ORIGINAL ARTICLE

Cytotoxicity, Proliferation and Migration Effects of 2,6-bis-(4-hydroxyl-3-methoxybenzylidine)cyclohexanone (BHMC) on Human Liver Cancer, HepG2 Cells

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ABSTRACT

Introduction: Natural bioactive substances have become increasingly noticeable for their capability to eliminate and counteract cancer throughout time. Curcumin, a bioactive compound derived from the rhizomes of turmeric, is well known for its therapeutic effect in inducing anti-inflammatory, anti-migration, and anti-proliferation activities. However, curcumin encounters several limitations that prevent it from reaching its maximum capabilities. One of the curcuminoid analogues, 2,6-bis-(4-hydroxyl-3-methoxybenzylidine)cyclohexanone (BHMC), was synthesized by removing the unstable β-diketone moiety and changing into double bonds while retaining the hydroxyl group to improve the curcumin's bioavailability. It is aims to investigate the cytotoxicity of BHMC especially on the proliferation and migration effects towards human liver cancer, HepG2 cells. **Methods:** MTT assay was performed to determine the cytotoxicity of BHMC and curcumin on HepG2 and Hs27 cells. Next, Hoechst 33342 and Propidium Iodide staining were executed to observe the morphological changes on HepG2 cells treated with BHMC and curcumin. Further analysis on the migration rate of HepG2 cells upon treatment with BHMC and curcumin was measured using scratch assay. **Results:** At lower concentration, BHMC demonstrated approximately 3-7 times higher toxicity effect towards HepG2 cells compared to curcumin. BHMC also specifically targets HepG2 cells with a selectivity index of up to 6 units which clearly demonstrate its cytotoxic selectivity towards Hs27 cells. Further examination reveals that BHMC induces cytotoxicity via late-stage apoptosis. BHMC also enhanced the inhibition of the migration effects by 4.2, 7.2, and 7.6% throughout incubation period compared to the untreated and curcumin. **Conclusion:** Despite the pronounced toxicity of BHMC on HepG2 cells, BHMC was demonstrated more selective cytotoxic on Hs27.
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Keywords: BHMC; Curcumin Analogue; Apoptosis; Migration; Hepatocellular Carcinoma Cells

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INTRODUCTION

Cancer is known to be one of the leading causes of death worldwide. According to Sung et al. (1) based on data from the International Agency for Research on Cancer (IARC), approximately 19.3 million new cases of cancer and 10.0 million deaths were reported in 2020. With these new cases, liver cancer has been reported to be the sixth most diagnosed and second most common cause of premature death from cancer. Despite the reducing incidence and mortality rates of liver cancer in some Eastern Asian countries, an increasing rate was continuously observed in many previously low-incidents countries worldwide such as US, Australia, and several European countries. This has created an alarming situation to across the world.

In Malaysia, liver cancer ranked as the eighth most common cause of cancer in both gender (2) and the third leading cause of cancer death among men (3). There are several risks factors identified that may contribute to the development of liver cancer including hepatitis B virus, hepatitis C virus, fatty liver disease, diabetes, and obesity. The poor prognosis has made it more difficult for diagnosis and treatment leading to poor survival rate. Despite the advancement of treatment, it has been reported that some of the treatments were only effective in less than one-third of the patients with drug resistant observed approximately six months after starting the regimen (4). Therefore, further research is needed to find better treatments with less adverse effects.

Thus, on this note, several studies have reported the novel anticancer effects possessed by some of the medicinal plants. The bioactive compounds extracted from the medicinal plants play important role in executing their potential effects. Curcumin or diferuloylmethane is one of the most well-known phytochemicals found in the rhizome of Curcuma longa (turmeric), a member of the Zingiberaceae family. The well-established research on this compound have reported its numerous beneficial effects in terms of pharmacology, such as antioxidant, anti-inflammatory, anti-migration, and anti-cancer activities (5). Curcumin's ability to regulate a range of cytokines, growth factors, transcription factors, kinases, and enzymes demonstrates that it has an extensive variety of pharmacological effects (6). Numerous studies showed that curcumin suppressed several cancer cells proliferation, transformation, invasion, metastasis, angiogenesis, and chemo-resistance while also inducing apoptosis (7). In another study by Prasad et al. (8) stated that curcumin can regulate a wide variety of molecular targets, including transcription factors, growth factors, receptors, inflammatory cytokines, enzymes, cell survival molecules, metastatic molecules, and apoptotic molecules, enabling it to modulate a variety of biochemical and molecular cascades.

However, curcumin has its own limitation that is poor bioavailability, due to the low chemical stability, low cytoplasmic availability, low water solubility, and low cellular uptake (9) which makes it not recommended for therapeutic potential (10). With the idea that chemical synthetic able to enhance the efficacy and safety of the known natural metabolites, further chemical modification is needed to improve the bioavailability, stability and selectivity of the curcumin (11). Thus, variety of strategies to enhance the curcumin's bioavailability has been done including nano formulations, the addition of adjuvants, liposomes, phospholipid complexes, and structural alterations to enhance its therapeutic effects while preserving its safety profile (12). A curcumin analogue known as 2,6-bis(4-hydroxy-3-methoxybenzylidene) cyclohexanone (BHMC) (Figure 1 (B)) has later been synthesized to improve the curcumin's bioavailability while preserving its therapeutic benefits. BHMC is synthesised by removing the unstable β-diketone moiety, converting into conjugated double bonds, and keeping the phenolic -OH functional group of curcumin (13,14). This modification is essential to ensure better selective cytotoxic effects on cancer and normal cell lines. Further research on BHMC has reported its efficacy in exerting its cytotoxic effect on MCF-7, an oestrogen dependent cell as well as on MDA-MB-231, a triple negative breast cancer (TNBC). It also showed better in vitro cytotoxicity and in vivo antitumor effect on murine 4T1 TNBC cells compared to its parental compound, curcumin via preventing cell growth, metastasis, inducing apoptosis, and decreasing inflammation at considerably lower concentrations (15). The cytotoxic selective effect of BHMC on normal breast cancer cell line, MCF10A makes it more suitable to be developed as one of the effective anticancer agents.

