

BRUCEA JAVANICA (L.) MERR. EXTRACT DOWNREGULATES MMP2 EXPRESSION AND REDUCES ENDOTHELIAL DENUDATION-INDUCED ARTERIAL NEOINTIMAL HYPERPLASIA IN MICE

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Abstract

This study evaluated the therapeutic potential of *Brucea javanica* (L.) Merr. (BJ) extract against vascular smooth muscle cell (VSMC) proliferation and migration, and endothelial denudation-induced neointimal hyperplasia in mice. Primary VSMCs isolated from mouse embryonic aortas were used, while guidewire injury-induced neointimal hyperplasia was utilized. In VSMCs, BJ (10 - 30 µg/mL) treatment reduced VSMC proliferation, migration, and lamellipodia formation. Further, Western blot showed reduced matrix metalloproteinase-2 (MMP2) expression, without affecting total or phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2). Meanwhile, in mice, BJ (30 mg/kg/day) treatment mitigated neointimal formation, as indicated by the artery intima-to-media ratio (0.313 ± 0.056) compared to injured controls (0.630 ± 0.089 , $p = 0.013$). Interestingly, the reduction of the intima-to-media ratio was followed by a reduction in matrix MMP2 expression in the area (1.342 ± 0.085 -fold compared to control) compared to untreated (1.687 ± 0.056 -fold compared to control, $p = 0.0071$). Taken together, BJ extract contains predominantly flavonoids, with quercetin as the major constituent, and inhibited VSMC proliferation and migration; this was accompanied by reduced MMP2 expression without changes in ERK1/2 expression or activation. In mice, BJ extract reduced neointimal hyperplasia and MMP2 expression in intimal and medial arteries.

Rezumat

Acest studiu a evaluat potențialul terapeutic al extractului de *Brucea javanica* (L.) Merr. (BJ) asupra proliferării și migrării celulelor musculare netede vasculare (CMNV), precum și asupra hiperplaziei neointimale induse prin denudare endotelială la șoarece. Au fost utilizate CMNV primare, izolate din aortă embrionară de șoarece, respectiv un model de hiperplazie neointimală indusă prin leziune. La nivelul CMNV, tratamentul cu BJ (10 - 30 µg/mL) a redus proliferarea, migrarea și formarea lamelipodiilor. În plus, analiza prin Western blot a evidențiat o scădere a expresiei metaloproteinazei matriceale 2 (MMP2), fără modificarea formei totale sau fosforilate a kinazelor modulate de semnale extracelulare 1/2 (ERK1/2). La șoarece, administrarea BJ (30 mg/kg/zi) a atenuat formarea neointimei, așa cum indică raportul intimă/medie de la nivel arterial ($0,313 \pm 0,056$), comparativ cu animalele lezate netratate ($0,630 \pm 0,089$; $p = 0,013$). Reducerea raportului intimă/medie a fost asociată cu o diminuare a expresiei MMP2 în zona respectivă (creștere de $1,342 \pm 0,085$ ori față de martor), comparativ cu lotul netratat (creștere de $1,687 \pm 0,056$ ori față de martor; $p = 0,0071$). În concluzie, extractul de BJ conține preponderent flavonoide și a inhibat proliferarea și migrarea CMNV, efect asociat cu scăderea expresiei MMP2, în absența unor modificări ale expresiei sau activării ERK1/2. La șoarece, extractul de BJ a redus hiperplazia neointimală și expresia MMP2 la nivelul intimei și mediei arteriale.

Keywords: *Brucea javanica*, MMP2, arterial hyperplasia, proliferation-migration, vascular smooth muscle cells

Introduction

After having a stent, restenosis remains an unresolved, recurring issue after every coronary procedure (1). It is just a continuation of the same vascular challenge, primarily tightening of blood vessels, expansion, and remodeling of the extracellular matrix caused by vascular smooth muscle cells (VSMCs) (2). Even with the use of drug-eluting stents, re-occlusion of the blood vessel where the stent is placed remains a challenge caused by neointimal hyper-

plasia, using hyperplasia as an indicator for drug-eluting stents. This is the reason for the need to explore alternative therapy.

Restenosis is a multifactorial pathological process driven primarily by persistent inflammation and oxidative stress, with the inflammatory cytokine IL-1β serving as a key inducer (2, 3). Under inflammatory conditions, VSMCs undergo phenotypic switching from a quiescent, contractile state to a synthetic, proliferative, and migratory phenotype (4). This

transition is characterized by heightened proliferation, migration, and excessive extracellular matrix deposition, ultimately leading to neointimal hyperplasia and vascular re-occlusion (5). Therefore, therapeutic strategies that reduce oxidative stress and inflammation, thereby preventing VSMC proliferation and migration, represent a promising approach to mitigate neointimal formation and restenosis (6).

Current treatment options, including drug-eluting stents, have substantially reduced but not eliminated restenosis, largely due to the complex, multi-target nature of the underlying pathology (7). Natural remedies, particularly those derived from traditional medicine, offer a compelling alternative because their rich content of bioactive compounds provides combined antioxidant, anti-inflammatory, and anti-proliferative properties, enabling multi-targeted therapeutic action (8, 9). Among the traditional Southeast Asian medicinal plants, *Brucea javanica* (L.) Merr. (BJ) is well recognized for its diverse active constituents, including flavonoids and quasinsoids (10). Flavonoids such as quercetin and isoquercetin, reported in BJ, are known to exert antioxidant, anti-inflammatory, and endothelial protective effects (7). However, their potential in addressing cardiovascular challenges, particularly restenosis, remains inadequately explored (11).

One major inflammatory cytokine involved in vascular diseases is IL-1 β . Considering that IL-1 β is a

strong promoter of matrix metalloproteinase-2 (MMP2), a matrix-degrading enzyme that facilitates VSMC proliferation and migration and initiates neointimal formation, it is imperative to determine whether BJ modulates these phenotypes associated with MMP2 (11). We thus hypothesized that BJ modulates MMP2 expression in both VSMCs (*in vitro*) and in a neointimal hyperplasia mouse model (*in vivo*) (5, 12). Therefore, this study aimed to evaluate the therapeutic potential of BJ extract against VSMC proliferation and migration *in vitro*, and against endothelial denudation-induced neointimal hyperplasia in male BALB/c mice *in vivo*, while also demonstrating proteins associated with VSMC dysfunction and neointimal formation.

Materials and Methods

Materials

We collected and characterized BJ seeds from Lombok Island, Indonesia (ID: 33/UN18.F7/LBL/2025). Primary and secondary antibodies used were provided in Table I. Male BALB/c mice (8 - 10 weeks old, 20 - 25 g) were obtained from the University of Mataram, Indonesia. A guide wire 0.014" (Abbott, #1001780-HC) was applied for femoral artery endothelial denudation under Avertin anesthesia (Sigma-Aldrich, #T48042), and elastin staining, Verhoeff's stain (Sigma-Aldrich, #HT25A-KT).

Table I

List of antibodies used in this study

Target Antibody	Host Species	Supplier	Catalogue Number
Anti-pFAK	Rabbit	Cell signalling	#3283
Anti-total FAK	Rabbit	Cell signalling	#3285
Anti-pERK1/2	Rabbit	Cell signalling	#9101
Anti-total ERK1/2	Rabbit	Cell signalling	#4696
Anti-MMP2	Rabbit	Proteintech	#10373-2-AP
Anti- β -actin	Mouse	Milipore	#MAB1501
Anti- α -tubulin	Mouse	GeneTex	#GTX112141
Goat anti-rabbit IgG (HRP-conjugated)	Goat	Thermo Fisher Scientific	#31460
Goat anti-mouse IgG (HRP-conjugated)	Goat	Thermo Fisher Scientific	#31430

BJ extraction and preparation for treatment

The seeds were air-dried, powdered, and extracted with 96% ethanol using ultrasonicator-assisted extraction (UAE), then evaporated to obtain a solid extract (13). For *in vitro* use, BJ extract was diluted in 0.1% DMSO (v/v) and diluted to 10, 20, and 30 μ g/mL. Quiescent VSMCs were stimulated with the inflammatory cytokine IL-1 β (10 ng/mL) to trigger proliferation and migration. The treatment groups (BJ 10, 20, 30 μ g/mL) were administered for 24 hours (antiproliferative activity) and 6 hours (antimigration activity), while the extract for the *in vivo* experiment was suspended in 0.5% CMC-Na.

