.00	99.63 ± 0.79
	0.1% DMSO	98.09 ± 0.45	97.65 ± 0.61
	15 µM cisplatin	72.06 ± 6.13	79.67 ± 2.60
	25 µM	69.97 ± 3.73[c]	80.13 ± 0.94[d]
	50 µM	61.55 ± 3.08[a,c]	78.15 ± 0.62[b,d]
48 Hours	Untreated	99.63 ± 0.51	99.63 ± 0.16
	0.1% DMSO	99.33 ± 0.17	99.33 ± 0.16
	15 µM cisplatin	66.11 ± 0.55[a]	79.77 ± 2.43[b]
	25 µM	69.51 ± 2.43[a]	78.31 ± 0.86[b]
	50 µM	46.67 ± 6.66[a]	71.86 ± 0.43[b]

Data are presented as mean ± S.E.M and represent three independent experiments. [a,b] Different superscript letters indicate significant difference at ($p < 0.05$) compared between cell lines at 24 and 48 hours using Independent-Samples T-test. [c,d] Different superscript letters indicate significant difference at ($p < 0.05$) compared between time points using Two Way ANOVA

CHAPTER 5

DISCUSSION

Curcumin is the main active component of turmeric that exhibits various pharmacological and biological activities, including anti-inflammation and anti-tumour modulated in in vitro and in vivo studies. Curcumin, a natural compound derived from the plants, has demonstrated excellent anti-cancer effects with minimal toxicity and is widely used to treat cancer (Bimonte et al., 2016). Curcumin possessed range of inhibitory mechanisms in cancer development encompasses initiation, progression, promotion and metastasis stages. Besides, cytotoxicity of curcumin has been well-established and confirmed in various cancerous cell lines, including chondrosarcoma, colorectal cancer, breast cancer, and hepatocellular carcinoma cells (Guo et al., 2013; Lee et al., 2012; Wang et al., 2012). The studies demonstrated that curcumin significantly reduce the growth of cancer cells in a concentration-dependent manner. Population of hepatocellular carcinoma, J5 cells are reduced with increasing concentrations of curcumin. The findings also indicated that the morphology does not change instead, the apoptotic bodies were observed. In fact, curcumin is toxic to the cancer cells at a concentration higher than 100 μM and induced necrosis at that range of concentrations rather than apoptosis at lower concentrations. Hence, morphology and staining analysis were conducted to further illustrate the mode of cell death following curcumin treatment.

Nevertheless, several preclinical and clinical studies have highlighted the limitation of curcumin, which has low bioavailability and low aqueous solubility. Due to the limitation, numerous drug delivery approaches have been introduced, including nanoformulations, liposomes, micelles, structural analogues and

hyaluronic (HA)-conjugated polyamidoramine particularly in the pancreatic cancer's treatment (Gupta et al., 2013; Kesharwani et al., 2015). Several analogues and derivatives were rapidly synthesised to overcome the bioavailability and solubility properties.

BHMC is one of the several analogues synthesised by modifying the diketone moiety of the parent compound, curcumin into conjugated double bonds and at the same time retaining the phenolic group (Harun et al., 2018). BHMC served as a potent anti-hyperalsic agent in a study by Ming-Tatt et al. (2013). Neuropathic pain is often implicated in diseases such as AIDS, multiple sclerosis and also cancer. Therefore, the study reported that treatment of BHMC in CCI-induced mice showed that BHMC concentration ranges of 0.03, 0.1 and 1.0 mg/kg remarkedly suppressed the hyperalgesic responses. Other than that, BHMC possessed anti-inflammatory activity as reported by Tham et al. (2010). Nitric oxide is one of the key role accounted in the pathogenesis of inflammation and cancer and BHMC significantly inhibited the synthesis of Nitric Oxide (NO) via suppression of iNOS gene and also secretion of cytokines in this study,

BHMC was proven to be cytotoxic selective towards tumour cells as elucidated in several studies. In study by Yeap et al. (2021), cytotoxic effect of BHMC was evaluated towards different subtypes of breast cancer, MCF-7 which is ER-positive and MDA-MB-231 which is negative. One of the indicator of cytotoxicity, cell viability was measured and determined following treatment of BHMC. The findings indicated that BHMC induced apoptosis in MCF-7 cell lines and reduced cell viability at 50% with lower concentration in comparison with MDA-MB-231 as well as normal cell lines, MCF-10A. The findings are also supported by comparing the selectivity index (SI) of BHMC towards each cell lines.

