RESEARCH ARTICLE

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Cytotoxicity and Apoptosis Studies of Brucein D against T24 Bladder Cancer Cells

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Abstract

Objective: Brucein D (BrD), a quassinoid isolated from Brucea javanica fruit, reportedly demonstrates anti-cancer activity. This study's objective is to evaluate the cytotoxicity of Brucein D and its ability to induce apoptosis in T24 bladder cancer cells. **Methods:** We investigated the cytotoxic activity of BrD against the T24 cell through the induction of apoptosis in vitro. This cytotoxic activity was evaluated with MTT assay and followed by Calcein-AM/PI viability staining. Apoptotic activity was determined with Hoechst 33342 nuclear staining and DNA fragmentation. Doxorubicin and docetaxel were used as a positive control. Evaluation of apoptotic-related gene expression, *Bax, Bak, Bcl2*, and *p53* was also performed using semi-quantitative PCR analysis. Statistical analysis was conducted using One-way ANOVA followed by post hoc test Turkey's HSD (Honestly Significance Difference). **Results:** Results show that BrD had high toxicity against T24 bladder cancer cells with an IC_{50} value of 7.65 ± 1.2 µg/mL but relatively less toxic to 1BR3 normal skin fibroblast cells compared to the doxorubicin and docetaxel treated cells. The viability assay shows that BrD significantly increases the percentage of dead cells relative to control in a dose-dependent manner. Furthermore, the percentage of cells with apoptotic appearance was significantly higher in group treated with BrD IC_{50} (56.04±3.09%) compared to control (9.42±2.88). The result was similar to doxorubicin IC_{50} (58.97±12.31) but lower than docetaxel IC50 (74.42±9.79). DNA fragmentation in gel electrophoresis was also observed in T24 cells treated with BrD. Apoptosis was also verified by an alteration in the expression of apoptosis-related genes, upregulation of *Bax, Bak,* and *p53*, and downregulation of *Bcl-2*. **Conclusion:** BrD has shown a cytotoxic effect against T24 bladder cancer cells. Hence, it is a promising natural compound for the management of bladder cancer by induction of apoptosis through activation of the intrinsic pathway, with low toxicity to normal cells.

Keywords: apoptosis- Brucea javanica- Brucein D- T24 bladder cancer cell line

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Introduction

Bladder cancer is one of the most common malignancies in the urinary tract besides prostate cancer. Global Cancer Statistics (GLOBOCAN) 2018 ranked bladder cancer in 10th position as the most common cancer worldwide, with an estimated 549.000 new cases annually, and almost 200.000 deaths [1]. The incidence in men was around 2 to 6 times higher than in women, and the risk increased with age, with the age-specific curve rise higher after the age of 50 years [2].

Local data from the leading hospitals in Indonesia shows the incidence of bladder cancer rising by 15% each year. The incidence in Indonesia was higher in men than women (6:1,5). Most of bladder cancer cases occur at ages over 60 years. A common pathological finding, more than 70%, was transitional cell carcinoma. Moreover, more than 60% cases are diagnosed as late-stage bladder cancer [3].

The main problem with this cancer was the recurrence rate, even if diagnosed with early-stage or non-muscle invasive bladder cancer (NMIBC) from initial TURBT staging, 60-70% will relapse and 25% will be grown into a more invasive form [4]. One effort to lower the recurrence rate is intravesical chemotherapy. Still, the success rate was only 30% with lots of complications,

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such as irritation, an allergic reaction, and bone marrow suppression [5]. Nusa Tenggara Barat General Hospital has 90 new cases annually [6], and 27 patients undergo intravesical chemotherapy, commonly men aged 60 and above [7].

Brucea javanica, an old traditional Chinese medicinal herb, has been widely known as a traditional Chinese medicinal plant since 250 years ago, recorded in a Chinese medical book called Supplementation to the Compendium of Chinese Materia Medica by Zhao Xue-Ming, for treatment of dysentery, malaria, gastric ulcer, ulcerative colitis, and lately for leukemia and other cancer [8; 9]. It spreads from Africa to Asia including Indonesia. It is known as buah makassar or buah wali, and mainly found as shrubs or bushes, with the rest grows in herbal plant industries [10]. This herb has many special functions such as anti-inflammation, anti-diabetes, anti-malaria, and anti-cancer [11; 12; 13].

Brucein D (BrD), one of the most abundant active quassinoid compounds in Brucea javanica, shows a cytotoxic activity in cancer cells, it has anti-proliferative and apoptogenic effects, thus inhibiting the cancer cell growth mostly in pancreatic, lungs, and mammary cancer [14, 15, 16]. Unfortunately, there is still very limited information available about the potential biological activity of this chemical compound, especially for bladder cancer treatment. To the best of our knowledge, no research has been done on the apoptosis-related activity of BrD in bladder cancer cells.