Non-specific and specific extract characterization

Non-specific characterization of BJ extract was performed, including determination of water content, loss

on drying (LOD), total ash, and acid-insoluble ash. Water content was measured by drying 1 g of extract at 105°C to constant weight; LOD was determined by heating the sample at 105°C for 30 minutes, repeated until no further weight change. Total ash content was obtained by incinerating 1 g of extract at 600°C until carbon-free residue remained. Acid-insoluble ash content was determined by boiling total ash with 25 mL dilute sulfuric acid for 5 minutes, followed by filtration, washing, and ignition to constant weight. Experiments were done in triplicate and expressed as mean $\pm$ SD.

Specific characterization included organoleptic evaluation, phytochemical screening, total flavonoid content (TFC), total phenolic content (TPC), Fourier Transform Infrared (FTIR) spectroscopy, Ultra-

High-Performance Liquid Chromatography (UHPLC), and Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) analysis. Organoleptic parameters were described based on physical appearance, color, odor, and taste. We conducted phytochemical screening using established protocols (14). TPC and TFC were measured using spectrophotometric analysis with standard gallic acid (λ 765 nm) and quercetin (λ 415 nm), respectively (15).

FTIR spectra were recorded from 4000–400 cm^{-1} using KBr pellets. UHPLC analysis was performed on a C18 column using a gradient mobile phase (water and acetonitrile, each with 0.1% formic acid). The UHPLC method was validated according to ICH Q2(R2) guidelines (Table II). LC-HRMS analysis was conducted on a Thermo Fisher Scientific Orbitrap Q Exactive Plus with an ESI source coupled to an Ultimate 3000 UHPLC system.

Table II

Validation parameters for quercetin quantification by UHPLC according to ICH Q2(R2) guidelines

Validation Parameter	Result
Linearity range	0.5 - 50 $\mu\text{g/mL}$
Calibration equation	$y = 40.375x + 291.95$
Coefficient of determination (R^2)	0.9975
Limit of detection	0.15 $\mu\text{g/mL}$
Limit of quantification	0.45 $\mu\text{g/mL}$
Accuracy (recovery)	98.2 - 101.5%
Precision	< 2.0%

Isolation and primary culture of mice embryonic VSMCs

VSMCs were isolated from the thoracic aortas of BALB/c embryonic mice, as previously described by Hamdin *et al.* (16). Briefly, aortae were dissected from the aortic arch to the renal level, cleaned of surrounding tissue, minced, and digested in serum-free Dulbecco's modified Eagle medium (DMEM) containing collagenase type I (0.5 mg/mL) and elastase type III (0.125 mg/mL) at 37°C for ~15 min. We stopped digestion with DMEM supplemented with 20% FBS, then collected cells by centrifugation, resuspended them, and plated them on fibronectin-coated dishes. VSMCs were maintained in standard growth medium and subcultured after 3-4 days (16). For the experiment, VSMCs were grown in DMEM (Gibco) with 10% FBS (Gibco), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin at 37°C and 5% CO_2 in a humidified chamber. We used VSMCs from passages 3 to 6 for the experiments.

Cell Viability Assay of BJ (MTT)

To assess the effect of BJ on cell viability, VSMCs were seeded in 24-well plates (1.6×10^4 cells/well), subjected to serum starvation for 24 hours, and subsequently incubated with the specified concentration (0, 10, 20, 30, 40, and 50 $\mu\text{g/mL}$) of BJ for 24 hours. MTT was subsequently added to the medium at a concentration of 1 mg/mL. Following 2 hours of incubation, the crystal was solubilized in 200 μL of DMSO. We measured optical density at 570 nm and normalized it to the measurement at 650 nm. Cell viability was expressed as a percentage relative to the control group.

VSMC proliferation assay (CCK-8)

VSMC proliferation was assessed through the manufacturer's instructions using a colorimetric Cell Counting Kit-8 (Dojindo Laboratories, #CK04-05). VSMCs were seeded onto 96-well plates at a density

of 5×10^3 cells/well. Thereafter, cells were maintained in quiescence with 0.2% FBS for 24 hours (16). VSMCs were assigned to 4 groups: (1) untreated control, (2) BJ 10 $\mu\text{g/mL}$, (3) IL-1 β 10 ng/mL alone, (4) IL-1 β + BJ (10, 20, and 30 $\mu\text{g/mL}$). VSMCs were treated with inflammatory cytokine IL-1 β and/or BJ for 24 hours. We added CCK-8 reagent and incubated for an additional 4 hours. We used a microplate reader (Bio-Rad, USA) to measure absorbance at 450 nm. VSMC proliferation was calculated as the percentage of the untreated control (17).

Wound healing assay

To evaluate the migration-inhibitory effect of BJ, VSMCs (2.5×10^5 cells) were seeded into 6-cm culture dishes until 90–100% confluence. VSMCs were serum-starved for 24 hours. Before scratching, VSMCs were treated with mitomycin-C (10 $\mu\text{g/mL}$) for 2 hours. We created a scratch wound using a sterile pipette tip A200. VSMCs were treated with or without IL-1 β and/or co-treated with BJ (10, 20, and 30 $\mu\text{g/mL}$) for 6 hours. We captured wound images at 0 and 6 hours, and quantified migration as the percentage of wound closure relative to baseline.

Immunocytochemistry (ICC) and phalloidin staining of VSMCs

To evaluate protein fluorescence, expression, and localization, mouse embryonic VSMCs (1×10^3 cells/mL) were seeded into μ -Slide chambers (Ibidi VI). We added 30 μL of cell suspension to each chamber and allowed the cells to adhere. Quiescent VSMCs were subsequently treated with or without IL-1 β or BJ extract (10, 20, and 30 $\mu\text{g/mL}$) for 10 minutes. Cells were then washed, fixed with 4% paraformaldehyde, permeabilized with 0.1% Tween-20, and blocked with 1% BSA. Primary antibody against pERK1/2 was applied overnight at 4°C, followed by Alexa Fluor 488-conjugated secondary antibody (green). Subsequently, the cells were counterstained

with DAPI and Alexa Fluor 594-conjugated phalloidin (red) for 5 minutes to visualize nuclei and the actin cytoskeleton, respectively. Fluorescence images were captured using a fluorescence microscope (Olympus IX71).

VSMC treatment and protein extraction for Western blot

VSMCs were seeded (1×10^5 cells) in 6-well plates. Serum-starved VSMCs were treated with or without IL-1 β for stimulation and/or BJ extract (10 μ g/mL). Proteins were collected at 0, 5, 10, and 30 minutes for kinase evaluation and at 0, 6, 12, and 24 hours for MMP2 analysis. Following treatment, protein was extracted from cells, and concentrations were assessed (5). Proteins were then stored at -20°C for further analysis.

Western blot analysis

For the Western blot analysis, 30 μ g of mouse embryonic VSMC protein was separated on SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% TBST and treated with specific primary antibodies. We then incubated the membranes with HRP-conjugated secondary antibodies. Protein bands were visualized with ECL and analyzed using ImageJ (NIH) (18).

In vivo treatment protocol

All animal experiments were approved by the Ethics Committee of the Faculty of Medicine and Health Sciences, University of Mataram (No. 122/UN18.F8/ETIK/2025). Animal care and experimental procedures were conducted in accordance with the ARRIVE 2.0 guidelines. Mice were housed individually in single cages under controlled environmental conditions (25°C, 12-hour light/dark cycle) with ad libitum access to standard chow and water. The inclusion criteria for mice were healthy male BALB/c, aged 8-10 weeks, and weighing 20- 25 g. Mice were excluded when complications occurred after modeling. Prior to surgery, mice were anesthetized with Avertin and recovered on a heating pad to maintain body temperature. We determined the sample size using the Resource Equation formula ($E = N - T$). We randomized animals in each group using GraphPad. The randomized animals were allocated into three groups: (1) Uninjured control, (2) Injured control, and (3) Injured + BJ treatment at 30 mg/kg/day. BJ extract was suspended in 0.5% CMC-Na and administered by oral gavage for 14 days post-injury. The uninjured group did not undergo wire insertion, while injured groups received vehicle (CMC-Na 0.5%) or BJ treatment. After 14 days, mice were euthanized with an overdose of Avertin, and femoral arteries were collected for histological analysis.