It is statistically significant that BHMC is highly selective towards MCF-7 cell lines at longer time point. Furthermore, BHMC also demonstrated higher cytotoxic effect towards breast cancer cells by having lower IC_{50} compared to the parent compound, curcumin. The cytotoxic effect of BHMC towards the cancer cell lines is believed attributable through the down regulation of miRNAs involved in the apoptosis.

In addition, other study by (Razak et al., 2017), anti-tumour effect of BHMC towards Murine 4T1 TNBC cells in mice models were demonstrated. In vitro cytotoxicity assay was conducted to compare the cytotoxic effect of curcumin and its derivative, BHMC. Subsequently, it was reported that BHMC has greater folds of cytotoxic effect compared to curcumin. The IC_{50} value of BHMC determined by MTT assay is significantly lower than curcumin towards 4T1 cells. Besides, further in vivo study in mouse models were also conducted to determine the anti-tumour effect of both compounds and it is later revealed that mice with BHMC-treated exhibit less mitotic cells in the tumour, which remarked a low tumour burden attributable by anti-proliferative properties of both compounds. Meanwhile, study by Tham et al. (2010), reported on the selective inhibitory mechanism possessed by BHMC. Cytotoxic effect of BHMC was also elucidated on U937 and RAW 264.7 cells. The findings from the study also indicated that BHMC reduce cell viability in a concentration dependent manner where it significantly reduced RAW 264.7 and U937 at concentration 25 µM and higher.

In this study, cytotoxic effect of BHMC were compared with the parent compound, curcumin and its demonstrated on two types of cell lines which are HepG2 and normal Hs27. The findings indicated that BHMC and curcumin exert their cytotoxic effect on both cell lines at different concentrations and also time points. BHMC caused more than 50% of cell death particularly at 48 hours on

HepG2 cell but with 3 times lower concentrations compared to curcumin. Thus, anti-proliferative effect exerted by BHMC was greater compared to curcumin. These findings are supported by a study on cytotoxic effect of BHMC on the same cell lines where the value of IC_{50} determined by MTT assay is 3 times lower than curcumin. However, there is a drawback in the study where BHMC was observed toxic to the normal mouse fibroblast cells, 3T3. (Alwi et al., 2019), Thus, normal human fibroblast, Hs27 cells is included in this study to establish and observed the percentage of cell death at the same concentration ranges of BHMC and curcumin. Besides that, there are significant differences in the cell viability of BHMC towards normal Hs27 cells. Lower percentage of cell deaths are observed in Hs27 cells following treatment of both compounds. BHMC demonstrated minimal cytotoxicity towards normal cells, Hs27 compared to its effect on cancer cells, HepG2. Thus, the cyto-selective effect on Hs27 was observed to be similar with its parental compound, curcumin.

According to Bernabé-Pineda et al. (2004), the aromatic groups in curcumin accounts for its hydrophobicity which makes it less soluble in water. Hydrogenation, methoxylation, and unsaturation of the diketone moiety are each important factors in the activity of modified curcuminoids, which possess improved anti-cancer and anti-inflammatory properties compared to curcumin. Series derivatives of curcumin was synthesised by modifying the aforementioned structure via conjugating with phenolic double bonds. In addition, the existence of an active methylene group and β-diketone moiety has been suggested attribute to unstable form of curcumin, along with poor absorption and rapid metabolism in physiological conditions (Aggarwal et al., 2017). Potent biological activities and cytotoxic effect of BHMC is believed linked to the modified structure from the parent compound, curcumin. Curcumin

consisted of enol moiety in which it is modified to increase the bioavailability, whereas BHMC has α-ß unsaturated bis-enone system which is highly responsible for the improved biological and pharmacological properties (Razak et al., 2017). This will in turn lead to enhance anti-proliferative and cytotoxic effect towards cancer cells.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

BHMC mediated greater cytotoxic effect in HepG2 cells by reducing the cell viability and modulate higher percentage of cell death at lower concentration compared to curcumin. Besides, BHMC also possessed a selective cytotoxicity by causing high toxicity towards cancer cells and exert minimal toxicity towards normal cell lines, Hs27 due to less cell death observed at similar concentrations.

