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CYTOTOXIC EFFECT OF CURCUMIONIS ANALOGUE, BHMC ON HUMAN LIVER CANCER, HEPG2 CELLS

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BY

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CYTOTOXIC EFFECT OF CURCUMIONIS ANALOGUE, BHMC ON HUMAN LIVER CANCER, HEPG2 CELLS

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ABSTRACT

Introduction: Hepatocellular Carcinoma (HCC) is the fourth most common cause of cancer deaths**.** It is challenging to develop effective HCC therapies due to the chemotherapeutic resistance. Curcumin is a polyphenol compound found in the rhizome of *Curcuma longa*. It has been linked to several health benefits and it attracted a lot of interest in the scientific and medicine worlds due to its strong anti-angiogenic, anti-inflammatory, antioxidant, and anti-proliferative activities. Several derivatives were synthesized in order to overcome the limitations of curcumin. One of these analogs that syntheses is 2,6-bis-(4-hydroxy-3-methoxybenzylidene) cyclohexanone (BHMC). The synthesis of BHMC was referred to via eliminating the unstable β-diketone moiety and modifying it into a conjugated double bond while retaining phenolic OH functional group. **Objective:** This study generally aimed to investigate the cytotoxic effect of BHMC and curcumin on human liver cancer, HepG2 cells. **Methodology:** HepG2 cells were treated with 20, 15, and 10μM concentration of BHMC and 50 and 25μM concentration of curcumin for 24, 48, 72 hours. The morphological changes in the nucleus were detected by Hoechst staining and the cell apoptosis was determined by propidium iodide staining. **Results and Discussion:** The morphological transformations of HepG2 cells were detected under fluorescence microscopes viewed at magnification of 40x. These morphological changes include DNA fragmentation, formation of apoptotic bodies, condensation of nucleus, and cell shrinkage. BHMC treatment showed Induced the apoptotic cells higher compared curcumin at 24 hours. **Conclusion:** Nevertheless, this study illustrates that BHMC has a cytotoxic effect against human liver cancer, HepG2 cell lines.

Keywords: BHMC; Curcumin; HepG2; Apoptosis

KESAN SITOTOKSIK ANALOG CURCUMIONIS, BHMC PADA KANSER HATI MANUSIA, TALIAN SEL HEPG2

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ABSTRAK

Pengenalan: Karsinoma Hepatoselular (HCC) ialah punca keempat paling biasa kematian kanser. Adalah mencabar untuk membangunkan terapi HCC yang berkesan kerana rintangan kemoterapi. Kurkumin ialah sebatian polifenol yang terdapat dalam rizom *Curcuma longa*. Ia telah dikaitkan dengan beberapa manfaat kesihatan dan ia menarik banyak minat dalam dunia saintifik dan perubatan kerana aktiviti anti-angiogenik, anti-radang, antioksidan dan anti-proliferatif yang kuat. Beberapa derivatif telah disintesis untuk mengatasi batasan kurkumin. Salah satu analog ini yang disintesis ialah 2,6-bis-(4-hydroxy-3-methoxybenzylidene) cyclohexanone (BHMC). Sintesis BHMC dirujuk melalui penyingkiran bahagian β-diketone yang tidak stabil dan mengubah suainya menjadi ikatan berganda terkonjugasi sambil mengekalkan kumpulan berfungsi fenolik OH. **Objektif:** Kajian ini secara amnya bertujuan untuk menyiasat kesan sitotoksik BHMC dan kurkumin pada kanser hati manusia, sel HepG2. **Metodologi:** Sel HepG2 dirawat dengan kepekatan 20, 15, dan 10μM BHMC dan kepekatan kurkumin 50 dan 25μM selama 24, 48, 72 jam. Perubahan morfologi dalam nukleus telah dikesan oleh pewarnaan Hoechst dan apoptosis sel ditentukan oleh pewarnaan propidium iodide. **Keputusan dan Perbincangan:** Transformasi morfologi sel HepG2 telah dikesan di bawah mikroskop pendarfluor yang dilihat pada perbesaran 40x. Perubahan morfologi ini termasuk pemecahan DNA, pembentukan badan apoptosis, pengeluaran nukleus, dan pengecutan sel. Rawatan BHMC menunjukkan Terinduksi sel apoptosis lebih tinggi berbanding curcuimn pada 24 jam. **Kesimpulan:** Namun begitu, kajian ini menggambarkan bahawa BHMC mempunyai kesan sitotoksik terhadap kanser hati manusia, garisan sel HepG2.

Kata kunci: BHMC; Curcumin; HepG2; Apoptosis

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LIST OF ABBREVIATIONS

BHMC	2,6-bis-(4-hydroxyl-3-methoxybenzylidine) cyclohexanone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
HBV	Hepatitis B virus
HepG2	Human liver hepatocellular carcinoma
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
NF-κB	Nuclear factor kappa B
PBS	Phosphate Buffer Saline
PI	Propidium Iodide

CHAPTER 1

INTRODUCTION

1.0 Study Background

Cancer is a set of diseases involving abnormal cell growth which can invade the surrounding tissues and spread to other parts of the body. Metastasis is the primary reason for death from cancer disease (Zamrus et al., 2018; Syed Alwi et al., 2019). According to the World Health Organization, cancer would lead to around 10 million deaths in 2020. In the same year, most causes of death with cancer were liver, lung, stomach, breast, colon, and rectum. Globally, the most fatal type of cancer and it classifies as sixth rank of incidence is liver cancer (McGlynn et al., 2021). The incidence and mortality of liver cancer vary based on age, gender, and geographic (El-Serag., 2020). Liver cancer can appear before the age of 20 years in countries with higher incidence rates compared to low risk countries, which incidences with liver cancer will be rare before age 50. Besad that, incidence rates in men are higher than females (Bosch et al., 2004). In Malaysia, there were 43,837 new cases and 26,395 mortality has been reported due to liver cancer (Raihan et al., 2018).

Hepatocellular carcinoma (HCC) is a type of primary liver cancer that causes 670,000 deaths annually. Commonly, HCC develops in the patients with viral infection such as hepatitis B and hepatitis C or due to some behavioral risk factors, which are alcohol consumption, obesity, and tobacco. Increase the risk of developing HCC in chronic hepatitis B infection patients to 44% and hepatitis C virus to 21% cause (Baecker et al., 2018). Besides, Symptoms differ in each stage of hepatocellular carcinoma. Usually, HCC patients show no symptoms during the early stage. In contrast, most of the HCC symptoms will appear in advanced stages which include jaundice, fever, and chills, enlarged liver, weight loss and triad in the right upper quadrant pain (Bartlett et al.,

2005). The signs and symptoms may be related to decompensation cirrhosis in the terminal stage. In addition, the most common symptom reported is abdominal pain which arises from enlarged tumor mass (Zabora et al., 2001). There are several therapies of HCC used to help to increase the survival rate of patients and allow them to live a normal life span. These therapies include surgical resection, various locoregional treatments such as percutaneous ethanol injection (PEI), trans-arterial chemoembolization (TACE), radiofrequency ablation (RFA), radioembolization (Raza & Sood, 2014). Furthermore, there is another option for the patients whose cancer cannot be treated with surgery or have not responded to the local therapies (American Cancer Society, 2021).

