Antioxidant and anti-inflammatory effects of the extract from rhizomes of *Belamcanda chinensis* (L.) DC. on human keratinocyte HaCaT cells

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Abstract. Chronic oxidative stress and inflammation play critical roles in the development of various skin disorders, and plant-derived polyphenols have attracted considerable attention as potential therapeutic agents due to their antioxidant and anti-inflammatory properties. In the present study, the antioxidant and anti-inflammatory activities of *Belamcanda chinensis* (L.) DC. (BC) rhizome extract on human skin keratinocytes were investigated. The chemical constituents of the BC extract were analyzed using high-performance liquid chromatography coupled with mass spectrometry, which identified five major polyphenolic compounds. The antioxidant capacity of the extract was evaluated using ultrafiltration-liquid chromatography targeting 2,2-diphenyl-1-picrylhydrazyl radicals. In lipopolysaccharide-stimulated HaCaT keratinocytes, BC extract significantly reduced the expression of inflammatory mediators, including cyclooxygenase-2 and inducible nitric oxide synthase and modulated the signal transducers and activators of transcription 3/nuclear factor-κB (NF-κB) signaling pathway. Furthermore, molecular docking analysis revealed strong binding affinities between NF-κB and the five identified polyphenols (tectoridin, iridin, genistein, tectorigenin and irisflorentin), supporting their potential biological relevance. In conclusion, the present findings demonstrated that BC extract exerts notable antioxidant and anti-inflammatory effects through polyphenol-mediated modulation of NF-κB signaling, providing a scientific basis for its potential use in anti-inflammatory drug development.

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Introduction

The skin serves as the outermost barrier of the human body and is particularly susceptible to various external stressors. Among these, harmful environmental factors such as ultraviolet (UV) radiation, air pollution and environmental toxins are major contributors that can disturb the cutaneous redox balance, resulting in oxidative stress and subsequent inflammation. This inflammatory response may accelerate the progression of various skin disorders, including photoaging, psoriasis, pigmentary abnormalities and skin tumors (1-4). Inflammation is a natural response of the body to infections, tissue injuries, chemicals, endotoxins and other factors. An adequately regulated inflammatory response is beneficial as it helps protect the body from harmful stimuli; however, when inflammation becomes uncontrolled, it can damage tissue structures and functions, leading to further deterioration in various diseases, such as psoriasis and atopic dermatitis (5-7).

Cyclooxygenase-2 (COX-2) is typically expressed at low or undetectable levels in normal tissues under physiological conditions; however, it can be induced by pro-inflammatory cytokines, growth factors, oncogenes, carcinogens and tumor promoters, indicating a role for COX-2 in inflammation and the regulation of cell proliferation (8-10). Compounds that inhibit COX-2 activity or expression have been widely investigated and utilized as therapeutic targets, particularly in cancer prevention and anti-inflammation strategies (11).

Inducible nitric oxide synthase (iNOS) serves a role in the development of various inflammatory conditions (12). iNOS is minimally expressed or virtually undetectable in normal resting cells but is induced by inflammatory cytokines or a combination of these cytokines and bacterial polysaccharides. Often, a synergistic effect is observed between different external stimuli, such as pro-inflammatory cytokines, bacterial

components (e.g., lipopolysaccharide), and environmental stressors, which is mediated by the nuclear factor-kappa B (NF-κB) signaling pathways (13).

Signal transducers and activators of transcription 3 (STAT3) serves a crucial role in regulating cell survival and inflammation. STAT3 influences gene expression related to survival, proliferation and angiogenesis by interacting with other transcription factors, such as NF-κB. (14,15) (NF-κB plays a crucial role in host defense and the inflammatory response to microbial and viral infections (16); upon exposure to external signals such as bacterial lipopolysaccharide (LPS), tumor necrosis factor-α or other inflammatory mediators, NF-κB is activated. Once activated, this transcription factor promotes the expression of various genes involved in inflammation, including *COX-2*, *iNOS* and specific cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) (17,18). STAT3 and NF-κB are transcription factors that can increase the expression of pro-inflammatory enzymes and mediators; consequently, the STAT3 and NF-κB pathways have been suggested as potential targets for controlling inflammation (19,20).

Belamcanda chinensis (L.) DC. (BC) is a traditional Chinese medicinal herb, and its dried rhizome is widely used in China to treat inflammation, asthma and throat disorders (21-24). It is also effective against several bacterial, fungal and viral organisms, and has been used as a folk medicine in China (25). Thus, it can be inferred that the presence of essential chemical compounds with known anti-inflammatory properties has played a notable role in the prevalent use of this herb in traditional medicine (26).

In recent years, increasing attention has been directed toward integrating natural phytochemicals with conventional pharmaceuticals to enhance therapeutic efficacy and reduce adverse effects (27,28). In this context, BC extract was selected for testing in the present study, due to its well-documented antioxidant and anti-inflammatory activities, as well as its potential applicability in the development of combination therapies (29,30). Notably, *Belamcanda chinensis* has been traditionally used as a key component of multi-herb formulations such as Shegan Mahuang Tang and Qing Yan Li Ge Wan, which are prescribed for the treatment of respiratory and inflammatory conditions including asthma, cough, pharyngitis, and throat inflammation. Unlike previous studies (31,32) that primarily focused on evaluating the efficacy of individual standard compounds, the present study provided a distinctive approach by identifying the specific bioactive constituents within the BC extract that predominantly contribute to its molecular interactions. This enabled the exploration of potential competitive or synergistic relationships among the components, thereby offering a more comprehensive understanding of the overall pharmacological potential of the extract and supporting its relevance for future drug development.

Materials and methods

Plant extract preparation. BC is native to Cheongju, Chungcheongbuk-do, Korea, and the plant material was obtained from the Herbmaul e-shop (https://aromainherb.net/shop-info/company.html, plant code, 1211.90-1999). The purchased rhizomes were harvested, washed with water, cut into pieces and air dried at 60-70˚C for 72 h. They were then sealed in polyethylene bags with silica gel and stored at -20˚C until further use.

Extraction and purification of BC phenolic compounds. The plant polyphenols were extracted using a modified method (33). First, 200 g rhizomes of BC was steeped in 10 l of 70% ethanol for 4 days at room temperature, and filter paper was used to remove contaminants from the extract (Whatman Qualitative No. 6). Afterwards, the extract was concentrated at 45˚C using a rotary evaporator (N-1110; Eyela; Tokyo Rikakikai Co., Ltd.) until the volume was reduced to 500 ml. Following this, 500 ml hexane was used three times to wash the concentrated extract to remove fatty particles. Then, 250 ml of ethyl acetate was used three times to extract the remaining extract, yielding the ethyl acetate fraction. The residue was eluted using silica gel solvent (40x2.5 cm) and ethyl acetate, followed by dehydration with $MgSO_4$ to remove the residual water. The extract was concentrated at a pressure between 100 to 130 mbar and stored at -80˚C to produce a mixed polyphenol powder, which was 6.88% of the raw material, or 48.2 g.

