

RESEARCH ARTICLE

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Hypotolide Promotes Breast Cancer Stem Cells Apoptosis and S-phase Cell Cycle Arrest

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Abstract

Objective: Breast cancer stem cells (BCSCs) develop apoptosis resistance by expressing pro and antiapoptotic proteins. The induction of apoptosis is an important mechanism of action for many anticancer agents. However, recently chemotherapy-induced chemoresistance and increased relapse phenomenon due to BCSCs population-targeted therapy failure. Therefore, treatment using a new compound was supposed to inhibit apoptosis resistance and increase the possibility of cancer apoptosis in the BCSCs population. This study aimed to investigate the effects of the hypotolide, isolate a compound from *Hyptis pectinata* on apoptosis induction in BCSCs. **Methods:** The cytotoxic activity was analyzed using MTT assay. Annexin V-Propidium Iodide measured apoptosis under flow cytometry. Cell cycle arrest was assessed by flow cytometry. The molecular target, key protein, and molecular mechanism of hypotolide targeting BCSCs were determined by bioinformatic analysis. **Result:** Hypotolide inhibited BCSCs cell growth with an IC₅₀ value of 55 µg/mL. The hypotolide cytotoxic effect by apoptosis induction up to 32% through S-phase cell cycle arrest in a dose-dependent manner through regulation of SRC, EGFR, and MAPK1 signaling pathway. **Conclusion:** Taken together, hypotolide has potential for treating BCSCs by targeting SRC, EGFR, and MAPK1 signaling. Further investigation of the molecular mechanisms involved is required to develop hypotolide as a BCSC-targeted drug.

Keywords: Apoptotic- bioinformatics- breast cancer stem cells- hypotolide- targeted therapy

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Introduction

Breast cancer (BC) is one of the most common malignant tumors in women worldwide, with about 20% of all deaths in developed countries [1-3]. Chemotherapy is the current standard therapy to treat BC. However, there is evidence that this therapy's effectiveness is less due to the breast cancer stem cells (BCSCs) population [1, 4]. BCSCs are one of the major causes of the development of chemoresistance in triple-negative breast cancer (TNBC) patients [5]. A previous study reported that TNBC-subtype MDAMB-231 cells had the highest population of CD44⁺/CD24⁻ cells, with a median value, was 72.1% [6]. BCSCs, a subgroup of cancer cells, is responsible for chemoresistance and cancer relapse, as it has the ability to self-renew, apoptosis resistance, and differentiate into the heterogeneous lineages of cancer cells in response to chemotherapeutic agents [7]. Previous studies reported that survival of BCSCs is regulated by the balance between pro and anti apoptosis factors [8-10]. Thus, it is urgent to identify a novel potential agent for the BCSCs targeted.

Drug discovery in the era of information technology has become more accessible, faster and directed to molecular targets with the aid of artificial intelligence,

cheminformatics, and data mining, as well as high throughput screening [11]. One application is using an integrated bioinformatics approach to obtain molecular targets, identify the key proteins, and understand the molecular mechanisms of a drug candidate [12, 13]. Hence, drug development for certain diseases, such as cancer, can be performed faster and more strategically using integrated bioinformatics analysis.

Indonesian medicinal herbs show promising anti-cancer properties, including hypotolide, due to their capability to induce cancer apoptosis on MCF-7 and T47D breast cancer cells [14, 15]. Previous studies reported that hypotolide might possess strong cytotoxic activity on MCF-7 and T47D breast cancer cells through apoptotic induction [16, 17]. In addition, *Hyptis pectinata* extract was also reported to induce late and early cell apoptosis leading to cell cycle arrest and cell death on MCF-7 cells [14]. Recently, studies on hypotolide-targeted BCSCs have yet to be published.

In this study, we combine bioinformatics and in vitro work. An in vitro study is carried out to measure the effects of hypotolide on BCSCs population under apoptosis and cell cycle analysis. In addition, a bioinformatic approach is performed to identify molecular targets, key proteins, and

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molecular mechanisms of hyptolide targeted at BCSCs. This study is expected to be the basis for developing hyptolide as a BCSC-targeted drug for overcoming chemotherapy resistance in breast cancer therapy. This study aimed to investigate the effects of the hyptolide, isolate a compound from *Hyptis pectinata* on apoptosis induction of BCSCs.