In recent years, researchers tried to find other alternative ways to cure liver cancer by using bioactive compounds like curcumin. Curcumin is also called diferuloylmethane, which belongs to the polyphenol group that derived from the rhizome of Curcuma longa in 1815. It has gained attention from medicine and scientific worlds for its biological properties, which are anti-tumor, anti-inflammatory, anti-proliferative and antioxidant (Giordano & Tommonaro, 2019). According to Ali et al (2006), since curcumin has antioxidant and anti-inflammatory activities, it has the ability to prevent cancer cells via suppressing the promotion of tumor, inhibit the growth and induce apoptosis. Moreover, curcumin is active in various signaling pathways include inhibit the signaling by NF-κB which is responsible to regulate expression of many genes such as COX-2 enzyme. Also, anti-proliferation activities of curcumin on cancer cells by suppressing the IκB Kinase which induces NF-κB activation inhibition (Hartojo et al., 2010). In spite of, the effectiveness of curcumin as treatment for cancer however there are some limitations in its bioavailability. These are poor bioavailability of curcumin which involves poor water solubility, low oral absorbability, and rapid metabolic rate (Oglah et al., 2020).

Recently, many studies have been done to increase the bioavailability of curcumin with adjuvants such as liposomes, nanoparticles, and phospholipid complexes (Sohn et al., 2021). However, several derivatives of curcumin were synthesized to overcome limitations. One of these analogs that syntheses is 2,6-bis-(4-hydroxy-3-methoxybenzylidene) cyclohexanone (BHMC). The synthesis of BHMC was referred to the chemical structure of curcumin by eliminating the unstable β-diketone moiety and modifying it into a conjugated double bond while retaining phenolic OH group. In addition, the β-diketone moiety which made the curcumin to have a low bioavailability was exchanged with stable cyclohexanone was exchanged with stable cyclohexanone in order to improve the therapeutic effects of curcumin (Harun et al., 2018). Nevertheless, BHMC was studied in hyperalgesia, inflammation, and murine breast cancer. BHMC showed the ability to inhibit the pro-inflammatory, induce anti-hyperalgesic, and prevent the cancer cell invasion (Lee et al., 2012).

1.1 Objective of Study

1.1.1 General Objective

This study generally aimed to investigate the effect of BHMC and curcumin on human liver cancer, HepG2 cells.

1.1.2 Specific Objective

To achieve the indicated general objective, the following specific objectives were focused in this study:

1. To observe the morphological changes in HepG2 cells by using Hoechst staining.
2. To observe the death cells after treatment with BHMC and curcumin on HepG2 cells by using Propidium Iodide staining.

1.2 Hypothesis of Study

It is hypothesized that BHMC significantly induces the apoptosis cell population and will also show an increase in change of morphological features of the nucleus in HepG2 cell lines.

1.3 Problem statements

The third greatest cause of cancer-related death worldwide is primary liver cancer. Most patients are diagnosed in the late stages (Yang et al., 2020). Besides that, the present therapeutics have shown some major limitations, including resistance to chemotherapy and harm to normal cells caused by radiation therapy. Therefore, it led to a lot of research into ways to improve the current treatment for cancer and less side effects particularly in using natural anticancer compounds. BHMC is a curcumin's analogue that is synthesized to improve bioavailability. Nevertheless, there is still a lack of studies that have investigated its effects in liver cancer cells.

1.4 Justification of Study

Many derivative analogs of curcumin have been synthesized to overcome the poor bioavailability of it. BHMC has shown anti-proliferative effects in different cell lines such as MDA-MB and MCF-7 of breast cancer. Thus, it would be more beneficial also to look at its effect on liver cancer, HepG2. Since liver cancer is one of the most common cancer mortality. Which cause 830000 deaths in 2020 (World Health Organization, 2022).

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of Hepatocellular Carcinoma

Liver cancer is the fifth most frequent type of cancer around the world, and it is considered a fourth leading cause of cancer-related mortality (Calderaro et al., 2019). Globally, Hepatocellular Carcinoma (HCC) is one type of primary liver cancer which is the fourth most common cause of cancer deaths. Annually, HCC causes 670,000 deaths around the world (Raihan et al., 2018). Based on the global statistics, the HCC mortality is indicating an increasing trend and it is estimated to extend to a higher rate by 2030 (Azit et al., 2021). According to El-Serag. (2020), the mortality and incidence of liver cancer differ based on age, region, and gender. The incidence rates in men are higher compared to women in most countries. Moreover, incidence rates of liver cancer and age are directly correlated by increasing the lifetime of humans. Over the time the geographic map has varied in HCC incidence due to the migrant populations from low- risk countries such as Asian, African to Western Europe which led to raise the risk of HCC compared to their countries of origin. In Malaysia, liver cancer is one of the ten most common causes of cancer for both gander and one of the reasons for early death. Malaysia in 2018, reported around 43,837 new cases and 26,395 deaths due to liver cancer (Raihan et al., 2018; Vaskular et al., 2022).

2.1.1 Risk Factor

Viral infection plays a main risk factor in developing HCC compared to behavioral risk factor. Worldwide, the chronic hepatitis B infection (HBV) causes 44% cases as well as hepatitis C virus (HCV) cause 21% cases of HCC. On the other hand, behavioral factors such as tobacco, alcohol consumption, obesity (Baecker et al., 2018). HBV is a DNA virus that can integrate into the host genome and infects hepatocytes. Later, it will increase the risk of HCC by promoting the mutations in liver cells even in absence of cirrhosis. Unlike HBV, HCV does not integrate into the genome host and is likely producing the tumors via fibrosis, repetitive damage, and regeneration. The relationship between HCC and HCV has been confirmed (Llovet et al., 2021). Around 90% of HCV infection that is associated with HCC cases has been preceded by cirrhosis (McGlynn et al., 2021). According to Llovet et al. (2021) heavy alcohol consumption is an established risk factor for liver cancer, cirrhosis, and alcoholic liver disease. Approximately 150, 629 cases of HCC can be linked to excessive alcohol intake. In many studies, increase the risk of HCC in HBV carriers who are alcoholics (≥3 drinks/day). Those who consume alcohol are more likely to be obesity. In 2012, there were 51,760 cases of HCC in the world. In the same study, 73,279 cases of tobacco smoking can be attributed to liver cancer and cirrhosis (Baecker et al., 2018).

2.1.2 Symptoms and Current Management of HCC

According to Cahill & Braccia. (2004), The patients showed asymptomatic symptoms in the early stage of the disease. Unfortunately, 80% of the patients diagnosed with hepatocellular carcinoma are in the advanced stage. Common symptoms during advanced stages of HCC. Usually, the patients present with enlarged liver, abdominal discomfort, vomiting, chills and fever, nausea, and jaundice, diarrhea. Among 90%- 95% of patients with HCC will be diagnosed with

weight loss, triad in the right upper quadrant pain, and palpable mass (Bartlett et al., 2005). In a study conducted by (Lin et al., 2004), signs and symptoms in the terminal stage will be related to decompensated cirrhosis which include hepatic encephalopathy, variceal bleeding, peripheral edema, ascites and dyspnea. As reported by Zabora et al. (2001), HCC patients were diagnosed with the third highest level of psychiatric distress among other cancer patients.