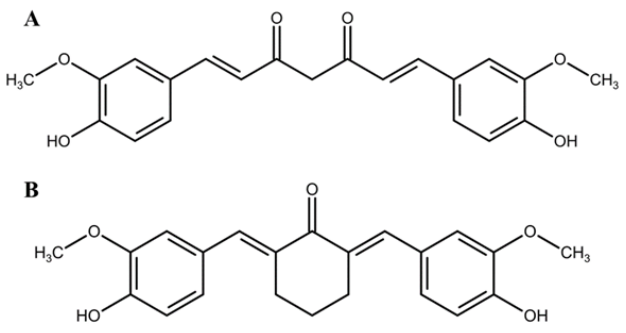

Figure 1: Chemical structure of (A) curcumin and (B) BHMC.

According to Syed Alwi et al. (5), BHMC was also observed to exhibit greater cytotoxicity effect on HepG2 cells compared to curcumin. This has been further confirmed with the morphological analysis that demonstrated signs of apoptosis such as cell shrinkage, membrane blebbing, and the production of apoptotic bodies in HepG2-treated BHMC. Despite the known effectiveness of BHMC in exerting better toxicity on HepG2 compared to curcumin, there is yet any extensive report on the mechanism that elucidating the treatment pathways. Moreover, the greater cytotoxic and anticancer effect of BHMC as well as being able to be more selective in suppressing the inflammatory mediators was postulated due to the presence of α,β-unsaturated bis-enone system in BHMC that replace the enol moiety in curcumin. Therefore, the anticancer effect of BHMC on human liver cancer cells, HepG2 will be further investigated and compared with its parental compound, curcumin. This is hoped to give us more insight on the how the chemical modification able to maintain the safety of the natural metabolites but enhance the efficacy, bioavailability, and selectivity of the compound.

MATERIALS AND METHODS

Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM) containing 4.5g/L Glucose, along with L-Glutamine and sodium pyruvate, 0.25% Trypsin/EDTA solution with phenol red, dimethyl sulfoxide (DMSO), penicillin streptomycin mixed solution, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were all procured from Nacalai Tesque (Kyoto, Japan). Phosphate buffer saline (PBS) tablets and Hoechst 33342 staining solution were acquired from Oxoid, Thermofisher Scientific (Massachusetts, USA). Fetal Bovine Serum (FBS) was obtained from Tico Europe (Amstelveen, Netherlands). Absolute ethanol denatured (99.6%) and sodium hydroxide were purchased from Systerm Chemicals (Selangor, Malaysia). Propidium iodide was sourced from BD Bioscience (New Jersey, USA). Paraformaldehyde and Bovine Serum Albumin (BSA) were bought from

Sigma-Aldrich (Missouri, USA).

Preparation of Compounds

Curcumin, the parental compound, with purity ≥ 98% (HPLC) (CAS: 458-37-7) (Figure 1 (A)) was bought from Nacalai Tesque (Kyoto, Japan). Meanwhile, BHMC was kindly supplied by Associate Professor Dr. Lam Kok Wai, Faculty of Pharmacy, Universiti Kebangsaan Malaysia (UKM). It was produced synthetically from curcumin structure as described in Razak et al. (15) (Figure 1 (B)) with purity ≥ 99.9% through HPLC analysis. Prior to being diluted to the desired concentration for the experiments, BHMC and curcumin were first dissolved in 100% DMSO as a stock at a concentration of 50 µM. The final DMSO concentration in each experiment was held constant at 0.1%.

Cell Lines and Cell Culture

The human liver cancer cell line, HepG2 (HB-8065), and the normal human fibroblast cell line, Hs27 (CRL-1634), were acquired from the American Type Culture Collection (ATCC) located in Virginia, USA (16,17). The HepG2 cells originated from a 15-year-old Caucasian male teenager, while the Hs27 cells were obtained from the skin of a newborn. Both cell types are characterised as adherent cells. HepG2 cells have a replication rate of 48 hours, whereas Hs27 cells double in number every 36 hours. The culture process involved incubating the HepG2 and Hs27 cell lines in a growth medium that contained 10% foetal bovine serum (FBS) and 1% penicillin streptomycin solution in DMEM. The cell culture was performed at a temperature of 37˚C using an Eppendorf Galaxy 170R/S CO2 Incubator (Hamburg, Germany), which maintained a 5% carbon dioxide (CO2) environment. Both cell lines were allowed to grow until they reached 80-90% confluency before being utilised in subsequent assays.

***In Vitro* Growth Inhibition Assay**

The MTT assay was carried out using previously reported methods by Azmy et al. (18), with slightly improvisations. The MTT assay measured how mitochondria dehydrogenase metabolised 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to produce an insoluble formazan precipitate, which is only found in live cells to determine the IC_{50} value from the dose and time-response graphs. In brief, 4X104 HepG2 and Hs27 cells were seeded in triplicate into a 96-well plate containing 100 µL of complete growth media. Next, the cells were treated with BHMC or curcumin at various concentrations (0.78, 1.56, 3.13, 6.25, 12.50, 25.00 and 50.00 µM) into designated well. After incubation time (24, 48 and 72 hours), 20 µL of 5 mg/mL MTT solution was added to each well followed by further incubation for 4 hours in 5% Eppendorf Galaxy 170R/S CO2 Incubator (Hamburg, Germany). Then, media containing MTT solution was removed and 100 µL of 100% DMSO was added into each well to allow the solubilisation of blue formazan crystals. The plate was measured using a Tecan Infinite F50 microplate reader (MΔnnedorf, Switzerland) at 570nm for the test wavelength and 630nm for the reference wavelength. In order to attain substantial quantitative analysis, the experiments were duplicated three times concurrently for each concentration and contrasted with untreated group. The percentage of cell viability will be calculated by using the formula below:

$$\text{Cell Viability (\%)} = \frac{\text{Absorbance of treated group-Absorbance of Blank}}{\text{Absorbance of untreated group-Absorbance of Blank}} \times 100$$