Materials and Methods

Plant Material

The herbs of *Hyptis pectinata* were collected in April 2021 from Tawangmangu, Karanganyar Central Java, Indonesia (Latitude 7°40'39.3"S; Longitude 111°08'09.4"E). The biologist from the Ecology and Biosystematics Laboratory, Faculty Science and Mathematics, Universitas Diponegoro, Semarang, Indonesia identified and verified the plants. For biological determination, the herbs of *Hyptis pectinata* were dried with circulated at 40°C and renewed of air oven until completely dehydrated.

Extraction and Isolation Procedure

Hyptis pectinata was cleaned and air-dried to constant weight at room temperature for three days before being ground into powder in a blender. The powder of *Hyptis pectinata* (500 g) was extracted by maceration method using ethanol for 72 h (3 cycles) based on [18] with slight modification. Furthermore, the solutions were filtered through Whatman no.1 filter paper and evaporated under reduced pressure (100 psi) in a rotary vacuum evaporator (IKA HB 10 basic) at 40°C to produce the crude extracts. The extracts were dissolved in water for 24 hours, and the partitioned water-methanol was evaporated until dry using a laboratory freeze dryer LyoQuest Telstar® under a 0.1 mbar pressure for 24 h. Hyptolide was isolated in 1.7% yield from extracts of methanol.

Cell culture

MDAMB-231 (ECACC #92020424) was maintained in Dulbecco's Modified Eagle's Medium (DMEM)-high glucose (Gibco, USA). The BCSCs were cultivated in DMEM F-12 (Gibco, USA). These mediums were supplemented 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). Cells were cultivated at 37°C under 5% CO₂. Culture media were renewed every two to three days, and cells were subculture when confluent of 80-90%. For assays, only cells with >90% viability, passage number <10, and in the log growth phase were used.

BCSCs isolation and validation

MDAMB-231 cells were analyzed for the presence of BCSCs by flow cytometry. CD44 and CD24 antibodies conjugated to magnetic microbeads (Millenia Biotec Inc, CA) were used to obtain BCSCs from MDAMB-231 cells. The cells population with CD44⁺ CD24⁻ were classed as BCSCs. The BCSCs 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 (Multani Biotec Inc, CA) [19]. 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 flow cytometry (BD Biosciences, Franklin Lakes, New Jersey) with anti-CD44-FITC and anti-CD24-PE monoclonal antibodies (BD Biosciences, Franklin Lakes, New Jersey). In addition, BCSCs population was also confirmed by atmosphere capability assay. Mammosphere from the BCSCs population of as much as 1×10⁵ cells / ml was planted on an ultralow attachment well plate. The number of cell collections (diameter > 50µm) for each well was morphologically evaluated under a microscope on days 0, 3 and 7, respectively.

Cell viability assay

The cell viability assay was determined using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay according to [20, 21] with slight modification. Briefly, the density of 5×10³ cells/well was seeded into 96 well-plate and incubated at 37°C under 5% CO₂ for 24 hours. Subsequently, cells were treated in a triple with hyptolide (5-500 µM) and exposed for 24 hours. Untreated cells were regarded as negative controls. After treatment, cells were treated with 0.5 mg/mL of MTT (Biovision, #Cat K299-1000) and incubated further for four hours. MTT formazan was soluble using 100µl DMSO and incubated for 15 minutes. After incubation, the absorbance was measured by ELISA reader (Biorad iMark™ Microplate Reader) at λ 595 nm. The absorbance was transformed into a percentage of cell viability by comparing the treated group with the untreated group at a particular time course. To calculate IC₅₀ value, linear regression between concentration (x) and % cell viability (y), giving the equation y = Bx + A. Using the linear equation of this graph for y = 50 value x point becomes IC₅₀ value, that is the concentration that prevents the cell growth of 50%. The data of this study was carried out with three replication experiments.