6.2 Recommendations

In this study, cytotoxic effect of curcumin and its analogue, BHMC are elucidated on the viability of human hepatocellular carcinoma, HepG2 and human fibroblast, Hs27 cell lines. However, for Hs27 cells, the IC_{50} value of BHMC and curcumin is not yet obtained. Therefore, it is necessary to determine the IC_{50} value determined by MTT assay before conducting Trypan Blue Exclusion Assay. It is also suggested to evaluate and establish the cytotoxic effect of BHMC in other normal cell lines such as human and rat normal hepatocytes in future study. In addition, it is suggested to determine mode of cell death caused by BHMC and curcumin via Annexin V-FITC and Hoechst staining.

REFERENCES

Abdel-Hamid, N. M., Abass, S. A., Mohamed, A. A., & Muneam Hamid, D. (2018). Herbal management of hepatocellular carcinoma through cutting the pathways of the common risk factors. In *Biomedicine and Pharmacotherapy* (Vol. 107, Issue January, pp. 1246–1258). https://doi.org/10.1016/j.biopha.2018.08.104

Aggarwal, C. B. B., Diego, S., Kunnumakkara, A. B., Kunnumakkara, A. B., Bordoloi, D., Padmavathi, G., Monisha, J., Roy, N. K., Prasad, S., & Aggarwal, B. B. (2017). *Curcumin , the golden nutraceutical : multitargeting for multiple chronic diseases*. https://doi.org/10.1111/bph.13621

Aggarwal, V., Tuli, H. S., Varol, A., Thakral, F., Yerer, M. B., Sak, K., Varol, M., Jain, A., Khan, M. A., & Sethi, G. (2019). Role of reactive oxygen species in cancer progression: Molecular mechanisms and recent advancements. *Biomolecules*, *9*(11). https://doi.org/10.3390/biom9110735

Alwi, S. S. S., Zahari, S., Haron, A. S., & Alexander, H. R. (2019). Cytotoxic Effect of 2,6-bis(4-Hydroxy-3-Methoxybenzylidene) cyclohexanone (BHMC) and Curcumin on Human Liver Cancer Cells, HepG2. *Malaysian Journal of Medicine and Health Sciences*, *15*(July), 44–50.

Amalraj, A., Pius, A., Gopi, S., & Gopi, S. (2017). Biological activities of curcuminoids, other biomolecules from turmeric and their derivatives – A review. *Journal of Traditional and Complementary Medicine*, *7*(2), 205–233. https://doi.org/10.1016/j.jtcme.2016.05.005

Anand, P., Kunnumakkara, A. B., Newman, R. A., & Aggarwal, B. B. (2007). Bioavailability of curcumin: Problems and promises. *Molecular Pharmaceutics*, *4*(6), 807–818. https://doi.org/10.1021/mp700113r

Anwanwan, D., Singh, S. K., Singh, S., Saikam, V., & Singh, R. (2020). Challenges in liver cancer and possible treatment approaches. In *Biochimica et Biophysica Acta - Reviews on Cancer* (Vol. 1873, Issue 1). Elsevier B.V. https://doi.org/10.1016/j.bbcan.2019.188314

AS, S., & RA, S. (2008). Curcumin: preventive and therapeutic properties in laboratory studies and clinical trials. *Antioxidants & Redox Signaling*, *10*(3). https://doi.org/10.1089/ARS.2007.1769

Ashour, A. A., Abdel-Aziz, A. A. H., Mansour, A. M., Neslihan Alpay, S., Huo, L., & Ozpolat, B. (2014). Targeting elongation factor-2 kinase (eEF-2K) induces apoptosis in human pancreatic cancer cells. *Apoptosis*, *19*(1), 241–258. https://doi.org/10.1007/s10495-013-0927-2

Balogh, J., Iii, D. V., Gordon, S., Li, X., Ghobrial, R. M., & Jr, H. P. M. (2016). Jhc-3-041. *Journal of Hepatocellular Carcinoma*, *Volume 3*, 41–53. https://www.dovepress.com/hepatocellular-carcinoma-a-review-peer-reviewed-article-JHC

Barzegar, A., & Moosavi-Movahedi, A. A. (2011). Intracellular ROS Protection Efficiency and Free Radical-Scavenging Activity of Curcumin. *PLoS ONE*, *6*(10), 26012. https://doi.org/10.1371/journal.pone.0026012

