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Molecular Docking and the Protective Effects of β-Caryophyllene on ER Stress in Rat Aortic Smooth Muscle Cells Induced by Tunicamycin

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Abstract

This study aimed to investigate the cytoprotective effects of β-caryophyllene (BCP) against endoplasmic reticulum (ER) stress-induced apoptosis in rat aortic smooth muscle cells (RASMCs), confirm target protein interactions via molecular docking, and evaluate BCP's pharmacokinetic/ADMET profiles through *in silico* analysis. The cytotoxic potential of BCP in RASMCs was assessed using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. Reactive oxygen species (ROS) production and apoptosis were evaluated using DCFH-DA and DAPI staining, respectively. Protein expression was analyzed through immunocytochemistry, while gene expression was assessed using real-time polymerase chain reaction (RT-PCR). Our findings from various cell-based experiments demonstrate that BCP exerts a cytoprotective effect against tunicamycin-induced toxicity in RASMCs. BCP significantly inhibits ROS production and apoptosis caused by tunicamycin-induced ER stress by downregulating ER stress response marker genes (CHOP, GRP78 and EIF2A) and smooth muscle cell markers (αSMA). Molecular docking studies further confirmed the mechanism of action of BCP. Additionally, pharmacokinetic/ADMET profiles determined through *in silico* studies suggest that BCP holds potential as a therapeutic agent for treating cardiovascular diseases (CVDs). This study highlights BCP as a promising candidate for CVDs treatment, demonstrating its protective effects against tunicamycin-induced toxicity in RASMCs and its ability to inhibit key ER stress response markers.

Keywords: β-caryophyllene, Cardiovascular diseases, ER stress, Reactive oxygen species, Aortic smooth muscle cells

Introduction

Cardiovascular diseases (CVDs) are conditions that affect the heart and circulatory system, with atherosclerosis being their primary underlying cause. According to the report from the World Health Organization, CVDs are the leading cause of death worldwide. In the past year, approximately 17.9 million people died from CVDs, representing one-third of all global deaths [1]. More recently, the concept that arterial smooth muscle cells play a crucial role in regulating numerous vascular pathologies has been increasingly recognized in the present day [2]. Several studies suggest that endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) impact many physiological processes in various vascular cell types [3,4]. Under normal conditions, the UPR remains inactive as BiP/glucose-regulated protein 78 (GRP78) binds to the luminal domains of ER transmembrane proteins [4]. When the ER lumen accumulates unfolded

or misfolded proteins, BiP/GRP78 dissociates to assist in the folding process, initiating the UPR signaling cascade. The dissociation of GRP78 is the prevailing theory for UPR activation, although other unidentified mechanisms may also play a role [3,4]. Moreover, the accumulation of unfolded proteins such as eukaryotic initiation factor-2α (eIF2α), C/EBP homologous protein (CHOP), as well as GRP78 has also been implicated in triggering the UPR, which may induce ER stress in arterial smooth muscle cells [3,5]. Additionally, another molecular indicator for investigating vascular cell injury is the expression of smooth muscle cell (SMC) markers. Myosin heavy chain 11 (MYH11) and alpha-smooth muscle actin (αSMA) are two commonly used molecular markers of SMC, especially in indicating thoracic aortic dissection and patent ductus arteriosus diseases [6]. Therefore, investigating alterations in characteristic molecular markers has recently become a reliable and convenient tool for studying the potential of therapeutic agents for CVDs.

β-caryophyllene (BCP) is a bicyclic sesquiterpene natural compound and one of the most common components of essential oils found in various plants such as black pepper, mint, basil, thyme and cannabis [7]. BCP is a volatile compound with low solubility in water and is known for its potential pharmaceutical benefits, including analgesic, antioxidant, antimicrobial and anti-inflammatory properties [8,9]. Classified as a member of the cannabinoid family, BCP has the ability to interact with cannabinoid receptors and exhibit pharmacological activities through the endocannabinoid system [9,10]. Recent studies have emphasized the protective role of BCP in animal cells, highlighting its beneficial effects against various diseases [8,10]. Based on this, we hypothesize that BCP may offer benefits for CVDs by reducing reactive oxygen species (ROS) production and decreasing apoptosis of aortic smooth muscle cells. Therefore, the aim of this study was to investigate the cytoprotective effects of BCP against ER stress inducer-induced apoptosis in rat aortic smooth muscle cells (RASMCs). Additionally, the potential effect of BCP on target proteins was confirmed through molecular docking studies. Pharmacokinetic/ADMET profiles of BCP were also determined *in silico*.

Materials and methods

Cell culture and cytotoxicity assay

RASMCs were cultured in high glucose DMEM medium (Nacalai tesque, INC., Kyoto, Japan) with the addition of 10% (v/v) fetal bovine serum and 1% antibiotic (penicillin/streptomycin; Nacalai Tesque, Inc., Kyoto, Japan) and incubated in a humidified tissue culture incubator containing 5% CO_2 at 37 °C. For cytotoxicity assay, 10^4 cells per well of RASMCs were plated in the conditioned medium for overnight in a 96-well plate. Subsequently, the cells were exposed to BCP at the concentration of 1, 50, 100, 250, 500 and 1,000 μM for 24 h. The cytotoxicity of test compounds was evaluated using the MTT (Wako Pure Chemical Industries Ltd., Osaka, Japan) assay. The absorbance of each well was recorded at 570 nm using a microplate reader (Bio-Rad Laboratories, Inc., California, USA).