Apoptosis Assay

Annexin-V - Propidium Iodide (PI) using flowcytometry was used to determine the apoptosis effect of hyptolide on BCSCs cells according to [22, 17] with slight modification. Cells (2×10⁵ cells/well) were seeded in a 6 well plate and incubated at 37°C. Then, adherent cells were treated with 13.75, 27.5, and 55 µM of hyptolide for 24 h, the control well was treated with 0.1% DMSO. Cells were detached and washed twice with cooled PBS. Subsequently, the cells were collected and resuspended in cold 1x binding buffer and Annexin V and PI (BD Bioscience, #Cat556547) were added into the binding buffer and incubated for 10 min at room temperature in the dark. Analysis was performed on a BD Accuri C6 (BD Biosciences, Franklin Lakes, New Jersey). The tests were performed in three independent experiments. The percentage of cell death includes early apoptosis, late apoptosis and necrosis which is displayed in a bar graph. Furthermore, the acceleration of cell death by the test compound solution is known by comparing between single and combination treatments

with untreated cells.

SPSS version 22.0 (SPSS Inc., Chicago, IL, USA).

Cell cycle Analysis

BCSCs cell (2×10^5 cells/well) were inoculated into 6-well plates and incubated for 24 h. Then, the cells were treated with each group treatment for 24 h. After treatment, cells were harvested, fixed under cold ethanol 70%, and washed twice in PBS. The collected cells were stained with PI 5 μ L (BD Biosciences, #Cat 559341), incubated at 4°C for 30 min. Then, cells were washed twice in PBS and re-suspended in 300 μ L PBS for cell cycle detection using flowcytometry BD Accuri C6. The percentage of the cell cycle distribution is displayed as a bar graph [23].

Acquisition of direct target proteins, indirect protein targets, and BCSCs regulatory genes

Direct target protein (DTP) of hyptolide were searched from SEA prediction (<https://sea.bkslab.org/>) and SWISSTargetPrediction (<https://swisstargetprediction.ch>). BCSCs regulatory genes were retrieved from PubMed with keywords “breast cancer stem cells”. A Venn diagram between all DTP and BCSCs regulatory genes was constructed using Venny 2.1 (<http://bioinfogp.cnb.csic.es/tools/venny/>). The overlapping genes were considered as hyptolide targets (HT) in BCSCs [24, 25].

Protein-protein interaction (PPI) network and KEGG-pathway enrichment of the HT

PPI network analysis among HT was conducted with STRING-DB v11.0 with confidence scores >0.7 and visualized by Cytoscape software. Genes with a degree score of more than 10, analyzed using CytoHubba plugin, were selected as hub proteins. Analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were conducted using WebGestalt with FDR <0.05 selected as the cut-off value [26].

Statistical Analysis

All experimental data are presented as mean $\pm$ standard deviation and analyzed using One way ANOVA. A $p < 0.05$ was considered to indicate a statistically significant difference. The statistical analysis was performed with

Results

BCSCs isolation and characterization

The MDAMB-231 cells were used to isolate the BCSCs population based on the expression of CD44⁺/CD24⁻ by magnetic cell sorting. The BCSCs morphology as the adherent cells at the base of the flask with spindle-like cell morphology and showed the lengthening of the actin filament. To confirm the purities of the BCSCs isolated cell population, we assessed the cell-surface antigen expression of CD44 and CD24 under flowcytometry analyses. The purities of the BCSCs isolated were to 98.70% and MDAMB-231 were to 89.00% that express CD44 and the lack of CD24 expression (Figure 1). High-level CD44 expression has been associated with cancer progression, whereas low-level CD24 expression has been associated with nondifferentiated cells [27].

Cell viability assay

The cytotoxic activities of hyptolide were first determined individually on BCSCs cells. As expected, the cytotoxic activity of hyptolide effectively increased in a dose-dependent manner with IC₅₀ value of 55 μ M (Figure 2). Hyptolide induce morphological changes in BCSCs, the high concentration of hyptolide induce cell to shrink and bubbling, indicating cell death.