Bernabé-Pineda, M., Ramírez-Silva, M. T., Romero-Romo, M., González-Vergara, E., & Rojas-Hernández, A. (2004). Determination of acidity constants of curcumin in aqueous solution and apparent rate constant of its decomposition. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, *60*(5), 1091–1097. https://doi.org/10.1016/S1386-1425(03)00342-1

Bimonte, S., Barbieri, A., Leongito, M., Piccirillo, M., Giudice, A., Pivonello, C., De Angelis, C., Granata, V., Palaia, R., & Izzo, F. (2016). Curcumin anticancer studies in pancreatic cancer. In *Nutrients* (Vol. 8, Issue 7). https://doi.org/10.3390/nu8070433

Bush, J. A., Cheung, K. J. J., & Li, G. (2001). Curcumin induces apoptosis in human melanoma cells through a Fas receptor/caspase-8 pathway independent of p53. *Experimental Cell Research*. https://doi.org/10.1006/excr.2001.5381

Center, M. M., & Jemal, A. (2011). International trends in liver cancer incidence rates. *Cancer Epidemiology Biomarkers and Prevention*, *20*(11), 2362–2368. https://doi.org/10.1158/1055-9965.EPI-11-0643

Chedid, M. F., Kruel, C. R. P., Pinto, M. A., Grezzana-Filho, T. J. M., Leipnitz, I., Kruel, C. D. P., Scaffaro, L. A., & Chedid, A. D. (2017). HEPATOCELLULAR CARCINOMA: DIAGNOSIS AND OPERATIVE MANAGEMENT. In *Arquivos brasileiros de cirurgia digestiva : ABCD = Brazilian archives of digestive surgery* (Vol. 30, Issue 4, pp. 272–278). https://doi.org/10.1590/0102-6720201700040011

Chen, Y., Wang, G., Xu, H., Wang, H., & Bai, D. (2021). Identification of a Novel Metastasis-Related miRNAs-Based Signature for Predicting the Prognosis of Hepatocellular Carcinoma. *Journal of Oncology*, *2021*, 6629633. https://doi.org/10.1155/2021/6629633

Choudhuri, T., Pal, S., Das, T., & Sa, G. (2005). Curcumin selectively induces apoptosis in deregulated cyclin D1-expressed cells at G2 phase of cell cycle in a p53-dependent manner. *Journal of Biological Chemistry*, *280*(20), 20059–20068. https://doi.org/10.1074/jbc.M410670200

Clark, T., Maximin, S., Meier, J., Pokharel, S., & Bhargava, P. (2015). Hepatocellular Carcinoma: Review of Epidemiology, Screening, Imaging Diagnosis, Response Assessment, and Treatment. In *Current Problems in Diagnostic Radiology* (Vol. 44, Issue 6, pp. 479–486). Mosby Inc. https://doi.org/10.1067/j.cpradiol.2015.04.004

Colagrande, S., Inghilesi, A. L., Aburas, S., Taliani, G. G., Nardi, C., & Marra, F. (2016). Challenges of advanced hepatocellular carcinoma. In *World Journal of Gastroenterology* (Vol. 22, Issue 34, pp. 7645–7659). Baishideng Publishing Group Co., Limited. https://doi.org/10.3748/wjg.v22.i34.7645

Daher, S., Massarwa, M., Benson, A. A., & Khoury, T. (2018). Current and future treatment of hepatocellular carcinoma: An updated comprehensive review. In *Journal of Clinical and Translational Hepatology* (Vol. 6, Issue 1, pp. 69–78). Xia and He Publishing Inc. https://doi.org/10.14218/JCTH.2017.00031

Duarte, V. M., Han, E., Veena, M. S., Salvado, A., Suh, J. D., Liang, L.-J., Faull, K. F., Srivatsan, E. S., & Wang, M. B. (2010). Curcumin Enhances the Effect of Cisplatin in Suppression of Head and Neck Squamous Cell Carcinoma via Inhibition of IKKβ Protein of the NFκB Pathway. *Molecular Cancer Therapeutics*, *9*(10), 2665–2675. https://doi.org/10.1158/1535-7163.MCT-10-0064

Eaton, L. (2003). World cancer rates set to double by 2020. *BMJ (Clinical Research Ed.)*, *326*(7392), 728. https://doi.org/10.1136/bmj.326.7392.728/a

Feng, T., Wei, Y., Lee, R. J., & Zhao, L. (2017). Liposomal curcumin and its application in cancer Physical property. *International Journal of Nanomedicine*, *12*, 6027–6044.