Animal model of endothelial denudation-induced neointimal hyperplasia

Before femoral endothelial denudation, mice were anesthetized intraperitoneally with Avertin. Under the microscope, the left femoral artery was identified and incised with a 0.014" guide wire, as explained in Hamdin *et al.* (16). The flexible guide wire was inserted approximately 1 cm into the left femoral artery of the mice and passed 3 times to induce endothelial denudation. Mice were then placed on a heating pad to recover. Following endothelial injury, mice were treated with or without BJ (30 mg/kg/day) for 14 days.

Intima-media (I/M) ratio and immunohistochemistry (IHC)

At post-injury, 14 days, the mice were humanely euthanized with an overdose of Avertin, and the femoral arteries were harvested and processed by fixing in 10% formaldehyde and paraffin embedding. Serial cross sections (4 μ m) were elastin-stained, and a light microscope (Olympus BX53) was used to take pictures. For each animal, we analyzed 3 sections spaced 150 μ m apart. We measured the intima (I) and media (M) areas and calculated the I/M ratio (16). For IHC, sections were prepared for MMP2 antibody staining as described by Hamdin *et al.* (16). The quantification of the I/M ratio and MMP2 expression was analyzed with ImageJ version 1.54g from 6 animals for each group.

Statistical analysis

All experiments used a minimum of 3 ($n = 3$ to 6) distinct biological replicates, including proliferation, migration, ICC, lamellipodia protrusion, western blot, and I/M ratio. The data are presented as mean $\pm$ SD (analytical and characterization data) and mean $\pm$ SEM (biological data). For factorial experimental design (proliferation and migration assay with $\pm$ IL-1 β $\times$ BJ dose), two-way ANOVA was applied, while for single-factor comparisons (western blot densitometry and I/M ratio). One-way ANOVA was utilized, followed by Tukey's post-hoc test, while Student's t-test analysis was specifically used for lamellipodia formation. GraphPad Prism version 10.4.1 was used for statistical analysis, and significance was set at $p < 0.05$.

Results and Discussion

BJ extract characterization

BJ seed was collected from Sesaot, Narmada District, West Lombok Regency, at coordinates (-8.543658°, 116.24168°). Once samples are collected and processed into solid extract, the simplicia and extract yields are then quantified (Table III). From 3 kg of BJ seeds, 0.9 kg of dry simplicia was obtained (30% yield). Subsequently, sonication of 903 g of simplicia using 96% ethanol yielded 95.6 g of crude ethanolic extract, representing an extract yield of 10.5%,

comparable to the 10.03% yield reported by Almira *et al.* (13) using UAE with 96% ethanol.

Sonication offers advantages such as reduced extraction time and lower solvent consumption compared to conventional maceration, although yield varies with parameters such as sonication duration, liquid-

solid ratio, and extraction cycles. For comparison, Amini *et al.* (19) reported a maceration-based yield of 7.4%, which falls within reported ranges for BJ extracts. Minor discrepancies may be attributed to seed maturity, geographical origin, drying temperature, and solvent-to-sample ratio (20).

Table III

Extraction yield of BJ seed simplicia and ethanolic extract

Extraction Stage	Initial Weight	Final Weight	Yield (%)
Drying process	3000 g	900 g	30.0
UAE extraction	903 g	95.6 g	10.5

Beyond extraction yield, we further evaluated the quality and safety of BJ seed extract using non-specific and specific standardization parameters. Non-specific parameters, which include determination of water content, LOD, total ash content, and acid-insoluble ash levels, were performed in triplicate (Table IV). Measurement of water content in a plant extract is essential across various fields, as high moisture levels can promote mold growth and accelerate the degradation of bioactive compounds (21). The water content of the BJ seed ethanolic extract obtained in this study was $2.32 \pm 0.200\%$. This relatively low water content is favorable, as it minimizes the risk of fungal contamination and helps maintain the extract's stability and quality during storage (21).

Table IV

Non-specific standardization parameters of BJ extract (mean $\pm$ SD, $n = 3$)

Parameter	Result (%)
Water content	2.32 ± 0.200
Loss on drying	3.37 ± 0.055
Total ash content	2.03 ± 0.218
Acid soluble ash content	1.78 ± 0.690

LOD is a critical parameter that reflects the amount of volatile substances, including water and other volatile compounds, lost during drying (21). The LOD values for the three replicates averaged $3.37 \pm 0.055\%$ (Table IV), indicating minimal loss of volatile compounds and supporting batch-to-batch

consistency. Together with the low water content, this result suggests good extract stability and a reduced risk of microbial deterioration. The total ash content of the BJ seed extract was $2.03 \pm 0.218\%$ (Table IV), while the acid-insoluble ash content was $1.78 \pm 0.690\%$ (Table IV). These low values indicate a low mineral content and minimal contamination with sand, soil, or other siliceous materials, reflecting good extract purity (21). Overall, the non-specific standardization parameters indicate that the BJ ethanolic extract meets acceptable quality standards for further development as a phytopharmaceutical preparation.

Following the non-specific evaluation, we conducted specific standardization parameters, including organoleptic tests (physical appearance, color, odor, taste), bioactive compound quantification, and phytochemical profiling (Table V). The BJ seed extract was a viscous extract with a brownish-yellow to dark brown color, no typical odor, and a bitter taste, consistent with the findings of Amini *et al.* (19). The TFC of BJ extract was 34.98 ± 0.440 mg QE/g, indicating that BJ seeds are a rich source of flavonoids, compounds known for their antioxidant and anti-inflammatory activities (22, 23). The flavonoid content is comparable to previous studies, although differences may occur due to extraction solvent, method, and seed origin (20).

Table V

Specific standardization parameters of BJ ethanolic extract

Parameters	Result
Physical appearance	Viscous extract
Color	Brownish yellow to dark brown
Odor	Not typical
Taste	Bitter
Total flavonoid content (mg QE/g)	34.98 ± 0.440
Total phenolic content (mg GAE/g)	115.10 ± 0.426

Other than TFC, the TPC of BJ extract was 115.10 ± 0.426 mg GAE/g (Table V), indicating that the extract is rich in phenolic compounds. Phenolics are known for their strong antioxidant activity through free radical scavenging, metal ion chelation, and inhibition of lipid peroxidation (24). The high phenolic content (115.10 mg GAE/g) compared to flavonoid

content (34.98 mg QE/g) suggests that the extract contains a substantial proportion of non-flavonoid phenolics, such as phenolic acids and other polyphenolic derivatives. This finding is consistent with previous studies on BJ, where phenolic compounds have been identified as major contributors to the antioxidant capacity of the seeds (25). This high

phenolic content also supports the traditional medicinal use of BJ seeds against oxidative stress-related diseases.

To complement the quantitative analysis of flavonoids and phenolics, we performed a qualitative phytochemical screening to identify the broader classes of secondary metabolites present in the extract. Phytochemical screening of the BJ extract revealed the presence of flavonoids, triterpenoids, saponins, and tannins (Table VI). However, alkaloids were negative. This result is consistent with previous phytochemical studies on BJ seeds that consistently

reported the absence or very low levels of alkaloids (25). The major constituents of BJ seeds are flavonoids and phenolic compounds, while alkaloids are not characteristic of this species (25). Therefore, the negative finding for alkaloids is expected and does not diminish the medicinal value of the extract. Standardization based on internationally accepted quality control guidelines is essential to ensure consistent quality, safety, and reproducibility, particularly because phytochemical composition may vary with extraction conditions and seed origin (26).

Table VI

Preliminary phytochemical screening of the BJ extract

Secondary Metabolite	Reagent	Observed Result	Conclusion
Alkaloids	Dragendorff's Mayer's Wagner's	No characteristic precipitate observed Alkaloids were not detected	-
Flavonoids	Shinoda test	Red/orange coloration observed Flavonoids were detected	+
Triterpenoids	Liebermann–Burchard test	Dark/black coloration observed Triterpenoids were detected	+
Saponins	Froth test	Persistent foam observed Saponins were detected	+
Tannins	FeCl ₃ test	Greenish coloration observed Tannins were detected	+

Phytochemical profiling of BJ extract by FTIR, LC-HRMS, and UHPLC

The phytochemical profiling of BJ extract was further elucidated using FTIR spectroscopy (Figure 1). The FTIR spectrum showed a broad absorption band at 3391 cm⁻¹, characteristic of O-H stretching of hydrogen-bonded hydroxyl groups, typically associated with polyphenolic compounds, including flavonoids and phenolic acids (27). Aromatic C=C stretching vibrations were confirmed by absorption bands at 1636 cm⁻¹ and 1515 cm⁻¹, which are diagnostic of aromatic ring systems typically found in flavonoids and phenolic compounds. Importantly, no distinct absorption bands characteristic of alkaloid functional groups were observed, supporting the negative alkaloid result from phytochemical screening (Table VI) and consistent with previous reports that alkaloids are not characteristic constituents of BJ seeds (13). Collectively, the FTIR spectral features strongly support the presence of flavonoids and phenolic compounds in the BJ extract, which corroborates the quantitative TFC and TPC results (Table V). However, FTIR spectroscopy provides valuable information on functional groups; it does not allow definitive identification or quantification of individual compounds. Therefore, complementary analytical techniques such as LC-HRMS and UHPLC are recommended for unambiguous identification and structural elucidation of individual bioactive constituents (28).