Apoptosis and cell cycle analysis

Analysis by Annexin-V PI flowcytometry assay after treatment of cells with various concentration of hyptolide for 24 h, showed apoptosis of BCSCs cells in dose-dependent manner. Interestingly, in the group receiving hyptolide 55 μ M increase in cell death up to 32% (Figure 3A). In addition, we also evaluated the cell cycle progression under hyptolide treatment. We found that hyptolide induced S-phase cell cycle arrest in dose-dependent manner (Figure 3B).

Acquisition on DTP and BCSCs regulatory genes

The molecular target of hyptolide compound (Figure 4A) in the inhibition of BCSCs using integrated bioinformatics

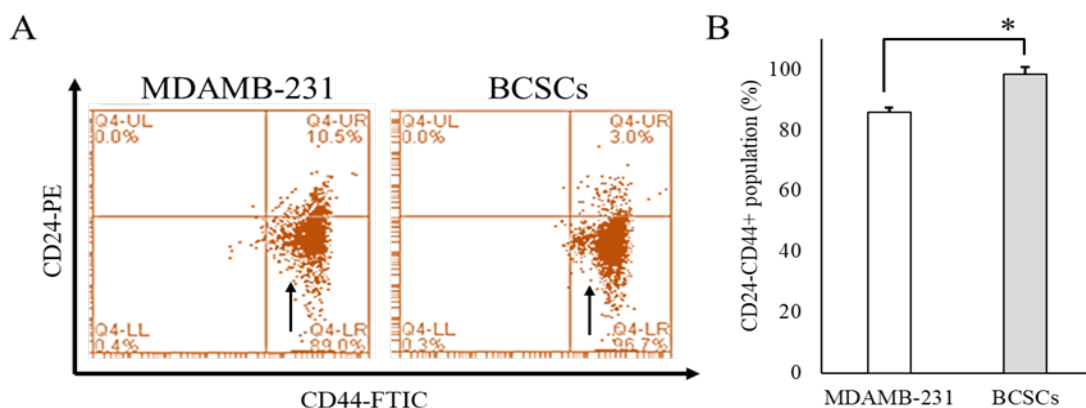


Figure 1. Characterization and Validation of Clone BCSCs. (A) Flowcytometry detection of CD44 and CD24 markers on the surface of MDAMB-231 and BCSCs cells. (B) Percentage of MDAMB-231 and BCSCs positive CD44 and negative CD24 cells. (* $p < 0.05$).

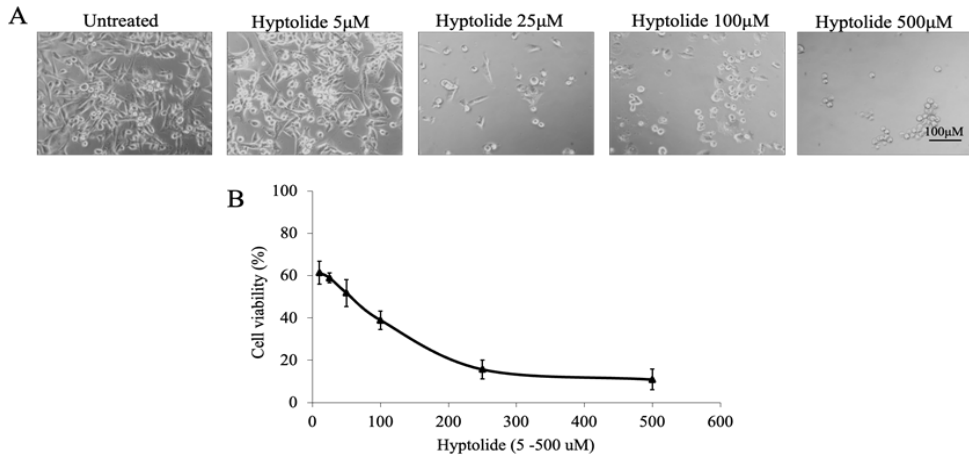


Figure 2. Cytotoxic Effects of Hyptolide on BCSCs. (A) morphological changes of BCSCs under hyptolide treatment for 24 h and (B) cytotoxic graph cell viability of hyptolide at 24 h. Cell viability profiles are presented from the mean $\pm$ standard error (SE) of 3 experiments. Scale bar: 100 μ m

was evaluated. We obtained 61 DTP of hyptolide under SEAprediction and SWISSTargetPrediction that 26.7% protein was kinase protein (Figure 4B). Furthermore, the

