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Unveiling the Potential of *Hyptis Capitata* and *Hyptis Brevipes* Compounds in Overcoming Apoptosis Resistance and Inducing Targeted Cell Cycle Arrest in Breast Cancer Stem Cells

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Abstract

Breast cancer stem cells (CSC), as a kind of tumor cells, are able to regenerate themselves, leading to apoptosis resistance and cancer relapse. In this study, we investigated the potential of Hyptis Capitata and Hyptis Brevipes compounds in preventing apoptosis resistance and inducing targeted cell cycle arrest in CSC. The cytotoxic effects of the compounds were evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay and the cell cycle distribution and apoptosis induction were assessed through flow cytometry analysis. The phytochemical characterization of *Hyptis bravipes* extract (HBE) and *Hyptis capitata* extract (HCE) conducted under thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analysis. The results revealed that Hyptis Brevipes extract (HBE) exhibited strong cytotoxicity towards CSCs, with an IC50 value of 89 µg/mL. In contrast, Hyptis Capitata extract (HCE) showed weak growth inhibition, with an IC50 value greater than 500 µg/mL. Subsequent analysis demonstrated that HBE induced cell cycle arrest in the G2/M and S phases and significantly increased apoptosis in the CSC population. Furthermore, phytochemical characterization using TLC and HPLC revealed that HBE and HCE share similar constituent compounds, with HBE exhibiting a more diverse compound content and stronger cytotoxic effects. These findings provide valuable insights into the potential of Hyptis Capitata and Hyptis Brevipes compounds as targeted agents against breast cancer stem cells and contribute to understanding their chemical profiles and pharmacological properties.

Keywords: Hyptis bravipes, Hyptis capitata, CSC, Apoptosis resistance, Cell cycle arrest

Introduction

Breast cancer is the cancer with the highest incidence in the world, accounting for 24.5 % of all cancer cases [1]. The death rate of breast cancer continues to rise to 6.9 % in 2022 [2]. The main factor contributing to the increase in breast cancer deaths is the occurrence of recurrence and chemoresistance due to the development of resistant apoptosis in the cancer stem cell (CSC) population [3,4]. Currently, breast cancer is treated with first-line chemotherapy, doxorubicin, and paclitaxel [5]. Previous studies have shown that the presence of CSC populations in breast cancer patients leads to treatment failure, resulting in recurrence and death [6,7]. There is a need for compounds with minimal side effects targeting CSCs. Herbal medicines

are used more frequently in cancer treatment because they accurately target cancer cells and cause less damage to healthy cells. Moreover, anticancer drugs from natural products have shown diverse ways of inducing cell death [8]. Throughout history, researchers have investigated various plant species for their anticancer properties. Flavonoids, carotenoids, alkaloids, and phenolics are some of the most extensively researched plant chemicals known for their wide-ranging medicinal effects and ability to combat tumors [9]. Our previous study reported that Hyptolide, a lactone compound from Hyptis pectinate, strongly inhibits cancer growth and metastatic triple-negative breast cancer [10]. α-β unsaturated lactone is a compound belonging to the group of secondary metabolites that has anticancer activity in several types of cancer cells and can target CSC [11].

One of the components of α-β unsaturated lactone that has been studied in CSCs is the compound hiptolide from the plant Hyptis pectinata (L.) Poit, but the activity of this compound is moderate [12-15]. Our previous study found that α-β unsaturated lactone compounds from other plant families of hyptis such as Hyptis capitata and Hyptis brevipes have a stronger antioxidant potential than Hyptis pectinata. 1 characteristic of CSCs is that they have a low level of reactive oxygen species (ROS), which allows them to resist apoptosis and senescence, leading to high progression [16-20]. We have successfully isolated α-β unsaturated lactone compounds from the plants Hyptis capitata and Hyptis brevipes, including bravipolite A and bravipolite D. However, the anticancer effects of these α-β unsaturated lactone isolates from Hyptis capitata and Hyptis brevipes on CSC populations have not yet been explored. Therefore, in this study, we evaluated the anticancer effects of Hyptis capitata and Hyptis brevipes on CSC populations.