The most common treatment that use for HCC disease such as surgical resection, liver transplantation, radiofrequency ablation, chemoembolization, cryosurgery and percutaneous ethanol injection (Zhu., 2003). The surgical resection is one of the best treatments for HCC patients because the mortality can be less than 5%. Although the survival has been increased, most of the patients will still have only 5 year survival. Moreover, percutaneous ethanol injection, cryotherapy and radiofrequency ablation is a local ablative therapy which is useful for maintaining the remaining liver function when used in surgery and for the patients with multiple lesions. The final option that is used in HCC is liver transplantation, but it must be used for patients with inoperable liver (Carr., 2004). According to a study conducted by Geschwind, Ramsey, Choti, Thuluvath, & Huncharek (2003), the purpose of chemoembolization treatment is to preserve the liver functional for a long time and transfer highly dose concentration of chemotherapy immediately into the tumor cells but have not been supported via randomized experiments. The new therapeutic option for HCC is selective internal radiation therapy which transfers a high radiation dose to the tumor that feeds on arteries while avoiding the normal liver parenchyma. The survival rate will increase into 63% but unfortunately this treatment will have a side effect on the patients (Geschwind et al., 2004). One of these agents that are used to treat HCC is sorafenib, which is known as multikinase inhibitors of receptor tyrosine kinases and raf-kinases (Abou-Alfa et al., 2006). It is used to inhibit the cell proliferation of cancer, promote the apoptosis, and angiogenesis of tumors. The results

showed the efficacy of sorafenib by decreasing the death risk by 30% in the advanced stage of HCC (Chen et al., 2017). As stated by Keating (2017), sorafenib gives adverse side effects to the HCC patients such as hand-foot skin reactions, weight loss, anorexia, diarrhea, alopecia, and dry skin. However, prolonged sorafenib exposure in HCC cells results in sorafenib resistance and tumor development (Chen et al., 2017).

2.2 General Introduction to Curcumin

Phytochemicals are substances that are found in the plants such as curcumin, lutein and thymoquinone. Curcumin or 1,7-bis (4-hydroxy- 3-methoxyphenyl) -1,6- heptadiene-3,5-dione is also known as diferuloylmethane that can be found in the rhizome of *Curcuma longa*. Many years ago, *curcuma longa* has been used in Asian medicine and it was called in Hindi as halid , in English called turmeric and Japanese as ukon (Sharma et al., 2005). Curcumin is a crystalline compound which appears as a bright orange color (Lestari & Indrayanto., 2014). Commercially, turmeric contains around 77% of curcumin with other two related compounds which are 17% desmethoxycurcumin, and 6% bisdemethoxycurcumin (Anand et al., 2007). These three components belong to the diarylheptanoids group and together are called curcuminoids (Lestari & Indrayanto., 2014). Curcumin possesses properties diverse in the scientific and medicine worlds due to anti-angiogenic, anti-inflammatory, antioxidant, and anti-proliferative activities (Hewlings & Kalman., 2017). The anti-inflammatory and antioxidant properties of curcumin can lead to the ability to inhibit cancer cells (Anirudhan & Binusreejayan., 2016). According to Hu et al. (2015), it has been utilized as treatment for hepatic disorders, anorexia, inflammation, and biliary as well as used for rheumatism and sinusitis as treatment in Indian medicine.

2.2.1 Biological Activities of Curcumin

Carcinogenesis consists of three stages such as initiation, promotion, and progression. All of these stages are related to each other. The promotion of cancer because of inflammatory and oxidative tissue damage. Since curcumin has antioxidant and anti-inflammatory properties, it has the ability to prevent cancer cells via suppressing the promotion of tumor. Curcumin also can inhibit the growth and induce apoptosis in various cell types (Ali et al., 2006). As reported by Kuo et al. (1996), curcumin is considered as an agent of apoptosis. A study contacted BY Chen et al. (1999); Chen and Huang (1998); Hanif et al. (1997), rat treated with low concentration of curcumin showed cell cycle arrests in the cell proliferation in G0-G1/G2/S cell cycle phase, while high concentration has been induced apoptosis in A7r5 cells. A number of the hallmarks of apoptosis such as fragmentation, DNA laddering, apoptosis specific cleavage of ribosomal RNA and chromatin condensation in human hepatocellular carcinoma (Hep G2) cells, human colon cancer cell (HT-29) and kidney cancer cell after treated with curcumin (Jiang et al., 1996). In another study by Tong et al. (2006), human bladder cancer cells were treated with curcumin, the result showed arrests in the S, G2/M phase. Besides, curcumin is active in various signaling pathways include inhibit the signaling by NF-κB which is responsible to regulate expression of many genes such as COX-2 enzyme (Ail et al., 2006). This enzyme is responsible for malignant transformation and inflammation. Curcumin also has anti-proliferation properties on tumor cells via suppressing the IκB Kinase which induces NF-κB activation inhibition (Hartojo et al., 2010). Curcumin also induces apoptosis in many cancer cells but not all. Colorectal carcinoma cells (COLO205) showed delay in appearance of apoptotic DNA ladders and detect cell cycle arrest in G1 phase (Chen et al., 1996).

2.2.2 Derivatives and analogues of Curcumin

Curcumin showed efficacy as a therapeutic agent for several diseases, but it has limitations in the bioavailability due to low oral absorbability, rapid metabolic rate, and poor water solubility (Oglah et al., 2020). In the other study conducted by Anand et al. (2017), poor bioavailability of curcumin due to low serum level, short half-life, and limit in tissue distribution, and rapid metabolism. Many derivatives of curcumin have been developed to improve the bioavailability via structural modifications. Curcumin was combined with piperine which is recognized as inhibitor intestinal glucuronidation and hepatic. In the same study, results showed the effect of this combined has increased the bioavailability into 2000% in humans. Another analogue which is EF24 that showed excellent enhancement on the bioavailability and more potent in anti-tumor in both vivo and vitro. EF24 could inhibit cancer growth via induced apoptosis and cell cycle arrest by multiple pathways including inhibiting NF-κB and HIF-1α (Adams et al., 2005). According to Verma et al. (1997), the result of combining curcumin and genistein was shown inhibited in the cellular proliferation of breast carcinoma (MCF-7) cell lines. Recently, another way has emerged which is drug delivery systems such as nanoparticles, micelles complexes, liposomes, and phospholipid complexes. The delivery systems based on nanoparticles will be more suitable for hydrophobic agents which could overcome the poor water solubility in the curcumin (Adams et al., 2005). Another example for drug delivery systems is liposomes. As reported by Li et al. (2005), Liposomes could carry both hydrophobic and hydrophilic molecules. In addition, demonstrated that liposomal curcumin could inhibit both the growth of pancreatic carcinoma and tumor angiogenesis. On the other hand, phospholipid and micelles complexes could enhance the drug absorption in gastrointestinal which lead to improved bioavailability compared to curcumin alone.