The primary goal of anti-neoplastic treatments is to eliminate the cancer cells without affecting the normal cells, which can be very difficult to obtain by chemotherapy or radiotherapy. Even in non-muscle invasive bladder cancer with the intravesical doxorubicin instillation had several adverse effects like bone marrow suppression, nausea or vomiting, hair loss, and cardiotoxicity [17], and only has limited improvement in preventing recurrence and progression [18]. Intravesical docetaxel was also being investigated for tumor recurrence, BCG failure, or high-risk non-muscle invasive bladder cancer, but experts say it is better to perform radical cystectomy [19, 20]. Docetaxel is also used as intravenous second-line chemotherapy for bladder cancer. It has an acceptable efficacy and is well-tolerated, but still had a moderate adverse effects like gastrointestinal disturbance, neuropathy, and myelosuppression [21].

This study aims to investigate the BrD's-related cytotoxicity and apoptosis in T24 bladder cancer cell line. The primary basis of this research is to assess the cytotoxicity by determine the IC_{50} and the cell viability assay; to assess the apoptosis event by determine the apoptotic cell morphology, DNA fragmentation, and the alteration of apoptosis-related gene expression, *Bax*, *Bak*, *p53* tumor suppressor gene, and Bcl-2

Materials and Methods

Brucein D (BrD)

BrD is a bioactive quassinoid compound isolated from Brucea javanica fruit. BrD was purchased from Aktin Chemicals, Inc (Chengdu, P.R.China), with purity >98% by HPLC, molecule weight 410.4150 g/mol, in white crystal powder form, and identified by TLC and NMR (www.aktinlab.com). BrD solution was prepared using a dilution technique with distilled water and DMSO to produce a solution of 100 µg BrD with 0.1% DMSO and gradually diluted to make the concentration needed required for the experiment. The solution was placed in a sterile and air-tight container at 4°C.

Reagents

The MTT cell viability assay (Thermo Fisher) from BioVision, Inc. (Milpitas, USA). McCoy's medium, FBS (Fetal Bovine Serum) of South American Origin, and Penicillin from Gibco Life Technologies (Thermo Fisher). The fluorescents probe Calcein-AM (Thermo Fisher), Propidium Iodide (Sigma Aldrich), and Hoechst33342 (Dojindo) were purchased from Dojindo, Japan.

Cell Line T24 Bladder Cancer

The bladder cancer cell line T24 was purchased from the ECACC (European Collection of Authenticated Cell Cultures) with cat-pack number 85061107-1 VL. The cells were grown with McCoy's medium augmented with 10% FBS and 1% Penicillin. Cells were incubated at 37°C and a 5% CO_2 humidified incubator (Forma Steri - Cycle, Thermo Fisher). T24 cells were seeded in T-25 cell culture flasks with a density of 0.8 x 10^6 cells/mL. After 80-90% confluence was achieved, cells were then separated into two groups, the control group and the BrD-treated group, which were treated with gradual concentrations of BrD from 0.01–100 µg/mL. The control group was divided into three groups, one without any intervention (negative control) and two other group, each containing doxorubicin and docetaxel with a concentration of 0.01–100 µg/mL, and later consistent with IC_{50}.

Cell Line 1BR3 Normal Skin Fibroblast Cell

The Normal cell line 1BR3 was purchased from the ECACC (European Collection of Authenticated Cell Cultures) Cat number 90011801. The cells were grown in EMEM (EBSS) + 2mM Glutamine + 1% Non Essential Amino Acids (NEAA) + 15% FBS. Cells were seeded in cell culture flasks with a seeding density of 5 x 10^4 cells/mL using 0.05 trypsin/EDTA and incubated in 37°C and 5% CO_2 humidified incubator (Forma Steri-Cycle, Thermo Fisher).

Cytotoxicity assay and IC_{50} determination

MTT cytotoxic assay was performed to estimate the cytotoxicity of BrD. T24 cells were cultured in 96-well cultured plates at a density of 1 x 10^4 cells/well and incubated for 24 h. The culture media were then removed and substituted with media containing BrD, doxorubicin, and docetaxel with ascending concentrations from 0.01-100 µg/mL. A 50 µL serum-free medium containing 50 µM of MTT reagent was added after incubation for 72 h. The 96-well plates were maintained at 37°C with 5% CO_2 for 3 hours, then the solution was dispensed and changed with 150 µL of MTT solvent and undergo a 15 min rotation. The transformation of yellow Tetrazole (MTT dye) to purple formazan in

metabolically active cells reveals the number of live cells. A UV-Vis microplate reader (Thermo Fisher, Multiskan GO Spectrophotometer) was used to measure the color change in absorbance at 590 nm related to MTT activity. Cytotoxicity (%) was estimated according to the equation by calculating the average absorbance of the control group at 590 nm ($A_{590contro}$) and the average absorbance of the treated group ($A_{590\ treated}$) with various concentration of BrD, doxorubicin, and docetaxel from the triplicate experiment. Half-maximal inhibitory concentration was defined as the concentration of BrD that inhibited 50% of the T24 cells (IC_{50}). IC_{50} calculation tools by GraphPad Prism software version 9.3.1 (471) were used [22].

$$Cytotoxicity\ (\%) = \frac{A_{590\ treated\ cells}}{A_{590\ control}}$$

Effect of BrD on T24 cells Morphology

T24 cancer cell line was grown in a 60-mm tissue culture dish and treated with each substance and concentration separately (0.01-100 µg/mL) for 24 h. Cells were examined under phase contrast / bright field microscope (Zeiss primovert, Germany) at 20x magnification, and photographs were taken using a Magna-Fire digital camera for analysis of cell morphology.