The phenomenon of chemoresistance and resistant apoptosis in CSCs is caused by the ability of CSCs to inactivate drugs, alter target cells, suppress the accumulation of drugs entering cells through increased efflux pumps, and overexpress regulatory proteins such as OCT4, Nanog, and SOX2 [21,22]. To date, chemotherapy approaches have only been able to kill non-CSC cell populations and cause CSCs to enter a dormant state, leading to relapse and resistant apoptosis [23,24]. The ability of CSCs to evade apoptosis mechanisms is due to their ability to decrease ROS levels and counterbalance with their own antioxidants, thereby suppressing proapoptotic proteins such as caspase-3, Bax, and Bcl-x, and inducing proapoptotic proteins such as Bcl2 and Bcl-xl [25-27]. Therefore, this study aims to explore the potential of Hyptis capitata and Hyptis brevipes extract in inducing CSC apoptosis.

Methods

Extraction of *Hyptis bravipes* and *Hyptis capitata*

Leaves of *Hyptis bravipes* and *Hyptis capitata* deposited in the Department of Chemistry at Universitas Diponegoro, Indonesia, were harvested outside the flowering period. These leaves were dried in an oven (model MA-037) with air renewal and circulation at 37 °C until fully dehydrated. The dried leaves were then ground into a fine powder using a blender. An extract was prepared from 500 g of this powder by mixing it with ethanol 70 % in a 1:10 (w/v) ratio, shaking continuously for 24 h at 37 °C, and then filtering. The filtrate was evaporated under rotary vacuum evaporator to obtain the ethanolic crude extract, which was stored at 4 °C. Prior to use, the extract was reconstituted in dimethylsulfide to the desired concentrations [10,13].

Characterization of *Hyptis bravipes* and *Hyptis capitata* Extract

Hyptis bravipes extract (HBE) and *Hyptis capitata extract* (HCE) were characterized under thin layer chromatography (TLC) with the extracts dissolved in Methanol. Both extracts were eluted on silica plates with Chloroform: Diethyl ether (4:1) and analyzed under UV lamp light. Analysis using high-performance liquid chromatography (HPLC) was also performed by identifying the retention time and UV spectral

pattern of α,β-Unsaturated δ-lactone in the extracts on HPLC with a detection λ of 206 nm. The scanning spectrum pattern was set at a wavelength of 200 - 400 nm. A mixture of Acetonitrile: 0.1 % KH_2PO_4 (40:60 v/v) on a LiChrospher C18, 250×4 mm column was used isocratically.

Cell culture

MDA-MB-231 human breast cancer cell line was obtained from the American Type Culture Collection (#HTB26 ATCC, Manassas, VA, USA). MDAMB-231 is cultured in high glucose Dulbecco's modified Eagle's Medium (DMEM) (Gibco, USA) enriched with 10 % fetal bovine serum (Gibco, USA), 12.5 µg/mL amphotericin B (Gibco, USA), 150 µg/mL streptomycin, and 150 IU/mL penicillin (Gibco, USA). The cells were cultured at 37 °C and 5 % CO_2.

Breast cancer stem cell (CSC) isolation and validation

MDAMB-231 cells were analyzed for the presence of CSC by flow cytometry. CD44 and CD24 antibodies conjugated to magnetic microbeads (Miltenyi Biotec Inc, CA) were used to obtain BCSCs from MDAMB-231 cells. The cell population with $CD44^+$ $CD24^-$ was classed as CSC. The CSC population was isolated based on the cell surface expression of CD44 and CD24 by magnetic-activated cell sorting (MACS) system with anti-CD44 and anti-CD24-biotin combined anti-biotin microbeads (Miltenyi Biotec Inc, CA). Positive selection was performed using MS columns, and negative selection using LD columns (Miltenyi Biotec Inc, CA). The positive $CD44^+$ $CD24^-$ phenotype was confirmed by flowcytometry (BD Biosciences, Franklin Lakes, New Jersey) with anti-CD44-FITC and anti-CD24-PE monoclonal antibodies (BD Biosciences, Franklin Lakes, New Jersey) [28,29].