Hoechst 33342 Staining

Morphology observation was performed via Hoechst 33342 staining with minor modifications to the methods mentioned by Sun et al. (19). First, a 6-well plate with a coverslip was seeded with 3 mL of HepG2 cells at a cell density of 3X105 cells/mL. Next, the cells were treated with 3 mL of BHMC (10, 15 and 20 µM), curcumin (25 and 50 µM), 15 µM cisplatin and 0.1% DMSO respectively and incubated at 37˚C in 5% Eppendorf Galaxy 170R/S CO2 Incubator (Hamburg, Germany) for 24, 48 and 72 hours. Then, 10 µg/mL Hoechst 33342 stain solution was prepared in PBS. Following incubation period, the plate was fixed with 1 mL of fixation buffer (4% Paraformaldehyde, 1 M of NaOH, PBS) into each well of 6-well plate then incubated for 1 hour at 37˚C in 5% CO2 incubator. After incubation time, the fixation buffer was removed and the plate was stained with 1 mL of 10 µg/mL Hoechst 33342 stain solution followed by incubation for another 15 minutes at 37˚C in 5% CO2 incubator. The coverslip was transferred and mounted onto glass slide in dark. The slide was observed under Leica DM2500 digital fluorescence microscope (Leica Microsystems CMS GmbH, Heerbuug, Switzerland) and image showing hallmarks of apoptosis was captured.

Differential Propidium Iodide Staining

Pre-fixation Propidium Iodide Staining

Morphological pre-fixation PI staining was determined using the Hezel et al. (20) method with some modification to investigate the stage of apoptosis, which was followed by BHMC or curcumin induction. With the impermeability of PI to live cells, it is widely used as pre-fixation PI staining to detect dead cells in populations by binding to DNA through intercalation between the bases. Briefly, 3 mL of complete growth media containing 1X106 HepG2 cells/well were seeded into coverslips in a 6-well plate. Next, the cells were treated with 10, 15 and 20 µM of BHMC, 25 and 50 µM of curcumin, 15 µM cisplatin, 0.1% DMSO and untreated cells into each flask followed by incubation of 24 hours. On the next day, the plate was washed once with PBS followed by addition of 3 mL of 5 µg/ml of PI stain solution and incubated for 1 hour in 5% Eppendorf Galaxy 170R/S CO2 Incubator (Hamburg, Germany) at 37°C. Then, the stain solution on the plate was removed

and washed once with PBS after the incubation period followed by fixation with 3 mL of Fixation Buffer containing Paraformaldehyde, Sodium Hydroxide and PBS. After incubation of 1 hour, the slide was observed under Leica DM2500 digital fluorescence microscope (Heerbuug, Switzerland) and image was captured.

Post-fixation Propidium Iodide Staining

Post-fixation PI staining was performed in accordance to method by Hezel et al. (20) with minor adjustment. In post-fixation PI staining, the dye can intercalate with the nuclear and nucleic acid in the cytoplasm of living cells. The protocol is similar to the pre-fixation of propidium iodide staining section up to the treatment of BHMC or curcumin. On the next day, the plate was washed once with PBS followed by fixation with 3 mL of Fixation Buffer containing Paraformaldehyde, Sodium Hydroxide and PBS then incubated at 37°C for 1 hour in 5% Eppendorf Galaxy 170R/S CO2 Incubator (Hamburg, Germany). Following that, the plate was washed once with PBS followed by addition of 3 mL of 5 µg/ml of PI stain solution. After incubation of 1 hour, the Leica DM2500 digital fluorescence microscope (Heerbuug, Switzerland) was used to observe the slide and image was captured.

Cisplatin is one of chemotherapeutic drug that has been used for liver cancer treatment. Cisplatin was used in pre-fixation and post-fixation propidium iodide (PI) staining as it induces DNA cross-linking and other cellular damage, leading to membrane permeabilization in non-viable cells. This allows PI to intercalating with DNA after entering the cells. PI dye act as marker for late-stage apoptosis and necrosis due to damaged and leaky membrane (21,22). As pre-fixation PI staining only able to cross the damaged membrane, post-fixation PI staining was performed to observe the detail structure of cells. It was performed to further confirm the Hoechst 33342 staining on the morphological changes.

Migration Scratch Assay

To assess the migration effect of BHMC on HepG2 cells, scratch assay was performed in accordance to method by Alexander et al. (23) and Abruzzese et al. (24) with minor improvisation. In short, HepG2 cells were seeded at 1x106 cells/well in triplicate into 6-well plate containing 3 mL complete growth media. Next, the plate is then scratched into a single straight line using SPLScar™ scratcher (Gyeonggi-do, South Korea). Under an inverted microscope, the scratch area on the plate was observed at 0 hour. Subsequently, the cells were treated with 10, 15, and 20 µM of BHMC, 25 and 50 µM of curcumin, 15 µM cisplatin, 0.1% DMSO, and untreated cells in designated wells using serum-free media (DMEM). The plates were observed under inverted microscope after 24, 48 and 72 hours of incubation in 5% Eppendorf Galaxy 170R/S CO2 Incubator (Hamburg, Germany) at 37°C. The data was captured using digital camera, DinoEye 2.0 Dino-Lite connected to the Motic AE31 Inverted Biological Microscope (California, USA) and computer. The data was then analysed using Image J to quantitatively measure the cells migration at each time point. All experiments were performed in triplicate and the standard error of mean was calculated. Percentage of migration was calculated as below:

$$\text{Percentage of Migration} = \frac{Do - Dn}{Do} \times 100\%$$

Do = Initial distance
Dn = Measured distance at incubation time

Statistical Analysis

GraphPad Prism 8 (GraphPad Software) was utilised to analyse the data. To compare BHMC and curcumin with the untreated, a one-way analysis of variance (ANOVA) was performed, followed by Dunnett's post hoc test. Statistical significance was determined at a level of $p<0.05$. The data were presented as mean ± S.E.M. and represent of three independent experiments.