2.3 2,6-bis-(4-hydroxy-3-methoxybenzylidene) cyclohexanone (BHMC)

2,6-bis-(4-hydroxy-3-methoxybenzylidene) cyclohexanone (BHMC) is one of the synthetic curcuminoid analogs which are synthesized to improve the bioavailability of curcumin. The synthesis of BHMC was referred to the chemical structure of curcumin via eliminating the unstable β-diketone moiety and modifying it into a conjugated double bond while retaining phenolic OH functional group which is in charge of antioxidant properties in curcumin, while the β-diketone moiety which made the curcumin to have a low bioavailability was exchanged with stable cyclohexanone to enhance the therapeutic effects of it (Harun et al., 2018). Nevertheless, The BHMC was used to study hyperalgesia, inflammation, and murine breast cancer. (Lee et al., 2012). BHMC showed the ability to inhibit the pro-inflammatory cytokines and signaling pathway of inflammation which consists of the p38 and mitogen-activated protein kinase and nuclear factor, and activator protein (Tham et al., 2010). It is also proven to induce anti-hyperalgesia impact in mice models with neuropathic pain (Ming-Tatt., 2013). Besides that, BHMC has been shown to decrease in the number of mitotic cells in 4T1-challenged mice which could be related to anti-proliferative effects possessed via BHMC. Moreover, the metastasis of 4T1 cells in the lung showed reduction which could indicate the anti-metastatic effect possessed via BHMC (Razak et al., 2017). In the same study, it stated that BHMC prevented the cancer cell invasion due to inhibition of invadopodia-related proteins and molecules invadopodia formation. The invadopodia-related proteins such as MT1-MMP, β-PIX, and MMP-9.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents

In the table below **(Table 3.1)**, listed all reagents and chemicals that were used in this experiment. Besides that, the name of the manufacturer.

Table 3.1: List of the chemicals and reagents used in the experiment

NO	Chemicals and reagents	Manufacturer
1	Dulbecco's Modified Eagle Medium (DMEM)	Nacalai Tesque (Kyoto, Japan)
2.	(0.25%) Trypsin-EDTA with Phenol Red	
3.	Dimethyl Sulfoxide (DMSO)	
4.	Fetal Bovine Serum (FBS)	
6	Trypan Blue	
7	Penicillin Streptomycin Mixed Solution	
7.	Phosphate Buffer Saline (PBS)	Oxoid (Hampshire, United Kingdom)
8	Hoechst 33342 Staining Solution (20mM)	Thermo Fisher Scientific (Massachusetts, USA)
10	Propidium Iodide Staining Solution	BD Biosciences (California, USA)

3.2 Instruments

The instruments used in this study included biohazard cabinet class II from ESCO (Changi, Singapore), CO2 incubator, water bath, micropipettes (volume ranges from 0.5µl to 1,000 µl) from Eppendorf Research Plus (Hamburg, Germany), serological pipette from Lab Serv (Longford, Ireland), pipet aid from Thermo Scientific (Massachusetts, USA), 6- well plate, centrifuge tubes (15 ml and 50 ml) from Fisher Scientific (Massachusetts, USA) , cryovial, laboratory Freezers (-30C° and -80 C°), microscope slides, coverslip, petri dish, centrifuge from Jora-aki Technology Sdn. Bhd (Cheras, Selangor), flask (T25 and T75), fluorescence microscope, and inverted microscope. All of these instruments were supplied via the laboratories of the Faculty of Medicine and Health Sciences, University Putra Malaysia (UPM).

3.3 Cell Line

Human hepatocellular carcinoma cell line (HepG2) was purchased from American Type Culture Collection (ATCC). HepG2 cells were cultured in DMEM that being supplemented with 10% of FBS and 1% Penicillin-Streptomycin solution. Cells were grown in T25/T75 tissue culture flasks and incubated in a 5% carbon dioxide (CO2) incubator at 37°C.

3.4 Cell Culture

3.4.1 Stock Cell Thawing

The cryovial containing Human hepatocellular carcinoma cell lines (HepG2) was removed from -80°C freezer. The cryovial was then swirled gently in the water bath for a minute until the content was thawed. The cryovial was then disinfected thoroughly with 70% alcohol before transferred to biosafety cabinet class II. Next, the thawed cells were transferred into a 15 ml centrifuge tube that

contained 1-2 ml of pre-warmed complete growth media and was added slowly to avoid osmotic shock. Then, proceeded with the centrifugation at 15000 rpm for 5 minutes. The cells were immediately transferred into the T25 labeled culture flasks. Finally, the flask was maintained in a 5% CO2 incubator at 37°C.

3.4.2 Cell Maintenance

It is necessary to change the medium when intensity is reduced and medium changes the color from red to yellow. The media was changed accordingly every few days to provide enough nutrients and a suitable environment for the cells. The spent medium from the flasks was decanted aseptically into a waste beaker. The cells were washed twice with 2mL phosphate buffer saline (PBS). Then, 4 mL of complete growth medium was added into T25 flasks and placed into a 5% CO2 incubator at 37°C.

3.4.3 Subculture of Cells

Once cells reach 70-80% confluency, the cells need to be split into a new flask. The old medium was discarded and washed with 2 mL phosphate buffer saline (PBS) twice. Then, the flasks were swirled gently before PBS was removed after each wash. In order to detach the cells from the flask, 1mL of trypsin was added into the flask and incubated for 5-7 minutes. After 5 minutes of incubation, the flask was gently tapped and examined under an inverted microscope to make sure all the cells were detached from the flask. Next, 2mL of complete growth media were added into the flask to stop the activity of trypsin on the cells and transferred into a 15mL centrifuge tube. The tubes were then centrifuged at 15,000 rpm for 5 minutes. The pellet of cells from the centrifugation was suspended in 1 mL of complete growth media and transferred equally into 2

new different T25 flasks containing 4 mL complete growth media. Both flasks were incubated in a 5% CO2 incubator at 37°C.

3.4.4 Cell Counting and Cell Seeding

Cell counting is a method to calculate the cell number or quantification of cells. After the cells were trypsinized and centrifuged as mentioned in section 3.4.3, the supernatant was discarded, and the pellet was resuspended with 1 mL complete growth media. Added 10 µl of trypan blue was mixed with 10 µl of the cell suspension by using the hemocytometer and observed under inverted microscope. The number of cells which were viable (unstained) and non-viable (stained) was determined. Total number of viable cells per 1 mL can be calculated by using the formula as below:

Total Number of Viable Cells:

$$\frac{Number\ of\ viable\ cells\ in\ 4\ quadrants}{4} x\ 2^n\ x\ 10^4$$

Were,

n= number of dilutions

After calculation, the amount of cell suspension and complete growth media required were used to calculate a new cell suspension density by using formula below:

Dilution Formula:

$$M_1V_1 = M_2V_2$$

Were,

M_1 = initial concentration
V_1 = initial volume
M_2 = final concentration
V_2 = final volume

Then, a sterile petri dish was used to transfer the new cell suspension density. A new density cell suspension was placed into 6-well plates sequentially. The plates were incubated in a 5% CO2 incubator at 37˚C for 24 hours before the treatment day.

3.5 Treatment

3.5.1 Curcumin, BHMC Preparation

The powder form of curcumin was dissolved in Dimethyl Sulfoxide (DMSO) to make (50mM) curcumin as a stock solution. The stock needs to be diluted with complete growth media. The stock amount that needs to be used can be calculated using M1V1 = M2V2. Then, (50mM) curcumin was diluted in complete growth media to get the final concentration of (50µM) curcumin. The (50µM) curcumin needs to be diluted to produce other concentration by serial dilution. The concentrations of curcumin that have been selected for this study were 50µM and 25µM.

Likewise, powder form of BHMC was dissolved in DMSO to make (20mM) BHMC as a stock solution. The stock needs to be diluted with complete growth media to produce other concentrations via serial dilution. The concentrations of BHMC that have been selected for this study were 20µM, 15µM and 10µM.