Calcein-AM and PI fluorescence staining was used for viability assay and determining the apoptotic characteristics [23]. T24 cells were seeded in 2 ml media containing 10% FBS and 1% Penicillin with a density of 3 x 10^4 cells/well in a 35 mm dish at 37 oC and 5% CO_2 atmosphere. After 24 h of incubation, the medium was then substituted with a medium containing each BrD, doxorubicin, and docetaxel at the IC_{50} concentration and left for further incubation for 72 h. Next, the cells were rinsed with PBS and stained with 5 µM of each calcein-AM and PI. After being incubated for 15 min at 37°C, the stained cells were observed with an inverted fluorescence microscope (Axio observer Z1, Zeiss). Live cells are marked as ones that emitted green fluorescence, while dead cells emitted red fluorescence [24]. Additional cell analyses were performed with Fiji ImageJ software [25].

Analysis of BrD-induced DNA condensation by Hoechst33342

Hoechst33342 fluorescence dye staining was used to analyze the apoptotic events in cells [26, 27]. T24 cells were cultured at a seeding density of 0.3 x 10^6 cells/mL in 35 mm tissue culture dishes. After incubation for 24 hours, the medium was removed and replaced with a control medium (non-treated) and a medium containing BrD, doxorubicin, and docetaxel at the IC_{50} concentration. Next, after incubation for 72 h at 37°C with 5% CO_2 the medium was replaced with 40 µM Hoechst33342 culture medium. The cells were then incubated at 37°C with 5% CO_2 for 20 minutes, washed twice with phosphate-buffered saline (PBS), and examined with an inverted fluorescence microscope and 350 nm excitation filters. Images were obtained with 20x magnification and analyzed with Fiji ImageJ to assess the proportion of apoptotic nuclei. The corrected total cell fluorescence (CTCF) equation was used to calculate the fluorescence intensity in condensed nuclear DNA. CTCF = Integrated density - (Area of selected cell x mean fluorescence of background reading) [25].

Assessment of Apoptosis with DNA fragmentation

A biological characteristic of apoptosis is the condensation and fragmentation of nuclear and cytoplasmic DNA [28, 29]. The cells were seeded in 24-well plates at a seeding density of 5 x 10^4 cells/mL for 24 h. The next day each medium was replaced by BrD, doxorubicin, and docetaxel medium at IC50 concentration and incubated for 72 h. After that, DNeasy Kit (Qiagen, USA) was used to extract the total cellular DNA, and then the extracted genomic DNA was separated by electrophoresis in 1.5% agarose gel (80V, 40 min). As a standard DNA ladder, we use a 1 kb DNA size marker (GeneRuler 1kb DNA Ladder, Thermo Scientific). The agarose gels were recorded using a GelDoc imaging system (Bio-Rad, UK).

Isolation of T24 cells RNA and Semi-Quantitative PCR Analysis

Extraction of RNA from all groups of T24 cells was performed using the RNAeasy Mini Kit (Qiagen, USA) [30]. Cells were seeded in 24-well plates with a density of 5 x 10^4 cells/mL and then placed for 24 h incubation. The day after, the medium was discarded and replaced by a medium containing each BrD, doxorubicin, and docetaxel at IC_{50} concentration and incubated for the next 72 h. The total RNA was extracted from T24 cells in accordance with the fabricator's protocols. The conversion into cDNA was performed using PrimeScript 1st strand cDNA synthesis kit (Takara, Japan), and the PCR was performed using TopTaq Master Mix PCR Kit (Qiagen, USA). The expression of *p53*, *Bak*, *Bax*, and *Bcl2* genes was measured using suitable primers documented in GenBank data. The primers (IDTDNA) were purchased from Fasmac, Japan. Analysis of the semi-quantitative PCR results determined with Image Lab software (Bio-Rad, USA) relative to the housekeeping gene (GADPH).

Statistical Analysis

One-way ANOVA followed by post hoc test Tukey's HSD (Honestly Significance Difference) was conducted for multiple comparisons between treatment groups and control groups. The experiment was conducted in triplicate, and the results were defined as the mean ±SD. All statistical analyses were performed using IBM® SPSS® Statistics version 25. A p-value <0.05 was considered as statistically significant.

Results

Morphological examination of the T24 cells

Morphological characteristics of T24 cells after treatment with graded concentrations of each substance obtained by a brightfield microscope showed a morphological condition of dead cells, either by apoptosis or by necrosis, like loss of plasma membrane integration,

cell fragmentation, and shrinkage [29]. The percentage of dead cells increased in line with the higher concentration of BrD, doxorubicin, and docetaxel, while in the control group, the morphological characteristics of T24 cells were normal (Figure 1).

Cytotoxicity assay and IC_{50} determination

The cytotoxicity of BrD on T24 cells was determined using the MTT assay according to the method by Mosmann [31]. Dilution of BrD solution into graded concentrations (0.01 – 100 µg/mL) followed by non-linear regression of the dose-response inhibition curve in GraphPad Prism to determine the effective doses. BrD showed an IC_{50} value of 7.65 ± 1.2 µg/mL, described as high cytotoxicity. According to the National Cancer Institute (NCI) guidelines, $IC_{50} \leq 20$ µg/mL is correlated with high cytotoxicity, between 21 and 200 µg/mL are moderate, 201-500 µg/mL are low, and $IC_{50} \geq 500$ µg/mL is considered to have no toxicity (Figure 2) [32, 33].