Ferenci, P., Fried, M., Labrecque, D., Bruix, J., Sherman, M., Omata, M., Heathcote, J., Piratsivuth, T., Kew, M., Otegbayo, J. A., Zheng, S. S., Sarin, S., Hamid, S. S., Modawi, S. B., Fleig, W., Fedail, S., Thomson, A., Khan, A., Malfertheiner, P., … Le Mair, A. (2010). Hepatocellular carcinoma (HCC): A global perspective. *Journal of Clinical Gastroenterology*, *44*(4), 239–245. https://doi.org/10.1097/MCG.0b013e3181d46ef2

Fujisawa, S., Atsumi, T., Ishihara, M., & Kadoma, Y. (2004). *Activity of Curcumin and Related Compounds*. *570*, 563–569.

Grazie, M. Le, Biagini, M. R., Tarocchi, M., Polvani, S., & Galli, A. (2017). Chemotherapy for hepatocellular carcinoma: The present and the future. In *World Journal of Hepatology* (Vol. 9, Issue 21, pp. 907–920). Baishideng Publishing Group Co. https://doi.org/10.4254/wjh.v9.i21.907

Guo, L. Da, Chen, X. J., Hu, Y. H., Yu, Z. J., Wang, D., & Liu, J. Z. (2013). Curcumin inhibits proliferation and induces apoptosis of human colorectal cancer cells by activating the mitochondria apoptotic pathway. *Phytotherapy Research*, *27*(3), 422–430. https://doi.org/10.1002/ptr.4731

Gupta, S. C., Sung, B., Kim, J. H., Prasad, S., Li, S., & Aggarwal, B. B. (2013). Multitargeting by turmeric, the golden spice: From kitchen to clinic. In *Molecular Nutrition and Food Research* (Vol. 57, Issue 9, pp. 1510–1528). https://doi.org/10.1002/mnfr.201100741

Hahn, Y. Il, Kim, S. J., Choi, B. Y., Cho, K. C., Bandu, R., Kim, K. P., Kim, D. H., Kim, W., Park, J. S., Han, B. W., Lee, J., Na, H. K., Cha, Y. N., & Surh, Y. J. (2018). Curcumin interacts directly with the Cysteine 259 residue of STAT3 and induces apoptosis in H-Ras transformed human mammary epithelial cells. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-23840-2

Harun, S. N. A., Israf, D. A., Tham, C. L., Lam, K. W., Cheema, M. S., & Hashim, N. F. M. (2018). The molecular targets and anti-invasive effects of 2,6-bis-(4-hydroxyl-3methoxybenzylidine) cyclohexanone or BHMC in MDA-MB-231 human breast cancer cells. *Molecules*, *23*(4), 1–19. https://doi.org/10.3390/molecules23040865

Heffeter, P., Jungwirth, U., Jakupec, M., Hartinger, C., Galanski, M., Elbling, L., Micksche, M., Keppler, B., & Berger, W. (2008). *Resistance against novel*

anticancer metal compounds : Differences and similarities. *11*, 1–16. https://doi.org/10.1016/j.drup.2008.02.002

Hewlings, S., & Kalman, D. (2017). Curcumin: A Review of Its Effects on Human Health. *Foods*, *6*(10), 92. https://doi.org/10.3390/foods6100092

Kesharwani, P., Xie, L., Mao, G., Padhye, S., & Iyer, A. K. (2015). Hyaluronic acid-conjugated polyamidoamine dendrimers for targeted delivery of 3,4-difluorobenzylidene curcumin to CD44 overexpressing pancreatic cancer cells. *Colloids and Surfaces B: Biointerfaces*, *136*, 413–423. https://doi.org/10.1016/j.colsurfb.2015.09.043

Lee, H. P., Li, T. M., Tsao, J. Y., Fong, Y. C., & Tang, C. H. (2012). Curcumin induces cell apoptosis in human chondrosarcoma through extrinsic death receptor pathway. *International Immunopharmacology*, *13*(2), 163–169. https://doi.org/10.1016/j.intimp.2012.04.002

