https://doi.org/10.31925/farmacia.2026.3.7 **ORIGINAL ARTICLE**

BETULINIC ACID AND BETULIN DEMONSTRATE ANTITUMOR ACTIVITY AGAINST DETROIT 562 PHARYNGEAL CARCINOMA CELL LINE

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Abstract

Although there have been advances in current treatments for OPSCC (oropharyngeal squamous cell carcinoma), its management remains challenging due to the toxicity of current treatments, resistance and recurrence. These limitations have prompted the search for novel, innovative therapeutic strategies to optimize beneficial effects and minimize toxic effects. Betulinic acid (BA) and betulin (BET) are triterpenoids derived from natural sources that have received considerable attention for their activity against cancer cells. Although the antitumor effects of both compounds are well documented in the scientific literature, data regarding their activity in pharyngeal cancer are limited. In light of these premises, the present study aims to characterize the effects of BA and BET in terms of their cytotoxic activity and apoptotic/necrotic cell death patterns, their impact on nuclear and mitochondrial morphology, and alterations in mitochondrial membrane potential.

Rezumat

Deși s-au înregistrat progrese în tratamentele actuale pentru OPSCC (carcinom scuamos orofaringian), gestionarea acestei afecțiuni rămâne dificilă din cauza toxicității tratamentelor actuale, a rezistenței și a recidivelor. Aceste limitări au determinat căutarea și nevoia urgentă de strategii terapeutice noi și inovatoare pentru a optimiza efectele benefice și a le minimiza pe cele toxice. Acidul betulinic (BA) și betulina (BET) sunt triterpenoide derivate din surse naturale, care au beneficiat de o atenție sporită datorită activității lor împotriva celulelor canceroase. Deși efectele antitumorale ale ambilor compuși sunt bine documentate în literatura științifică, datele privind efectele lor asupra cancerului faringian sunt limitate. Având în vedere aceste premize, prezentul studiu își propune să caracterizeze efectele BA și BET din perspectiva activității lor citotoxice și a tipului de moarte celulară (apoptotă/necroză), a impactului lor asupra morfologiei nucleelor și a mitocondriilor, precum și a modificărilor potențialului membranei mitocondriale.

Keywords: betulinic acid, betulin, pharyngeal cancer, cytotoxicity

Introduction

Oropharyngeal squamous cell carcinoma (OPSCC) denotes those cancers that develop in the soft palate, palatine tonsils, tongue base, areas surrounding the epiglottis and pharyngeal wall (1). OPSCC is a subtype of head and neck squamous cell carcinoma (HNSCC)

and is part of a group of diseases that ranks seventh among the most prevalent forms of cancer worldwide, with nearly 700,000 new cases and 350,000 deaths each year (1, 2). Radiotherapy, anticancer medication therapy and surgery are all part of the multidisciplinary treatment strategy for HNSCC. However, the 5-year survival rate among patients is decreased by 50% due to metastasis to the regional lymph nodes. The predominant risk factors include smoking, alcohol consumption, autoimmune disorders and HPV (1-3). Although there have been advances in current treatments for OPSCC, its management remains challenging due to the toxicity of modern remedies, resistance and recurrence (4). Furthermore, OPSCC is a heterogeneous disease, with differences between HPV-positive and HPV-negative cases in terms of genetic profile and clinical outcomes, highlighting the need to develop personalized therapies. At the molecular level, a central role in tumour development is played by the dysregulation of signalling pathways, namely those involved in cell proliferation, apoptosis and inflammation (2, 4). These limitations have prompted the search for novel, innovative therapeutic strategies to optimize beneficial effects and minimize toxic effects. Hence, natural compounds are a promising alternative because they can target multiple oncogenic pathways and have a low toxicity profile.

Betulinic acid (BA) and betulin (BET) are triterpenoids derived from natural sources, such as the outer bark of birch trees (BA at 2 - 3%; BET at 10 - 30%). They differ structurally at the C-28 position, where BET has a hydroxyl group, whereas BA has a carboxyl group. This difference is also reflected in their aqueous solubility: 0.02 µg/mL for BA and 0.08 µg/mL for BET. Due to their limited solubility, several delivery techniques are being explored to enhance their bioavailability, as these compounds are of great interest because of their multiple therapeutic benefits, including antibacterial, anti-inflammatory and antiviral activities (5-7). However, their anticancer potential has attracted considerable attention in both *in vitro* and *in vivo* models. The features that make BA and BET particularly intriguing for cancer research are their selective action, meaning they are more toxic to tumour cells than to normal cells (6, 7). Although the antitumor effects of both compounds are well documented in the scientific literature, data regarding their effects in pharyngeal cancer are limited.