Cytoprotective assay

RASMC cells (10^4 cells per well) were seeded into 96-well plates overnight. They were then pretreated with BCP (100, 250 and 500 μM) for 2 h. Subsequently, the cells were induced with tunicamycin (Tu; 1 μg/mL) in the presence or absence of BCP for the following 24 h in serum-free medium. Afterward, the MTT solution was added for 4 h and then measured at 570 nm.

Apoptotic assay by DAPI staining

RASMCs (5×10^4 cells per well) were cultured in 8-well slide chambers (Watson® Bio Lab, Kyoto, Japan) overnight. Subsequently, they were treated with BCP (100 and 250 μM) for 2 h. Following the pretreatment, the RASMCs were exposed to Tu (1 μg/mL) with or without BCP for 24 h in serum-free medium. After the specified treatments, the cells were fixed with 4% paraformaldehyde (PFA) for 10 min and stained with 4,6-diamidine-2-phenylindole, dihydrochloride (DAPI; LGC Seracare, USA) for 30 min. Finally, imaging was performed using a Nikon ECLIPSE E200 fluorescence microscope (Nikon, Tokyo, Japan). The percentage of apoptotic cells was calculated as (number of apoptotic cells/total number of cells)×100.

Reactive oxygen species (ROS) production by DCFH-DA assay

RASMCs (5×10^4 cells per well) were cultured in 8-well slide chambers overnight. The cells were then pre-treated with BCP (100 and 250 μM) for 2 h, followed by induction with Tu (1 μg/mL) in the presence or absence of BCP for 24 h under serum-free conditions. After the specified treatments, the cells were fixed with 4% PFA and stained for intracellular ROS production by the oxidation of DCFH-DA. Specifically, the cells were incubated with DCFH-DA (50 μM) for 1 h in dark conditions. The fluorescence of DCF was detected using a fluorescence microscope (Nikon, Tokyo, Japan). The intensity of DCF was analyzed using the ImageJ program (https://imagej.nih.gov/ij/download.ht).

Immunofluorescence assay

RASMCs (5×10^4 cells per well) were cultured in an 8-well slide chamber overnight. Subsequently, the cells were pre-treated with BCP (100 and 250 μM) for 2 h, followed by induction with Tu (1 μg/mL) in the presence or absence of BCP for the next 24 h under serum-free conditions. After the 24-hour incubation period, the cells were fixed with 4% PFA for 15 min, permeabilized with 0.2% Triton X-100 for 10 min and then blocked with 3% bovine serum albumin (BSA) for 1 h. The slides were then incubated with a primary αSMA antibody (sc-58669, Santa Cruz Biotechnology, Texas, USA) at a 1:200 dilution at 4 °C overnight. Subsequently, the cells were incubated with fluorescent secondary antibodies Alexa Fluor 488 (LGC Seracare, USA) at a dilution of 1:500 at room temperature for 1.5 h. Nuclei were stained with DAPI. Finally, images were captured using a Nikon ECLIPSE E200 fluorescence microscope (Nikon, Tokyo, Japan).

Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR)

RASMCs (10^6 cells per plate) were cultured in P100 culture dishes overnight. Subsequently, the cells were pre-treated with BCP (100 and 250 μM) for 2 h, followed by induction with Tu (1 μg/mL) in the presence or absence of BCP for the following 24 h under serum-free conditions. Total RNA was extracted using Sepasol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan) following the manufacturer's instructions. Subsequently, cDNA was synthesized using the PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara Bio Inc., Japan). After cDNA synthesis, real-time PCR was conducted using Luna Universal qPCR Master Mix (New England Biolabs Inc., Japan) to quantify mRNA expression with a real-time PCR detection system (Bio-Rad Laboratories, Inc., California, USA) using SYBR green. The amplification conditions included 15 sec at 95 °C and 30 s at 62 °C for 45 cycles. GAPDH was utilized as the internal control following the $2^{-\Delta\Delta Ct}$ method. All primer sequences were presented in our previous study [11].

Molecular docking and determination of pharmacokinetic/ADMET profiles

An *in silico* experiment was conducted for molecular docking and the determination of pharmacokinetic/ADMET profiles of BCP and relevant target proteins. The three-dimensional structures of GRP78 and EIF2A were downloaded from the Protein Data Bank (PDB) with PDB codes 3LDL and 8DYS, respectively [12,13]. The chemical structure of BCP was obtained from the PubChem database (CID_20831623) for further analysis [14].

Simulations of the molecular docking process of BCP and the target protein were conducted using the Genetic Optimisation for Ligand Docking (GOLD) program version 5.3.0 software and Discovery Studio 2021 software [15]. The protein structure was prepared by initially removing water molecules, followed by adding hydrogen atoms to the protein models using the GOLD setup process. The docking site is situated at the active site of the target protein structures, positioned on the ligand. A Genetic Algorithm (GA) search process was then carried out at the most accurate level (slow mode) and the molecular docking was evaluated using the Chem Score fitness function [16]. PLP fitness scores were utilized to assess the docking of BCP at the binding site of the target proteins. Furthermore, Discovery Studio 2021 software was employed to identify the hydrophilic, hydrophobic, electrostatic and van der Waals forces resulting from the interaction between target proteins and BCP [17]. Physicochemical and pharmacokinetic profiles of BCP were assessed to evaluate the drug-like attributes of the chemical compound using the SwissADME web tool [18].