High-performance liquid chromatography (HPLC) and LC-mass spectrometry (MS/MS) analysis. A 10 µl sample of the extract powder was diluted in 70% ethanol to a concentration of 1,000 µg/ml was used for compound identification. The Ultra Quadrupole Time of Flight LC-MS/MS System (X500R; SCIEX, Inc.) and Nexera Lite HPLC system (Shimadzu Corporation) were used for HPLC and LC-MS/MS. Both systems operated in the positive ion mode with the voltage set at -4.5 kV. Using electrospray ionization and multi-scan in the m/z 100-2,000 range, mass spectrometry settings were carried out in positive ion mode. 500˚C was the desolvation temperature, 5,500 V was the spray voltage, 50 psi was the ion source gas pressure, 30 psi was the curtain gas pressure and 80 V was the declustering potential. For MS/MS spectra, the collision energy was fixed at 35±15 V. SCIEX OS software (3.0.0) was used to generate the obtained ion chromatogram data. The solvents used were, distilled water (solvent A) and acetonitrile with 0.1% formic acid (solvent B). A Prontosil C18 column (length: 250 mm, inner diameter: 4.6 mm, particle size: 5 µm; manufactured by Bischoff Chromatography and distributed by Phenomenex Inc. was used, and a gradient system was employed for analysis at a flow rate of 0.5 ml/min. The solvent B conditions for the mobile phases were as follows: 10-15% for 0-10 min, 20% for 20-30 min, 40% for 30-40 min, 70% for 40-50 min and 95% for 50-60 min. A wavelength of 284 nm and a temperature of 35˚C were used for the analysis.

Antioxidant activity using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) binding HPLC technology. A 1:1 (v:v) mixture of 0.2 mg/ml DPPH (MilliporeSigma) and 1 mg/ml (1,000 ppm) BC sample was allowed to react for 15 min at room temperature. A 0.45 µm syringe filter was used to filter the mixture before HPLC analysis, and methanol was used as a control in place of the DPPH reagent. Identification of the reacting chemical composition was made by analyzing the chromatographic peak values and standard curve results from the samples and controls that underwent the DPPH reaction. This enabled the identification of the key antioxidant elements found in the phenolic compounds of BC.

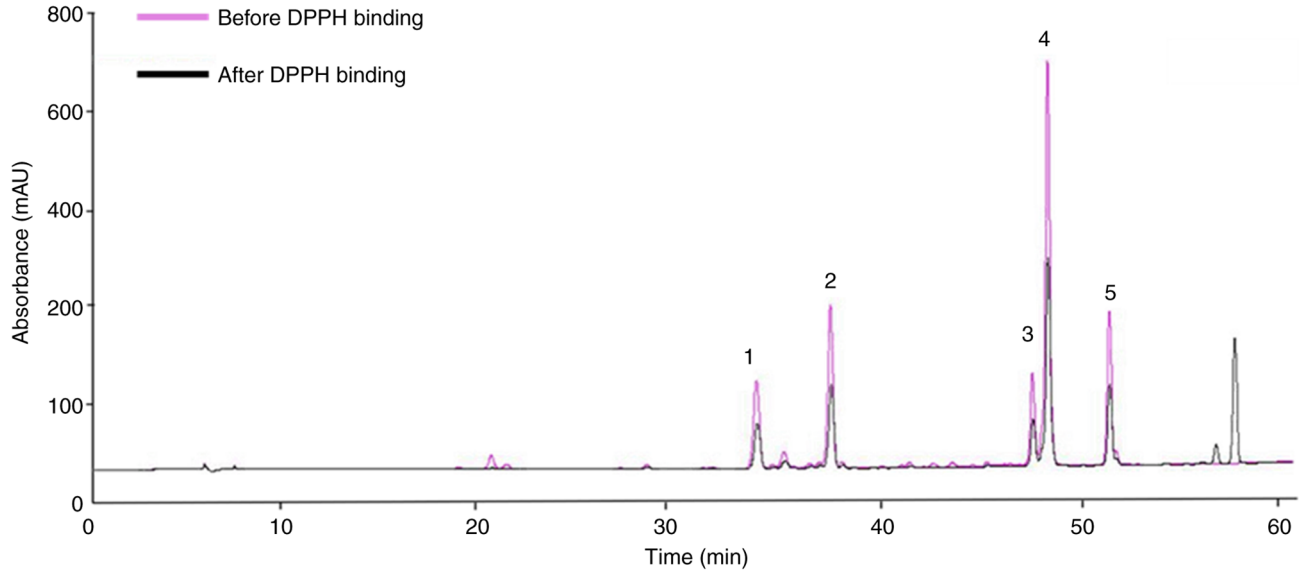

Figure 1. High performance liquid chromatography chromatograms of phenolic compounds in BC. The pink line represents the original chromatogram at the start of the *Belamcanda chinensis* (L.) DC., whereas the black line depicts the chromatogram after reacting with the DPPH solution. The detected compounds at the 254 nm wavelength are: 1) Tectoridin; 2) iridin; 3) genistein; 4) tectorigenin; and 5) irisflorentin. DPPH, 2,2-diphenyl-1-picrylhydrazyl; mAU, milli absorption unit.

Cell culture. The human keratinocyte HaCaT cell line, which is widely used as an *in vitro* model to study skin inflammation and oxidative stress due to its stable phenotype and well-characterized inflammatory signaling responses, was purchased from AddexBio Technologies (cat. no. T0020001). The cell line identity was verified by matching the supplier-provided STR profile with the HaCaT reference profile from Ubigene (https://www.ubigene.us/). The cells were grown in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) with 100 U/ml penicillin and 100 g/ml streptomycin. The cells were incubated at 37˚C in a humidified environment with 5% CO_2.

Cell viability assay. HaCaT cells ($5x10^3$) were seeded into 96-well plates and treated with BC extract dissolved in dimethyl sulfoxide (DMSO) until fully solubilized at various concentrations (0, 0.5, 1, 2.5, 5, 7.5, 10, 12.5, 25, 50, 75 and 100 μg/ml) for 24 h, either with or without 1 μg/ml lipopolysaccharide (LPS; MilliporeSigma), which was prepared by dissolving LPS in sterile phosphate-buffered saline (PBS) to ensure complete solubility, with 0.01% of DMSO alone used as the vehicle control. MTT reagent (0.5 mg/ml, dissolved in PBS) was added into each well for 2 h at 37˚C and then the optical density was measured at 450 nm using a microplate reader (Synergy HTX; BioTek; Agilent Technologies, Inc.).