Given this, the present study aims to characterize the *in vitro* effects of BA and BET in terms of cytotoxic activity and effects on nuclei and mitochondria.

Materials and Methods

Reagents and instruments

BA and BET, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability kit, trypsin–EDTA solution, phosphate-buffered saline (PBS) and trypan blue solution 0.4%, Acridine orange and Propidium iodide solutions were obtained from Sigma Aldrich, Merck KGaA (Darmstadt, Germany). The cell culture medium Eagle's Minimum Essential Medium (EMEM) and foetal bovine serum (FBS) were acquired from ATCC (American Type Cell Collection, Lomianki, Poland). Hoechst 33342 dye and MitoTracker™ Red CMXRos were obtained from Thermo Fisher Scientific (Waltham, MA, USA), while JC-1 Dye was bought from Invitrogen, Carlsbad, CA, USA. The penicillin 100 U/mL–streptomycin 100 µg/mL antibiotic solution and dimethyl sulfoxide (DMSO) were obtained from PanBiotech (Aidenbach, Germany), 4% paraformaldehyde was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The Cytation 5 plate reader, Lionheart FX automated microscope and Gen5™ Microplate Data Collection and Analysis Software (v3.14) were obtained from BioTek Instruments Inc. (Winooski, VT, USA). The Olympus IX73 inverted microscope, together with the cellSens Dimensions software (v.1.8), was obtained from Olympus (Tokyo, Japan).

Cell culture conditions

Detroit 562 (pharyngeal carcinoma) cells used in the present study were obtained from the American Type Culture Collection, Lomianki, Poland. The cells were grown in EMEM, their specific cell culture medium. The antibiotic solution (Penicillin-streptomycin mixture, 1%) and 10% FBS were used to prepare the medium. Afterward, cells were maintained in a humidified incubator at 37°C and 5% CO_2 throughout every experiment. The stock solution was prepared by dissolving BA and BET in DMSO. Then, the working solutions were prepared by diluting the stock solutions in EMEM. The final DMSO concentrations of the working solutions did not exceed 0.5% (v/v). All working solutions were visually inspected before application to the cells and showed no visible signs of precipitation, turbidity, or phase separation; the solutions appeared homogeneous throughout preparation and use.

Cell viability assay

The MTT test was used to determine Detroit 562 cells' viability. Cells were cultured at a density of 1 x 10^4 *per* well in clear flat-bottom 96-well plates, and were left to attach until they reached the desired confluence. Afterward, cells were stimulated with BA and BET at 5, 10, 25 and 50 µM for 24 h. Post-treatment, 10 µL of MTT solution was added to each well, and the plates were then incubated for 3 h in a humidified incubator at 37°C and 5% CO_2. After the incubation period, 100 µL of MTT solubilization solution was added and incubated for another 30 min at room temperature. The final step was to read the absorbance at 570 nm and 630 nm using the Cytation 5 microplate reader (8).

Bright-field analysis of cell morphology

The effect of BA and BET at 5, 10, 25 and 50 µM on Detroit 562 cell morphology was examined after 24 h of treatment. Cells were seeded at a density of 1 x 10^4 *per* well in clear flat-bottom 96-well plates. After the treatment period, cells were imaged at 20× magnification using the Lionheart FX automated microscope and the Gen5™ Microplate Data Collection and Analysis Software (Version 3.14) (9).

Mitochondrial staining using MitoTracker™ Red CMXRos and nuclear morphology analysis with Hoechst 33342

To examine the morphology of the mitochondria and nuclei of the Detroit 562 cell line, the cells were seeded at a density of 1 x 10^4 cells *per* well in clear flat-bottom 96-well plates and were kept at standard culture conditions until they reached the required confluence. Subsequently, the cells were treated with BA and BET at 5, 10, 25 and 50 µM for 24 h. MitoTracker™ Red CMXRos was dissolved in DMSO to prepare a 1 mM stock solution, then diluted in the specific cell culture medium to obtain a final working solution of 300 nM. The protocol was then followed as described by Dan *et al*. (10).