RESULTS

Cytotoxicity of BHMC and Curcumin on Growth Inhibition of HepG2 and Hs27 Cells Determined by MTT Assay

To evaluate the cytotoxic effects of BHMC and its parent compound, curcumin, on HepG2 and Hs27 cells, MTT assay was performed. Inhibitory concentration that kills 50% of HepG2 and Hs27 cells population (IC_{50}) were measured. The calculated IC_{50} values were tabulated in Table I as mean ± S.E.M. from three independent trials. Our data showed that BHMC or curcumin induced their toxicity effects in concentrations- and time- dependent manners. Table I showed a significant difference in the IC_{50} value of BHMC or curcumin across the incubation period. At 24 hours incubation, BHMC significantly kills HepG2 cells with a 3-fold less concentration compared to curcumin with IC_{50} value of 16.73 ± 0.69 µM. At longer incubation period of 48 and 72 hours, greater cytotoxic

Table I: Cytotoxicity of BHMC and curcumin on HepG2 and Hs27 cells reflected by IC_{50} values at incubation time of 24, 48 and 72 hours by using MTT assay.

Incubation Time (Hours)	IC_{50} ± S.E.M. (µM)				Selectivity Index (SI)	
	HepG2		Hs27			
	BHMC	Curcumin	BHMC	Curcumin	BHMC	Curcumin
24	16.73 ± 0.69[a, b]	46.03 ± 0.26[c, d]	34.32 ± 2.84	>50.00 ± 0.00	2.05	1.09
48	4.77 ± 0.61[a]	26.00 ± 2.79[c]	28.50 ± 3.68	>50.00 ± 0.00	5.97	1.92
72	4.67 ± 0.15[b]	18.07 ± 1.92[d]	28.04 ± 8.95	>50.00 ± 0.00	6.00	2.77

Data from three independent experiments are presented as mean ± S.E.M. [a,b,c,d] Similar superscript letters indicate significant differences ($p<0.05$) compared within same compounds at incubation time of 24, 48, 72 hours.

effects were observed which BHMC significantly kills HepG2 cells with IC_{50} values as low as 4.77 ± 0.61 µM and 4.67 ± 0.15 µM, respectively. Meanwhile, BHMC was observed to be selective cytotoxic in normal fibroblast, Hs27 cells with IC_{50} values of approximately 7-fold less toxic compared to the IC value of HepG2 cells treated BHMC. The selective cytotoxic potential of BHMC was further confirmed with the selectivity index (SI) value of more than 2 indicating BHMC is considered to have the selective cytotoxic activity towards cancer and normal cells. In our study, we want to focus on BHMC effect in HepG2 cells. Curcumin was included as it is the parental compound of BHMC. It can be seen that BHMC did have significant effect when treated on HepG2 cells.

Effect of BHMC and Curcumin on Morphological Changes in HepG2 Cells using Hoechst 33342 Staining

To observe the influence of BHMC and curcumin on the morphological alterations in apoptosis in HepG2 cells, Hoechst 33342 staining was performed. Hoechst dye has the ability to cross the cell membranes of the living and dead cells and bind to the DNA fragments that are released from the nucleus, allowed it to label the total number of cells (25). Hoechst 33342 dye was selected from other Hoechst dyes due to the presence of lipophilic ethyl group which significantly higher permeability to cells especially living cells (26). Figure 2 reveals the disclosed features of apoptosis including nuclear shrinkage (NS), chromatin condensation (CC), nucleus margination (MN), membrane blebbing (BL), and apoptotic bodies (AB) at 40X magnification marked under fluorescence microscopes by arrow. After 24 hours of treatment with 10 µM BHMC, HepG2 cells started to exhibit nuclear shrinkage and nuclear margination. The presence of apoptotic bodies was observed to increase with the increased concentrations of BHMC at 15 and 20 µM. Longer period of 48 hours incubation demonstrated more cells population underwent late stage of apoptosis at a concentration as low as 10 µM. However, despite the presence of membrane blebbing after prolonged treatment of 72 hours, no apoptotic bodies were observed.

Similar apoptosis features of chromatin condensation, membrane blebbing and apoptotic bodies were observed in HepG2 cells treated curcumin at 25 and 50 µM. Comprehensively, our data revealed that these features were seen to increase upon higher concentrations and longer incubation treatment when treated with BHMC or curcumin. Thus, these characteristics were in parallel with the IC_{50} obtained for HepG2 cells treated BHMC or curcumin. Although BHMC and curcumin able to exert their toxicity via apoptosis induction, a relatively higher concentrations of curcumin were needed to execute this force.