Western blotting. HaCaT cells ($5x10^5$) were seeded into 6-well plates and treated for 24 h with BC extract (0.5, 1 and 2 μg/ml), which was dissolved in DMSO, either with or without 1 μg/ml LPS at 37˚C. DMSO was used as the vehicle control. Cell pellets were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and then centrifuged at 11,000 x g for 10 min at 4˚C to remove debris. Equal amounts of protein (10 μg), as determined by a bicinchoninic acid protein assay, were separated via Mini-PROTEAN TGX 4-20% gels (Bio-Rad Laboratories, Inc.) and transferred onto PVDF membranes in iBlot™ 3 Transfer Stacks (Thermo Scientific; Thermo Fisher Scientific, Inc.) using the iBlot 3 Western Blot Transfer System (Thermo Scientific; Thermo Fisher Scientific, Inc.). After blocking with EzBlockChemi (ATTO Corporation), the membranes were incubated overnight at 4˚C with the following primary antibodies, each diluted to 1:1,000: COX-2 (cat. no. ab15191; Abcam), iNOS (cat. no. ab283655; Abcam), phospho-STAT3 (cat. no. 9131; Cell Signaling Technology, Inc.), STAT3 (cat. no. 9139; Cell Signaling Technology, Inc.), phospho-p65 (cat. no. 3031; Cell Signaling Technology, Inc.), p65 (cat. no. 8242; Cell Signaling Technology, Inc.), phospho-IκB-α (cat. no. 2859; Cell Signaling Technology, Inc.), IκB-α (cat. no. 9242; Cell Signaling Technology, Inc.) and β-actin (cat. no. A5441; MilliporeSigma). The membranes were incubated 2 h at room temperature with the following second antibodies, each diluted to 1:5,000: Horseradish peroxidase (HRP)-conjugated secondary antibodies to anti-rabbit (cat. no. A120-101P; Bethyl Laboratories, Inc.) and anti-mouse (cat. no. A90-116P; Bethyl Laboratories, Inc.). The blots were visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific; Thermo Fisher Scientific, Inc.) and semi-quantified by densitometry using ImageJ software (version 1.54p) (National Institutes of Health) with β-actin as the loading control.

Molecular docking. The protein structure was obtained from the Protein Data Bank (PDB; https://www.wwpdb.org/) using the search ID 4Q3J (NF-κB). The 3D compound structures of tectoridin (ID: 5281810), iridin (ID: 5281777), genistein (ID: 5280961), tectorigenin (ID: 5281811) and irisflorentin (ID: 170569) were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). Docking analysis was performed using the default settings of UCSF Chimera (version 1.18; https://www.cgl.ucsf.edu/chimera/) and AutoDock Vina

Table I. High-performance liquid chromatography-MS/MS data of phenolic compounds from *Belamcanda chinensis* (L.) DC.

Peak No.	Rt, min	Formula	Compound	UV max (nm)	Precursor ion $[M+H]^+$	MS/MS	(Refs.)
1	34.20	$C_{22}H_{22}O_{11}$	Tectoridin	263, 331	463	301 ($C_{16}H_{12}O_6$) $[M+H-C_6H_{12}O_5]^+$, 286 ($C_{15}H_{10}O_6$) $[M+H-CH_2]^+$	(36-38)
2	38.14	$C_{24}H_{26}O_{13}$	Iridin	264	523	361 ($C_{18}H_{16}O_8$) $[M+H-C_6H_{12}O_5]^+$, 346 ($C_{17}H_{14}O_8$) $[M+H-CH_2]^+$	(36,37,39)
3	48.40	$C_{15}H_{10}O_5$	Genistein	327, 259	271	243 ($C_{14}H_{10}O_4$) [M+H-CO], 215 ($C_{13}H_{10}O_3$) [M+H-CO]	(40,41)
4	48.74	$C_{16}H_{12}O_6$	Tectorigenin	338, 269	301	286 ($C_{15}H_{10}O_6$) $[M+H-CH_2]^+$	(37,38)
5	52.81	$C_{20}H_{18}O_8$	Irisflorentin	321, 266	387	372 ($C_{19}H_{15}O_8$) $[M+H-CH_3]^+$, 357 ($C_{18}H_{12}O_8$) $[M+H-CH_3]^+$, 341 ($C_{18}H_{12}O_7$) $[M+H-O]^+$, 195 ($C_9H_6O_5$) $[M+H-RDA]^+$	(42,43)

Rt, retention time; MS, mass spectrometry.

(https://vina.scripps.edu/). To ensure reproducibility, detailed grid box parameters were applied for each receptor-ligand pair. NF-κB was docked with tectoridin (center: 8.98975, 30.8691, 54.2354; size: 36.5078, 53.8813, 36.2329), iridin (center: 8.99029, 28.355, 52.8342; size: 36.5068, 48.8537, 39.0972), genistein (center: 9.20401, 31.1588, 53.1349; size: 35.0665, 54.4613, 39.7049), tectorigenin (center: 8.99103, 30.7641, 53.8333; size: 36.506, 53.6719, 38.3141) and irisflorentin (center: 8.9912, 29.8613, 53.1376; size: 36.506, 50.9934, 39.7055). These parameters were carefully selected to comprehensively cover the active binding region and ensure consistent and reproducible docking conditions across all ligands. The docking results, including the interactions of amino acids, were displayed using Discovery Studio 2021 Client (https://www.3ds.com/products/biovia/discovery-studio). The binding affinity was calculated based on the total intermolecular energy and the predicted free energy of binding. All experiments were conducted in triplicate, with a root-mean-square deviation (RMSD) of ≤2 Å.

Statistical analysis. Western blot experimental data were analyzed using GraphPad Prism software (version 5.0; Dotmatics). The data are expressed as the means ± standard deviation of triplicate samples. Statistical significance was determined using one-way analysis of variance followed by Dunnett's multiple comparison test, comparing each treatment group with the control. Screening of antioxidant potential data are presented as the mean ± standard deviation of triplicate samples. Two-way ANOVA was used for statistical analysis, followed by Tukey's HSD test. Means with different superscripts (A-E) within the same column and different superscripts (a-c) within the same row indicate significant differences at $P<0.05$. $P<0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

Separation and characterization of polyphenol compounds in BC. HPLC-MS/MS was used to qualitatively analyze the compounds present in the BC extract. UV-visible spectroscopy and HPLC retention time yielded a total of five major peaks (Fig. 1). HPLC was used to identify the five compounds contained in the extract at a wavelength of 284 nm. Although the present study did not use authentic reference standards, the identified compounds were confirmed by matching the observed MS/MS spectra with SCIEX OS 3.0.0 system library data. Additionally, the fragmentation patterns of the precursor ions were carefully analyzed and compared with previously published literature to ensure accurate compound identification. Previous reports have demonstrated biological functions of these compounds and, notably, several have been investigated for antitumor potential. For example, tectoridin and tectorigenin