Lee, W. H., Loo, C. Y., Young, P. M., Traini, D., Mason, R. S., & Rohanizadeh, R. (2014). Recent advances in curcumin nanoformulation for cancer therapy. In *Expert Opinion on Drug Delivery* (Vol. 11, Issue 8, pp. 1183–1201). Informa Healthcare. https://doi.org/10.1517/17425247.2014.916686

Li, S., Yang, F., & Ren, X. (2015). Immunotherapy for hepatocellular carcinoma. In *Drug discoveries & therapeutics* (Vol. 9, Issue 5, pp. 363–371). https://doi.org/10.5582/ddt.2015.01054

Liu, G. Y., Sun, Y. Z., Zhou, N., Du, X. M., Yang, J., & Guo, S. J. (2016). 3,3′-OH curcumin causes apoptosis in HepG2 cells through ROS-mediated pathway. *European Journal of Medicinal Chemistry*, *112*, 157–163. https://doi.org/10.1016/j.ejmech.2016.02.019

Llovet, J. M., Montal, R., Sia, D., & Finn, R. S. (2018). Molecular therapies and precision medicine for hepatocellular carcinoma. In *Nature Reviews Clinical Oncology* (Vol. 15, Issue 10, pp. 599–616). Nature Publishing Group. https://doi.org/10.1038/s41571-018-0073-4

Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.-F., de Oliveira, A. C., Santoro, A., Raoul, J.-L., Forner, A., Schwartz, M., Porta, C., Zeuzem, S., Bolondi, L., Greten, T. F., Galle, P. R., Seitz, J.-F., Borbath, I.,

Häussinger, D., … Bruix, J. (2008). Sorafenib in Advanced Hepatocellular Carcinoma. *New England Journal of Medicine*, *359*(4), 378–390. https://doi.org/10.1056/nejmoa0708857

Marrero, J. A. (2005). Hepatocellular carcinoma. In D. L. Longo (Ed.), *Current Opinion in Gastroenterology* (Vol. 21, Issue 3, pp. 308–312). N Engl J Med. https://doi.org/10.1097/01.mog.0000159817.55661.ca

Mbese, Z., Khwaza, V., & Aderibigbe, B. A. (2019a). Curcumin and Its Derivatives as Potential Therapeutic Agents in Prostate, Colon and Breast Cancers. In *Molecules (Basel, Switzerland)* (Vol. 24, Issue 23). https://doi.org/10.3390/molecules24234386

Mbese, Z., Khwaza, V., & Aderibigbe, B. A. (2019b). Curcumin and Its Derivatives as Potential Therapeutic Agents in Prostate, Colon and Breast Cancers. In *Molecules (Basel, Switzerland)* (Vol. 24, Issue 23). NLM (Medline). https://doi.org/10.3390/molecules24234386

Ming-Tatt, L., Khalivulla, S. I., Akhtar, M. N., Lajis, N., Perimal, E. K., Akira, A., Ali, D. I., & Sulaiman, M. R. (2013). Anti-Hyperalgesic effect of a benzilidine-cyclohexanone analogue on a mouse model of chronic constriction injury-induced neuropathic pain: Participation of the κ-Opioid receptor and KATP. *Pharmacology Biochemistry and Behavior*, *114–115*, 58–63. https://doi.org/10.1016/j.pbb.2013.10.019

Mohamed, R., Raihan, R., Azzeri, A., & Shabaruddin, F. H. (2018). Hepatocellular Carcinoma in Malaysia and Its Changing Trend. *Euroasian Journal of Hepato-Gastroenterology*, *8*(1), 54–56. https://doi.org/10.5005/jp-journals-10018-1259

Nurgali, K., Jagoe, R. T., & Abalo, R. (2018). Editorial: Adverse effects of cancer chemotherapy: Anything new to improve tolerance and reduce sequelae? In *Frontiers in Pharmacology* (Vol. 9, Issue MAR, p. 245). Frontiers Media S.A. https://doi.org/10.3389/fphar.2018.00245

Qadir, M. I., Naqvi, S. T. Q., & Muhammad, S. A. (2016). Curcumin: A polyphenol with molecular targets for cancer control. In *Asian Pacific Journal of Cancer Prevention* (Vol. 17, Issue 6, pp. 2735–2739).