The LC-HRMS analysis provided definitive identification of the major secondary metabolites in the BJ extract, including quercetin, isoquercetin, bruceanin, and erucamide, along with other flavonoids, phenolic acids, and quassinoids (Figure 2 and Supplementary Data). Previous studies on BJ fruit have reported metabolites such as bruceine D, E, F, and bruceanic acid. In pharmacognosy, the medicinal 'fruit' of BJ is characterized as a dry indehiscent structure, with the seed as the bioactive component. This study extracted material from BJ seeds, whereas others referred to it as fruit (25). Discrepancies in phytochemical profiles may arise from differences in plant origin, extraction procedures, and analytical objectives, as geographic origin, climate, soil, and harvesting conditions influence quassinoid and flavonoid production (29).

Among the identified metabolites, quercetin emerged as a predominant flavonoid, confirmed by the MS/MS fragment at 303 m/z (Figure 2). Quercetin is a well-established dietary flavonoid with potent antioxidant, anti-inflammatory, and vascular protective properties. Its presence in BJ seeds is particularly significant, as it has been extensively reported to inhibit inflammatory cytokines such as IL-1β in vascular diseases (30). The identification of isoquercetin, detected alongside quercetin, further supports the extract's flavonoid-rich nature, including antioxidant and anti-inflammatory effects, and may contribute synergistically to the overall vascular protective activity of the BJ extract (30).

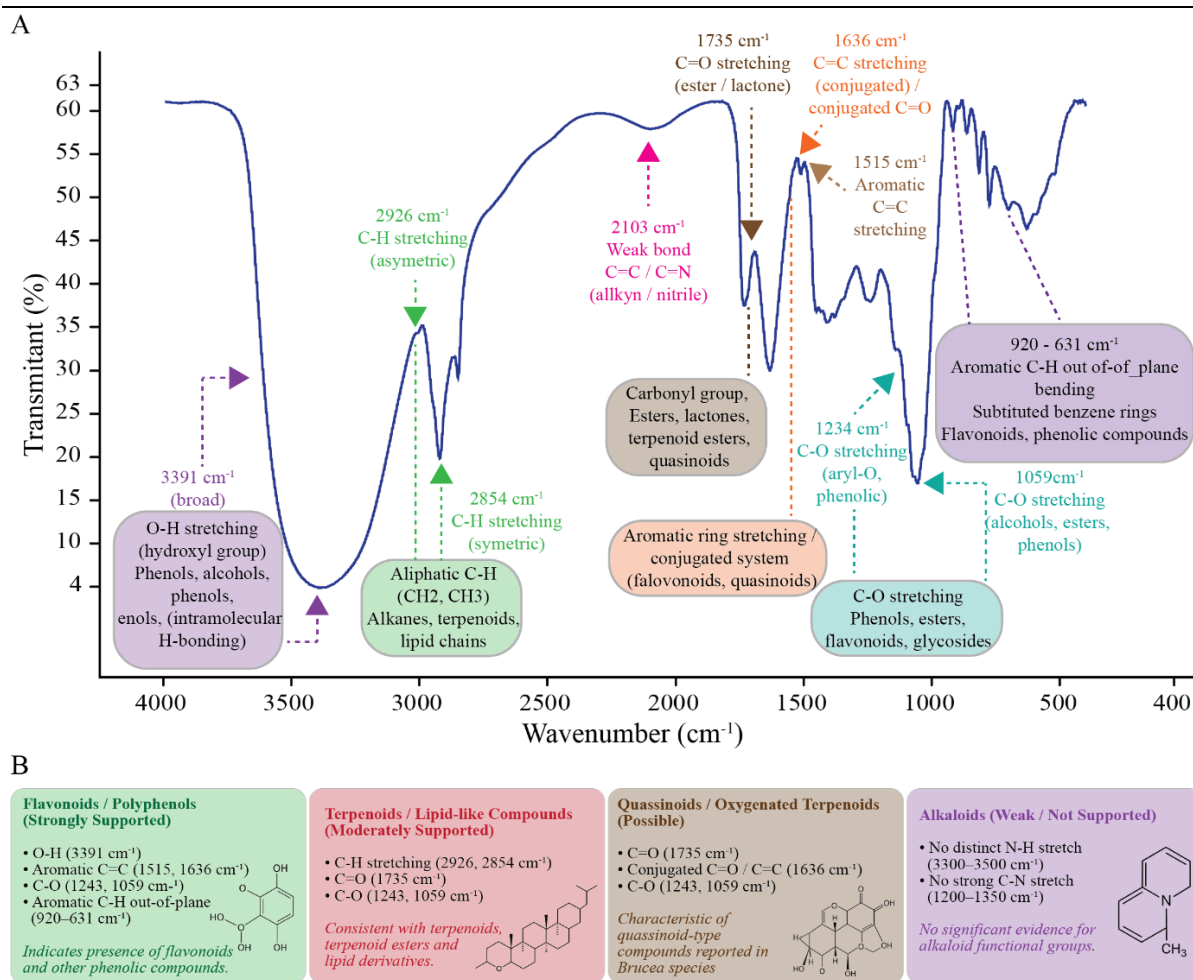


Figure 1.

FTIR characterization of the ethanolic extract. (A) Representative FTIR spectrum with the principal absorption bands and assigned functional groups. (B) Proposed phytochemical classes based on the FTIR spectral features, highlighting flavonoids/polyphenols, terpenoids, possible quassinoids, and the absence of significant alkaloid-related functional groups.

In addition to quercetin and isoquercetin, bruceantin was identified in this work, consistent with prior reports as a constituent of BJ fruits, and is known for anti-inflammatory and anti-proliferative activities (30). As a fatty acid amide, erucamide shows cytokine-modulatory activity and mitigates vascular oxidative stress. Quercetin and isoquercetin are known to be vascular protective and were identified by MS/MS at 303 m/z (Figure 2). These protective activities of quercetin and isoquercetin were also observed in the inhibition of IL-1 β -stimulated reactive vascular (31). These four compounds show multi-targeted activity of BJ. Combined anti-inflammatory, antioxidant, anti-proliferative, and anti-migratory activities were also observed in the biological studies (32), whereby BJ was noted to also reduce the IL-1 β -induced VSMC proliferation, migration, formation of lamellipodia, and MMP2 of the reactive vascular quiescence pathway (Figure 4, 5, and 7). Thus, observations suggest that BJ modulates the inflammatory and oxidative-stress-sensitive pathways

that are most likely responsible for the observed vascular remodeling and neointimal hyperplasia inhibition (32).

After qualitative confirmation of quercetin by LC-HRMS, we performed quantitative analysis using UHPLC coupled with a photodiode array detector. We identified quercetin by comparing retention time with a quercetin standard (Figure 3). The quercetin standard exhibited a retention time of 1.407 minutes, while the BJ seed extract samples (R1, R2, and R3) showed retention times of 1.374 minutes, 1.378 minutes, and 1.381 minutes, respectively (Figure 3). The close agreement between the standard and sample retention times confirms the presence of quercetin in the BJ ethanolic extract, consistent with the previously reported LC-HRMS findings. Minor retention-time shifts may be attributed to matrix effects or variations in mobile-phase composition and column temperature (33). Quantitative analysis was performed using an external calibration curve ($y = 40.375x + 291.95$), with an excellent coefficient of

determination ($R^2 = 0.9975$). The high R^2 value approaching 1.000 indicates exceptional linearity over

the tested concentration range and acceptable linearity over the calibration range (33).