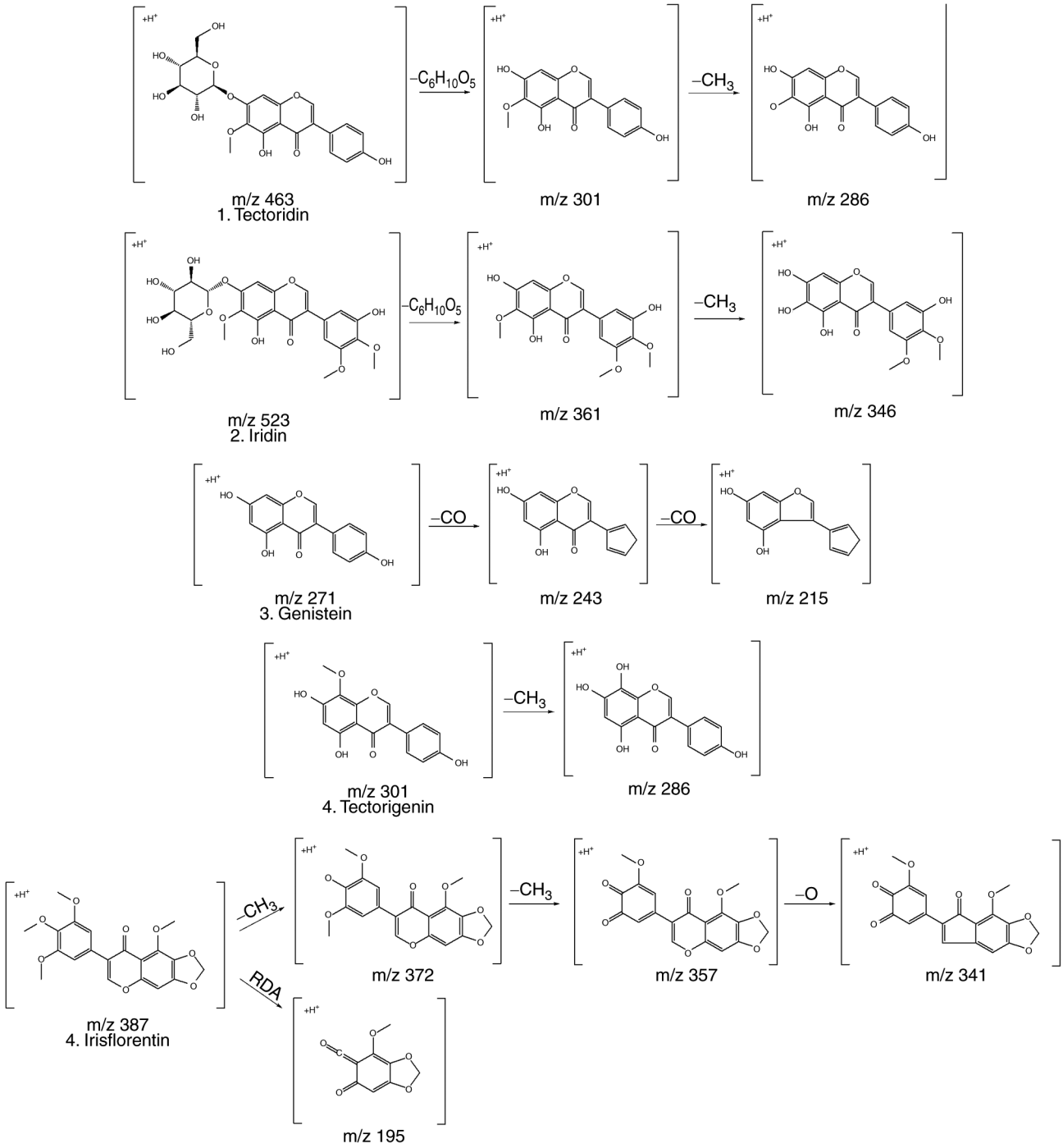

Figure 2. Fragmentation scheme of the phenolic compounds contained in *Belamcanda chinensis* (L.) DC.

have been reported to exhibit antiproliferative effects in breast and hepatocellular carcinoma models, while genistein is a well-studied isoflavone with known chemopreventive activity through modulation of estrogen signaling and induction of apoptosis. Iridin and irisflorentin have also shown anti-inflammatory and anticancer-related bioactivities *in vitro*, suggesting that the presence of these metabolites in BC may contribute to its potential therapeutic properties (34,35). Incorporating quantitative methodologies such as high performance LC-based quantification using authenticated reference standards would allow more precise determination of metabolite concentrations and could complement the qualitative approach used in the present study. The five phenolic compounds identified by MS, referred to in published sources, are shown in Table I. The five compounds identified, included tectoridin (36-38), iridin (36,37,39), genistein (40,41), tectorigenin (37,38) and irisflorentin (42,43). The purpose of this conceptualization was to characterize the compounds found and analyze the data using the molecular ions and mass patterns found in the LC-MS/MS data. Fig. 2 shows the results of predicting the fragmentation of the compounds based on these results; this fragmentation pattern was the basis for the compound formation. Therefore, the structural properties of the phenolic compounds found in this extract were investigated.

Table II. Screening of antioxidant potential of *Belamcanda chinensis* (L.) DC. compounds.

Peak no.	Compound	Before DPPH area absorbance (mAU)	After DPPH binding area absorbance (mAU)	Reactive area absorbance (mAU)
1	Tectoridin	3,720.58±6.03^{Bc}	1,915.86±1.40^{Bb}	1,804.71±7.33^{Ba}
2	Iridin	5,710±2.18^{Dc}	2,984.28±1.27^{Dd}	2,725.72±2.26^{Da}
3	Genistein	2,484.44±3.48^{Ac}	1,267.38±2.37^{Ab}	1,217.06±4.36^{Aa}
4	Tectorigenin	13,145.37±3.92^{Ec}	6,963.09±2.15^{Eb}	6,182.29±6.02^{Ea}
5	Irisflorentin	4,425.03±2.45^{Cc}	2,396.49±2.46^{Cb}	2,027.54±2.03^{Ca}

All values are expressed as mean ± SD (n=3). Statistical analysis was performed using SPSS software. Different superscripts within the same column (A-E) and different superscripts within the same row (a-c) indicate significant differences at P<0.05, based on Tukey's HSD test following a two-way ANOVA. DPPH, 2,2-diphenyl-1-picrylhydrazyl; mAU, milli-absorbance units.