3.6 Morphological changes using Hoechst 33342 staining

HepG2 cells (1×10^6cells/well) were seeded into a 6-well plate and treated with different concentrations of BHMC, curcumin for 24,48,72 hours. The cells were fixed in the fixation buffer (4% paraformaldehyde and 250uL NaOH in 50 mL PBS) for 30 minutes at the incubator. The cells were washed once with 1mL PBS and stained with 500µL Hoechst 33342 (10µg/mL) for 15 minutes at the incubator. The cells were washed again with PBS and cell morphology was observed under an inverted fluorescence microscope.

3.7 Observation of cell death using Propidium Iodide (PI) staining

HepG2 cells were plated at a density of 1×10^6cells/well in 6-well plates and then cells were treated with BHMC and curcumin for 24 hours. For pre-fixation PI staining, after incubation time, washed the cells slowly with 1mL PBS for one time and incubate with 500µL PI stain (5µg/mL) and follow with a fixation buffer with 1mL for 30 minutes, respectively. After 30 minutes, it was washed and viewed immediately under fluorescence microscope. For post-fixation PI staining, after incubation time, washed the cells slowly with 1mL PBS for one time and incubate with fixation buffer and following with PI (5µg/mL) for 30 minutes, respectively. After 30 minutes, it was washed and viewed immediately under fluorescence microscope.

CHAPTER 4

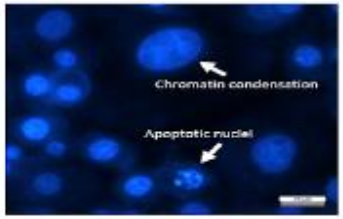
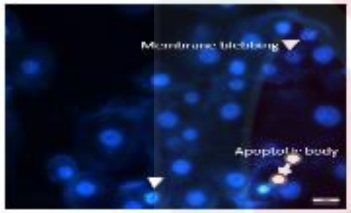
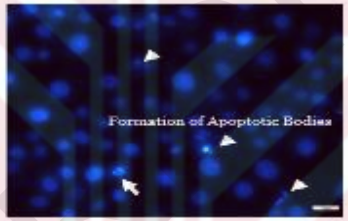
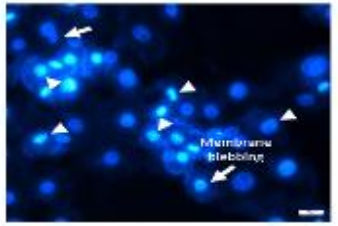
RESULTS

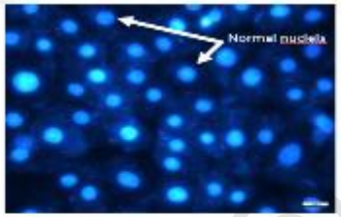
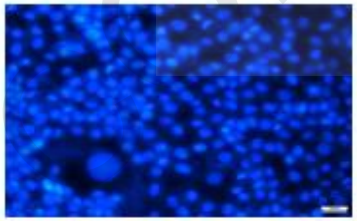
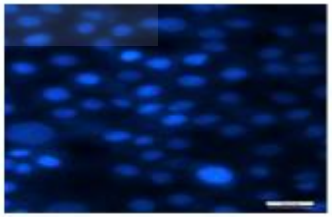
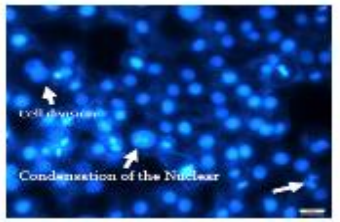
4.1 Cell morphological changes of HepG2 cells treated with Curcumin and BHMC by using Hoechst Staining

Hoechst 33343 staining was applied to observe the morphological changes of HepG2 cell lines. The HepG2 cell was exposed to several concentrations of curcumin (50,25µM) and BHMC (20,15,10µM) for 24, 48, and 72h. The previous concentrations for both compounds were obtained based on IC_{50} towards HepG2 cells (Syed Alwi et al., 2019). As shown in **Table 4.1**, after Hoechst staining, disclosed marked blebbing of cell membrane, chromatin condensation, condensation of the nuclear material, DNA fragmentation, cell shrinkage, apoptosis body formation, and death cell at 40x magnification under fluorescence microscopes.

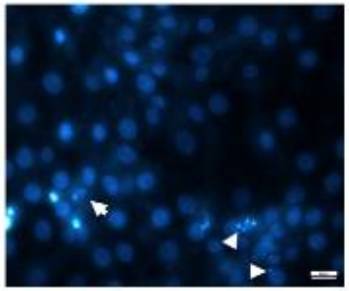
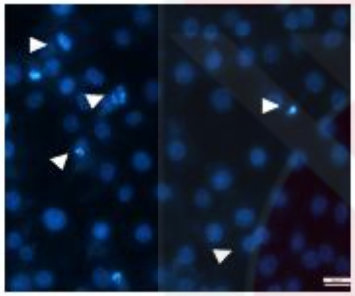
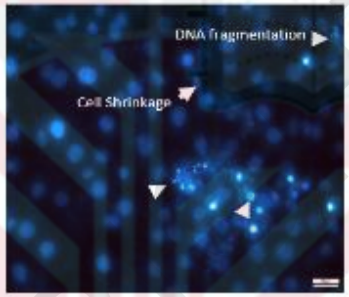
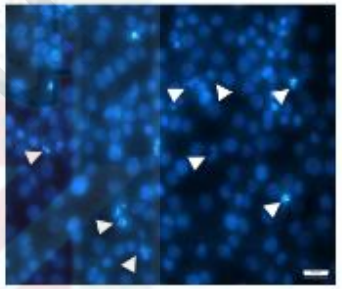
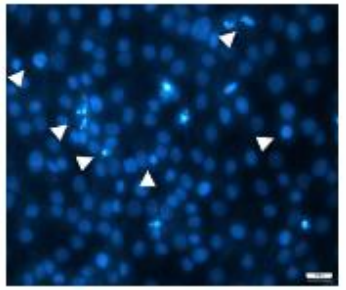
Table 4.1 Hoechst 33342 staining of HepG2 treated with Curcumin and BHMC for a) 24h, b) 48h, c) 72h. The arrows a) blebbing of cell membrane (BM), chromatin condensation (CC), and condensation of the nuclear material; b) DNA fragmentation, CC, cell shrinkage (CS), BM; c) apoptosis body formation and death cell, CC, BM, CS.