Cytotoxic assay

The effect of *Hyptis capitata* extract (HCE) and *Hyptis bravipes* extract (HBE) on CSC cell and normal vero cell proliferation was determined using MTT assay [30]. Briefly, 8×10^3 cells/well^2 of CSC and 5×10^3 cells/well^2 of vero cell were cultured in a 96-well plate for 24 h. Then, the treatment with 0 - 500 µg/mL of HCE and HBE was applied for 24 h. Untreated cells are considered as negative controls (DMEM containing DMSO 0.01 %). After the treatment, the medium containing the extracts was replaced by fresh complete medium and 0.5 mg/mL MTT (Sigma-Aldrich) was added for 4 h. Then, DMSO was added to dilute formazan crystals, and the OD of the supernatant was measured at λ595 nm under ELISA reader (Biorad iMarkTM Microplate Reader). The IC_{50} value was calculated through linear regression equation (Y= bX + a). The data for this study was conducted through 3 replication experiments [31].

Cell cycle analysis

MDAMB-231 breast cancer cells were cultured in the 6-well plates in the presences of HBE 45 and 90 µg/mL for 24 h. After treatment, the cells were incubated with BD Cycletest (BD Biosciences, USA) according to manufacture instructions. Finally, the percentage of cell distribution were determined by flow cytometry (DB Accury C6 plus, BD Biosciences, USA) [10].

Apoptosis analysis

The cells were treated with HBE 45 and 90µg/mL for 24 h. The quantification of apoptosis cells was measured by Annexin V-PI assay (BD Biosciences, USA). Briefly, after incubation the cells were harvested and incubated with 5µL Annexin V-FITC and 5µL PI (50µg/mL) for 30 min at 4 °C in the dark. Finally, the cells were analyzed by flow cytometry (DB Accury C6 plus, BD Biosciences, USA) [32].

Statistical analysis

Statistical analyses were accomplished with software SPSS 26.0 (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard deviation (SD). Data analysis used one-way ANOVA and continued with the Least Significant Difference (LSD) test with p-value < 0.05.

Results

Phytochemical characterization of HCE and HBE

To elucidate the possibility of HBE has the strong cytotoxic effect of HCE, we characterized the phytochemical properties of those extract using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The analysis using TLC revealed the presence of similar spots in HBE and HCE (**Figure 1**(**A**)). The presence of compounds with similar characteristics is indicated by spots with the same polarity. The TLC results provide crucial information that HBE and HCE share the same constituent compounds. The analysis also showed chromatogram peaks at a retention time of 5.007 min for HCE and 5.257 min for HBE (**Figures 1(B) - 1(C)**). The peaks at the longer retention time for HBE is due to its more polar compounds, causing the analyte to be retained longer compared to the compounds in HCE. Additionally, the HPLC results indicated that the methanol extract of HBE exhibited a greater number of chromatogram peaks compared to HCE suggesting a more diverse compound content in the methanol extract of HBE. These findings provide valuable insights into the similar and diverse compound compositions of the 2 Hyptis species, contributing to the understanding of their chemical profiles and potential pharmacological properties.