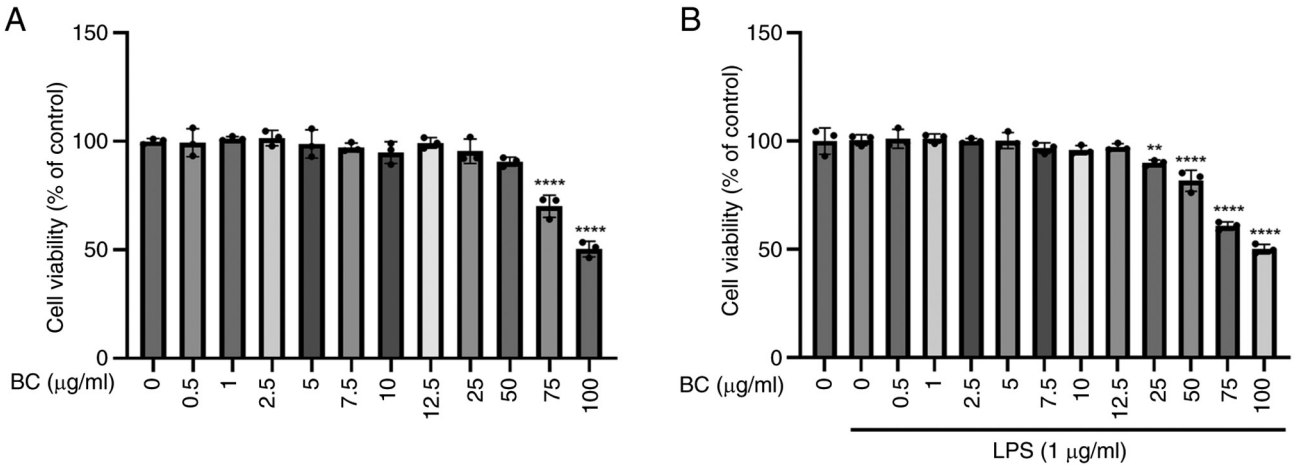

Figure 3. Effects of BC extract on HaCaT cell viability. (A) HaCaT cells were treated with BC (0, 0.5, 1, 2.5, 5, 7.5, 10, 12.5, 25, 50, 75 and 100 μg/ml) for 24 h. ****P<0.0001 vs. untreated group, respectively. (B) HaCaT cells were treated with BC (0, 0.5, 1, 2.5, 5, 7.5, 10, 12.5, 25, 50, 75 and 100 μg/ml) with or without LPS (1 μg/ml) for 24 h. **P<0.01; ****P<0.0001 vs. LPS-alone-treated group, respectively. MTT assay showed that BC extract was non-toxic to HaCaT cells at concentrations up to ~75 μg/ml. Each experiment was independently repeated at least three times with consistent results. BC, *Belamcanda chinensis* (L.) DC.; LPS, lipopolysaccharide.

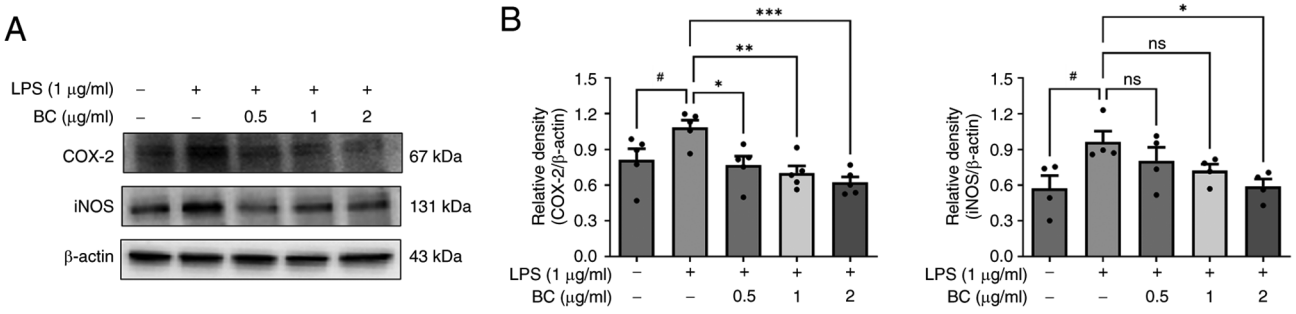

Figure 4. Effect of BC extract on the expression of COX-2 and iNOS. (A) HaCaT cells were treated with BC (0, 5, 1 and 2 μg/ml), with or without LPS (1 μg/ml), for 24 h. The expression levels of COX-2 and iNOS were assessed using western blotting. BC treatment decreased COX-2 and iNOS levels in a dose-dependent manner, indicating its anti-inflammatory effects. (B) The results are presented as the mean ± standard deviation of three independent experiments. $^{\#}$P<0.05 vs. untreated group; *P<0.05, **P<0.01, ***P<0.001 vs. LPS alone-treated group. BC, *Belamcanda chinensis* (L.) DC.; LPS, lipopolysaccharide; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; ns, not significant.

Screening of antioxidant polyphenolic compounds in BC extract. The DPPH reagent is mainly used to analyze radical scavenging or antioxidant effects of natural products. Antioxidants provide electrons to free radicals, causing a color change of the reagent from purple to yellow, which indicates its antioxidant capacity (44). Screening candidates for potential antioxidant effects among polyphenol compounds from BC extract was conducted by combining DPPH and HPLC methods. HPLC peak area values were compared and analyzed before and after the reaction