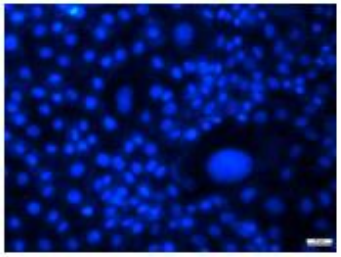
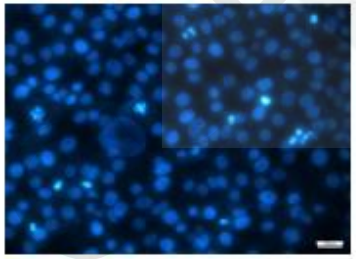
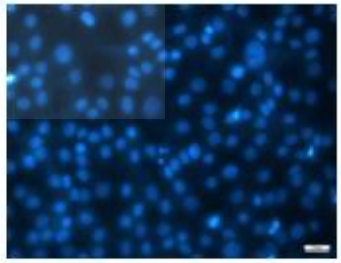
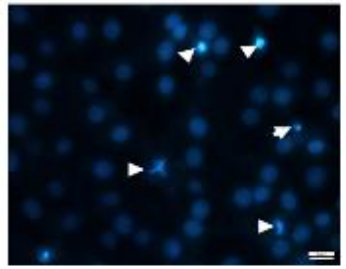
a) 24 hours

Treatment Concentration (μM)				
Curcumin		BHMC		
50μM	25μM	20μM	15μM	10μM
				

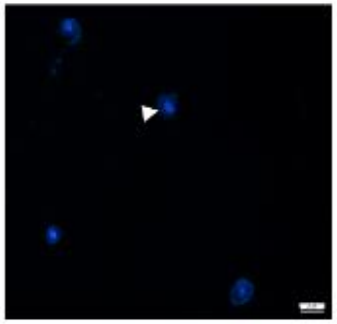
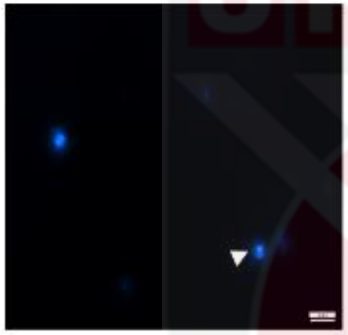
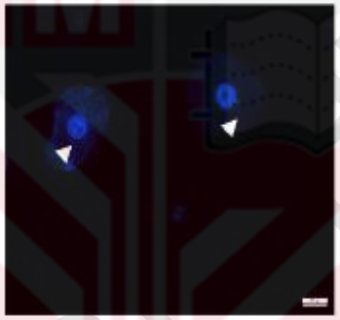
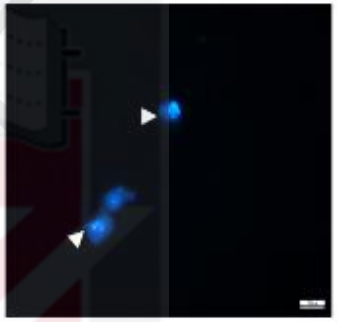
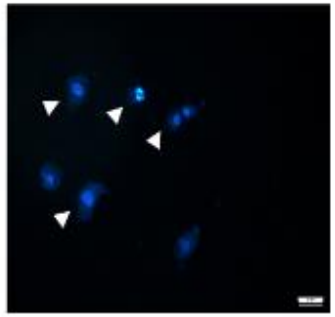
Negative control				Positive control
Untreated	Hoechst	No Staining	DMSO	Cisplatin
			0.1%	15μM
				

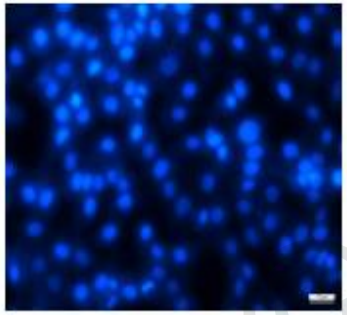
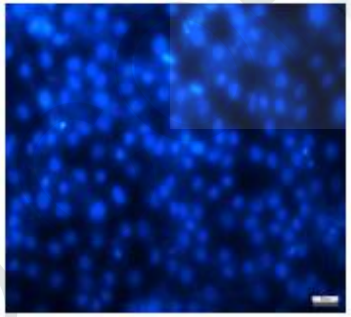
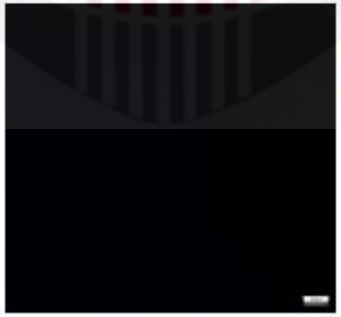
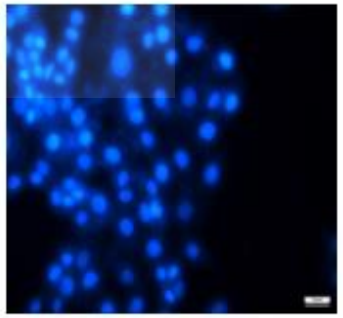
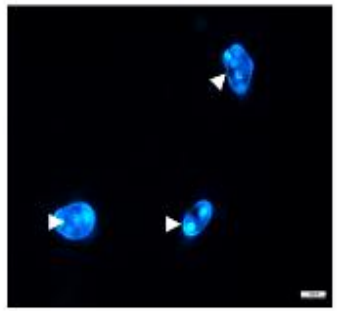
b) 48 hours

Treatment Concentration (µM)				
Curcumin		BHMC		
50µM	25µM	20µM	15µM	10µM
				

Negative control				Positive control
Untreated	Hoechst	No Staining	DMSO	Cisplatin
			0.1%	15µM
				

c) 74 hours

Treatment Concentration (μM)				
Curcumin		BHMC		
50μM	25μM	20μM	15μM	10μM
				

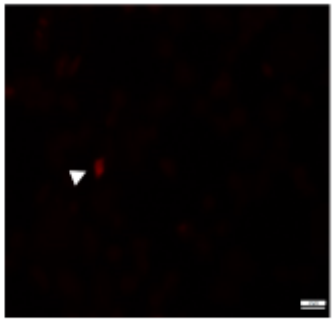
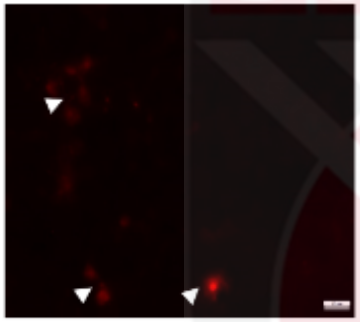
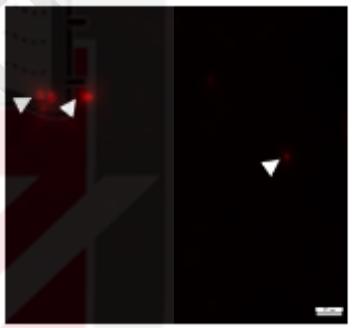
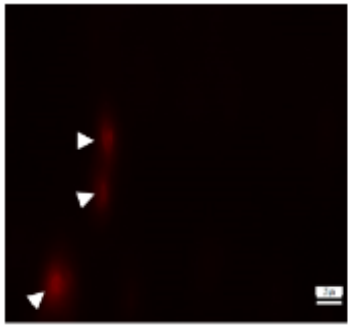
Negative control				Positive control
Untreated	Hoechst	No Staining	DMSO	Cisplatin
			0.1%	15μM
				