Mitochondrial membrane potential assay

Detroit 562 cells were stained with JC-1 to assess mitochondrial potential after 24 h of treatment with BA and BET at 5, 10, 25 and 50 µM. Cells were first seeded at a density of 1 x 10^4 cells *per* well in clear, flat-bottom 96-well plates and maintained under standard culture conditions until they reached the required confluence. After stimulation, the cells were washed with PBS, and 100 µL/well of JC-1 dye at 5 µM was added. Then, the cells were incubated for 45 min at 37°C and 5% CO_2. After incubation, PBS was used again to wash the cells. Next, the cells were imaged using the Lionheart FX automated microscope and the Gen5™ Microplate Data Collection and Analysis Software (Version 3.14). Afterward, the fluorescence was read at 590 and 530 nm on Cytation 5 (11).

Acridine Orange/Propidium Iodide (AO/PI) double staining

Apoptotic and necrotic morphological changes in Detroit 562 cells were analysed by AO/PI double staining after treatment with BA and BET at 5, 10, 25 and 50 µM for 24 h. Cells were seeded in 96-well plates at a density of 1 × 10^4 cells *per* well and allowed to adhere under conventional culture conditions. Following treatment, 100 µL of staining solution containing 10 µg/mL AO and 10 µg/mL PI (10% final combination) was added to each well in the respective culture medium. Plates were incubated for 10 min at room temperature in the dark. The fluorescent images were taken with an exposure time of 100 ms and a resolution of 2048 × 2048 pixels at 20× magnification with an automated microscope, the Lionheart FX. Image acquisition and quantitative analysis were done using Gen5 Microplate Data Collection and Analysis Software.

Statistical analysis

Each assay was carried out in three separate experiments using technical triplicates. The mean ± standard deviation (SD) was used to express the results. The Shapiro-Wilk test was employed to determine whether the data distribution was normal. As the data followed a normal distribution, statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. GraphPad Prism version 10.2.3 (GraphPad Software, San Diego, CA, USA; www.graphpad.com) was chosen for the analysis. Data results that were statistically significant were marked with "*": **** $p < 0.0001$.

Results and Discussion

Pharyngeal cancer is one of the most aggressive forms of head and neck cancer, characterized by a poor prognosis, diagnosis at advanced stages and resistance to treatment (12, 13). In this context, therapies based on natural compounds are being investigated, as they have fewer side effects and help overcome multidrug resistance, which poses a significant challenge in clinical anticancer practice (14). BA and BET are among the natural substances of interest that have demonstrated antitumor efficacy and activity against multidrug-resistant tumour cells in several studies (15, 16). However, their antitumoral effects in pharyngeal cancer remain scarcely documented. Therefore, the present study was designed to examine the cytotoxic effects of BA and BET on Detroit 562 pharyngeal cancer cells after 24 hours of treatment at concentrations of 5, 10, 25 and 50 µM.

A combination of complementary assays was carried out to attain this objective: (i) MTT assay to determine cell viability, (ii) automated bright-field morphological analysis to examine structural changes and cell number, (iii) MitoTracker and Hoechst 33342 cell staining to highlight mitochondrial and nuclear alterations, (iv) JC-1 dye cell staining to assess mitochondrial membrane integrity, (v) AO/PI double staining to visualize apoptotic and necrotic morphological changes. The concentrations (5 - 50 µM) and exposure time (24 h) used in this study were selected based on previously reported ranges in the literature that demonstrated antitumor activity (6, 17-19).

Cell viability assay

The MTT assay is a colorimetric assay frequently used in drug discovery studies to evaluate treatment toxicity and metabolic activity by assessing cell viability (8). Thus, the effects of BA and BET at 5, 10, 25 and 50 µM on the viability of Detroit 562 cells were established using the MTT assay. Both compounds significantly reduced the viability of the cells compared to the control at all tested concentrations in a dose-dependent manner – BA from 67% to 26% and BET

from 48% to 34% at 5 and 50 μM, respectively (Figure 1).