Statistical analysis

The data were presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by a post-hoc Tukey test. A *p-value* less than 0.05 was considered statistically significant, indicating significant differences between groups.

Results and discussion

Results

Cytotoxicity of BCP on RASMA cells

Illustrated in **Figure 1**, the viability of RASMC cells was evaluated following exposure to BCP across concentrations ranging from 1 to 1,000 µM over a 24-hour incubation period. The findings revealed that none of the BCP concentrations exhibited cytotoxic effects on RASMC cells, as cell viability remained above 90% compared to the control group (**Figure 1(A)**). Consequently, the concentrations of 100, 250 and 500 µM of BCP were chosen for subsequent experiments.

Cytoprotective effects of BCP against tunicamycin-induced ER stress in RASMCs cells

To explore the cytoprotective impact of BCP on RASMC cells under ER stress induced by Tu, the cells were pre-treated with non-toxic concentrations of BCP (100, 250 and 500 µM) prior to exposure to Tu at toxic levels (1 µg/mL). The results demonstrated a significant reduction in RASMC cell viability by 74.50 ± 0.62% with Tu treatment. However, pre-treatment with BCP for 2 h before Tu exposure led to enhanced cell viability. The combined treatment of BCP and Tu resulted in cell viabilities of 99.94 ± 7.11%, 102.35 ± 5.69% and 92.24 ± 5.68% at concentrations of 100, 250 and 500 µM, respectively, compared to Tu treatment alone (**Figure 1(B)**). These results indicate that BCP has the potential to shield cells from the toxicity induced by Tu.

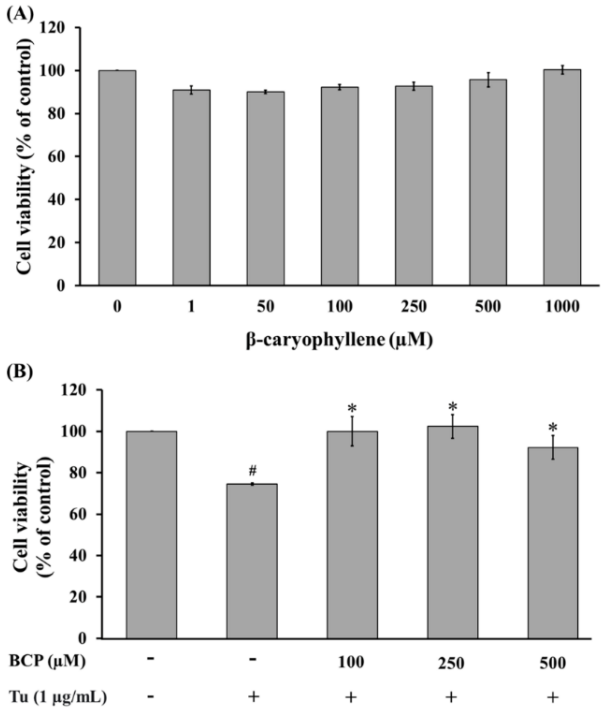

Figure 1 Effects of β-caryophyllene (BCP) on (A) cell viability and (B) the cytoprotective effect of BCP against tunicamycin (Tu)-induced ER stress in RASMCs cells. All values are presented as mean ± SEM (n = 4). Statistical significance was determined using Tukey post hoc analysis, with significance accepted when *p-value* < 0.05 (# compared to the control group, * compared to the Tu group).

BCP inhibits ROS production and apoptosis in tunicamycin-induced ER stress in RASMCs cells

Illustrated in **Figures 2(A) - 2(B)**, the intracellular ROS levels in RASMC cells were evaluated using the DCFH-DA probe. The group treated with Tu alone exhibited a significant increase in ROS levels by 142.75 ± 2.93% compared to untreated cells. However, pre-treatment with BCP at concentrations of 100 and 250 µM for 2 h followed by exposure to Tu (1 µg/mL) for 24 h notably decreased the ROS levels induced by Tu, in contrast to cells treated with Tu alone (**Figures 2(A) - 2(B)**). Elevated ROS production can trigger cell apoptosis. Hence, the percentage of apoptotic cells in response to Tu was determined through the DAPI staining assay. Tu treatment led to a substantial increase in the percentage of apoptotic cells to 25.79 ± 0.67%. Pre-treatment with BCP at concentrations of 100 and 250 µM for 2 h followed by exposure to Tu significantly reduced the percentages of apoptosis to 14.47 ± 1.00% and 6.95 ± 1.47%, respectively (**Figures 2(C) - 2(D)**). These results indicate that BCP mitigated Tu-induced toxicity by suppressing ROS production and apoptosis in RASMC cells.

BCP prevents ER stress-mediated cell death in RASMCs cells by reducing the ER stress response marker gene

Real-time RT-PCR was employed to analyze the gene expressions of CHOP, GRP78, ATF4 and EIF2A, as illustrated in **Figure 3**. The findings revealed that Tu notably elevated the mRNA levels of CHOP, GRP78 and EIF2A by approximately 2.84 ± 0.58, 3.06 ± 0.34 and 1.94 ± 0.14, respectively, while decreasing ATF4 expression by 0.51 ± 0.02 (**Figures 3(A) - 3(D)**). Pre-treatment with BCP in RASMC cells significantly attenuated the mRNA expression of CHOP, GRP78 and EIF2A compared to Tu treatment alone. However, all concentrations of BCP did not impact ATF4 mRNA expression relative to Tu treatment alone (**Figures 3(A) - 3(D)**).