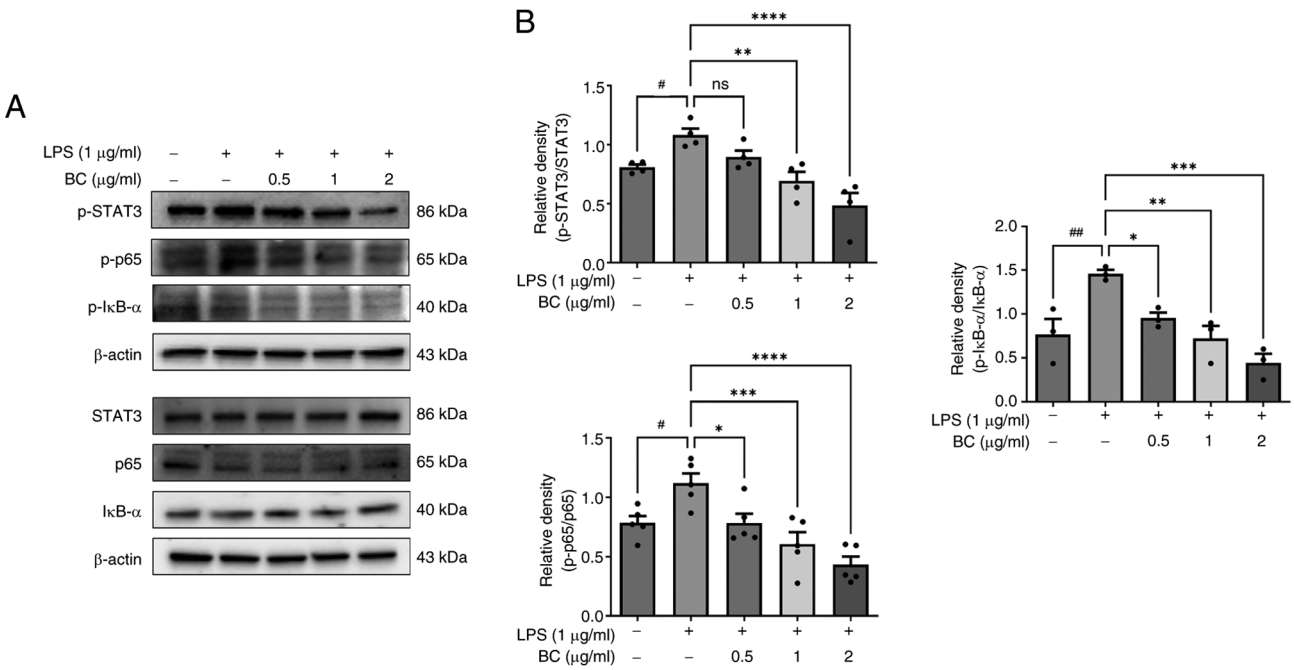

Figure 5. Effect of BC on the expression of STAT3 and NF-κB. (A) HaCaT cells were treated with BC (0, 5, 1 and 2 μg/ml), with or without LPS (1 μg/ml), for 24 h. The expression of STAT3, p65 and IκB-α was determined by western blotting. BC treatment dose-dependently reduced the levels of p-STAT3, p-p65 and p-IκB-α in LPS-stimulated HaCaT cells. (B) The results are presented as the mean ± standard deviation of three independent experiments. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. untreated group; *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. LPS alone-treated group. BC, *Belamcanda chinensis* (L.) DC.; LPS, lipopolysaccharide; ns, not significant; STAT3, signal transducers and activators of transcription 3; NF-κB, nuclear factor-κB.

between the polyphenol components and DPPH. According to the HPLC-MS/MS results after reacting DPPH with the BC extract, all selected polyphenol compounds in the extract displayed changes in peak area values, indicating their potential contribution to the antioxidant effect. Table II shows that the decrease in peak area after the reaction demonstrates the antioxidant effect of the polyphenol compounds, which reacted competitively with DPPH. The difference in area values (%) indicates a notable radical scavenging ability, and the difference between the reactive area of DPPH for each compound was as follows: Tectoridin (1,804.71 mAU), iridin (2,725.72 mAU), genistein (1,217.06 mAU), tectorigenin (6,182.29 mAU) and irisflorentin (2,027.54 mAU). Furthermore, post-hoc tests revealed statistically significant differences among the five compounds. The compounds that reacted notably with DPPH were tectorigenin and iridin; taken together, these initial screening results suggest that tectorigenin and iridin may represent particularly promising antioxidant candidates, although further validation is required to substantiate their potential. The present analytical approach has inherent limitations, as DPPH-based assays do not fully recapitulate physiological redox conditions (45). To address this limitation, additional experiments evaluating the inhibition of induced intracellular reactive oxygen species production may provide more biologically relevant evidence and could be incorporated to strengthen future investigations.

Effects of BC extract on the viability of HaCaT cells. The cytotoxicity of the extract was evaluated using a MTT assay on the HaCaT cells (Fig. 3). HaCaT cells were treated with BC extract at various concentrations: 0, 0.5, 1, 2.5, 5, 7.5, 10, 12.5, 25, 50, 75 and 100 μg/ml, both with and without 1 μg/ml LPS. LPS was included to establish an inflammation-stimulated cellular model and to assess whether BC extract exerted cytotoxic effects under inflammatory conditions. The results indicated that the BC extract was non-toxic at ~75 μg/ml. Consequently, the concentrations of 0.5, 1 and 2 μg/ml were selected for further experiments.

BC extract inhibits iNOS and COX-2 expression on LPS-induced HaCaT Cells. Increased levels of COX-2 or iNOS are linked to certain inflammatory skin disorders in humans, such as atopic dermatitis and psoriasis, where up-regulation of COX-2 contributes to inflammatory responses in atopic skin lesions and iNOS expression is elevated in psoriatic epidermal keratinocytes, implicating these enzymes in chronic skin inflammation and pathological changes. Since inflammation is closely associated with tumor promotion, substances with strong anti-inflammatory properties are anticipated to have chemopreventive effects on carcinogenesis, particularly during the promotion stage (46-48). Therefore, the ability of BC extract to suppress COX-2 and iNOS expression suggests its potential as an anti-inflammatory and chemopreventive agent against inflammation-associated skin disorders. To determine the effects of BC extract on iNOS and COX-2, the BC extract was administered at concentrations of 0.5, 1 and 2 μg/ml with LPS treatment. The results showed that iNOS and COX-2 protein expression decreased in a dose-dependent manner (Fig. 4). Although the current design provides initial insight, including a positive inhibitory control, future studies could further validate assay sensitivity and strengthen the findings by incorporating techniques such as reverse transcription-quantitative PCR to assess mRNA levels, enzyme activity assays for iNOS and COX-2, and the use of additional inflammatory stimuli or cytokine profiling to confirm anti-inflammatory effects.

Table III. Molecular docking of tectoridin, iridin, genistein, tectorigenin and irisflorentin with the NF-κB complex and their binding energies.

Binding ligand	Amino acid residue that interacts with NF-κB	Docking score, kcal/mol
Tectoridin	ASP194, ALA151, ARG26, GLU184, HIS187, SER192, GLU153 and GLY191	-8.0
Iridin	ASN240, ASP194, CYS149, GLU184, GLU233, HIS187, LEU236, PHE163 and TYR227	-8.3
Genistein	ASN240, ARG237, CYS149, LEU236, PRO147 and PHE146	-7.4
Tectorigenin	ASN240, ARG237, CYS149, GLU184, LEU236 and PHE146	-7.4
Irisflorentin	ASN240, ARG237, ARG239, CYS149, GLU184, LEU236 and PHE146	-6.9

NF-κB, nuclear factor-κB.