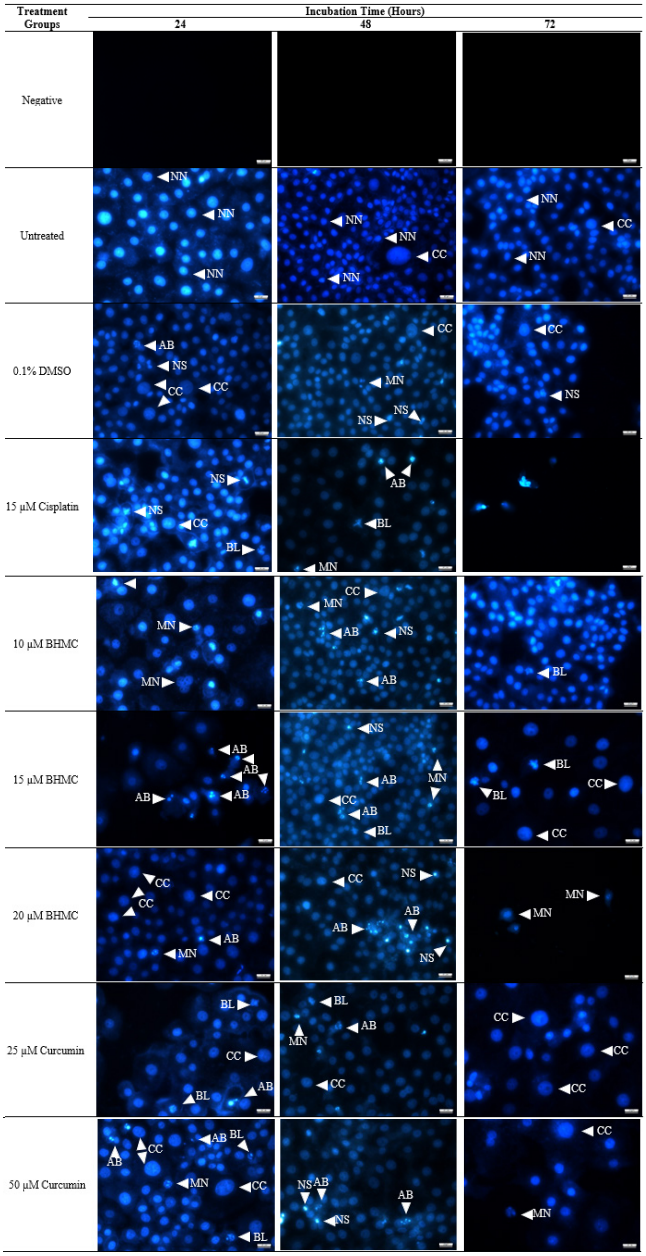

Figure 2: The Hoechst 33342 staining images of the HepG2 cells treated with BHMC and curcumin for 24, 48 and 72 hours. Nuclear shrinkage (NS), chromatin condensation (CC), nucleus margination (MN), membrane blebbing (BL), and apoptotic bodies (AB) are shown by arrows.

Effect of BHMC and Curcumin on Morphological Changes in HepG2 Cells using Differential Propidium Iodide Staining

Further morphological analysis on HepG2 cells treated BHMC or curcumin was assessed using different approach that is propidium iodide (PI) staining. Unlike Hoechst, PI is a red fluorescent dye that has been used as a marker to late-stage apoptosis and necrosis by binding to DNA of dead or dying cells with damaged or leaky membrane (21,22). Pre-fixation PI staining was used as PI permeable to cells with damaged membranes

to bind to DNA and RNA to differentiate the live and dead cells. Post-fixation PI staining showed more details structure as it can intercalate with the nucleic acids in both living and dead cells' cytoplasm and nucleus. Figure 3 shows the image of HepG2 cells treated with BHMC or curcumin in pre- and post-fixation PI staining at 24 hours. The number of HepG2 cells that was stained with PI was higher in post-fixation condition compared to pre-fixation. Lower number of stained HepG2 cells were observed in pre-fixation PI staining due to PI stain only indicate the dead cells resulted from membrane disruption as consequent of apoptosis. However, in post-fixation PI staining, higher number of HepG2 cells were seen that allowed to visualise the cells' morphology more detail. In HepG2 cells treated with selected concentrations of BHMC or curcumin, there were clear indications of apoptosis, such as nuclear shrinkage, chromatin condensation, cell disintegration, membrane blebbing and apoptotic bodies, as compared to the untreated group in post-fixation PI staining. The late stage of apoptosis was observed at concentrations of 15 and 20 µM of BHMC in post-fixation condition.

Effect of BHMC and Curcumin on Migration of HepG2 Cells using Scratch Assay

One of the characteristics of cancer is the capability of the cancer cells to migrate and infiltrate the surrounding area. Therefore, to investigate the effects of BHMC or curcumin on the proliferation and migration of HepG2 cells upon treatment, a migration scratch assay was conducted. The percentage of cell migration in HepG2 cells treated BHMC or curcumin for 24, 48, and 72 hours was measured using the scratch assay.

The HepG2 cells treated with BHMC and curcumin at 0 and 24 hours were portrayed in Figure 4. After 24 hours of treatment, it was observed that HepG2 cells treated with BHMC and curcumin do not exhibit any noticeable closure at any concentrations (10, 15, and 20 µM of BHMC) (25 and 50 µM of curcumin). Both treatment groups also do not exhibit any migrations or growth. Scratch assay is known as one of the semi-quantitative analyses that might not display visible closure. Therefore, further analysis to investigate the rate of cells migration was done using Image J as illustrated in Figure 5. Data obtained supported our findings by demonstrating significant decrease in the migration percentage upon treatment with BHMC or curcumin after 24 hours as compared to the untreated group. Both compounds have different effects depending on the concentration.

Figure 5 (A) revealed that 15 and 20 µM of BHMC demonstrated significant suppression of HepG2 cells proliferation and migration after 24 hours of treatment by approximately 2.5, 2.6 and 4.2% at 10, 15 and 20 µM, when compared to the untreated group, respectively. Further incubation of 48 hours demonstrated that BHMC substantially reduced the migration percentage