Both compounds significantly reduced cell viability in the Detroit 562 cell line, but their treatment responses differed. BA exhibited a more pronounced concentration-dependent effect; this pattern is consistent with a study conducted on FaDu pharyngeal carcinoma cells, where BA reduced cell viability from 66% to 20% at concentrations of 12.5 - 50 μM after 24 hours of treatment (20). BET exhibited a plateau effect at 5 and 10 μM in Detroit 562 cells, which persisted even at higher concentrations (25 and 50 μM), indicating comparable cytotoxicity. In another study conducted on the Detroit 562 cell line, BET at 5 and 10 μM reduced cell viability to approximately 75% and 65%, respectively (18). These differences between our findings and previous reports may be due to the conditions under which the experiments were conducted, such as the manufacturer of the compounds and the cell line passage number. Other studies suggest that BA generally has a more potent effect compared to BET. Güttler *et al.* reported that 10 μM BA suppressed the growth of MDA-MB-231 breast cancer cells by approximately 50% after 24 h of treatment (21). On the other hand, BET exhibited moderate activity against the same cell line, with viability remaining around 90% at 10 μM and approximately 75% even at 25 - 50 μM after identical exposure durations (22).

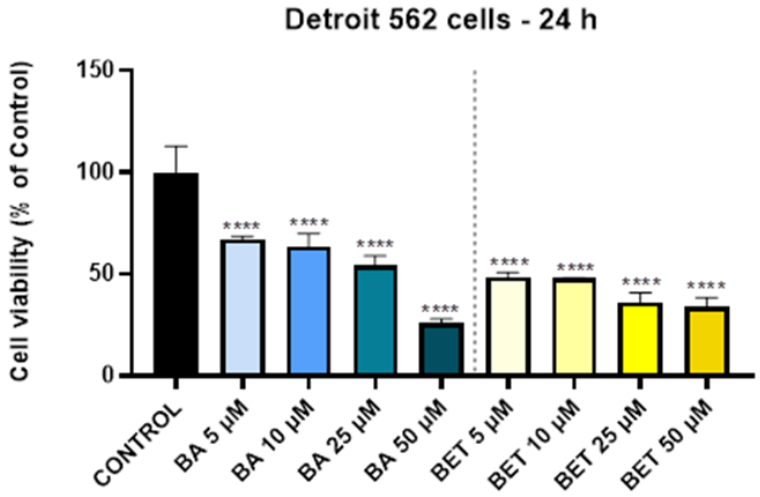

Figure 1.

Graphical representation of the effects of BA and BET on Detroit 562 pharyngeal carcinoma cells' viability at 5, 10, 25 and 50 μM after 24 h of treatment. Results were reported as percentages compared to the untreated cells (control group). One-way ANOVA and Dunnett's post hoc test were used to assess statistical significance between the BA- and BET-treated groups and the control (**** $p < 0.0001$). The experiments were performed in triplicate

Structurally, BET differs from BA by the presence of a hydroxymethyl group instead of a carboxylic acid group at the C-28 position. This difference may affect interactions with the mitochondrial membrane and other intracellular targets involved in apoptosis (7). Several scientific studies indicate that BA generally has a stronger and more consistent anticancer effect than BET in multiple experimental tumour models (23, 24). In the present study, BA confirms this, but only at the highest concentration. Since the MTT assay primarily reflects mitochondrial metabolic activity rather than direct cell death, these results should be interpreted alongside complementary apoptosis and morphological assays to accurately determine the underlying cytotoxic mechanisms.

Bright-field analysis of cell morphology

Microscopic analysis of Detroit 562 cell morphology was performed as a complementary test to the MTT assay to evaluate possible changes induced by the tested compounds following 24-hour stimulation. The morphological changes induced by BA and BET (Figure 2) were observable even at lower concentrations compared to the untreated group (control). BA at 5 and 10 μM caused pronounced cytosolic vacuolization, the most evident feature in this dosage range, along with a dose-dependent reduction in cell confluence. Furthermore, at 25 μM, cell rounding and shrinkage, debris and loss of cell-cell adhesion are more apparent. At the highest concentration, the observed alterations are substantial and include floating rounded cells, loss of adherence, disrupted cell-cell contact, markedly reduced confluence and cellular debris. BET treatment showed a similar trend, except that dysmorphologies were pronounced starting at 5 μM (rounded cells, loss of cell-cell contact, debris and detached floating cells).