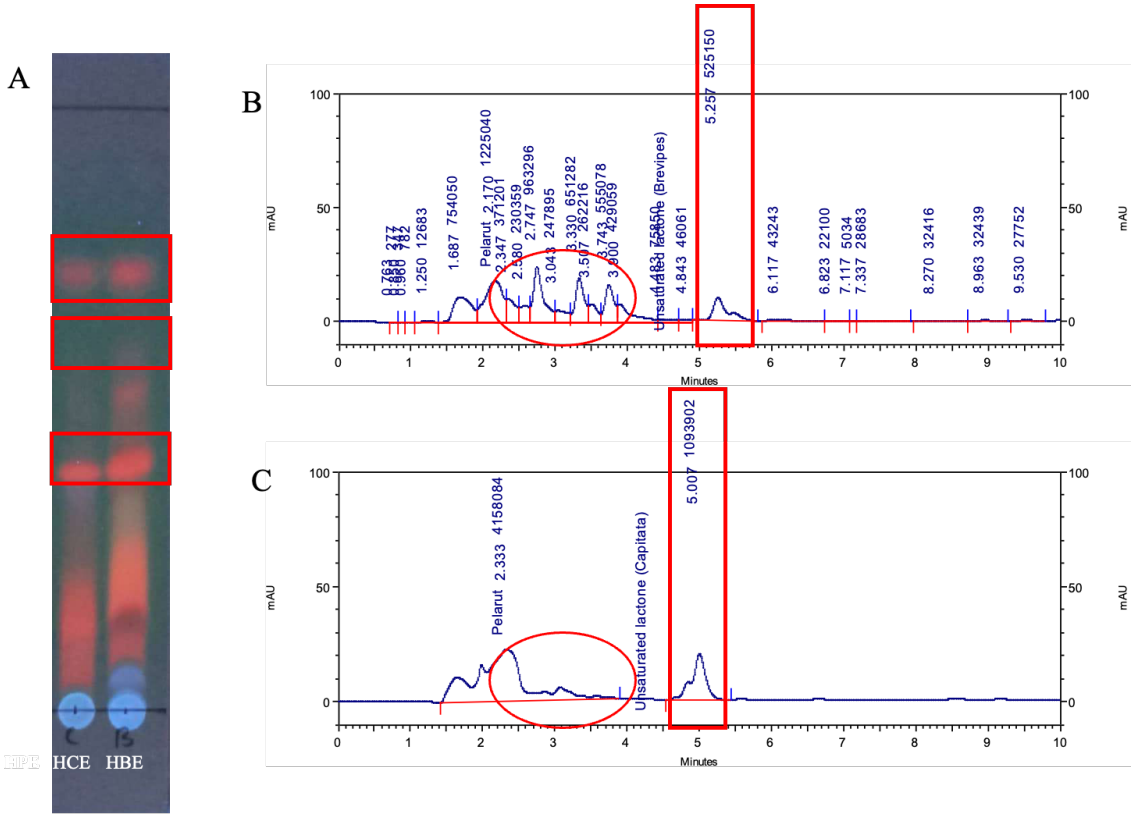

Figure 1 Phytochemical Characterization of HBE and HCE. (A) Thin Layer Chromatography (TLC) Analysis (B) High Performance Liquid Chromatography (HPLC) Analysis of HBE and (C) HCE.

HBE and HCE inhibited CSC cells proliferation

In this study we used CSC cells population characterized CD44+/CD24-. High-level CD44 expression has been associated with cancer progression, whereas low-level CD24 expression has been associated with nondifferentiated cells. Furthermore, the cytotoxic effect of HBE and HCE on CSC was analysed using MTT assay. We found that HCE exhibits weak growth inhibition of CSC cells, as indicated by an IC50 value greater than 500 µg/mL (**Figure 2(A)**). However, HBE shows strong cytotoxicity towards CSC, with an IC50 value of 89 µg/mL (**Figure 2**(**B**)). For the analysis of cytotoxicity of HBE and HCE on normal cells, we utilized Vero cells as the model. Interestingly, our findings indicated that HBE and HCE did not exhibit toxic effects on these normal cells, even at high concentrations. The cell viability remained above 80 %, demonstrating the safety of HBE and HCE in normal cell conditions (**Figures 2(C) - 2(D)**). This suggests that HBE may have a more potent effect on CSC cell population compared to HCE, highlighting its potential as a cytotoxic agent for CSC cell population. Therefore, in the next study we focus on exploring the potential of HBE in CSC.

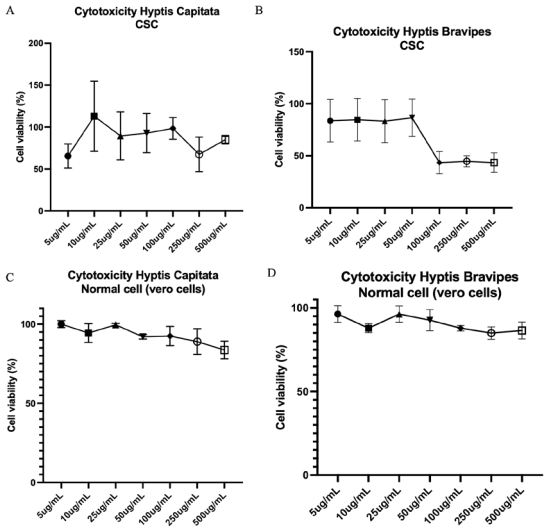

Figure 2 Cytotoxic effect HCE and HBE on CSC and vero cells for 24 h. (A) cytotoxic activity of HCE and (B) HBE on CSC population. (C) cytotoxic activity of HCE and (D) HBE on normal vero cells. The columns represent the mean ± SD 3 independent trials with at least 3 replicates. Statistical difference was analyzed using one-way ANOVA; $^{*}p < 0.05$.