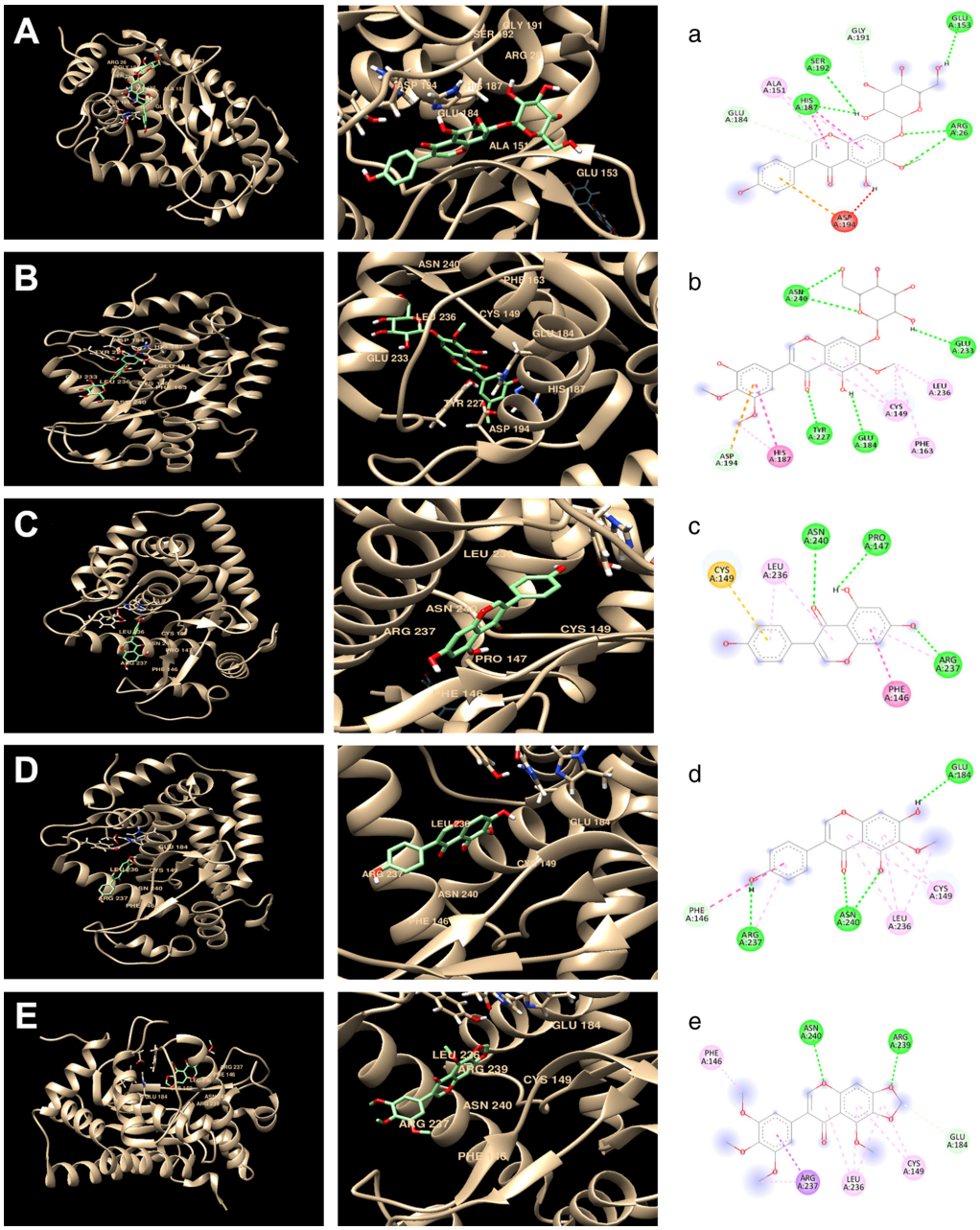

Figure 6. Molecular docking of phenolic compounds and NF-κB in BC, *Belamcanda chinensis* (L.) DC. (A) Tectoridin, (B) iridin, (C) genistein, (D) tectorigenin and (E) irisflorentin were all effectively bound to the 3D structure of NF-κB. The interaction of amino acids in the NF-κB with the ligand: (a) Tectoridin, (b) iridin, (c) genistein, (d) tectorigenin and (e) irisflorentin. NF-κB, nuclear factor-κB.

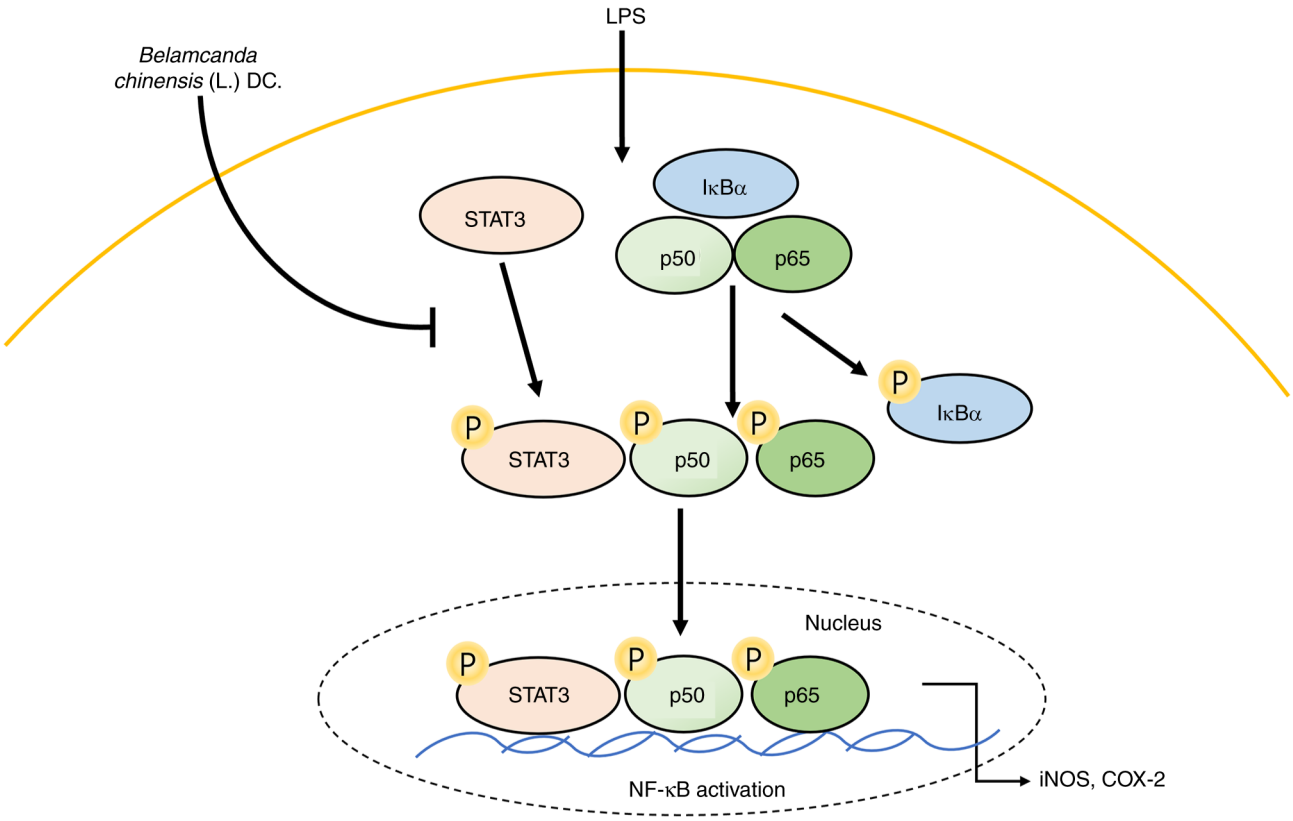

Figure 7. Schematic representation of the antioxidant and anti-inflammatory properties of BC, *Belamcanda chinensis* (L.) DC. on HaCaT cells. LPS, lipopolysaccharide; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase.