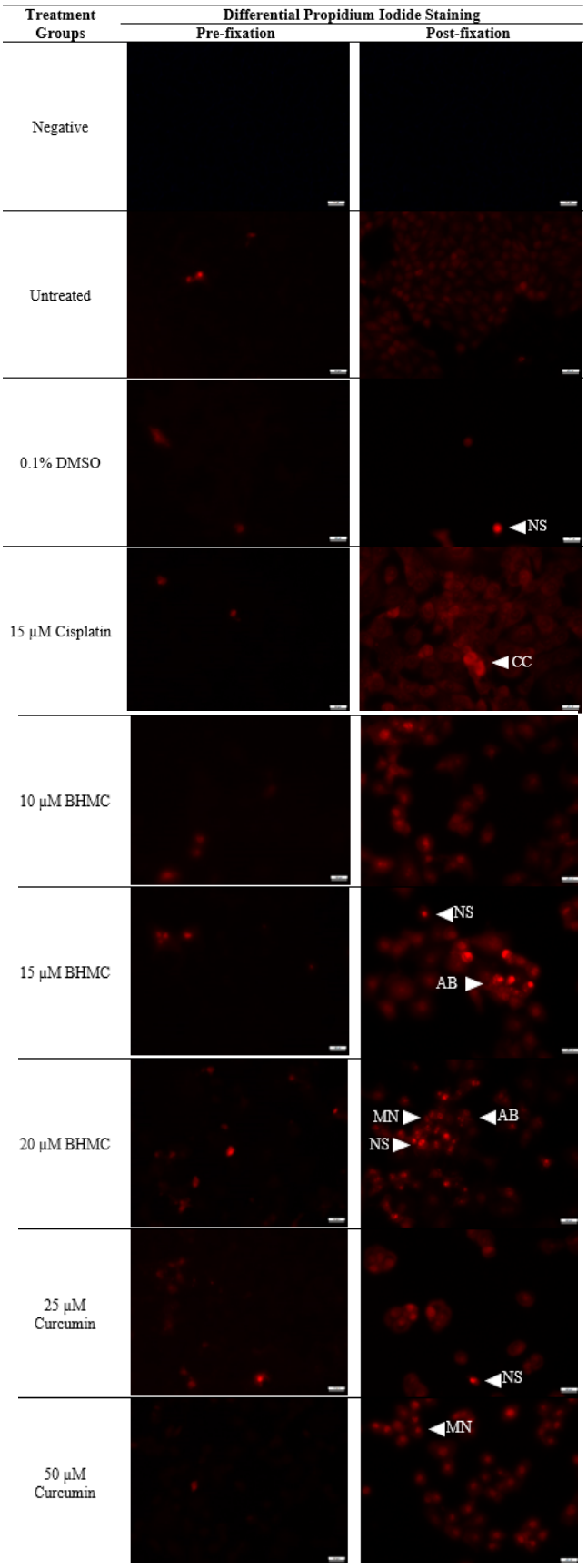

Figure 3: The differential Propidium Iodide staining images of the HepG2 cells treated with BHMC and curcumin for 24 hours. Scale bar, 25 µm (40x magnification).

Figure 4: The images of the HepG2 cells migration at 0 and 24 hours upon treatment of BHMC and curcumin. Arrows indicate the length of sides of scratch at each timepoints. Migration rates are expressed in percentage of migration after 24, 48 and 72 hours compared to initial area. Scale bar, 10 mm (20x magnification).

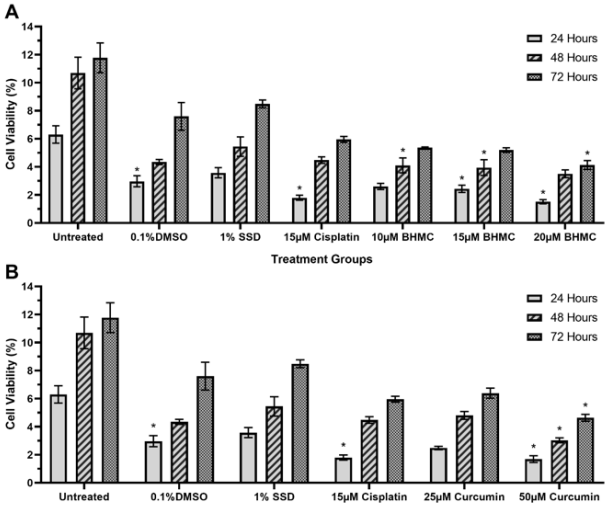

Figure 5: The effect of (A) BHMC and (B) curcumin on migration of HepG2 cells. Data are presented as mean ± S.E.M. and represent of three independent experiments. Statistically significant differences are indicated with *p<0.05 between treatment groups at same incubation time One-way ANOVA followed by Dunnett's post hoc tests compared to untreated group.

of HepG2 cells at lower concentrations which were 10 and 15 μM in comparison to the untreated group. BHMC at the concentrations of 10, 15 and 20 μM reduced the migration percentage up to 6.6, 6.7 and 7.2% after 48 hours of treatment, respectively. Meanwhile, after the 72 hours treatment, only 20 μM of BHMC significantly suppressed the migration of HepG2 cells at 6.4, 6.6 and 7.6% compared to the untreated group.

Comparatively, curcumin at a concentration of 50 μM significantly lowered the percentage of HepG2 cells migration compared to the untreated group at all incubation periods as shown in Figure 5 (B). However, no significant difference observed upon treatment at a concentration of 25 μM, at all incubation period (24, 48 and 72 hours). Thoroughly, the migration rate of HepG2 cells reduced by curcumin upon treatment with 25 and 50 μM at 24 hours were 2.6 and 3.9%, respectively.

Meanwhile, the positive group of cisplatin was observed to have significant antimigration effect at 24 hours of treatment. At 48 hours, curcumin reduced up to 5.9 and 7.7% when treated with 25 and 50 μM concentrations, respectively. Meanwhile, at 72 hours, HepG2 cells were reduced by 5.4 and 7.1% at 25 and 50 μM concentrations, respectively. Both BHMC and curcumin acted on time- and concentration-dependent manners in suppressing the HepG2 cells migration. However, BHMC was observed able to suppress cells migration at a concentration of as low as 15 μM, making it a good anti-migration agent compared to curcumin.

DISCUSSION

Numerous research has reported the potency of bioactive compounds with anticancer properties on various type of cancer cells. One naturally occurring phytochemical in turmeric known as curcumin is renowned for having several pharmacological activities, such as antioxidant, anti-inflammatory, anti-migration and anticancer properties (5). Its pharmacological characteristics are explained by its capacity to modulate a variety of cytokines, growth factors, transcription factors, kinases, and enzymes in signalling pathways involved in cellular proliferation, differentiation, and apoptosis (6). According to recent studies, curcumin may provide therapeutic benefits for a variety of illnesses, including cancer (27), Alzheimer's disease (28), Parkinson's disease (29), and cardiovascular conditions (30). Numerous research has looked at the potential of curcumin as an anti-cancer agent in recent years, concentrating on its ability to prevent cancer cells from proliferating (31), mutating (32), invading and metastasizing healthy tissue (33), and angiogenesis (34) while also leading them to die.