The IC_{50} for docetaxel and doxorubicin are 6.5 ± 1.61 µg/mL and 1.37 ± 0.733 µg/mL, respectively, which means all of the compounds are highly toxic. Despite the high toxicity of BrD in T24 cells, the result in Normal Skin Fibroblast cells 1BR3 indicates that BrD shows a cytotoxicity effect at 100 µg/mL concentration, compared to 1 µg/mL for docetaxel and doxorubicin. This signifies the low cytotoxicity of BrD in a normal cell (Figure 3).

Cell Viability assay with Calcein-AM and PI staining

Fluorescent staining with calcein-AM and PI were used to determine cell viability in order to distinguish between living and dead cells [30]. In living cells, intracellular esterase will transform calcein-AM to calcein. Calcein will remain in the cells and emits green fluorescence. Meanwhile, because of its impermeability, PI can penetrate into the cells only when there is a plasma membrane integrity disruption. Then PI will bind with the cell's DNA and emits red fluorescence [34]. In the BrD-treated group, the number of dead cells was increased in a concentration-dependent manner. Fiji ImageJ software was used for semi-quantitative analyses in accordance with the images captured from the fluorescence-inverted microscope and set for the chart with Microsoft Excel, then calculated for statistical analyses using Oneway ANOVA and Tukey's HSD multiple comparison test. The BrD 0.1 µg/mL treatment significantly increased the percentage of dead cells compared to the control group ($p<0.05$). Compared to the other chemotherapy drugs, BrD 0.1 µg/mL had a similar dead cell percentage compared to doxorubicin 0.1 µg/mL, but lower compared to docetaxel 0.1 µg/mL. In higher doses, BrD 1 µg/mL and 10 µg/mL had a significantly lower dead cell percentage compared to doxorubicin and docetaxel at the same concentration. At 10 µg/mL, the dead cell percentage of doxorubicin and docetaxel is almost 100%, while BrD is only 64% (Figure 4).

Effects of BrD in apoptosis

Morphological examination of apoptotic cells by Hoechst33342

Cell apoptosis is characterized by several hallmarks, one of which is chromatin condensation. The Hoechst33342 fluorescence dye can stain the condensed apoptotic cells [35]. T24 cells treated with BrD with IC_{50} concentration show condensed nuclei (Figure 5A). Chromatin condensation could be determined by measuring the fluorescence intensity with the Corrected Total Cell Fluorescence (CTCF) equation, analyzed using Fiji ImageJ. Based on the CTCF result, the fluorescence intensity in T24 cells treated with BrD showed a

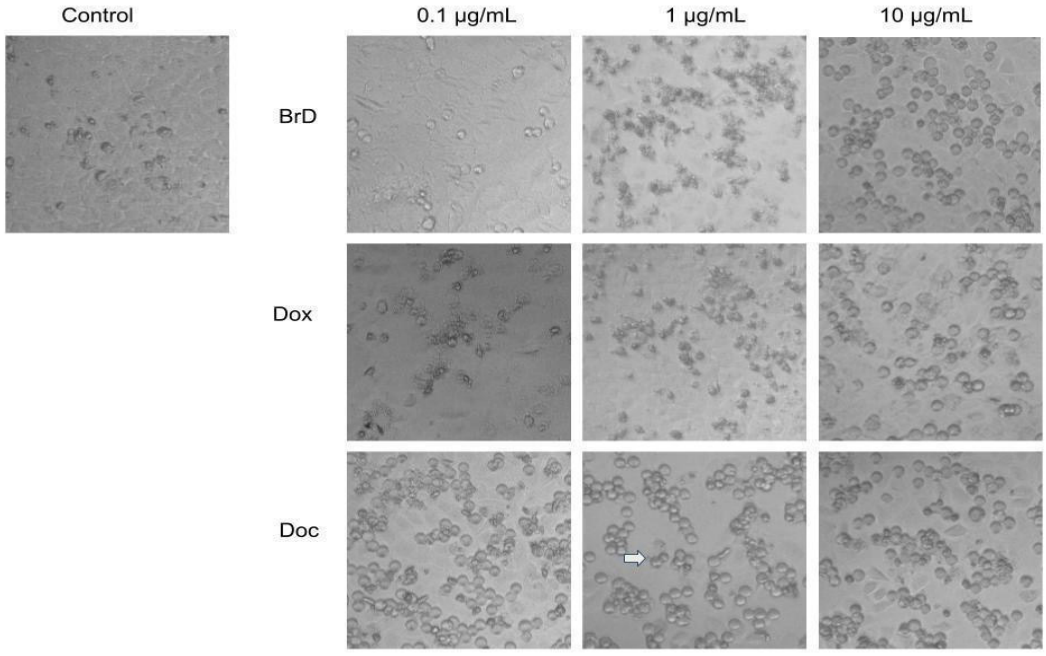

Figure 1. Cytotoxic effect of BrD in T24 Cells Treated for 72 h White Arrow Indicates Morphological Changes in Treated Cells. BrD = Brucein D, Dox = Doxorubicin, Doc = Docetaxel.