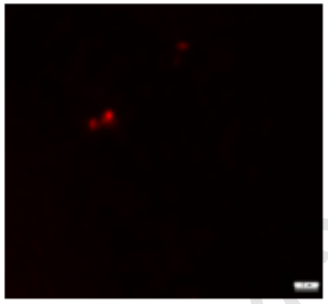
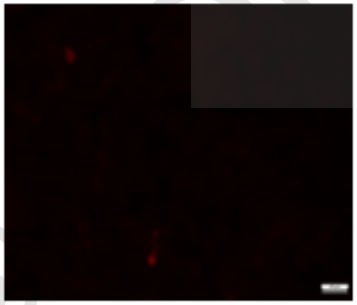
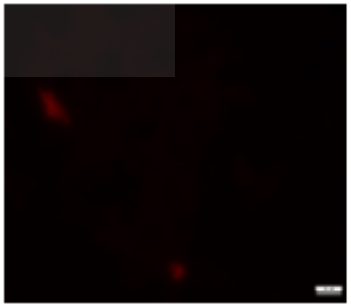
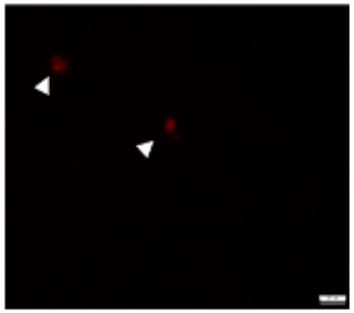
4.2 Curcumin and BHMC induce the apoptosis of HepG2 cells by Propidium Iodide Staining

The morphological changes related to apoptosis in HepG2 were detected PI staining for 24h. There were clear apoptotic features which are chromatin condensation, cell fragmentation and membrane blebbing shown on HepG2 treated with different concentration curcumin and BHMC compared to the untreated cells (control). Besides, the number of apoptotic cell populations was observed significantly increased in PI post-fixation compared to PI pre-fixation **(Table 4.2**).

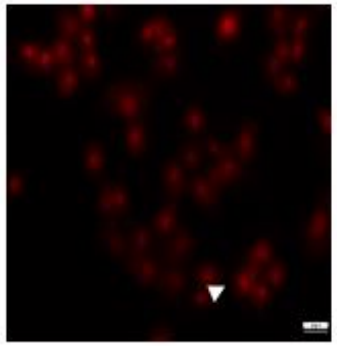
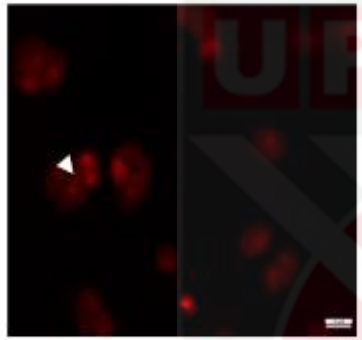
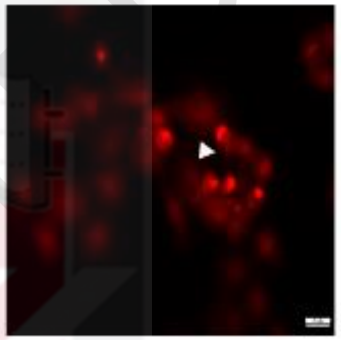
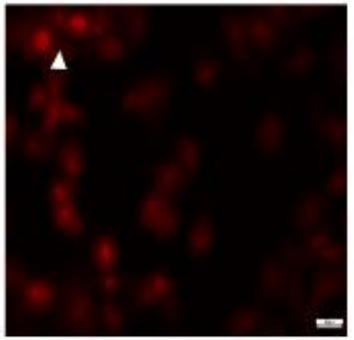
Table 4.2 Induction of apoptosis by Curcumin and BHMC in HepG2 Cell as detected by PI Staining for 24h under the fluorescent microscope (40x magnification). The white arrowhead indicates PI staining can be visible in the early and late stage of apoptosis.

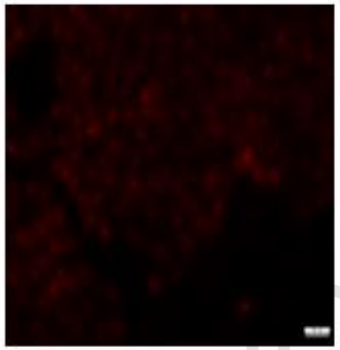
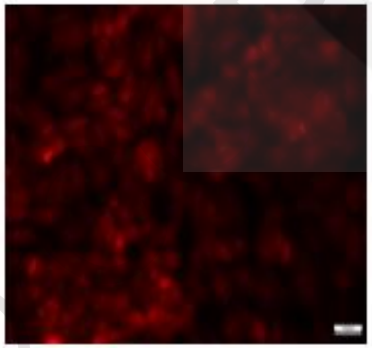
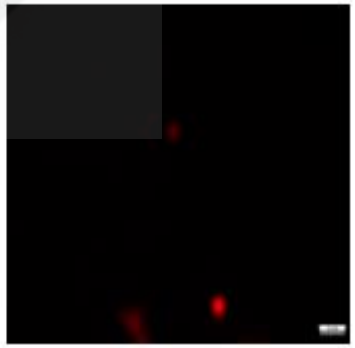
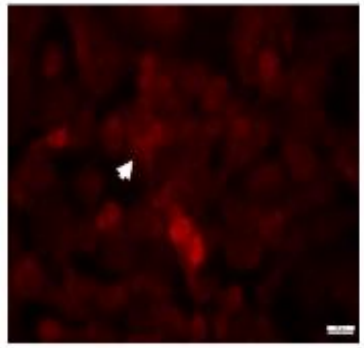
a) Pre-fixation PI staining

Curcumin		BHMC		
50µM	25µM	20µM	15µM	10µM
				

Negative control				Positive control
Untreated	PI	No Staining	DMSO	Cisplatin
			0.1%	15µM
				

b) Post-fixation PI staining

Curcumin		BHMC		
50µM	25µM	20µM	15µM	10µM
				

Negative control				Positive control
Untreated	PI	No Staining	DMSO	Cisplatin
			0.1%	15µM
				

CHAPTER 5

DISCUSSION

Hepatocellular carcinoma (HCC), is the second most common liver cancer-related mortality, and its frequency is rising worldwide. Most patients are diagnosed in the late stages (Yang et al., 2020). Moreover, 80% of HCC patients are currently detected at an advanced stage with a median survival of 6–8 months. The most well-established curative method for HCC is surgical resection followed by chemotherapy. Therefore, due to the spread and size of the tumor in the liver, blood arteries and other critical organs which make the operation on the liver might be difficult. In addition, a significant problem for more than 90% of HCC patients has been the recurrence of the tumor after surgery. However, it led to a lot of studies into ways to improve the current treatment for cancer and less side effects to the patients particularly by using natural anticancer compounds.

Curcumin is a phytochemicals compound reported to have various pharmacological properties such as anti-tumor, antioxidant, anti-inflammatory, and anti-proliferative. Several studies have reported that curcumin has shown limitations due to the poor bioavailability include rapid metabolic rate**,** rapid systemic elimination, low oral absorbability, and chemical stability (Lopresti, 2018; Oglah et al., 2020). Another way has emerged to improve the poor bioavailability of curcumin which is drug delivery systems such as nanoparticles, micelles complexes, liposomes, and phospholipid complexes (Adams et al., 2005). Thus to increase the bioavailability of curcumin, one of the synthetic curcuminoid analogues known as 2,6-bis-(4-hydroxy-3-methoxybenzylidene) cyclohexanone (BHMC) is produced by removing the unstable β-diketone

moiety and converting it into a conjugated double bond while keeping the phenolic OH functional group, which is responsible for curcumin's antioxidant properties (Syed Alwi et al., 2019). In a previous study, it was found that BHMC was more potent towards breast cancer cell lines MCF-7, MDA-MB-231, and SKBr-3 (Harun et al., 2018).