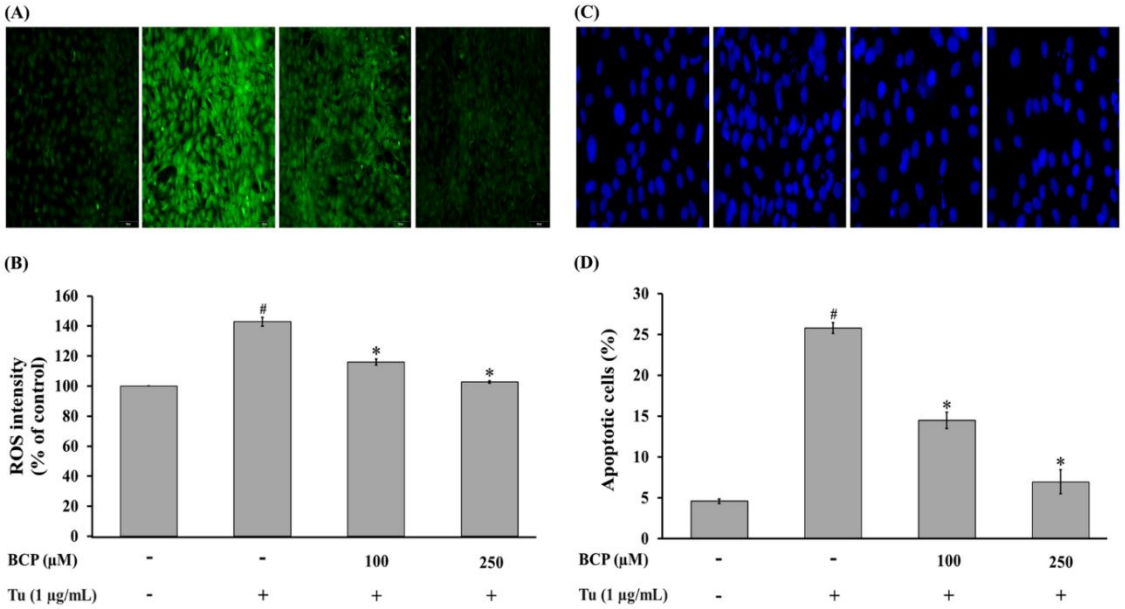

Figure 2 Effect of β-caryophyllene (BCP) on the reactive oxygen species (ROS) and apoptosis against tunicamycin (Tu) induced-ER stress in RASMCs cells. (A) The ROS levels were detected by fluorescence microscopy. (B) The ROS intensity was measured by ImageJ program. (C) Morphology of apoptotic nuclei stained with DAPI. (D) The percentages of apoptotic cells were calculated. All values are presented as mean ± SEM (n = 4). Statistical significance was determined using Tukey post hoc analysis, with significance accepted when *p-value* < 0.05 (# compared to the control group, * compared to the Tu group).

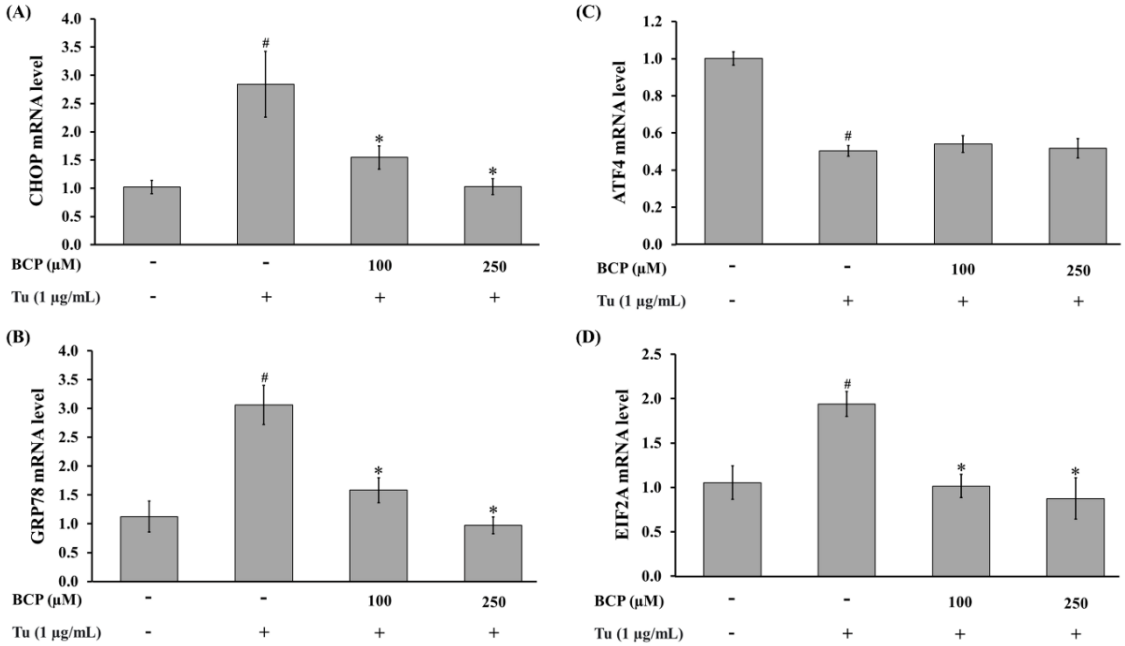

Figure 3 Effects of β-caryophyllene (BCP) on the mRNA expression of CHOP, GRP78, ATF4 and EIF2A on tunicamycin (Tu)-induced ER stress in RASMCs cells. The cells were pre-treated with BCP for 24 h followed by the ER stress inducer Tu for 24 h. (A) CHOP, (B) GRP78, (C) ATF4 and (D) EIF2A. All values are presented as mean ± SEM (n = 4). Statistical significance was determined using Tukey post hoc analysis, with significance accepted when *p-value* < 0.05 (# compared to the control group, * compared to the Tu group).