Rao, J., Xu, D. R., Zheng, F. M., Long, Z. J., Huang, S. S., Wu, X., Zhou, W. H.,

Huang, R. W., & Liu, Q. (2011). Curcumin reduces expression of Bcl-2, leading to apoptosis in daunorubicin-insensitive CD34+acute myeloid leukemia cell lines and primary sorted CD34+acute myeloid leukemia cells. *Journal of Translational Medicine*. https://doi.org/10.1186/1479-5876-9-71

Razak, N. A., Akhtar, M. N., Abu, N., Ho, W. Y., Tan, S. W., Zareen, S., Taj-Ud-Din, S. N. Bin, Long, K., Alitheen, N. B., & Yeap, S. K. (2017). The: In vivo anti-tumor effect of curcumin derivative (2 E,6 E)-2,6-bis(4-hydroxy-3-methoxybenzylidene)cyclohexanone (BHMC) on 4T1 breast cancer cells. *RSC Advances*, *7*(57), 36185–36192. https://doi.org/10.1039/c7ra06580a

Sa, G., & Das, T. (2008). Anti cancer effects of curcumin: Cycle of life and death. In *Cell Division* (Vol. 3, p. 14). BioMed Central. https://doi.org/10.1186/1747-1028-3-14

Sandur, S. K., Ichikawa, H., Pandey, M. K., Kunnumakkara, A. B., Sung, B., Sethi, G., & Aggarwal, B. B. (2007). Role of pro-oxidants and antioxidants in the anti-inflammatory and apoptotic effects of curcumin (diferuloylmethane). *Free Radical Biology and Medicine*, *43*(4), 568–580. https://doi.org/10.1016/j.freeradbiomed.2007.05.009

Shanmugam, M. K., Rane, G., Kanchi, M. M., Arfuso, F., Chinnathambi, A., Zayed, M. E., Alharbi, S. A., Tan, B. K. H., Kumar, A. P., & Sethi, G. (2015). The multifaceted role of curcumin in cancer prevention and treatment. In *Molecules* (Vol. 20, Issue 2, pp. 2728–2769). MDPI AG. https://doi.org/10.3390/molecules20022728

Sia, D., Villanueva, A., Friedman, S. L., & Llovet, J. M. (2017). Liver Cancer Cell of Origin, Molecular Class, and Effects on Patient Prognosis. *Gastroenterology*, *152*(4), 745–761. https://doi.org/10.1053/j.gastro.2016.11.048

SNA, H., DA, I., CL, T., KW, L., MS, C., & NF, M. H. (2018). The Molecular Targets and Anti-Invasive Effects of 2,6-bis-(4-hydroxyl-3methoxybenzylidine) cyclohexanone or BHMC in MDA-MB-231 Human Breast Cancer Cells. *Molecules (Basel, Switzerland)*, *23*(4). https://doi.org/10.3390/MOLECULES23040865

Tham, C. L., Hazeera Harith, H., Wai Lam, K., Joong Chong, Y., Singh Cheema, M., Roslan Sulaiman, M., Hj Lajis, N., & Ahmad Israf, D. (2015). The

synthetic curcuminoid BHMC restores endotoxin-stimulated HUVEC dysfunction:Specific disruption on enzymatic activity of p38 MAPK. *European Journal of Pharmacology*, *749*, 1–11. https://doi.org/10.1016/j.ejphar.2014.12.015

Tham, C. L., Lam, K. W., Rajajendram, R., Cheah, Y. K., Sulaiman, M. R., Lajis, N. H., Kim, M. K., & Israf, D. A. (2011). The effects of a synthetic curcuminoid analogue, 2,6-bis-(4-hydroxyl-3- methoxybenzylidine)cyclohexanone on proinflammatory signaling pathways and CLP-induced lethal sepsis in mice. *European Journal of Pharmacology*, *652*(1–3), 136–144. https://doi.org/10.1016/j.ejphar.2010.10.092

Tham, C. L., Liew, C. Y., Lam, K. W., Mohamad, A. S., Kim, M. K., Cheah, Y. K., Zakaria, Z. A., Sulaiman, M. R., Lajis, N. H., & Israf, D. A. (2010). A synthetic curcuminoid derivative inhibits nitric oxide and proinflammatory cytokine synthesis. *European Journal of Pharmacology*, *628*(1–3), 247–254. https://doi.org/10.1016/j.ejphar.2009.11.053