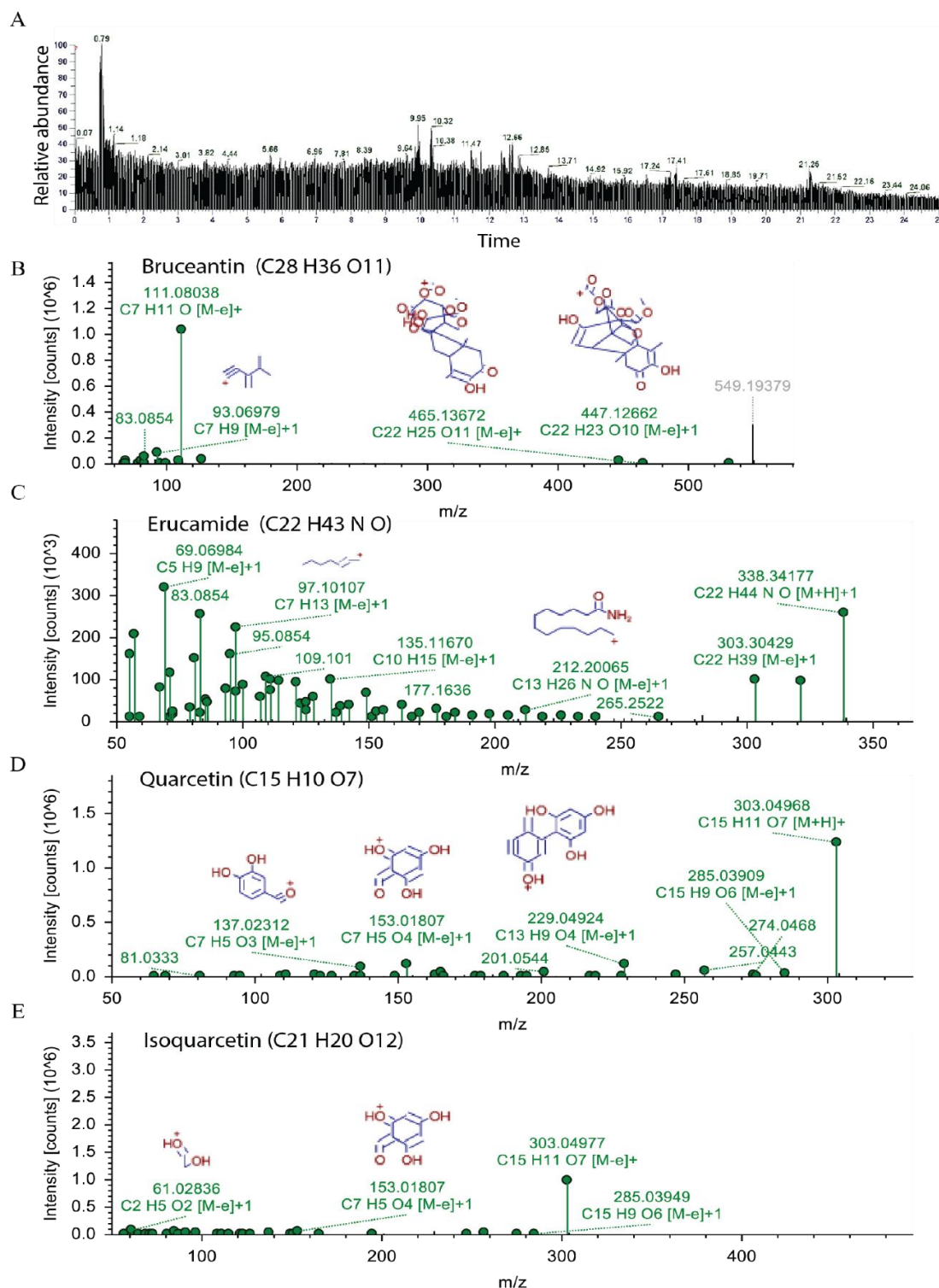


Figure 2.

LC-HRMS chromatographic and MS/MS spectral analysis of the ethanolic BJ extract. (A) Total ion chromatogram (TIC) of the ethanolic BJ extract showing multiple peaks corresponding to diverse secondary metabolites. (B–E) Representative MS/MS fragmentation spectra and proposed structures of four major compounds identified in the BJ extract: Bruceantin, Erucamide, Quercetin, and Isoquercetin. Each spectrum shows characteristic fragment ions used to confirm compound identity.

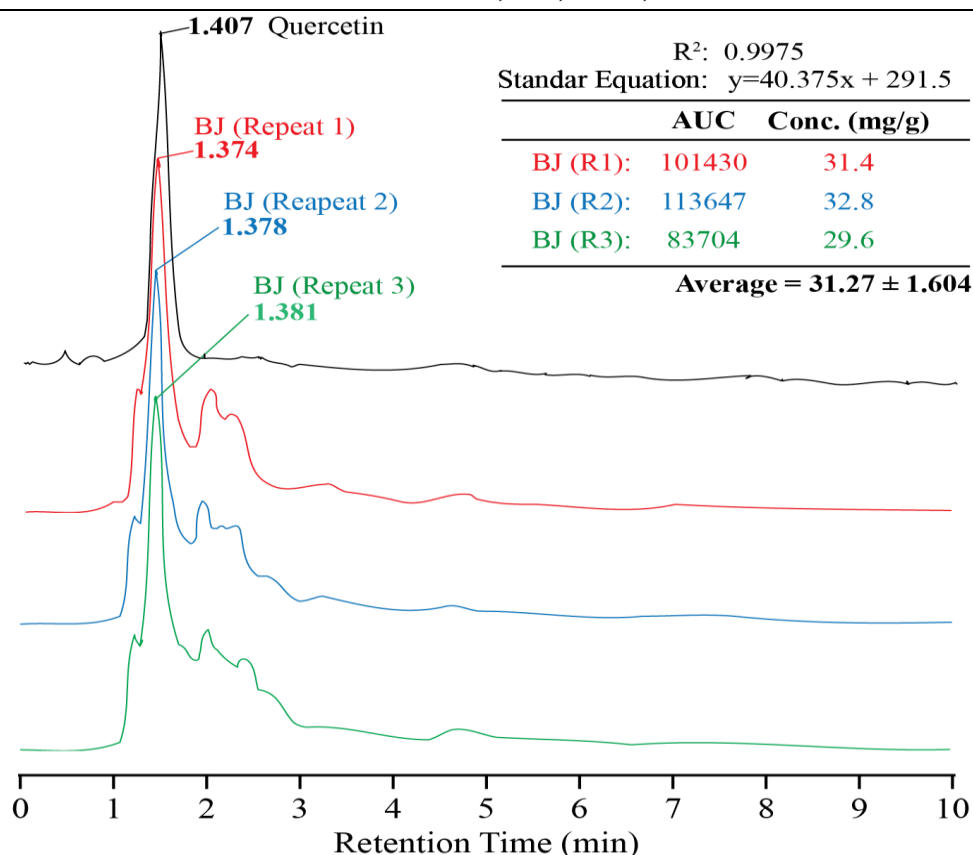


Figure 3.

UHPLC analysis of quercetin in the ethanolic extract. Representative UHPLC chromatograms of the quercetin standard and three independent extract replicates (BJ R1–R3), together with the calibration equation, AUC values, and the calculated quercetin content ($n = 3$, mean $\pm$ SD).

The quercetin concentrations obtained from triplicate measurements were 31.4, 32.8, and 29.6 mg/g, with a mean concentration of 31.27 ± 1.604 mg/g, indicating acceptable analytical reproducibility. On the other hand, the total flavonoid content of BJ extract was 34.98 mg QE/g (Table V). However, these values should not be considered directly comparable due to differences in analytical principles. UHPLC provides compound-specific quantification of quercetin, whereas the AlCl₃ colorimetric assay estimates total flavonoid content based on aluminum-chelation responses, which can vary depending on the chemical structure of individual flavonoids (34). The LC-HRMS profile further showed other flavonoid constituents, including isoquercetin and quercetin glycosides, supporting the characterization of BJ extract as containing a diverse flavonoid profile. This finding agrees with previous reports on BJ, where quercetin and its derivatives have been identified as dominant flavonoid components with potential vascular protective and anti-inflammatory activities (25). The relatively high quercetin concentration (31.27 mg/g) supports the traditional medicinal use of BJ and provides a quantitative basis for its biological activities, particularly the inhibition of VSMC proliferation, migration, and neointimal hyperplasia (35).