BCP prevent ER stress induced RASMCs cells injury by decreasing SMC markers

Figure 4(A) shows that MYH11 expression decreases significantly to approximately 0.50 ± 0.10 of the control following Tu treatment, suggesting a loss of the contractile smooth muscle cell phenotype, as MYH11 serves as a marker for mature contractile cells. Pre-treatment with BCP does not significantly alter MYH11 mRNA levels compared to Tu treatment alone (**Figure 4(A)**). As depicted in (**Figure 4B**, Tu treatment alone causes a marked upregulation of αSMA gene expression in RASMCs, with levels rising to approximately 2.24 ± 0.25 times those of the control group. These results indicate that Tu induces a phenotypic switch toward a more contractile or synthetic state, which is commonly associated with stress or pathological processes in smooth muscle cells. Pre-treatment with BCP at both 100 and 250 μM (prior to Tu exposure) significantly reduces αSMA mRNA levels compared to the Tu-only group (**Figure 4(C)**) demonstrates αSMA protein expression (red staining), where Tu-treated cells display intensified red fluorescence, confirming the increase in αSMA protein. In contrast, pre-treatment with BCP at 100 and 250 μM results in a progressive decrease in αSMA-positive staining, with 250 μM BCP showing the most substantial reduction. This finding is consistent with the mRNA data and confirms that BCP reduces αSMA expression at the protein level in a dose-dependent manner. Overall, BCP may mitigate some Tu-induced phenotypic changes by reducing αSMA upregulation, potentially limiting pathological smooth muscle cell remodeling; however, it does not restore all markers of the contractile phenotype, such as MYH11.

Molecular docking and determination of pharmacokinetic/ADMET profiles

Based on a molecular docking analysis against GRP78 and EIF2A, BCP exhibited the best-bound conformation and interactions in the active binding site of both GRP78 and EIF2A proteins (**Figures 5 - 6**). Therefore, BCP could potentially be used as a therapeutic agent to reduce ER stress, which is a therapeutic target in CVDs.

The physicochemical properties of the compound, including the partition coefficient (log P), hydrogen

bond acceptor (HBA), hydrogen bond donor (HDB), molecular weight (MW), total polar surface area (TPSA), molar refractivity (MR) and rotatable bond (RB), are crucial drug-like characteristics that are calculated for the selected compounds. *In silico* ADMET (absorption, distribution, metabolism, excretion and toxicity) profiles, including important physicochemical properties, pharmacokinetics and drug-likeness, are summarized in **Table 1**.

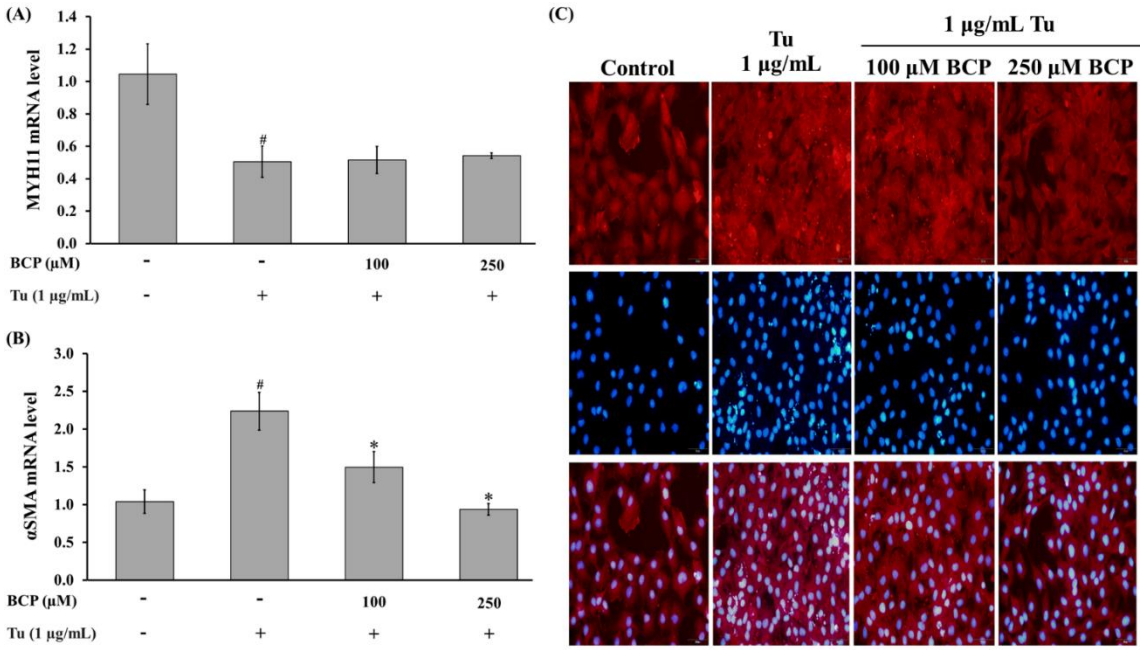

Figure 4 Effects of β-caryophyllene (BCP) on the expression of MYH11 and αSMA on tunicamycin (Tu)-induced ER stress in RASMCs cells. The cells were pre-treated with BCP for 2 h followed by Tu and BCP for 24 h. (A) MYH11 gene, (B) αSMA gene and (C) the αSMA proteins were detected by immunofluorescence. All values are presented as mean ± SEM (n = 4). Statistical significance was determined using Tukey *post hoc* analysis, with significance accepted when *p-value* < 0.05 (# compared to the control group, * compared to the Tu group).