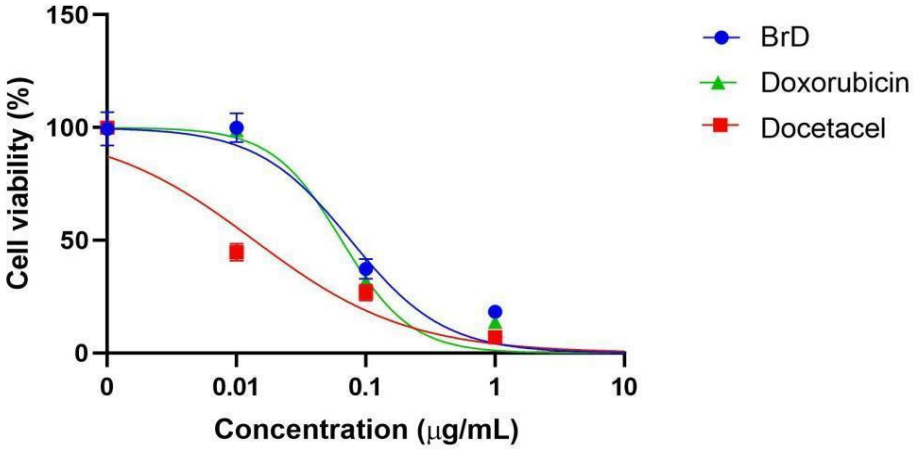

Figure 2. Dose-Response of BrD Treatment (0.01 – 100 μg/mL) in T24 cells for 72 h

significant increase (37043.69) compared to control. The result was similar in doxorubicin IC_{50} (36413.01) and docetaxel IC_{50} (41198.04) (Figure 5B).

Additionally, the apoptotic cells percentage observed in T24 cells treated with BrD IC_{50} (56.04±3.09 %) was significantly higher than control (9.42±2.88), and the result was similar with doxorubicin IC_{50} (58.97±12.31), but statistically lower compared to docetaxel IC_{50} (74.42±9.79) (Figure 5C).

DNA fragmentation analysis

Another key sign of cell apoptosis is the fragmentation of DNA. This figure can be observed by gel electrophoresis. The electrophoresis can separate nucleic acid DNA fragments based on their size [36; 37]. Small DNA fragments can be seen in DNA samples of T24 cells after 72 h treatment with BrD, doxorubicin, and docetaxel (Figure 5D)

Bax, Bak, Bcl-2, p53 gene expression

The apoptosis-related gene expression alteration can show the initiation of apoptosis or an early sign of the apoptosis process [38, 39]. Semi-quantitative PCR analysis and Image Lab software were used to analyze the effect of BrD in IC_{50} concentration on the pro-apoptotic and anti-apoptotic genes expression. After 72 h of BrD treatment, the expression of pro-apoptosis genes *Bax*, *Bak*, and *p53* increases while the anti-apoptosis gene *Bcl2* decreases. The result also demonstrates that BrD has a similar effect compared to doxorubicin and docetaxel as chemotherapy medicines (Figure 6).

Discussion

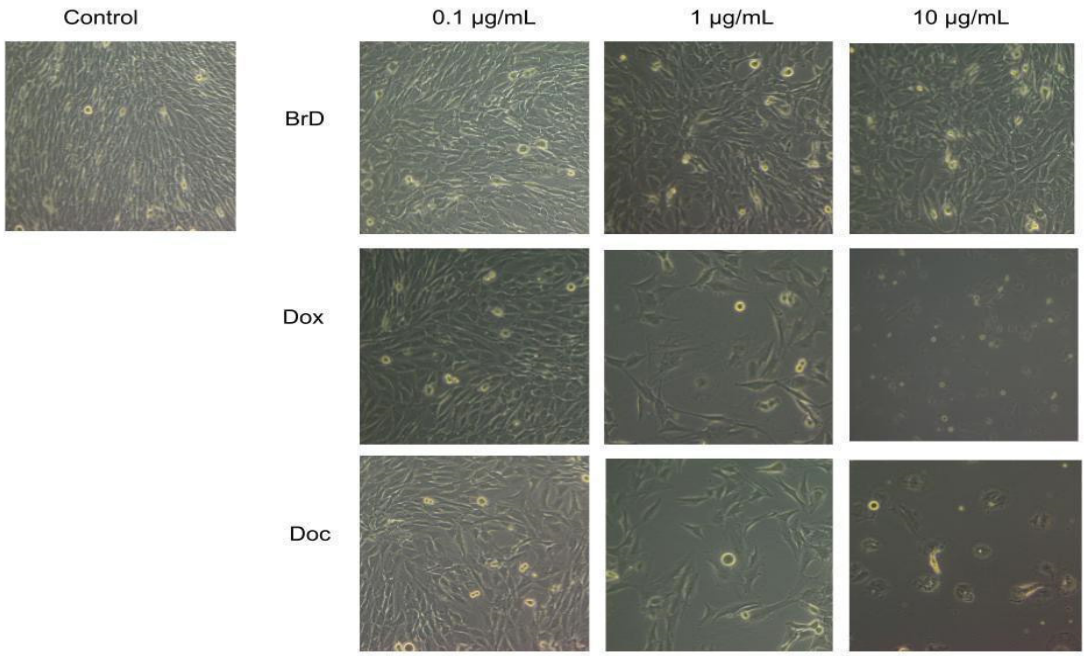

Figure 3. BrD's Cytotoxic eEects in 1BR3 Cells after Treatment for 72 h Compared to Doxorubicin and Docetaxel