BJ ethanolic extract reduced VSMC proliferation and migration

Before assessing the effects of BJ on VSMC proliferation, migration, and lamellipodia formation, we evaluated cell viability by MTT assay. BJ extract at doses of 10 to 30 μ g/mL minimally affected VSMC viability, confirming the validity of subsequent experiments (Figure 4A). Given that IL-1 β is a key inflammatory cytokine modulating VSMC phenotype, IL-1 β -stimulated VSMCs were used in subsequent experiments. We evaluated changes in VSMC proliferation with BJ treatment with and without IL-1 β . For the analyses without IL-1 β , BJ at 10 μ g/mL significantly impacted VSMC proliferation relative to the control group, suggesting that at this concentration, BJ has an effect on reducing VSMC proliferation to 0.75 ± 0.025 -fold of the control from the basal level (Figure 4B). For the analyses with IL-1 β , control group proliferation was significantly lower than the VSMC proliferation when IL-1 β was added, and this confirmed that IL-1 β was indeed stimulating VSMC proliferation, which is typical for the inflammatory condition of vascular disease. The VSMC proliferation was inhibited when IL-1 β and BJ were used together, although the effect was not dose-dependent. Ten μ g/mL BJ (brown bar) significantly

reduced proliferation compared with the IL-1 β proliferation control group, suggesting that BJ can counter proliferation. Unexpectedly, the other 2

higher concentrations (20 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$ BJ) showed the same reduction in proliferation (Figure 4B).

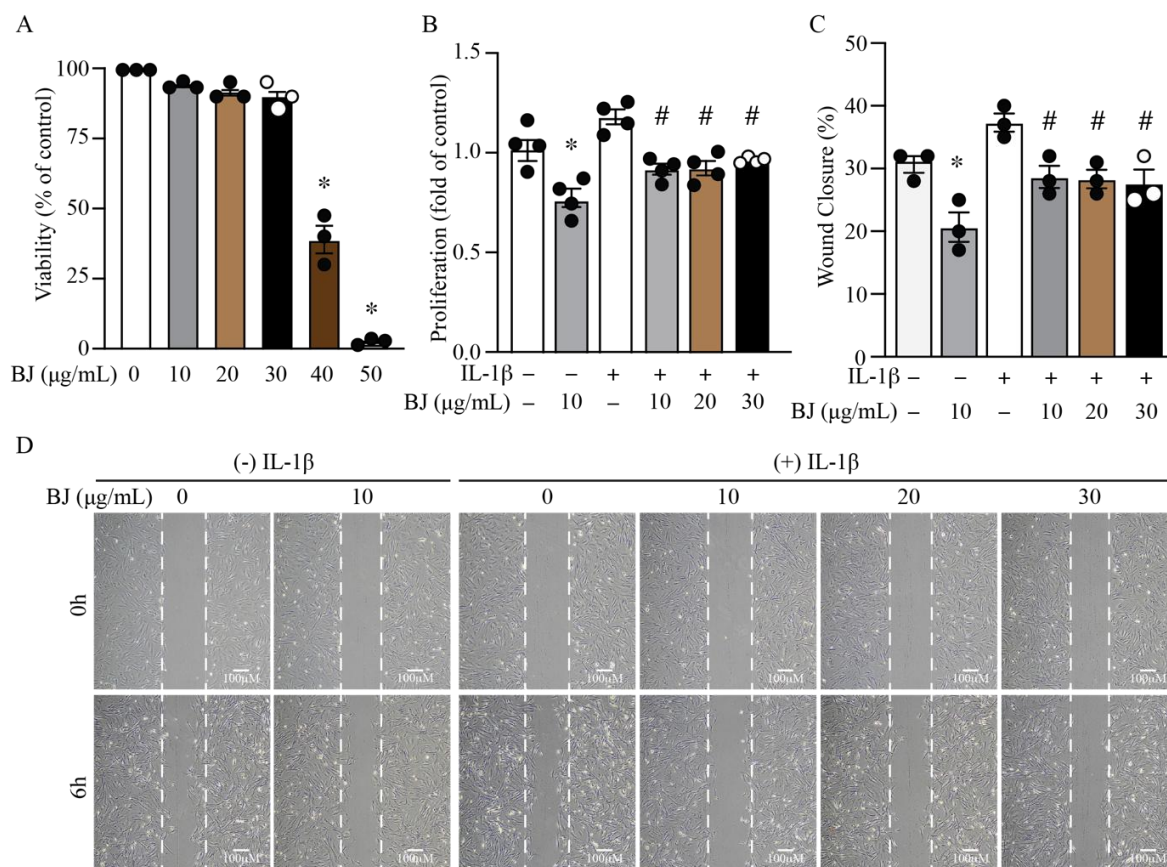


Figure 4.

Effect of BJ on VSMC viability, proliferation, and migration. (A) VSMCs were treated with increasing concentrations of BJ, and cell viability was assessed using an MTT assay. (B) VSMCs were treated with various concentrations of BJ (10, 20, and 30 $\mu\text{g/mL}$) in the presence or absence of IL-1 β (10 ng/mL) for 24 hours. Cell proliferation was assessed by measuring the fold change compared with the control group (no IL-1 β or BJ treatment). (C) Wound closure (%) at 6 hours. Cells were treated with different concentrations of BJ (0, 10, 20, and 30 $\mu\text{g/mL}$) in the presence or absence of IL-1 β (10 ng/mL). (D) Representative images of VSMC migration in a scratch wound healing assay at 0 hours and 6 hours. Dashed lines represent the initial wound area. Data represent the mean $\pm$ SEM, $n = 3$ to 4, and individual data points are shown as black circles. Statistical significance was determined using GraphPad Prism version 10.4.1 software *via* two-way ANOVA followed by post hoc comparisons; * $p < 0.05$ compared to the control group without IL-1 β or BJ treatment, # $p < 0.05$ compared to the IL-1 β alone group.

Several factors may explain the lack of a clear dose-dependent response. Unlike conventional pharmaceuticals, herbal extracts often show early saturation or plateau effects because of their polychemical nature (36). Unlike Western allopathic medicine, early saturation or plateau effects are commonly seen in herbal combinations or polychemicals (37). In these cases, low concentrations are effective in activating the primary pathways of polychemicals at higher concentrations and provide no additional effects because of the convergent inhibitory actions of different metabolites in a crude extract (37). Thus, the 10 $\mu\text{g/mL}$ concentration of BJ may explain the lack of

difference seen at higher concentrations of 20 to 30 $\mu\text{g/mL}$ (Figure 4B).

Additionally, IL-1 β -stimulated VSMCs appear to operate *via* a “threshold-dependent inflammatory cascade” whereby early pro-inflammatory cytokine signaling blockade is sufficient to blunt redirection or reverse the subsequent proliferative response (38). After this IL-1 β inflammatory effect was compromised, most likely at the 10 $\mu\text{g/mL}$ concentration of BJ, further dose increases would not provide additional anti-inflammatory effects. Also, higher concentrations of plant extracts may elicit the so-called compensatory or homeostatic responses in plants. In this case, the response would be to “control”

proliferation at a predetermined baseline and regain control *via* compensatory pathways (38).

Therapeutically, the absence of dose dependency indicates that BJ exerts remarkable anti-proliferative effects at low concentrations. This characteristic is beneficial for vascular purposes, as high vascular exposure may not be advisable. This behavior is typical of natural products, especially those that do not act primarily on the cell cycle but inhibit upstream inflammatory mediators (39). Thus, at low concentrations, BJ may suppress pathological VSMC hyperproliferation associated with vascular inflammatory phenomena, reinforcing its potential as a preventive agent against restenosis (25).

In addition to proliferation, VSMC migration also contributes to neointimal hyperplasia (40). Figures 4C and D show that BJ can inhibit this migration, as shown in the scratch wound-healing assay, in the presence or absence of the inflammatory cytokine IL-1 β . Initially, there were no significant differences in wound area, confirming that the scratches were similar in size across groups at the 0-hour assessment. At 6 hours, BJ treatment inhibited wound closure. In the absence of IL-1 β , VSMC migration was

reduced at all tested BJ concentrations (10, 20, and 30 μ g/mL), with similar effects across concentrations, while IL-1 β enhanced VSMC migration as expected due to its pro-inflammatory effects. In the presence of IL-1 β , VSMC migration was significantly enhanced. Despite the pro-migratory effects of IL-1 β , the 10, 20, and 30 μ g/mL concentrations of BJ inhibited migration in VSMCs exposed to IL-1 β (Figure 4C-D). This indicates that BJ provides some protection against inflammatory conditions on VSMC migration (5).