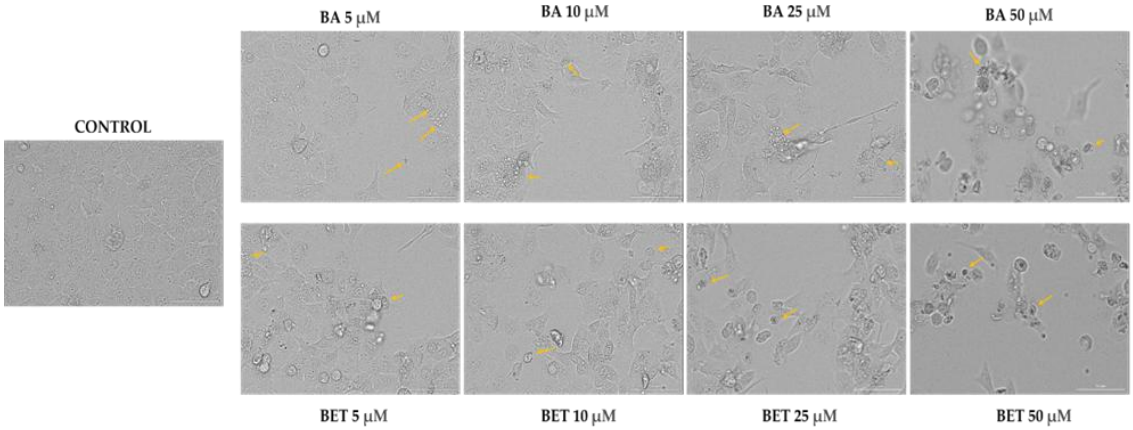

Figure 2.
Morphology of Detroit 562 cells after 24 h of treatment with BA and BET at 5, 10, 25 and 50 μM. Morphological alterations are indicated by yellow arrows. Scale bar = 100 μm

Bright-field microscopy is a mandatory standard for morphological observation of cells and for visualizing the cellular response induced by treatment (9). This approach gives valuable insights into cellular stress, cytotoxicity and changes in other cellular dynamics. In the present study, the morphological analyses were consistent with the MTT assay, confirming and further supporting the cytotoxic potential of the two tested substances. Treated cells exhibited clear dose-dependent morphological changes. More precisely, they displayed clear morphological modifications associated with cellular stress and loss of viability, including reduced adhesion, altered cell-cell interactions, and drastic shifts from the normal flat polygonal morphology to round or elongated phenotypes. Cells were more sensitive to BET treatment, as these changes were observed at the lowest concentration (5 μM), whereas with BA, they became somewhat more pronounced starting at 25 μM.

Mitochondrial staining using MitoTracker™ Red CMXRos and nuclear morphology analysis with Hoechst 33342

Next, to explore the possible mechanisms of action of BA and BET at concentrations of 5, 10, 25 and 50 μM on the Detroit 562 pharyngeal cancer cell line after 24 hours, changes in the mitochondria and nuclei were investigated (Figure 3). This assay was performed using the fluorescent dyes MitoTracker and Hoechst 33342. In both groups, the treatments affected the cells in a dose-dependent manner compared with the control group, in which the mitochondrial network maintained its reticular organization, the nuclei preserved their normal morphology, and the fluorescence was uniformly distributed. In the case of BA treatment, the lowest dose (5 μM) caused nuclear dysmorphology, accompanied by condensation of chromatin and mitochondria. At 10 μM, these signs became more evident, as evidenced by a more pronounced increase in fluorescence, along with cell shrinkage and decreased confluence. At 25 and 50 μM, these alterations became significantly more pronounced, with nuclear fragmentation occurring concurrently with the appearance of apoptotic bodies. The same pattern was observed in BET-treated cells; however, these signs were already noticeable at 5 μM.

Mitochondria are the pivotal organelles in maintaining cellular homeostasis. They function as central signalling centres that orchestrate cellular responses to stress and as reservoirs of proapoptotic molecules that control cell survival and apoptosis. Visualizing them is imperative for understanding the pathophysiological phenomena underlying energy production, oxidative stress and apoptosis (25). DNA content, its distribution and nuclear morphology are also important for appreciating cell cycle progression and for identifying drug-induced phenotypes. These objectives are typically achieved by fluorescence microscopy, which is commonly used in academic research (26). These include MitoTracker (for mitochondrial visualization) and Hoechst33342 (for nuclear visualization) fluorescent dyes, which remain preferred choices due to their simplicity, low cost and because they do not require genetic modifications. Moreover, they have the advantage of good permeability and low cytotoxicity (26, 27). Consistent with prior findings, BA and BET induced dose-dependent apoptosis-related morphological changes, including chromatin condensation, mitochondrial alterations, nuclear fragmentation and apoptotic body formation. Coricovac *et al*. treated A375 melanoma cells with BA (10 - 50 μM) and identified nuclear and mitochondrial changes using fluorescence microscopy. BA-stimulated cells exhibited dose-dependent cytotoxic effects, including nuclear fragmentation, apoptotic bodies and altered mitochondrial morphology (6). For BET, Hoechst staining showed that treatment with 5 μM and 10 μM resulted in nuclear condensation and apoptotic body formation in a dose-responsive manner (18).