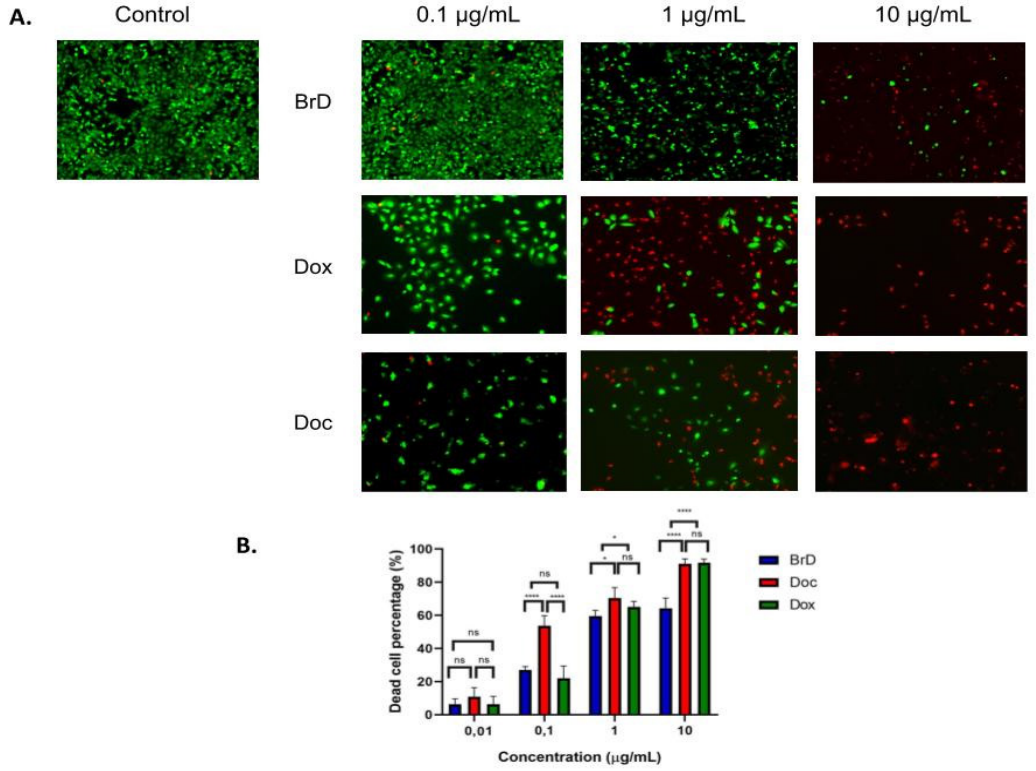

Figure 4. Viability assay by Staining with (A) Calcein AM (green) and PI (red) in T24 Cells Treated with a Graded Dose of BrD, Doxorubicin, and Docetaxel for 72 h. The percentage of cells stained with PI represents dead cells (B) The percentage of dead cells showed an increase in a dose-dependent manner (ns indicates non-significant, * significant difference p< 0.05, ** p<0.01, *** p<0.001, **** p<0.0001)