Despite the massive pharmacological properties and effective modulatory activity of curcumin (35), it is still not advocated for therapeutic purposes due to its poor bioavailability. Thus, to increase its

potency, bioavailability, stability, and selectivity, it is recommended to do some modification on the chemical structure. This structural modifications yield various derivatives that can maximise its anticancer activity, which can be the solution to its poor solubility, instability, and interference in several modalities of evaluation in vitro (5,15). Recent research provides even more credence to the notion that synthetic chemistry can improve the efficacy and safety of identified natural metabolites (11). In this study, BHMC has been produced by swapping out the β-diketone moiety for monocyclic ketone that has α,β-unsaturated bis-enone system in order to increase its biological activities such as antioxidant and anticancer (5,36). An analogue that contains conjugated enones or compounds that resemble enones, have the ability to engage with the Michael acceptor and react only with the target nucleophiles (36). Therefore, this structure may have contributed to the increased cytotoxicity of BHMC on HepG2 cells in vitro, an effect that was comparable to the earlier in vitro investigations on human liver cancer, HepG2 cells (5).

MTT assay was performed as benchmark to observe the difference between each tested compound on a cell line. Previous finding by Syed Alwi et al. (5) is in parallel with our data which BHMC exerted greater cytotoxic effect on HepG2 cells with IC_{50} values ~3-fold more toxic compared to curcumin after 24 hours treatment. Meanwhile, longer treatment durations of 48 and 72 hours demonstrated more effective cytotoxic effect of BHMC on HepG2 cells with approximately 7-fold more potent compared to curcumin. However, it is important to note that an effective anticancer treatment should not be able to harm cancer cells more than it already has (37). It also should not cause any adverse effects on the normal cells. Therefore, in an effort to identify whether these compounds are selective cytotoxic, normal Hs27 cells were treated with BHMC or curcumin. Interestingly, despite the great cytotoxic effect exhibited by BHMC on HepG2 cells, BHMC was observed to have less toxicity on normal Hs27 cells. This was further confirmed with the selectivity index (SI) of BHMC on Hs27 cells which was more than 2 after 24, 48 and 72 hours of treatment incubation period (Table 1). SI is a measurement tool used to determine a selective cytotoxic property of a compound with greater SI indicates more selectivity. If a compound has a SI larger than 2, it is suggested to demonstrate greater selective cytotoxic activity, whereas if it has a SI of less than 2, it is exhibiting simply general cytotoxic activity (38). Thus, for treatments on HepG2 and Hs27 cells that were executed in time- and concentrations-dependent manner, it was revealed that BHMC was more potent towards HepG2 cells and yet execute its selective cytotoxicity on normal Hs27 cells compared to curcumin.

Prior study by Syed Alwi et al. (5) reported that BHMC exhibited more cytotoxic effect on normal 3T3 cell in all concentration and time points than HepG2 cells. This effect was further supported with the presence of apoptosis features as morphological changes in 3T3-treated BHMC. In the current study, MTT assay was still performed on HepG2 cells to confirm if the data is reproducible. Meanwhile, the normal cell line used for our current study is Hs27 cells which proved that BHMC is cytotoxic selective compared to the 3T3 cells done by Syed Alwi et al. (5) in the previous study. Previous research has also discovered that BHMC not only exerted its selective cytotoxicity on normal cells but also on many cancer cell lines including MDA-MB-231 cells (39), 4T1 cells (15), HepG2 cells (5) and MCF-7 cells (40) in time- and concentration-dependent manner. Meanwhile, curcumin demonstrated to be more selective cytotoxic towards MDA-MB-231 cells compared to MCF-7 cells (40). The synthetic curcumin analogues with various chemical alterations and structural variations might demonstrate various forms of selective cytotoxicity against certain cells (41). Hence, it is expected that when exposed to different cell types for various exposure durations, various curcumin analogues would elicit varied selective cytotoxicity. Thus, to further confirm the mode of cell death triggered by BHMC or curcumin, Hoechst staining was performed. Several concentrations BHMC and curcumin were selected based on the IC_{50} values obtained from MTT assay. For BHMC, the concentrations selected were 10, 15, 20 μM while for curcumin, the concentrations selected were 25 and 50 μM.

The Hoechst 33342 dye is a type of dye that does not intercalate and binds to the minor groove of DNA in regions of living cells that are rich in A-T base pairs. It has the ability to visualise and analyse nuclei morphology by the emission of blue fluorescence after being excited by ultraviolet or blue light (26). Thus, the cytotoxic effects of BHMC or curcumin was further evaluated by looking at the morphological changes linked to apoptosis upon treatment with BHMC or curcumin. Apoptosis is known as a programmed cell death. It is a mechanism that allows cells to execute self-destruction. It is characterized by different stages of morphological and biochemical characteristics including DNA fragmentation, cell shrinkage, chromatin condensation, membrane blebbing and apoptotic bodies formation (42). Other than that, morphological changes in the current study also using Hoechst 3342 and propidium iodide staining which later observed via fluorescence microscope compared to previous study by Syed Alwi et al. (5) which do not used any staining and only observed using light microscope.

Based on our observations on HepG2 cells treated with BHMC or curcumin at selected concentrations, all the apoptotic characteristics were observed after 24, 48 and 72 hours of treatment. Our data demonstrated that HepG2 cells began to exhibit nuclear margination and nuclear shrinkage after 24 hours of treatment with 10 μM BHMC. It is noteworthy that when HepG2 cells

was treated with higher concentrations of BHMC (15 and 20 μM), there was an increase in the manifestation of late apoptosis markers such as the presence of apoptotic bodies in HepG2 cells. Longer treatment of 48 hours with BHMC demonstrated a more prominent features of late-stage apoptosis that were presented in all concentration. However, prolonged treatment of 72 hours showed lower frequency of late-stage apoptosis characteristic. This was suggested due to HepG2 cells has fully underwent apoptosis and were washed away as debris during the procedure. In normal human body condition, the existence of apoptotic bodies, nuclear condensation, and membrane blebbing, will subsequently lead to phagocytosis by neighbouring cells including macrophages and parenchymal cells (5).