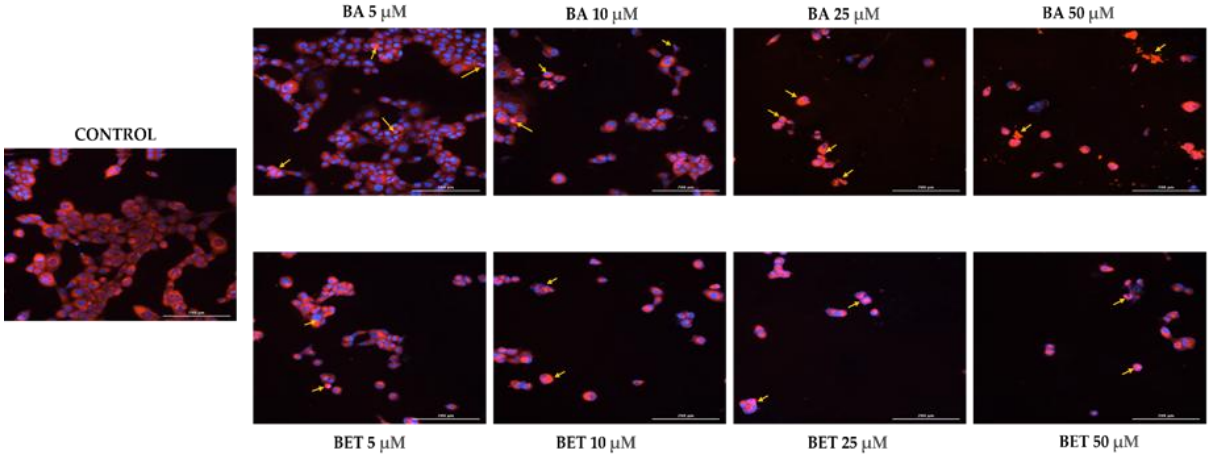

Figure 3.
Mitochondrial and nuclear morphology of Detroit 562 cells after 24 h of treatment with BA and BET at 5, 10, 25 and 50 µM. Morphological alterations are indicated by yellow arrows. Scale bar = 200 µm