DTP interactions indicated that proteins played a critical role in the molecular function mediated by hyptolide. In total, we retrieved hyptolide mediated proteins consisting

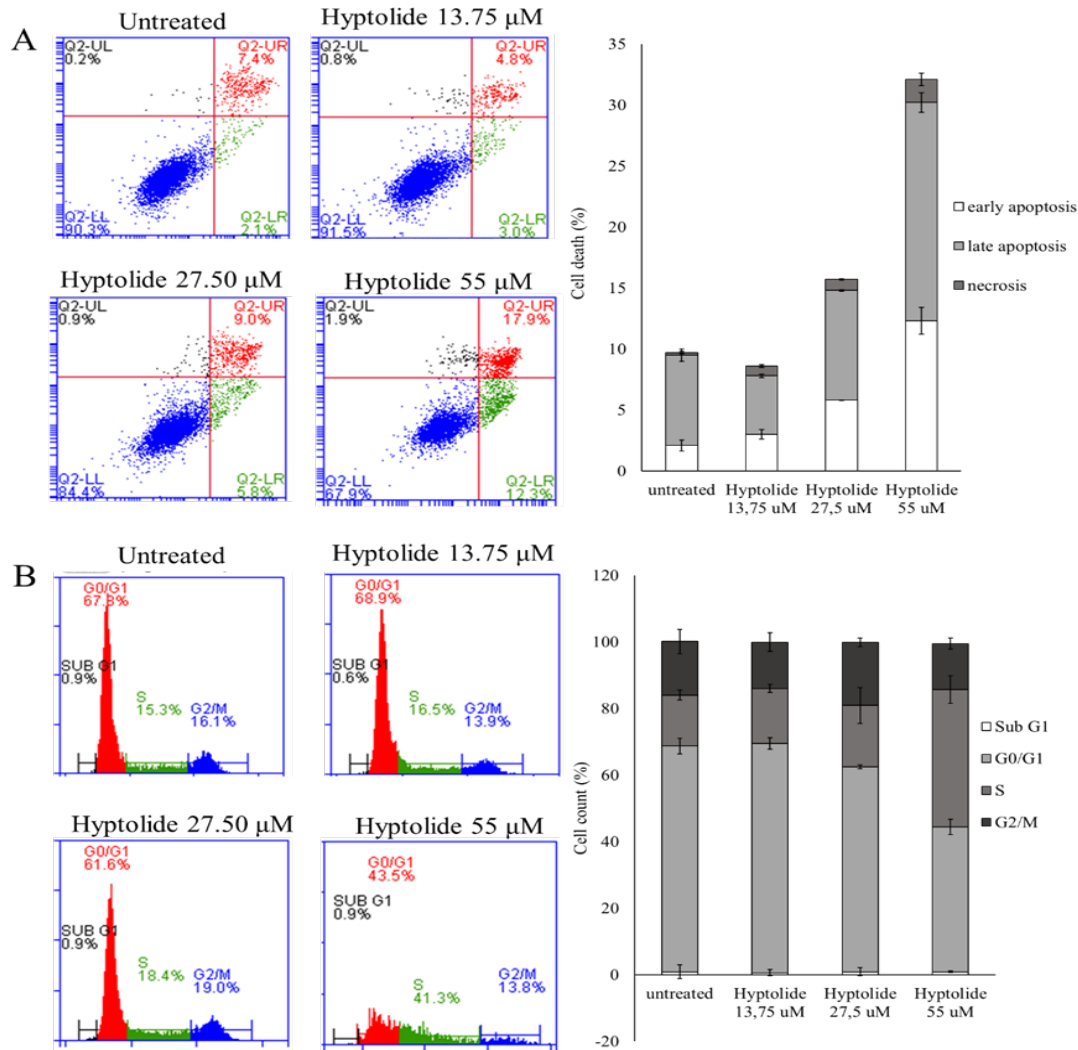
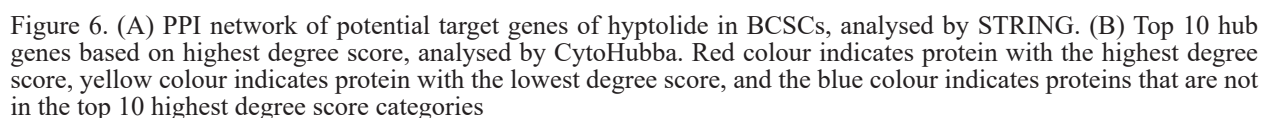
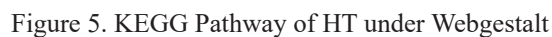
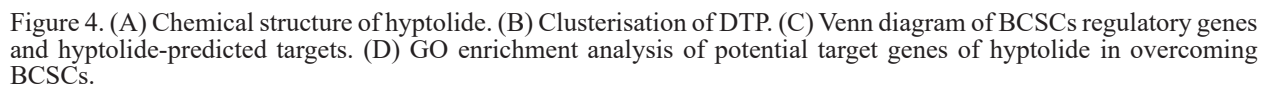