Mitigation of VSMC proliferation and migration remains a primary approach to managing neointimal hyperplasia and vascular injury restenosis (1). Knowledge of the mechanisms of their actions has increased, yet vascular remodeling targeted to the VSMC remains mostly unchanged with respect to therapeutic options. Because of the bioactive compounds and safety profiles, natural products have become viable options (41). The following data document BJ extract's potential for reducing injury-induced intimal hyperplasia in mice (Figure 8) and the analysis of its impact on kinase signaling in VSMCs (Figure 6).

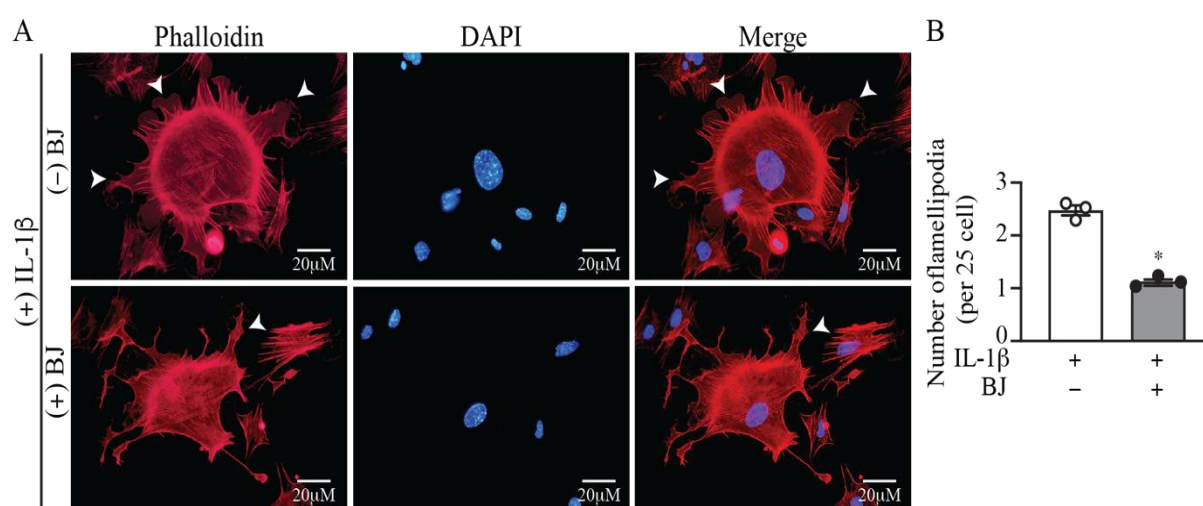


Figure 5.

BJ extract inhibits lamellipodia formation in VSMCs. (A) Representative immunofluorescence images of VSMCs stained with phalloidin (red) to detect F-actin and DAPI (blue) to label nuclei. Arrowheads indicate lamellipodia formation. VSMCs treated with IL-1 β alone show increased lamellipodia formation, while co-treatment with BJ extract markedly reduces this formation. Images were taken with a fluorescence microscope (Olympus IX71, 40 $\times$ magnification). Scale bar = 20 μ m. (B) Quantification of the number of lamellipodia *per* cell. IL-1 β significantly increased lamellipodia formation, whereas BJ extract significantly reduced this effect. Data are expressed as mean $\pm$ SEM (n = 3 individual experiments with 25 cells evaluated in each experiment). Data significance was determined using GraphPad Prism version 10.4.1 software *via* t-test analysis, * p < 0.05 compared to the IL-1 β alone group.

BJ extract inhibits IL-1 β -induced lamellipodia formation in VSMCs

To determine the effect of BJ on cytoskeletal remodeling, a lamellipodia formation assay in VSMCs was performed. Phalloidin staining was used to visualize the F-actin structures (42). The results showed that

IL-1 β treatment markedly increased lamellipodia formation, the actin-rich membrane protrusions (Figure 5A). In contrast, during co-treatment with BJ, there was a considerable reduction in the lamellipodia count, indicating that BJ has a potential effect to suppress cytoskeletal reorganization (Figure 5A).

We proceeded to quantify the results, which show that IL-1 β -stimulated cells significantly increased the count of lamellipodia formation *per cell* compared to the control. In contrast, BJ treatment significantly mitigated protrusion, resulting in a substantial reduction in lamellipodia formation (Figure 5B). These results show that BJ inhibits lamellipodia protrusion in VSMCs and demonstrate its potential anti-migratory effect on vascular cells (43).

The suppression of lamellipodia formation by BJ is mechanistically significant, as lamellipodia serve as the primary cellular structures driving VSMC migration during vascular remodeling (44). IL-1 β -induced VSMC migration results from cytoskeletal reorganization followed by actin polymerization (44). By inhibiting this process, BJ likely targets early cytoskeletal and motility-related signaling pathways, which correlates with its inhibitory effect in the wound-healing migration assay (Figure 4C-D). Thus, BJ may attenuate remodeling-associated phenotypic switching by interfering with cytoskeletal remodeling (11).

BJ effects on the activation of ERK and FAK in VSMCs

Activation of ERK1/2, a major MAPK pathway, determines VSMC proliferation and migration (11). To investigate the effect of BJ on ERK1/2 activation, we performed ICC to assess levels of phosphorylated ERK1/2 (pERK1/2). In the control group, VSMCs that did not receive IL-1 β treatment showed diminished expression of pERK1/2. Meanwhile, in the IL-1 β -treated group, pERK1/2 expression was higher and appeared as a strong green signal. Unexpectedly, BJ treatment at 10 to 30 μ g/mL did not affect pERK1/2 expression compared with the control (Figure 6A).

Quantification of relative pERK1/2 expression (Figure 6B) indicated that BJ treatment at 20 and 30 μ g/mL resulted in a slight, but not significant, difference compared to controls treated with IL-1 β only and 10 μ g/mL BJ treatment. These data indicate that BJ has minimal effect on IL-1 β -induced ERK1/2 phosphorylation in VSMCs (Figure 6A-B). To summarize, in this study, BJ treatment at 10 to 30 μ g/mL had minimal effect on pERK1/2 expression in VSMCs compared with the IL-1 β -alone group.

To further elucidate the underlying mechanisms, we analyzed the expression of phosphorylated and total FAK (Y397) and ERK1/2 by Western blotting (Figure 6C). VSMCs were treated with IL-1 β (positive control) and BJ at 0, 5, 10, and 30 minutes. It was found that no discernible changes in pFAK (Y397), total FAK, pERK1/2, or total ERK1/2 levels were observed following BJ treatment. As expected, IL-1 β groups showed increased pFAK (Y397) and pERK1/2 levels (Figure 6C). Densitometry analysis (Figure 6D-E) confirmed that pERK1/2 and pFAK (Y397) levels remained unchanged across all time

points for BJ-treated groups compared to controls. Collectively, these results demonstrate that BJ does not alter the phosphorylation state of FAK or ERK1/2 in VSMCs under the conditions of this study.

Our *in vitro* analyses demonstrated that BJ treatment did not markedly change FAK (Y397) or ERK1/2 phosphorylation (Figure 6). These kinases are fundamental in regulating VSMC proliferation and migration triggered by inflammation (11). The lack of substantial modulation suggests that BJ may not directly target these pathways, possibly because the crude extract has multiple targets (25). Although BJ treatment did not significantly alter FAK (Y397) or ERK1/2 phosphorylation in the experiments conducted, this does not mean these kinases are not involved in BJ effects. On the contrary, the data suggest FAK/ERK activation was not modulated within the time frame assessed. Candidate mechanisms may include inhibition of VSMC migration and proliferation through modulation of cytoskeletal rearrangements and extracellular matrix (ECM) remodeling (45). This failure to elicit clear canonical signaling underscores involvement of distinct downstream and non-FAK/ERK pathways through which BJ's anti-proliferative and anti-migratory effects were mediated. As the remodeling of the extracellular matrix is an essential mechanistic driver of the vascular injury response, we turned to MMP2, a key protease involved in ECM remodeling during vascular injury, which is known to control ECM degradation, VSMC migration, proliferation, and neointimal formation (1). These results support further investigation of MMP2 as a potential pathway mediating BJ's vascular-protective effects.