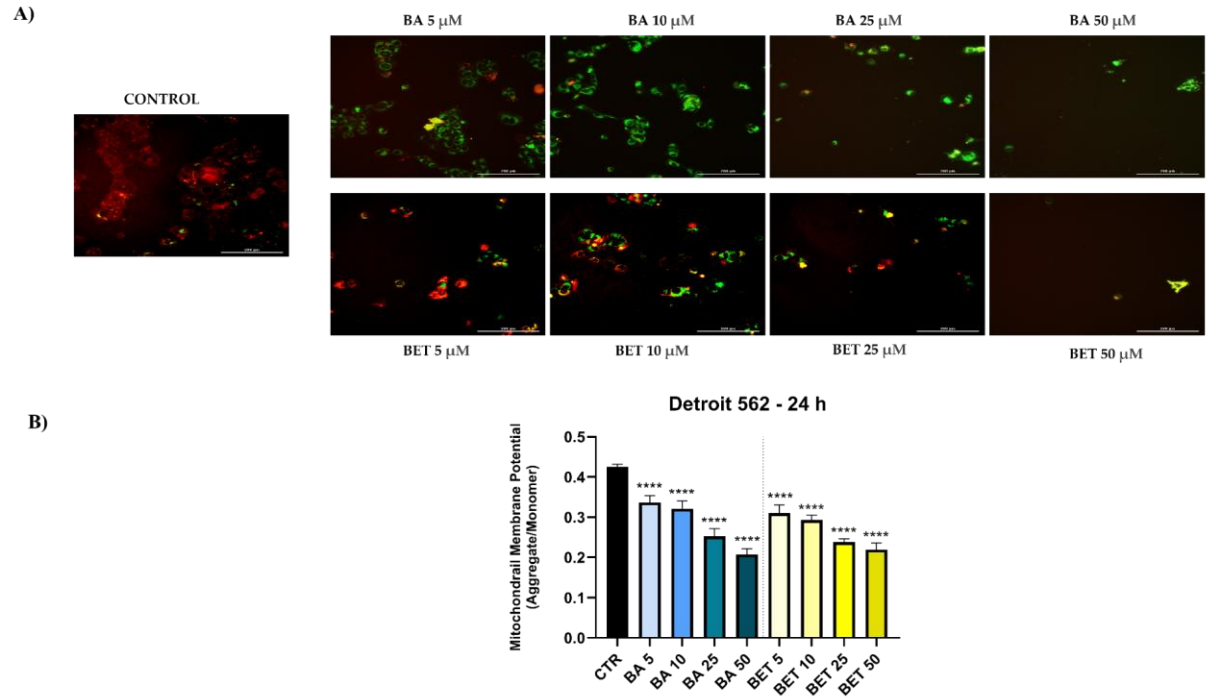

Figure 4.
A) Fluorescence images revealing JC-1 staining with BA and BET at 5, 10, 25 and 50 µM for 24 h on Detroit 562 pharyngeal carcinoma cells. Living cells emitted red fluorescence, whereas non-viable or necrotic cells emitted green fluorescence. An inverted fluorescence microscope was used to capture the images; the scale bar = 100 µm. B) The graph shows the ratio of JC-1 aggregates (red) to JC-1 monomers (green) as a measure of fluorescence intensity. The mean values ± SD of four separate measurements are represented in the data. The statistical differences in comparison with the control group were determined using a one-way ANOVA analysis and Dunnett's multiple comparisons post-test (**** $p < 0.0001$)

Mitochondrial membrane potential assay

Since we observed that BA and BET treatment induced cytotoxicity *via* the mitochondrial pathway, we further investigated the impact of our active substances on mitochondrial membrane potential, a hallmark of apoptosis-related cell death (6). The fluorescent dye JC-1 was used to highlight the effects of BA and BET (5 - 50 µM) after 24 h. Red fluorescence indicates unaffected cells, *i.e.*, a polarized membrane, whereas green fluorescence indicates dead cells with a depolarized membrane. The results show that green fluorescence predominates (dose-dependently) in cells treated with the two compounds, in contrast to the control, where red fluorescence predominates (Figure 4A). The ratio of JC-1 aggregates (red) to JC-1 monomers (green) was greater than 0.4 in untreated cells,

indicating a polarized membrane; meanwhile, in the treated groups, the ratio was less than 0.4 (Figure 4B).

BA and BET are considered mitocans, meaning they are agents that cause cell death by acting on mitochondria. Both are known to induce apoptotic cell death by altering mitochondrial membrane potential and interfering with several key mediators of cell death, including Bcl-2 family proteins, MAPKs, PI3K/AKT/mTOR, AMPK/mTOR and NF-κB (6, 7). Therefore, the decrease in mitochondrial membrane potential observed in the present study further supports their mitochondria-mediated proapoptotic effects.

Both BA and BET have demonstrated mitochondria-targeting anticancer activity in numerous *in vitro* studies conducted at concentration ranges similar to those used in the present study. BA was shown to induce mitochondrial dysfunction and loss of mitochondrial membrane potential in melanoma cells at concentrations from 1 - 50 μM (6), in lung cancer cells at 1 - 100 μM (28), and in cervical, breast, lung and colorectal cancer cells at approximately 22 μM (5) following 24 - 48 h exposure periods. Likewise, BET showed mitochondria-associated cytotoxic and proapoptotic effects in numerous cancer models, including neuroblastoma cells at 2.5 μM (29) and cervical, hepatic, prostate, lung, erythroleukemia and breast cancer cells at concentrations of approximately 2 - 226 μM after 48 h (30).

Acridine Orange/Propidium Iodide (AO/PI) double staining

The AO/PI double-staining was performed to assess whether BA and BET induced apoptosis in Detroit 562 cells at 5, 10, 25 and 50 μM after 24 h (Figure 5). Following the treatment period, untreated cells exhibited an even green fluorescence due to AO penetrating living cells, indicating intact morphology. The red fluorescent cells, as indicated by PI staining, appeared in a dose-dependent manner after BA and BET exposure, indicating loss of membrane integrity associated with late apoptosis and necrosis.