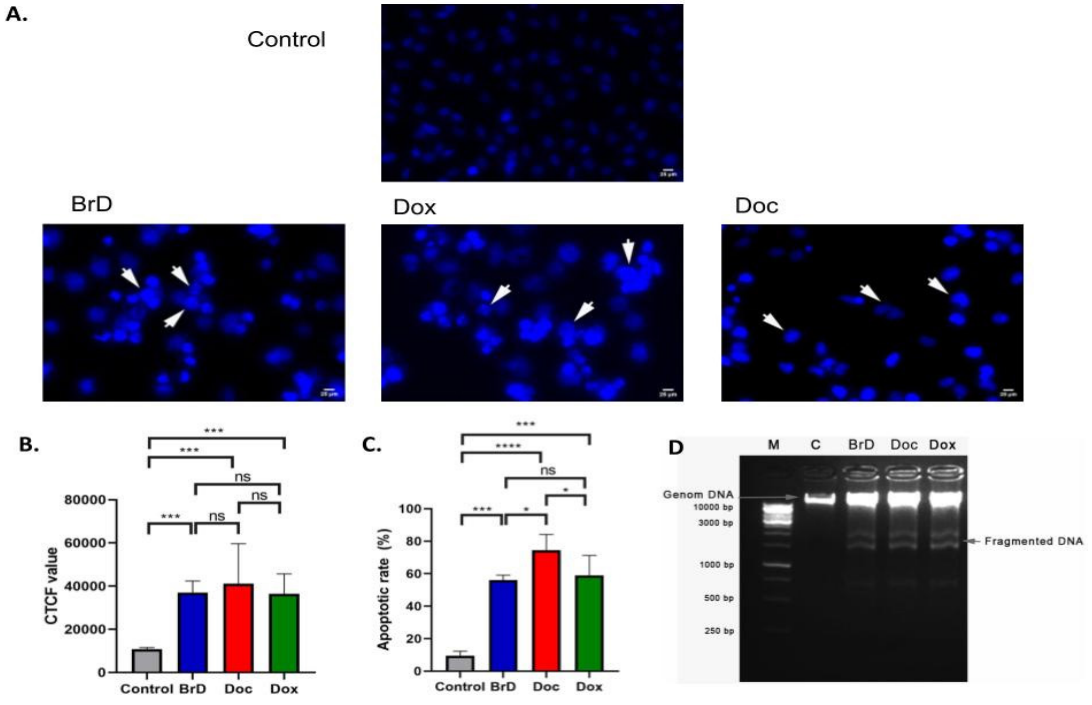

Figure 5. Apoptosis-Inducing Effects of T-24 Cells: (A) Hoechst33342 staining shows condensation of chromatin in apoptotic cells; arrowheads = apoptotic nuclei. (B) CTCF values of T-24 cells treated with BrD, doxorubicin, and docetaxel at IC50 concentrations, shows an increase in cell fluorescence that indicates an increase of chromatin condensation in all treatment group compared to control (C) Measurement of the apoptotic nuclei percentage in T24 cells treated with BrD, doxorubicin, and docetaxel in IC50 concentrations shows an increase of apoptotic nuclei percentage in all treatment group compared to control. (D) Fragmentation of T24 genomic DNA treated with BrD, doxorubicin, and docetaxel at IC50 concentration; a 1 kb DNA ladder (250–10,000 bp Thermo Fisher) was used as a basic measurement; M: 1 kb marker DNA ladder; C: control. Arrows indicate genomic DNA and fragmented DNA. (ns indicates non-significant, * significant difference p< 0.05, ** p<0.01, *** p<0.001, **** p<0.0001)

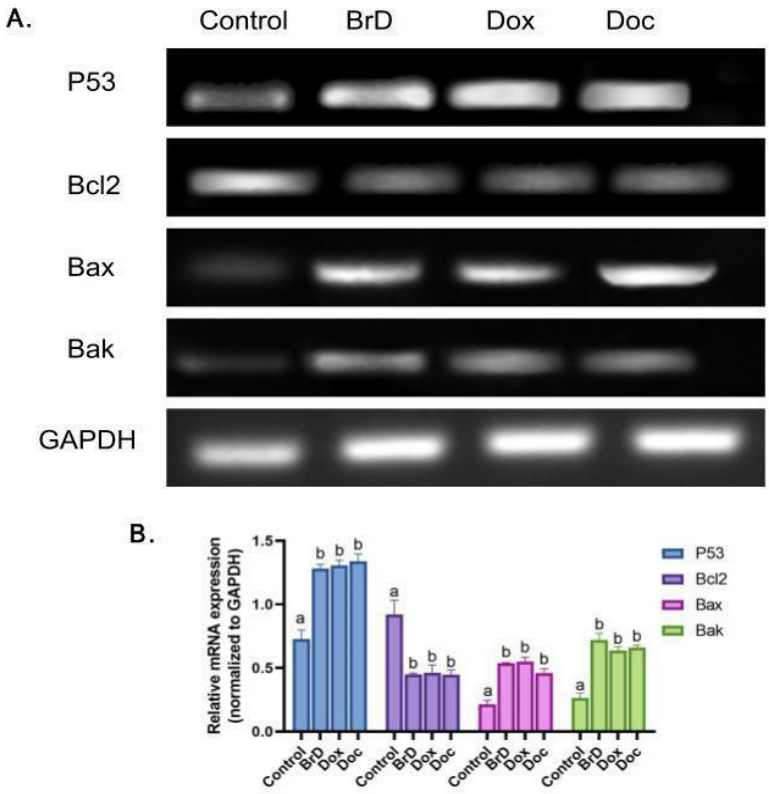

Figure 6. (A) Amplification of *Bax, Bak, p53,* and *Bcl2* genes compared to *GAPDH*. (B) Analysis of gene expression based on the expression of mRNA compared to *GAPDH* as the housekeeping gene shows that the expression of Bax, *Bak*, and *p53* was increased, and *Bcl2* was decreased in all treatment groups compared to control.

Our study show that BrD has high toxicity against T24 bladder cancer cell but relatively less toxic to 1BR3 normal skin fibroblast cells compared to the doxorubicin and docetaxel. There are increasing percentage of dead cells relative to control after BrD treatment. Moreover, percentage of apoptotic appearance was significantly higher in group treated with BrD compared to control.