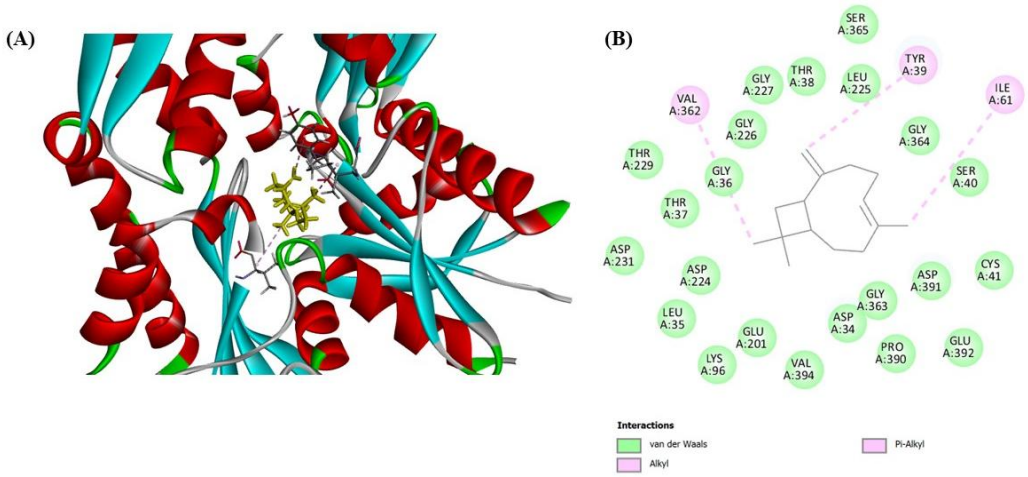

Figure 5 Graphical representation of (A) three-dimensional presenting molecular docked complex of BCP in the region of active binding site of GRP78 protein and (B) two-dimensional plot presenting binding interactions of the BCP with target GRP78 protein.

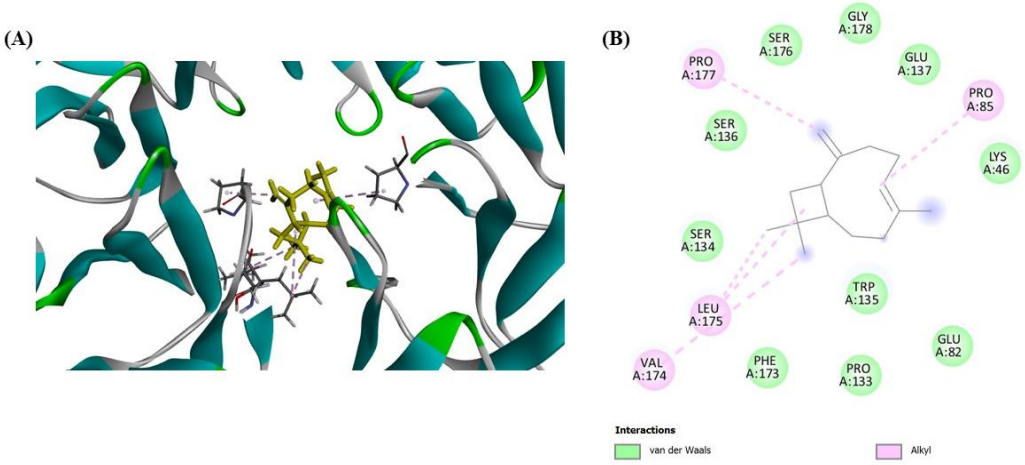

Figure 6 Graphical representation of (A) three-dimensional presenting molecular docked complex of BCP in the region of active binding site of EIF2A protein and (B) two-dimensional plot presenting binding interactions of the BCP with target EIF2A protein.

Table 1 Summary of *in silico* ADMET profiles estimated for BCP.

Descriptors	**β-caryophyllene**
Formular	$C_{15}H_{24}$
Molecular weight (MW)	204.35 g/mol
Number of rotatable bonds	0
Number of hydrogen bond acceptors (HBA)	0
Number of hydrogen bond donors (HBD)	0
Molar refractivity (MR)	68.87
Lipophilicity (Log P)	3.29
Water solubility (Log S)	–3.87 (Soluble)
Topological polar surface area (TPSA)	0.00 Å²
Pharmacokinetics	
- Gastrointestinal drug absorbtion (GI-DA)	Low
- Blood brain barrier (BBB) permeability	No
- P-glycoprotein (P-gp) substrate	No
- CYP1A2 inhibitor	No
- CYP2C19 inhibitor	Yes
- CYP2C9 inhibitor	Yes
- CYP2D6 inhibitor	No
- CYP3A4 inhibitor	No
Log Kp (skin permeation)	–4.44 cm/s
Drug-likeness	
- Lipinski rule (MW ≤ 500, logP ≤ 5, HBD ≤ 5, HBA ≤ 10)	Acceptable