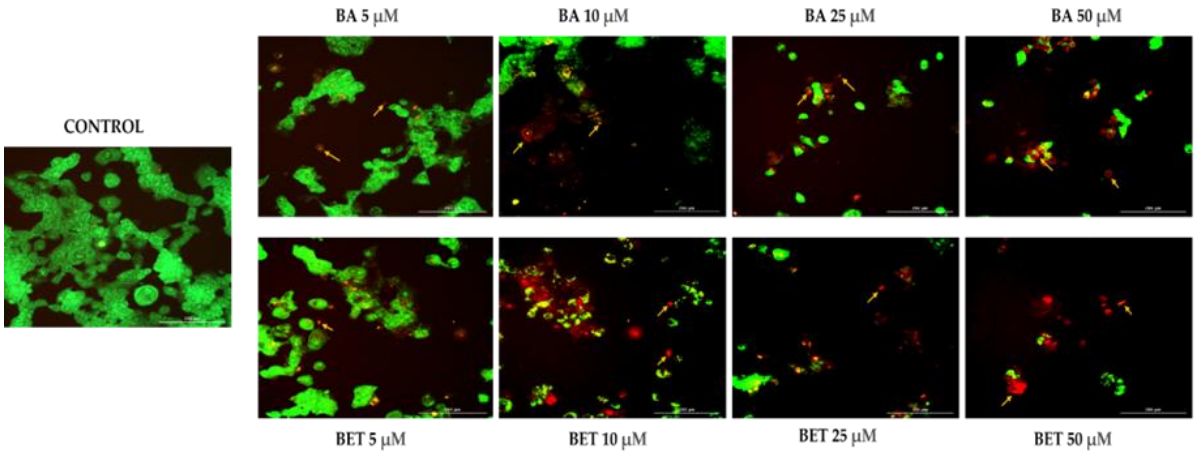

Figure 5.

Representative fluorescence images of the Detroit 562 cells stained with AO/PI after BA and BET at 5, 10, 25 and 50 μM after 24 h. Apoptotic and necrotic cells are indicated by yellow arrows. Scale bar = 200 μm

To further confirm BA- and BET-induced apoptosis in Detroit 562 cells, AO/PI double-staining was performed, as it is a reliable method that allows for differentiation of viable from non-viable cells. The results (Figure 5) showed a dose-dependent effect for both compounds, with an increase in apoptotic and secondary necrotic cells, as evidenced by chromatin condensation and PI-positive nuclei. The results are in agreement with previous observations on BA and BET derivatives. Vadivelu *et al*. showed that BA induced apoptosis in vascular smooth muscle cells in a time-dependent manner using AO/PI staining, with the number of apoptotic cells increasing after 24, 48 and 72 h of treatment (31). Csuk *et al*. demonstrated that cytotoxic BET-derived hydroxypropargylamines induced apoptotic cell death in human cancer cell lines; this was confirmed by AO/PI staining, annexin V-FITC assays and DNA laddering experiments (32). Despite these studies being performed in different cellular models, they still support the apoptosis-inducing potential of BA and BET-derived compounds.

The present paper has several limitations, one of which is that the selectivity of the tested compounds couldn't be assessed because no non-tumoral cell line was used. Also, the inclusion of multiple exposure intervals would provide a better understanding of the time-dependent effects of the compounds. Moreover, only one pharyngeal carcinoma cell line was investigated, which may limit the generalizability of the findings. Despite morphological observations and fluorescence-based assays, the exact molecular pathways underlying BA- and BET-induced cytotoxicity and apoptosis were not investigated. In this manner, a future perspective could be represented by examining oxidative stress (*e.g.*, through ROS assays) together with evaluating markers of apoptosis *via* PCR or Western

blot tests. In a nutshell, more advanced preclinical research is needed to establish the actual anticancer potential and safety profile, and to quantify the overall translational potential of the phytocompounds in pharyngeal cancer.

Conclusions

Both BA and BET exerted antitumoral activity against Detroit 562 pharyngeal carcinoma cells, by decreasing cell viability and inducing morphological, nuclear and mitochondrial changes associated with apoptosis-related cell death. At lower concentrations, BET was more active, whereas BA became more effective at the highest tested concentration. Alterations in mitochondrial membrane potential and AO/PI staining patterns indicate that mitochondrial dysfunction may contribute to the cytotoxic effects of both compounds. Collectively, these results provide evidence for the promising potential of BA and BET as natural compounds for further research in pharyngeal cancer. However, supplementary molecular and preclinical studies are required to elucidate their mechanisms of action, selectivity, safety profile and translational applicability.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

Not applicable.

AI use disclosure

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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