In order to examine the cytotoxic effects of curcumin and BHMC, HepG2 cells were examined for any morphological alterations associated with apoptosis. Apoptosis is defined by a variety of specific morphological changes in the cell's structure (D'Arcy, 2019). Hoechst 33342 and Propidium Iodide staining are a common procedure to recognize apoptosis. When apoptosis arises, the permeability of the cell membrane allows more Hoechst 33342 dye to enter the cell and attach to the nuclear DNA that led to make the apoptotic cells show bright blue (Wei et al., 2021; Tu et al., 2021). In this study, HepG2 cells were stained for 15 mins with 10 ug/mL Hoechst 33342, as described in chapter 3. After being treated with curcumin and BHMC, the HepG2 cells exhibited multiple apoptotic characteristics. Hoechst staining demonstrated the apoptosis characteristics such as membrane blebbing, chromatin condensation, DNA fragmentation, cell shrinkage, and body formation of apoptosis and death cells observed in HepG2 cells but not in untreated cells (control) during 24, 48, and 74 hours under fluorescence microscope at the 40x magnification. Furthermore, the results in **Table 4.1** showed a higher decrease in the cell number population was detected in 74 hours and rise in the formation of apoptotic bodies was observed in BHMC compared to curcumin especially in the incubation time 24 and 48 hours.

Previous research has demonstrated that curcumin altered the morphology of the HepG2 cell line (Liang et al., 2021). In 2007, Cao et al. provided evidence that curcumin-induced apoptosis is significantly triggered by oxidation (mitochondrial DNA damage). In another study done in 2021

by Liang et al, they found that curcumin induced apoptosis in HepG2 liver cancer cells to increase the time and concentration which could led to promoting the apoptosis by regulating ROS production.

On the other hand, a fluorescent double-stranded DNA dye that cannot cross cell membranes is called Propidium Iodide (PI). It produces red fluorescence after binding to DNA. PI dye could only stain the dead or late apoptotic cells as well as could not stain live cells or early apoptotic cells because the plasma membrane is still intact (Lee et al., 2012; Crowley et al., 2016). In this recent study, BHMC and curcumin induced apoptosis in HepG2 cells were analyzed by PI staining and viewed at magnification of 40x. HepG2 cells were treated with various concentrations of BHMC and curcumin for 24 hours. As shown in **Table 4.2,** it was easier to detect the cell death in the post-fixation PI staining. PI staining could be visible in the late stage of apoptosis such as DNA fragmentation mostly in 20 and 15μM of BHMC. In a previous study, they found that BHMC showed presence of cell shrinkage and membrane blebbing as well as necrotic or late apoptotic morphological features by using PI staining in breast cancer cell line, MCF-7 cells (Yeap et al., 2021). The current results are consistent with these previous reports. However, in this study, found that Hoechst 33342 and PI staining suggested that BHMC and curcumin could increase the permeability of cell membrane and induce apoptosis in HepG2 cells line.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The findings revealed that BHMC has shown cytotoxic effects by increasing the morphological changes of the nucleus such as blebbing of cell membrane, chromatin condensation, DNA fragmentation, cell shrinkage, and apoptosis body formation. BHMC also has shown to induce the death cell population on HepG2 more compared to curcumin.

6.2 Further Recommendation

In the future study, further investigation in PI staining to determine if BHMC can induce more cell death populations at 48 and 72 hours. Moreover, BHMC is recommended to be tested on other cancer cell lines.

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APPENDICES

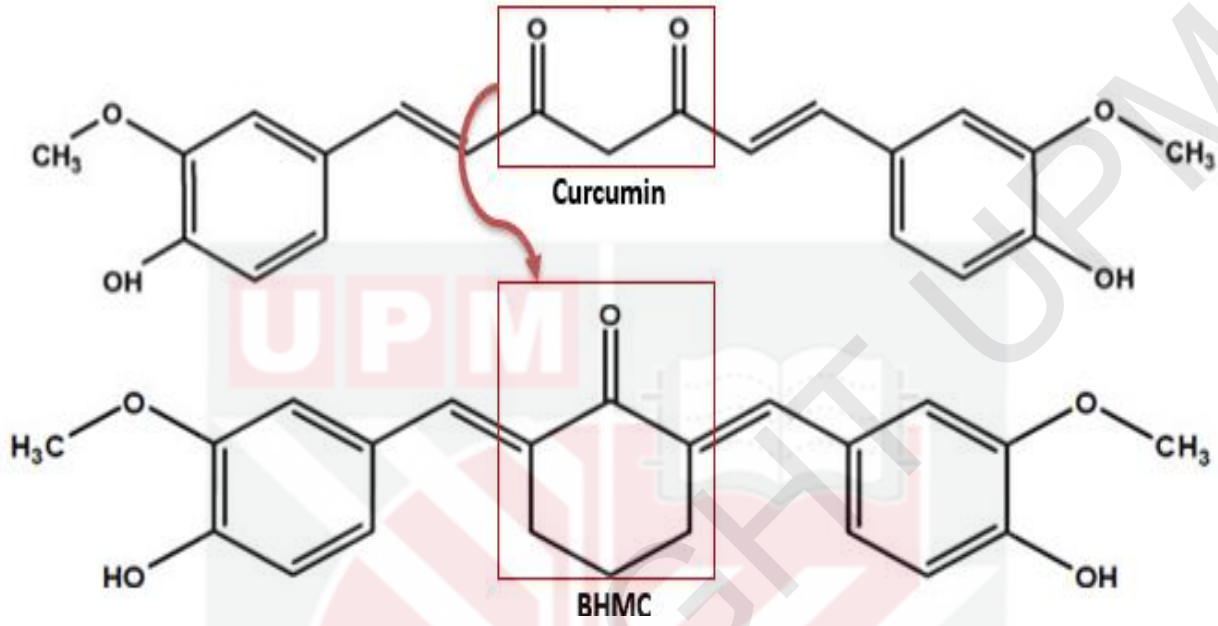

Figure 1: Chemical structure of curcumin and BHMC

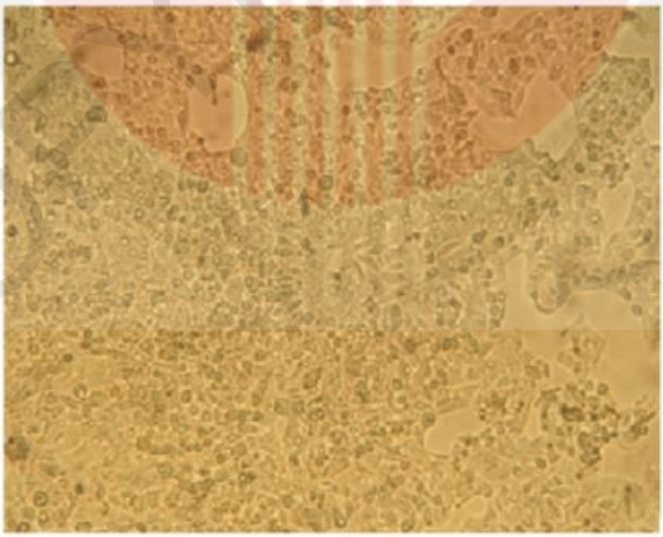

Figure 2: HepG2 cells, detected under light microscope at 10x magnification

(Sugesh et al., 2014)