Descriptors	β-caryophyllene
- Veber rule (RB ≤ 10, TPSA ≤ 140)	Acceptable
- Drug-likeness	Yes
- Bioavailability score	0.55

Discussion

CVDs are a major health problem globally. The development of CVDs, such as atherosclerosis, is a multifaceted process that encompasses a range of metabolic and signaling pathways. Recently, various studies suggested that ER stress signaling pathways play crucial roles in atherosclerosis and its related CVDs, mediating the activation of inflammatory response mechanisms and apoptotic signaling pathways [2]. BCP is a natural product that has been studied for its ability to influence ER stress, which has been associated with various diseases, including CVDs [10]. In this study, various methods of cell-based *in vitro* experiments and molecular docking *in silico* studies were conducted to explore the potential of BCP as a therapeutic agent for CVDs.

A preliminary cytotoxic screening using the MTT assay revealed that BCP did not exhibit cytotoxicity against RASMC cells, even at a high concentration (1,000 μM), suggesting that BCP is considered safe for aortic smooth muscle cells. Furthermore, to investigate the cytoprotective effect of BCP, Tu, one of the most common natural antibiotics produced by several bacteria, was used to induce ER stress in pre-treated RASMC cells in this study. Tu has been reported to inhibit the initial stages of N-linked protein glycosylation, catalyzed by DPAGT1 (UDP-GlcNAc: Dolichol phosphate N-acetylglucosamine-1-phospho transferase), through its action on the MFSD2A transporter, which contains a major facilitator domain [19]. This process results in significant protein misfolding, activating the UPR and ultimately triggering the apoptosis process [20]. According to our results, BCP has a protective effect on RASMC cells against the toxicity induced by Tu, potentially preserving aortic smooth muscle cell function and viability. This effect could help prevent cell damage or death during the development of atherosclerosis. However, to confirm the direct involvement of this mechanism of action, further studies are necessary. In particular, evaluating protein expression using techniques such as Western blotting or employing gene-editing technologies like CRISPR/Cas9 would provide stronger mechanistic evidence to support the role of this pathway in BCP-mediated protection.

Apoptosis of vascular smooth muscle cells has recently been recognized as a phenomenon occurring in the physiological remodeling of the vasculature, as well as in atherosclerosis disease [21,22]. ROS are crucial for cell signaling at physiological levels. However, elevated ROS levels can lead to oxidative stress, which, in turn, can cause apoptosis of vascular smooth muscle cells, implicated in CVDs [21,23]. Therefore, preventing the apoptosis of vascular smooth muscle cells and reducing ROS production is an effective strategy to slow the progression of cardiovascular diseases, especially atherosclerosis. The present study demonstrated that BCP significantly suppressed ROS production induced by Tu compared to the control group of Tu-treated cells alone and also significantly inhibited the apoptosis process in RASMC cells pre-treated with Tu. This finding is consistent with a previous report that showed BCP has significant anti-inflammatory and antioxidant effects in various experimental models. For example, in a rat model of isoproterenol-induced myocardial infarction, BCP exhibited cardioprotective properties by reducing oxidative stress—evidenced by increased cardiac antioxidant enzyme activity and decreased lipid hydroperoxides and lysosomal thiobarbituric acid reactive substances levels, both markers of oxidative heart damage [24]. Similarly, in a type 2 diabetic rat model, BCP corrected redox imbalance, preserved mitochondrial structure and protected hepatocytes from diabetes-related oxidative injury [25]. Additionally, in LPS-stimulated HaCaT cells, BCP's anti-inflammatory effects were reversed by a CB2 antagonist, confirming CB2's role in mediating this protection [26,27].

To conduct an in-depth investigation, real-time RT-PCR was used to determine the expressions of genes involved in ER stress response. CHOP, GRP78, ATF4

and EIF2A are commonly used as markers of ER stress, with GRP78 playing a critical role in regulating the UPR signaling network [28]. The primary objectives of UPR signaling responses are to decrease the accumulation of unfolded proteins in the ER and restore cellular balance. However, when these processes become excessive or prolonged, they can trigger apoptosis, autophagy and other forms of cell death, ultimately leading to disease [29]. According to our findings, BCP significantly decreased the mRNA expression of CHOP, GRP78 and EIF2A compared to Tu treatment alone. However, none of the BCP concentrations had an impact on ATF4 mRNA expression compared to Tu treatment alone. In this pathway of the UPR, generally, protein kinase RNA-like endoplasmic reticulum kinase (PERK) stimulates the phosphorylation of EIF2A, leading to increased translation of ATF4. ATF4, in turn, enhances the transcription of specific UPR target genes, such as CHOP. It is believed that CHOP plays a role in inducing apoptosis [28]. However, the expression of ATF4 is regulated by both transcriptional and translational processes. Under certain stressful conditions, ATF4 transcription can be suppressed, leading to a decrease in the amount of ATF4 mRNA available for translation [30]. In this context, EIF2A phosphorylation and translational control are activated or suppressed without triggering ATF4 and its downstream targets. Therefore, our results indicate that the ER stress-mediated apoptosis pathway is activated by the Tu-induction method and BCP has the ability to inhibit the apoptosis process via the GRP78/EIF2A/CHOP signaling pathway. Similarly, in the *in vivo* model of indomethacin -induced gastric ulcer, BCP alleviates gastric injury by suppressing GRP78/eIF2α/CHOP expression, thereby reducing ER stress and its downstream apoptotic effects. These results highlight the potential of BCP as a promising modulator of the ER stress response [31].