Similarly, the manifestation of nuclear shrinkage, chromatin condensation, nucleus margination, membrane blebbing and apoptotic bodies was also observed in HepG2 cells treated with 25 or 50 μM of curcumin in all incubation periods. Thus, while both BHMC and curcumin able to execute apoptosis as a means of its cytotoxicity, curcumin requires a relatively higher concentration to achieve this effect compared to BHMC. This aligns with the IC_{50} values observed in HepG2 cells treated with either BHMC or curcumin. Comparatively, our data showed that HepG2 cells treated BHMC was more potent in inducing late-stage of apoptosis feature with concentration as low as 15 μM at 24 hours and 10 μM at 48 and 72 hours than curcumin. Further analysis on the morphology changes of HepG2 cells treated with BHMC or curcumin were determined by differential PI staining which stained the dead cells. As similar to the Hoechst staining, HepG2 cells treated with BHMC at the concentrations of 15 and 20 μM demonstrated the late-stage characteristics of apoptosis including nuclear shrinkage, nuclear margination, and apoptotic bodies. However, in HepG2 cells treated with curcumin, only the margination of nuclear was observed at 50 μM as the late-stage apoptosis feature. The ability of BHMC to induce apoptosis was also reported in the MCF-7 cells with similar morphological characteristics. BHMC treatment caused decrease in the number of MCF-7 cells and resulted in severe deformation, including cell shrinkage and detachment, as well as progression to necrotic or late apoptosis stage (40). This was further confirmed by Syed Alwi et al. (5) in the morphological examination without any staining on HepG2 cells treated with BHMC that showed more apoptotic signs compared to HepG2 cells treated with curcumin. Thus, BHMC is suggested to be an efficient growth inhibitory agent that can kill HepG2 cells at low concentrations through apoptosis.

Cancer metastasis and invasion is one of the hallmarks of cancer in its development and progression (43). It only selectively invade nearby cells and tissues after spreading from their main cancerous site with angiogenesis occurs simultaneously for the nutrients and oxygen supplementation (44,45). To further validate on the antiproliferation and antimigration effects of BHMC on HepG2, scratch assay was performed. As illustrated in Figure 5 (A), quantitative analysis demonstrated BHMC at concentrations of 10, 15 and 20 μM significantly reduced the migration of HepG2 cells by approximately 6.6, 6.7% at 48 hours and 7.6% at 72 hours compared to the untreated group. In contrast, HepG2 cells treated with curcumin at a concentration of 50 μM significantly lowered the percentage of cells migration by approximately 7.1% throughout the incubation period compared to untreated cells (Figure 5 (B)). The findings indicated that BHMC could inhibit cell migration at all concentrations, particularly at 20 μM, and may possess greater anti-migration effect that is more significant than curcumin.

According to earlier research, curcumin also able to inhibit SCC-25 oral squamous cell carcinoma (46), MDA-MB-231 (47) and MCF-7 (48) breast cancer cells from migrating and invading. Our data coherent with the study by Harun et al. (49) that reported BHMC at a concentration of 12.5 μM suppressed the migration percentage of MDA-MB-231 breast cancer cells by forming invadopodia. By suppressing the expression of invadopodia-related proteins p-SRC, β-PIX, MMP9, and MT1-MMP, BHMC prevents the development of the cytoskeleton during the formation of invadopodia. Invadopodia formation was inhibited by BHMC as low as 1.6 μM, but at 12.5 μM, there was a noticeable reduction in the expression of the β-PIX, MMP9, and MT1-MMP proteins (49). Moreover, it was revealed that BHMC had a greater effect than curcumin at IC_{50} value in reducing HepG2 cells migration. An in vivo study by Razak et al. (15) reported that in breast cancer mice model, the proliferation and metastasis of breast cancer cells treated with BHMC reduced. In the same study, BHMC delayed the tumour formation at day 9, reduced the tumour size by 3 folds, lower cancerous mitotic number up to 3 folds, less metastatic activity compared to curcumin.

Additionally, a study by Yeap et al. (40) found that the expression of VEGF and SNAIL, two genes involved in the metastasis of cancer cells, is inhibited in breast cancer cells treated with BHMC as a result of miR-3195 and miR-30a-3p being markedly elevated. MMP9, TNF-α, IL-1β, IL-4, G-CSF, and NF-kB gene expression can all be greatly reduced by BHMC, however curcumin significantly reduces similar gene expression except MMP9 (15). VEGF, SNAIL and MMP9 is a crucial regulator for the ability of cancer cells to metastasize, this suggested that BHMC have higher anti-migration activity than curcumin. Hence, our results were consistent with earlier research on the anti-migration activity of BHMC in HepG2 cells, specifically at 20 μM up to 7.6% compared to curcumin, which requires a somewhat higher concentration of 50 μM with a reduction of only 7.1 %. Thus, our investigation revealed that BHMC not

only able to exert its selective cytotoxicity on cancer and normal cells, it also has impact on cellular proliferation and migration, which eventually lead the cells die via apoptosis.

CONCLUSION

All in all, our data demonstrated that, in comparison to curcumin, BHMC displayed greater cytotoxic to HepG2 cells in a concentration- and time-dependent manner. Moreover, HepG2 cells are more sensitive to the cytotoxic effects of BHMC compared to fibroblast Hs27 cells. It has also been shown that BHMC able to reduce the proliferation and migration of HepG2 cells as well as trigger apoptosis which can be seen from the prominent presence of apoptosis characteristics compared to curcumin. These observations were validated using the growth inhibition assay, morphological analyses, and migration scratch assay. We, the authors of this study aim to confirm the molecular target associated with the BHMC mechanism on HepG2 cells in the near future. These studies would provide further evidence supporting the ability of BHMC to induce apoptosis in liver cancer cells by regulating specific molecular targets associated to genes and proteins.

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