Figure 3. Effect of Hyptolide on (A) apoptosis and (B) cell cycle progression on BCSCs. The percentage of cell death and percentage of cell distribution were presented from the mean $\pm$ standard error (SE) of the 3 experiments (* $p < 0.05$).



of 61 DTP (Supplementary File 1) and 1284 BCSCs (Supplementary File 2) regulatory genes from PubMed. A Venn diagram generated 45 hyptolide targets in BCSCs or HT (Figure 4C, Supplementary File 3). To identify gene classes and predict the function of HT, we carried out GO and KEGG pathway enrichment analysis. GO analysis was aimed at checking the role of HT in biological processes, cellular components, and molecular functions. The results of GO analysis revealed the regulation of the biological process response to stimulus and the metabolic process by HT (Figure 4D). In addition, HT was located in the membrane and nucleus and served as a molecular function in protein, ion and nucleotide binding.

The results of the analysis of KEGG pathway enrichment revealed 6 pathways regulated by HT, including EGFR tyrosine kinase inhibitor resistance, progesterone-mediated oocyte maturation, non-small cell lung cancer, long-term potentiation, type II diabetes mellitus and TNF signaling pathway (Figure 5).

Analysis of PPI network and hub gene selection

Analysis of the PPI network (confidence level of 0.7) was conducted on HT, which consist of 45 nodes, 89 edges, a PPI enrichment value of $< 3.78 \times 10^{-11}$, and an average local clustering coefficient of 0.587 (Figure 6A). The top 10 genes with the highest degree scores were identified, including SRC, EGFR, MAPK1, MAPK14, PTK2, CDK1, MMP9, PRKCA, CREBBP, and MET (Figure 6B). These results indicated that those genes have a pivotal role in the PPI network, making them strong candidates for target genes.

Discussion

Breast cancer is a complex disease caused by a variety of factors leading to activation of multiple signaling pathways, including the *PI3K/Akt/mTOR*, *RAF/MEK/ERK* and ER pathways [4, 28]. BCSCs play an important role in cancer progression, relapse, metastatic, and drug resistance due to of their capacity to self-renew, apoptotic resistance and differentiate into many cancer cells lineages [29]. Recently, the limitedness of chemotherapy effectivity which make BCSCs difficult to eliminate. Thus, alternative potential therapy to induce BCSCs cells death is needed. The one of alternative therapy for cancer is using natural compound with multiple mechanism to kill cancer cells [4]. The concepts of natural chemotherapy are intended to reduce the side effects of a chemical chemotherapeutic agent [30]. Hyptolide is medicinal plants has strong potency of anticancer agent [17, 16]. However, in recent years the use of herbal medicine has been limited to natural chemotherapy [30]. The aim of this study was to interfere with BCSCs growth by administering different doses of hyptolide as natural chemotherapy to eliminate BCSCs populations. We examined the in vitro and bioinformatic analysis to determine whether BCSCs death following hyptolide administration was caused by apoptosis. We also analysis under bioinformatic assay to predict potential protein in the apoptosis cells of TNBC cells in the presence of hyptolide.