SMCs are the primary cell type in the pre-atherosclerotic intima. The altered phenotype of vascular smooth muscle cells is considered the primary pathophysiological change in CVDs. As atherosclerotic plaque develops, changes occur in the intimal SMC. These changes include increased proliferation, reduced contractility, higher production of proteoglycans and alteration of the expression of SMC markers such as αSMA and MYH11 protein [32]. According to a previous report, the expression of αSMA was found in all stages of atherosclerosis [33]. On the other hand, some researchers suggest that a decrease in the expression of MYH11 protein would lead to ER stress and cardiomyocyte damage [34]. Our results showed that after treating the cells with Tu, the expression of αSMA significantly increased, but the expression of the MYH11 gene significantly decreased compared to the non-treatment control group. BCP showed some potential beneficial effects, as the group pretreated with BCP significantly downregulated the expression level of αSMA in RASMC cells but did not alter MYH11 mRNA expression compared to Tu treatment alone. To validate the impact of BCP on αSMA, we conducted immunofluorescence analysis to assess αSMA protein expression in Tu-treated cells with or without BCP. The findings indicated that pretreatment with BCP before Tu exposure reduced the αSMA-positive signal in the cells compared to Tu treatment alone. Therefore, it may imply that BCP can prevent cell injury induced by ER stress by decreasing the expression of αSMA protein.

To confirm the interactions between BCP and the targeted proteins, *in silico* molecular docking was performed. Molecular docking can be valuable for elucidating the molecular mechanism of a bound ligand at the active site of a target protein. According to a molecular docking analysis against GRP78 and EIF2A, BCP exhibited a bounded conformation and interactions in the active binding site of both proteins. The evaluation of molecular docking of BCP at the active binding site of the GRP78 protein showed that BCP potentially generated a van der Waals interaction network with SER365, GLY227, THR38, LEU225, THR229, GLY226, GLY364, SER40, GLY36, THR37, ASP231, ASP224, LEU35, LYS96, VAL394, ASP34, GLY363, PRO390, ASP391, GLU392, CYS41 residue of the target protein. Additionally, VAL362, TYR39 and ILE61 were involved in alkyl and Pi-alkyl interactions of BCP with GRP78 (**Figure 5**). Furthermore, the interaction between BCP and EIF2A was also determined. BCP generated a van der Waals interaction network with SER176, GLY178, GLU137, SER136, LYS46, SER134, TRP135, PHE173, PRO133, GLU82 residues of the EIF2A protein. Moreover, PRO177, PRO85, LEU175 and VAL174 residues were involved in alkyl and Pi-alkyl interactions between BCP and EIF2A protein (**Figure 6**).

To identify the potential of drug-like compounds, pharmacokinetic and ADMET profiles provide crucial information for screening, designing and developing novel drugs. In this study, ADMET profiles of BCP were calculated using SwissADME. Physicochemical properties of BCP, including molecular weight (MW), partition coefficient (log P), hydrogen bond acceptor (HBA), hydrogen bond donor (HDB), total polar surface area (TPSA), molar refractivity (MR) and rotatable bonds (RB), essential drug-like characteristics calculated for selected compounds, are reported in **Table 1**. Based on the drug-likeness theory, the physicochemical properties of THC and CBD fell within acceptable ranges. The lipophilicity of BCP indicates that it is unlikely to cross the blood-brain barrier, showing a lower risk of causing CNS-related adverse effects. Additionally, the water solubility classes of BCP exhibited positive outcomes, suggesting that it can be absorbed in the blood and tissues [35]. Pharmacokinetic properties of BCP indicated that it was not a P-glycoprotein (P-gp) substrate; however, BCP showed inhibitory potential on CYP2C19 and CYP2C9. Regarding its drug-likeness, the present study suggested that BCP meets acceptable criteria according to the Lipinski rule and Veber rule, making it a potentially viable candidate as a drug-like compound. Nevertheless, these *in silico* studies are based on predictive models and do not account for complex biological factors such as metabolic transformation, tissue distribution, or long-term toxicity, all of which may significantly influence drug behavior *in vivo*. Therefore, while BCP exhibits favorable ADMET and drug-likeness characteristics *in silico*, further *in vivo* pharmacokinetic and toxicity studies are essential to validate these predictions and to better understand its pharmacokinetic profile under physiological conditions. Future studies should also provide more information on efficacy and safety in relevant animal models before clinical translation can be considered.

Conclusions

This study suggests that BCP has the potential to be an interesting candidate for treating CVDs. Various cell-based *in vitro* experiments were conducted and presented to demonstrate that BCP can protect RASMCs cells against toxicity induced by Tu. BCP also inhibits ROS production and apoptosis in tunicamycin-induced ER stress in RASMCs cells, which is one of the major pathologies in CVDs. The mechanism of action of BCP has been clarified; BCP significantly suppresses the expression of CHOP, GRP78 and EIF2A, ER stress response marker genes that are essential proteins in the ER stress-mediated cell death signaling pathway. Moreover, BCP also prevents ER stress-induced RASMCs cell injury by decreasing the αSMA-positive signal in the cells. *In silico* studies were performed to confirm the potential mechanism of BCP and its consideration as a drug-like compound. However, high-quality *in vivo* and clinical studies are still necessary to fully understand their efficacy and safety profiles.

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