HBE induced cell cycle arrest on CSC

The effect of HBE on cell cycle distribution on the CSC were explored to elucidate insights into the mechanism of its anti-proliferative activity. In CSC, 0.40 ± 0.10 % untreated cells were in Sub G1 phase, 58.76 ± 2.00 % in G0/G1 phase, 20.46 ± 0.91 % in S phase, 20.36 ± 1.45 in G2/M phase.

HBE on the ½ IC50 value (45µg/mL) induced the accumulation of CSC in G2/M phase up to 42.10 ± 0.82 ($p < 0.05$). Interestingly, in the IC50 dose (90 µg/mL) HBE induced S phase cell cycle arrest up to 51.36 ± 1.80 ($p < 0.05$) (**Figures 3(A) - 3(B)**). The dysregulation of the cell cycle at this phase can lead to apoptotic cell death, therefore too elucidate the mechanism of HBE involved in the regulation of cell cycle arrest that associated with cytotoxic activity, we next evaluated the effect of HBE on the apoptosis induction.

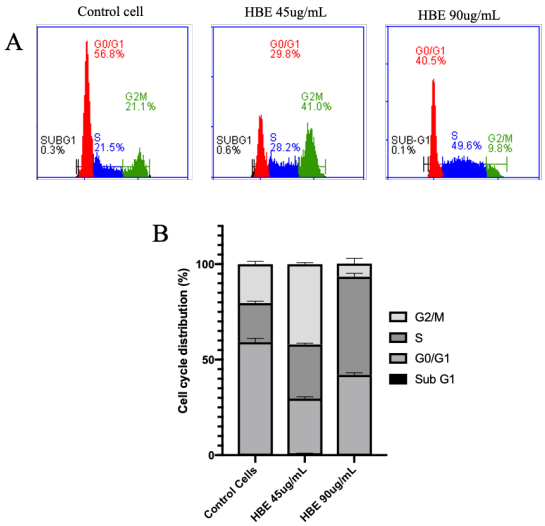

Figure 3 Cell cycle distribution of HBE CSC cells. The assay was conducted based on flow cytometry as describe in the method. (A) The flowcytometry profile of the treatments. (B) The percentage cell cycle distribution of each phase under different concentration treatment of HBE for 24 h on CSC cells. The columns represent the mean ± SD 3 independent trials with at least 3 replicates. Statistical difference was analyzed using two-way ANOVA; $^{*}p < 0.05$.

HBE induced apoptosis of CSC cell population

To explore whether the HBE-induced cell growth inhibition was also due to apoptosis, we evaluated the effect of HBE on cell apoptosis by flow cytometry analysis (**Figure 4(A)**). Apoptosis ranged from 16.00 ± 2.92 to 21.50 ± 4.43 % in CSC (**Figure 4(B)**). The treatment of CSC with HBE resulted in a significant increase the apoptosis cells. In the HBE 45 µg/mL induced cell death up to 19.96 ± 1.42 % and HBE 90 µg/mL also induced apoptosis up to 16.50 ± 0.44 %.

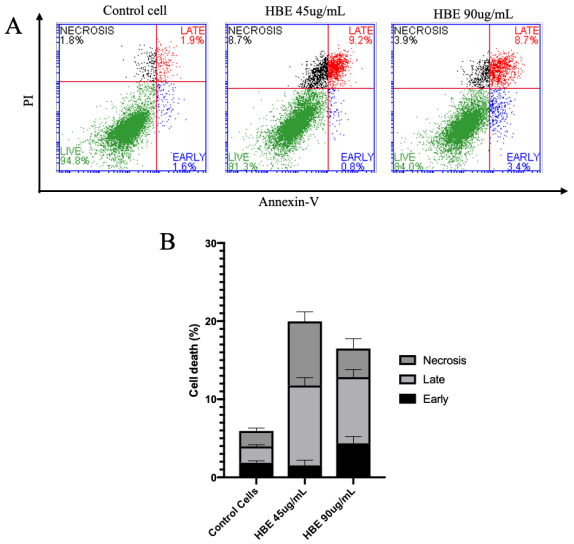

Figure 4 HBE-induced apoptosis of CSC cells. (A) Flow cytometry based assay was performed on 24 h treated cells. (B) The quantification of percentage apoptosis cells under HBE treatment for 24 h on CSC cells. The columns represent the mean ± SD 3 independent trials with at least 3 replicates. Statistical difference was analyzed using two-way ANOVA; $^{*}p < 0.05$.