Based on our result, BrD reduced cell viability in T24 cells in a dose-dependent manner, with the half-maximal inhibitory concentration (IC_{50}) value of 7.65 ± 1.2 µg/mL, and considered highly cytotoxic. Several studies of BrD with other cell lines shows that it reduced cell viability in time and dose dependent manner with various IC_{50} result, but mostly highly cytotoxic. It ranges between 0.5 to 2.7 µmol/mL and 1,0 to 6.0 µg/mL in human non-small cell lung cancer [15, 40], 1.1 to 5.8 µM in pancreatic adenocarcinoma [41, 42] and breast cancer cells [16], and also 6.37 in myeloid leukemia cell [43]. Even though the IC_{50} result was highly cytotoxic, our study found that in 1BR3 normal skin fibroblast cell, BrD shows its cytotoxicity at concentrations of more than 10 µg/mL, compared to 1 µg/mL for docetaxel and doxorubicin. This signifies the low cytotoxicity of BrD in normal cells. Other studies also have a similar result, IC_{50} of BrD in non-tumorigenic hepatocyte and pancreatic progenitor cells is more than 250 µM [41] and over 30 µM in normal skin fibroblast [42]. A study about the acute cytotoxicity of oral administration of Brucea javanica fruit also shows mild cytotoxicity and safe to be used as plant medicine [44], and administration of high dose BrD (3 mg/kg) in mice was associated with no obvious toxicity [14].

Apoptosis holds an important role in cancer prevention and treatment. In this study we use Calcein AM-PI fluorescence staining to distinguish live and dead cells, and the result shows that BrD increases the percentage of dead cells in a dose-dependent manner. The result is statistically equal to doxorubicin and docetaxel. Moreover, we use Hoechst33342 fluorescence dye for examination the apoptotic cell morphological transformation. Compared to the uniformly distributed blue cells in the control group, the cells treated with IC_{50} BrD concentration show distinct chromatin condensation and fragmented nuclei. Several other studies with other cell lines show a similar result, like in pancreatic cell lines, Capan-2 [41] and PANC-1 [42], or non-small cell lung cancer cell lines H460 [15]. They also show a typical apoptotic cell morphology in fluorescence-based image cellometer after BrD treatment. For further apoptosis assessment, we performed gel electrophoresis and found DNA fragmentation in the treatment group that does not appear in the control group. Other studies confirmed that there is no DNA fragmentation appears in normal cells, like fibroblast cell NIH-3T3 and umbilical vein cells (HUVEC) [45, 46].

The intrinsic pathway of the apoptosis process involved *Bcl2* family proteins together with the *p53* tumor suppressor gene as a regulator. The *Bcl2* family consists of pro-apoptosis proteins, like *Bak* and *Bax*, and anti-apoptosis proteins, like *Bcl2* and Bcl XL [47]. Recently, the *Bcl2* family protein and *p53* tumor suppressor gene have been widely investigated as new insight for various

cancer prevention and treatment. The *Bcl2* family proteins work in a unique way; they can bind each other, suppress one another or promote to keep the apoptosis process balance. In many cancers, high levels of anti-apoptotic proteins such as *Bcl2* were shown to contribute not only to tumor growth and progression but also to resistance to chemotherapy [48]. Due to the important role of these proteins, we investigated the expression of these markers at the gene level, using semi-quantitative PCR, to show the initiation process for early apoptosis signal. The mRNA expression has revealed an increase of *Bax*, *Bak*, and also *p53* gene expression after 72-h treatment of T24 cell with BrD. A similar result was also obtained in doxorubicin and docetaxel treatment. While *Bcl2* gene expression shows a decreased expression or down-regulation, which means there is inhibition of *Bcl2* gene expression. These results are similar to several other studies with other cell lines like HT29 colon cancer [9], Capan-2 human pancreatic adenocarcinoma cell [41], and A549 human lung cancer cell [40].

Limitation of the Study

No other studies have been conducted to evaluate the effects of BrD on bladder cancer. Further in-vitro studies are needed to analyze other apoptotic pathways. In addition, in-vivo studies are also needed using knocked-out mice to evaluate the effectiveness of BrD on bladder cancer.

In conclusion, overall, our study provided a novel outlook on the potential medicinal use of BrD as an alternative therapy for bladder cancer cells. The obtained result of this study showed that BrD has a strong cytotoxic effect against T24 bladder cancer cells leading to the apoptosis process of the cells observed by morphological change and by inducing the pro-apoptosis gene *Bak, Bax*, and *p53*, and also inhibiting anti-apoptosis gene *Bcl2*. In addition, the BrD effect in cytotoxicity and apoptosis in T24 bladder cancer cells are equal to commonly used chemotherapy for bladder cancer, doxorubicin, and docetaxel. These results indicated that BrD is a promising plant-based bioactive compound in the fight against cancer. However, further in vitro and especially in vivo studies for this bioactive compound in various apoptosis signals and markers or different pathways and proteins are still required to be investigated.

Author Contribution Statement

All authors drafted and reviewed the manuscript; P.I.N.: design of the work, execution of experiments, data collection, and data analysis and interpretation; H.R., P.P. and I.Y.: design of the work, final approval, and supervision; E.S.P.: execution of experiments, data collection, and data analysis and interpretation; L.H.: final approval and supervision; A.B.: supervision.

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Conflicts of interest

The authors declare that the publication of this article does not have any conflict of interest.

Scientific approval

This study is a part of PhD Thesis of Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.

Ethical statement

Ethical approval was obtained from the Ethics Committee of Faculty of Medicine, Hasanuddin University, Makassar, Indonesia (371/UN4.6.4.5.31/PP36/2021).

MIRDA standard reporting recommendation

The manuscript follows the MIRDA standard reporting recommendation [49]

Data availability

The raw data and further supplementary information could be received upon request from the corresponding author.

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