Tomeh, M. A., Hadianamrei, R., & Zhao, X. (2019). A review of curcumin and its derivatives as anticancer agents. In *International Journal of Molecular Sciences* (Vol. 20, Issue 5). MDPI AG. https://doi.org/10.3390/ijms20051033

Tuorkey, M. J. (2014). Curcumin a potent cancer preventive agent: Mechanisms of cancer cellkilling. *Interventional Medicine & Applied Science*, *6*(4), 139. https://doi.org/10.1556/IMAS.6.2014.4.1

Viatour, P., Merville, M. P., Bours, V., & Chariot, A. (2005). Phosphorylation of NF-κB and IκB proteins: Implications in cancer and inflammation. In *Trends in Biochemical Sciences* (Vol. 30, Issue 1, pp. 43–52). Trends Biochem Sci. https://doi.org/10.1016/j.tibs.2004.11.009

Wang, C. H., Wey, K. C., Mo, L. R., Chang, K. K., Lin, R. C., & Kuo, J. J. (2015). Current trends and recent advances in diagnosis, therapy, and prevention of hepatocellular Carcinoma. In *Asian Pacific Journal of Cancer Prevention* (Vol. 16, Issue 9, pp. 3595–3604). Asian Pacific Organization for Cancer Prevention. https://doi.org/10.7314/APJCP.2015.16.9.3595

Wang, W. H., Chiang, I. T., Ding, K., Chung, J. G., Lin, W. J., Lin, S. S., & Hwang, J. J. (2012). Curcumin-induced apoptosis in human hepatocellular carcinoma J5

cells: Critical role of Ca +2-dependent pathway. *Evidence-Based Complementary and Alternative Medicine*, *2012*. https://doi.org/10.1155/2012/512907

Wang, Y., Lu, J., Jiang, B., & Guo, J. (2020). The roles of curcumin in regulating the tumor immunosuppressive microenvironment (Review). *Oncology Letters*, *19*(4), 3059–3070. https://doi.org/10.3892/ol.2020.11437

Yang, J. D., Hainaut, P., Gores, G. J., Amadou, A., Plymoth, A., & Roberts, L. R. (2019). A global view of hepatocellular carcinoma: trends, risk, prevention and management. In *Nature Reviews Gastroenterology and Hepatology* (Vol. 16, Issue 10, pp. 589–604). Nature Publishing Group. https://doi.org/10.1038/s41575-019-0186-y

Yuandani, Jantan, I., Rohani, A. S., & Sumantri, I. B. (2021). Immunomodulatory Effects and Mechanisms of Curcuma Species and Their Bioactive Compounds: A Review. *Frontiers in Pharmacology*, *12*, 643119. https://doi.org/10.3389/fphar.2021.643119

APPENDICES

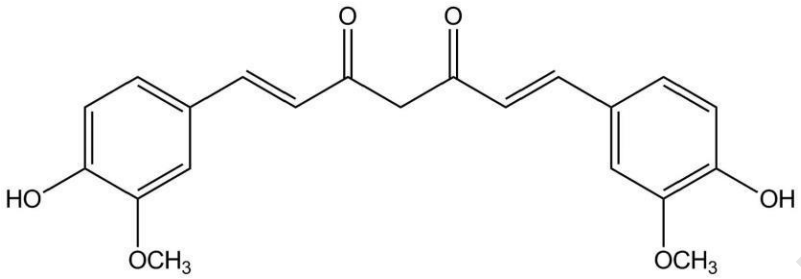

Figure 5: Chemical structure of curcumin

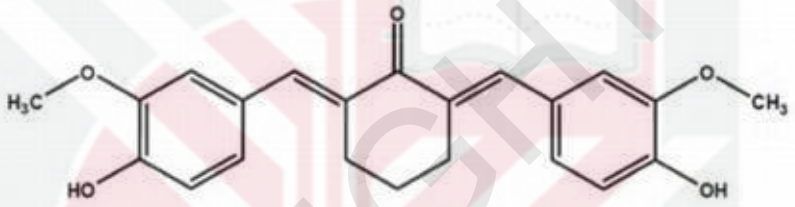

Figure 6: Chemical structure of 2,6-bis- 4-(hydroxyl-3-methoxy-benzylidine)-cyclohexanone (BHMC)