Discussion

The current study investigated the effectiveness of 2 genus of Hyptis against the population of CSC isolated from MDA-MB-231 human triple negative breast cancer (TNBC) cell line. TNBC cell is a subtype of breast cancer characterized as highly aggressive and poorly differentiated [33]. The CSC, on the other hand, are assumed to be responsible for cancer recurrence and breast cancer resistance to chemotherapy or radiation [33]. MDA-MB-231 cell proliferation have been reported to be inhibited by *Hyptis pectinata*. Therefore, it is interested in exploring the effect *of* other family of Hyptis including *Hyptis bravipes* (HBE) and *Hyptis capitata* (HCE) on the CSC population. According to studies, CD44+ cells displaying a more mesenchymal-like profile frequently enriche in cell proliferation, motility, and angiogenesis genes, and develop tumors in mice. In this study, the HBE cytotoxicity toward the CSC population stronger than HCE (**Figures 1(A) - 1(B)**), indicating that CSC were more sensitive to HBE than HCE. The data lead to explore the profile of HBE cytotoxic activity on the CSC. When exposed to HBE up to 90 µg/mL, CSC arrested at the phases of S and G2/M (**Figures 2(A) - 2(B)**) and apoptosis was increased, significantly (**Figures 3(A) - 3(B)**). However, 45 µg/mL of HBE (IC_{50}) halted the progression of CSC cells at S phase and induced more apoptosis compared with HBE doses 90 µg/mL, indicating that CSC gave a slightly different profile of cell cycle upon HBE treatment.

In this study, phytochemical profiling of the HBE and HCE revealed that those contains a similar compound based on TLC analysis due to have 3 similar spot (**Figure 4(A)**), however the HBE contains compound more polar than HCE (**Figure 4(B)**). Previous study shown that polar compounds can induce leukemia cell differentiation by modulating cell-surface potential, which is linked to the characteristics of CSC and their quiescent status [34,35]. Additionally, the inhibition of specific molecules by polar compounds has been found to reduce the stemness of prostate CSC, mobilize them into the cell cycle, and decrease their proliferation rates [36]. Furthermore, polar compounds have been demonstrated to inhibit the expression of CSC markers and pluripotency factors in colorectal cancer, leading to a decrease in the activity of aldehyde dehydrogenase (ALDH) enzymes, which are associated with CSC-related properties [37,38]. These findings highlight the potential of polar compounds such as HBE to interfere with the characteristics and behavior of CSC, ultimately contributing to the inhibition of CSC growth and the suppression of CSC-related properties. In this study we found that HBE induced different cell cycle phase arrest at different doses. HBE induced the S-phase cell cycle arrest in high doses. This results in line with previous study that showed that polar compounds can induce leukemia cell differentiation by modulating cell-surface potential, which is associated with the cell cycle and the treatment of cancer [34]. Additionally, the action of polar compounds involves the modulation of factors regulating the transition from the G1 to S phase of the cell cycle [39,40]. 1 limitation of this study is the lack of detailed investigation into the potential anti-cancer mechanisms of HCE and HBE, such as their effects on specific signaling pathways. Due to resource constraints, we did not explore these possible mechanisms in our research. Further studies are necessary to elucidate how HCE and HBE exert their anti-cancer effects at the molecular level. Furthermore, studies have demonstrated that cell cycle regulatory proteins are targets for the induced differentiation of transformed cells by polar compounds [35,38]. These findings highlight the potential of polar compounds to impact the cell cycle, specifically by influencing the transition to the S phase, and their role in the treatment of cancer through the induction of cell differentiation.

Conclusions

This study provides the new insight into the effects of HBE and HCE on the CSC. CSC were more sensitive to HBE compare to HCE. HBE increased the levels of S and G2/M cell cycle arrest and apoptosis CSC cells.

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