BC extract inhibits STAT3 and NF-κB expression on LPS-induced HaCaT Cells. Understanding the molecular mechanisms that underlie the cooperation between the interaction of STAT3 and NF-κB in inflammation may lead to the development of new chemopreventive and chemotherapeutic strategies (49). It was confirmed that the STAT3 and NF-κB activation were decreased when LPS-induced HaCaT cells were treated with BC extract (Fig. 5). Treatment with BC at concentrations of 1 and 2 μg/ml with LPS treatment significantly reduced the levels of p-STAT3, p-p65 and p-IκB-α; in addition, p-p65 and p-IκB-α levels were also decreased with 0.5 μg/ml BC. While these data suggest a potential inhibitory effect of BC on NF-κB signaling, incorporating functional assays such as NF-κB luciferase reporter activity or p65 nuclear translocation analyses would provide a more direct assessment of pathway inhibition and further reinforce the mechanistic interpretation in future studies. Based on these results, molecular docking between NF-κB and the five polyphenolic compounds contained in the BC extract was also conducted.

Molecular docking analysis of selected polyphenol compounds with NF-κB from extracts. The hyperactivation of NF-κB in a cellular system is linked to inflammation, cancer and various other human diseases; key components of the NF-kB cascade may regulate the translocation and activation of the NF-κB transcription factor (50). In the present study, the focus was on the NF-κB signaling pathway, examining the active sites of its key components and conducting molecular docking analysis to understand their interactions and functions. Molecular docking studies were performed with NF-κB protein complex and the five polyphenol compounds: Tectoridin, iridin, genistein, tectorigenin and irisflorentin. Notably, the PDB entry 4Q3J used in the present study indicated that these compounds primarily bind to the NF-κB subunit p65 (RelA), with minimal interaction with p50 or IκB-α, suggesting that the modulatory effects observed are mainly mediated through p65. The interactions between each protein and ligand with their complementary surfaces are depicted in Fig. 6. The ligand-protein docking was performed using the UCSF Chimera program (Fig. 6A-E), whereas the analysis of amino acid interactions was performed using the Discovery Studio program (Fig. 6A-E). Table III summarizes the amino acid residues involved in these interactions and their corresponding binding energy scores.

The docking analysis identified several active sites for tectoridin: ASP194, ALA151, ARG26, GLU184, HIS187, SER192, GLU153 and GLY191; this compound achieved a binding energy score of -8.0 kcal/mol. In the case of iridin, docking results showed active sites at ASN240, ASP194, CYS149, GLU184, GLU233, HIS187, LEU236, PHE163 and TYR227, and had the highest recorded binding energy score of -8.3 kcal/mol. For genistein, the docking analysis identified the following active sites: ARG237, ASN240, CYS149, GLU184, LEU236, PHE146, PRO147 and TYR227, leading to a binding energy score of -6.9 kcal/mol. Molecular docking of tectorigenin with NF-κB revealed active sites at ARG232, ALA228, CYS149, LEU236 and TYR227; this compound

achieved a binding energy score of -7.4 kcal/mol. Finally, for irisflorentin, the identified active sites interacting with NF-κB included ARG211, ASP223, ILE208 and LEU203, with a binding energy score of -6.8 kcal/mol, which was the lowest among the compounds analyzed. Furthermore, all five compounds exhibited an RMSD value of 0 Å, indicating that their docked conformations were identical to the crystallographic reference structures. This high degree of structural congruence validates the reliability of the docking simulations and suggests that the observed interactions are not computational artifacts but rather reflect true binding potential. Given that RMSD values <2 Å are typically considered indicative of successful docking poses (51,52), these results demonstrate the precision and stability of the predicted binding modes. Taken together, these findings highlight iridin as the most promising candidate for further investigation as an NF-κB inhibitor. Its high binding affinity and favorable interaction profile suggest potential anti-inflammatory effect and, based on the central role of NF-κB in cancer-related signaling pathways, possible antitumor properties through suppression of NF-κB-mediated transcriptional activity, including the downregulation of pro-survival and proliferation genes. However, it should be noted that the present study was performed on skin keratinocytes rather than a cancer cell line, and thus the antitumor potential of BC extract remains to be experimentally validated. Future studies could explore the effects of BC extract and iridin in relevant cancer cell models and *in vivo* systems to evaluate its chemopreventive or therapeutic potential, investigate the underlying molecular mechanisms, and assess pharmacokinetics and safety profiles (53,54). However, while *in silico* docking provides an initial mechanistic rationale, *in vitro* and *in vivo* validation, such as reporter gene assays, electrophoretic mobility shift assays or cytokine profiling, will be essential to confirm its biological efficacy and specificity. Ultimately, the present study supports the hypothesis that naturally occurring isoflavones, particularly iridin and tectoridin, may serve as potential lead compounds for the development of novel NF-κB-targeting therapeutics aimed at treating inflammation-related disorders or cancers.

In the present study, the aim was to investigate the anti-inflammatory effects of BC extract on LPS-induced HaCaT cells (Fig. 7). The present results showed a decrease in the expression of anti-inflammatory markers, specifically iNOS and COX-2, along with a significant reduction in the levels of proteins associated with the STAT3-NF-κB pathway. Based on these findings, NF-κB was selected as a candidate for the molecular docking analysis.

In conclusion, the present study confirmed the mechanism of action of the compounds present in BC through the simultaneous application of DPPH-HPLC. Through LC-MS/MS analysis, five compounds: Tectoridin, iridin, genistein, tectorigenin and irisflorentin were identified and quantified in the polyphenol extract of BC. The results showed that these compounds effectively contributed to antioxidant and anti-inflammatory activities by demonstrating marked reaction amounts and rates (%) as ligands for DPPH. Furthermore, the BC extract significantly reduced the expression of iNOS, COX-2, p-STAT3 and NF-κB proteins in LPS-induced HaCaT cells. The five selected BC compounds showed marked binding scores during molecular docking with NF-κB, a key inflammatory transcriptional regulator.

The antioxidant and anti-inflammatory properties of the compounds found in BC were evaluated to determine which exhibited the most notable potential. These findings serve as a valuable resource for identifying potential bioactive compounds with antioxidant and anti-inflammatory properties from natural sources. They also provide valuable data for the future application of BC in the food and pharmaceutical sectors. However, the present study has certain limitations, as it was conducted solely using the HaCaT cell line, which may not fully recapitulate the complex microenvironment of human skin *in vivo* or reflect the responses of different cell types present in actual tissue. To overcome this limitation, it is the aim to include experiments using normal primary keratinocytes in future studies to further validate and strengthen the physiological relevance of the present findings. In addition, the concentration range tested in the present study was relatively limited, and a more comprehensive dose response evaluation would provide a clearer understanding of concentration-dependent effects and further enhance the robustness of the present conclusions.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SEH participated in all aspects of the experimental design, performed the analysis and wrote the manuscript. HHK conducted the data analysis investigation, confirms the authenticity of all the raw data and reviewed and edited the manuscript. SHJ performed data curation and editing of the manuscript. GSK confirms the authenticity of all the raw data and participated in experimental design and project administration. KHH was responsible for funding acquisition, acquisition of data and supervision. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of the present manuscript, artificial intelligence tools were used to improve the readability and language of the manuscript, and subsequently, the authors revised and edited the content produced by the artificial intelligence tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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