In this study, BCSCs population was isolated from

MDAMB-231 breast cancer cells using MACS method to obtain the population that expresses CD44+ and CD24-. Previous study reported that CD44 and CD24 as potential surface markers in identification and isolation of cancer stem cells in various cancer cells [31, 7]. The high expression of CD44 has been associated with the potential of progression and metastasis [32]. In addition, CD24 is involved in cell adhesion that indicated CD24 could be a significant marker in cancer prognosis [31]. Taken together, the cell population in this study characterized by CD44+/CD24- showed positive BCSCs and could be used for further analysis.

Based on, cytotoxic assay we found that hyptolide has strong cytotoxicity on BCSCs with IC_{50} under $100 \mu M$. These findings are supported by the previous study that hyptolide inhibits breast cancers proliferation [25]. Thus, hyptolide could improve the therapeutic effect by sensitizing BCSCs and may provide a novel approach for cancer therapy. The cytotoxic activity of hyptolide in was further examined by measuring apoptosis profiles. Apoptosis assay results revealed that hyptolide significant induces cell death up to above 32% in BCSCs. Apoptosis is a crucial homeostatic process, which balances cell proliferation and cell death in order to maintain the appropriate cell number in the body [33]. Previous study reported that BCSCs displayed apoptotic resistance by upregulating the expression of anti-apoptotic proteins. In addition, to explored underlying another target of hyptolide we evaluated cell cycle analysis, hyptolide induced s-phase cell cycle arrest in dose-depedent manner. Interestingly, hyptolide did not induce a significant arrest in sub G1 signalling apoptosis. However, hyptolide was shown to significantly induce cell death. Therefore, the mechanism of hyptolide-induced cell apoptosis should be further explored. In this study, we evaluated for potential proteins that cause hyptolide-induced BCSCs cell death by using a bioinformatics approach.

Ten potential therapeutic targets of hyptolide action BSCS-targeted, including *SRC*, *EGFR*, *MAPK1*, *MAPK14*, *PTK2*, *CDK1*, *MMP9*, *PRKCA*, *CREBBP*, and *MET*. *MAPK* signaling pathway comminates with other pathways for example *PI3K/AKT* and mTOR. *MAPK* signaling is important for the maintenance of cancer stem cell propertied in BCSCs [34]. In addition, *MAPK* pathways are responsible for the apoptosis in cancer cells [35]. *MET*, also known as mesenchymal-epithelial transition factor gene or *MET* protooncogene, encodes a member of the receptor tyrosine kinase family of proteins [36, 37]. Upon binding to its ligand, namely hepatocyte growth factor (HGF), *MET* induces dimerization leading to activation of intracellular signaling, which is involved in cell proliferation, apoptosis, invasion, and migration. Moreover, the same author stated that *MET* signaling also communicates with other intracellular signaling mechanisms, including the *PI3K/AKT* and *MAPK* pathways [35]. On the other hand, activation of *EGFR* inhibits stemness in glioblastoma and colorectal cancer cells [38, 39]. Activation of *EGFR* inhibits metastasis by blocking β -catenin signaling and inhibiting *MMP9* expression and activity [40]. Taken together, the molecular mechanism of hyptolide in inhibition of BCSCs through

several signaling above needs to be explored further. These studies suggest that SRC, EGFR, and MAPK1 signalling are potential targets of hyptolide in inhibition of BCSCs, however the molecular mechanism involved need further investigation.

Author Contribution Statement

MZ and NDA conceptualized and designed the study; BC and NDA performed the experiments and collected the data; NDA analyzed and interpreted the results; NDA and BC prepared the initial manuscript; MZ reviewed and approved the final version of manuscript. .

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Data Availability

The study data is available with authors.

Conflict of Interest

Authors have no conflicts of interests to disclose.

Ethical Declaration

Not applicable because this study does not involve experiments on animals or human subject.

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