Impact of BJ extract on IL-1 β -induced MMP2 expression in VSMCs

Based on previous findings, including the effects of BJ on proliferation, migration, and the relative expression of pERK1/2 (ICC), there were no significant differences in response among the various BJ doses of 10, 20, and 30 μ g/mL (Figure 4B-D and 6A-B). Therefore, we selected a BJ concentration of 10 μ g/mL for the subsequent Western blot assays. The stimulation of VSMCs with IL-1 β resulted in significant, time-dependent (6-24 hours) increases in MMP2 protein expression, while β -actin remained unchanged (Figure 7A). Treatment with 10 μ g/mL BJ attenuated this induction at all time points analyzed. Densitometric quantification (Figure 7B) showed that MMP2 expression increased by approximately 1.3-fold at 6 and 12 hours post IL-1 β stimulation and remained elevated through 24 hours. Conversely, co-treatment with BJ significantly reduced MMP2 expression at 6, 12, and 24 hours compared to IL-1 β alone, indicating that BJ counteracts MMP2 upregulation in VSMCs (Figure 7).

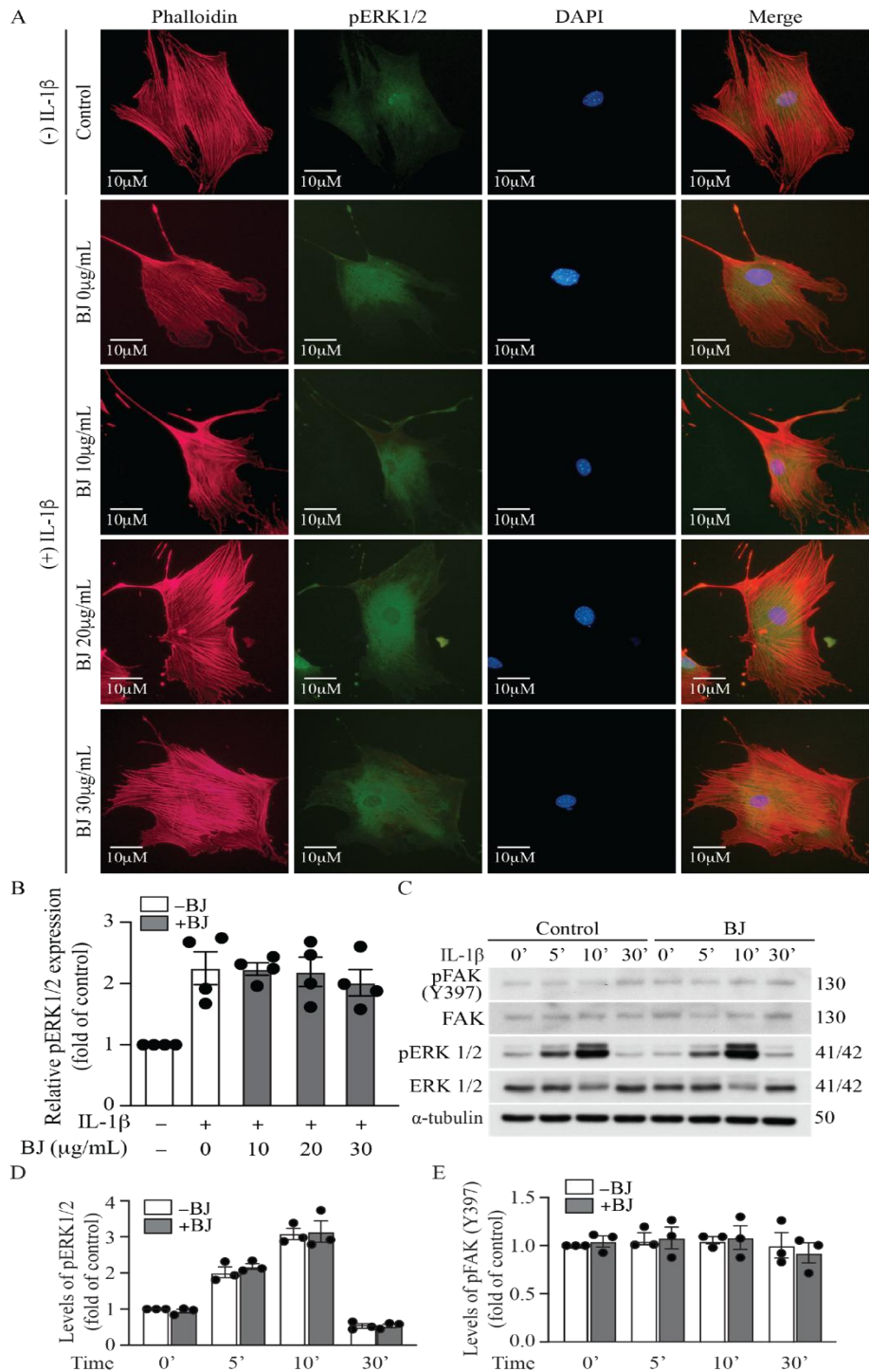


Figure 6.

Effect of BJ on ERK1/2 and FAK signaling in VSMCs under IL-1 β stimulation. (A) Representative immunofluorescence images of VSMCs treated with BJ (0, 10, 20, and 30 μ g/mL) in the presence or absence of IL-1 β . Phalloidin (red) labels F-actin filaments, pERK1/2 (green) indicates activated ERK1/2, and DAPI (blue) stains nuclei. Merged images show channel overlays. Scale bar = 10 μ m. (B) Quantification of relative pERK1/2 fluorescence intensity (fold change to control). Data are mean $\pm$ SEM (n = 4). (C) Western blot analysis of VSMC lysates treated with IL-1 β alone or BJ for the indicated times (0, 5, 10, and 30 min), showing expression of pFAK (Y397), total FAK, pERK1/2, total ERK1/2, and α -tubulin as the loading control. (D) Densitometric analysis of pERK1/2 normalized to total ERK1/2. (E) Densitometric analysis of pFAK (Y397) normalized to total FAK. Data are presented as mean $\pm$ SEM (n = 3). We determined statistical significance using GraphPad Prism version 10.4.1 via two-way ANOVA. We observed no significant differences between BJ-treated groups and IL-1 β controls for either kinase.

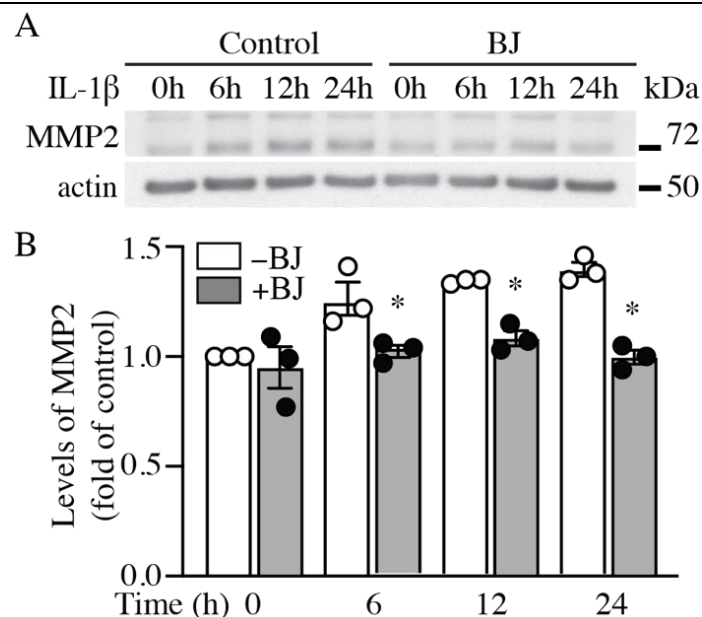


Figure 7.

BJ ethanolic extract suppresses MMP2 expression in VSMCs. (A) Representative Western blot showing MMP2 protein expression in VSMCs treated with IL-1 β (10 ng/mL) for the indicated time points (0, 6, 12, and 24 h), with or without BJ ethanolic extract. We used β -actin as a loading control. (B) Quantitative analysis of MMP2 protein levels normalized to β -actin and expressed as fold change relative to untreated control (0 h). Data are presented as mean $\pm$ SEM from $n = 3$ independent experiments. IL-1 β treatment significantly increased MMP2 expression over time, and BJ co-treatment markedly attenuated this increase. We determined statistical significance using GraphPad Prism version 10.4.1 via two-way ANOVA followed by post hoc comparison. * $p < 0.05$, IL-1 β co-treatment with BJ versus without BJ.

Consistent with its role as a pro-inflammatory mediator driving vascular remodeling (11), IL-1 β promoted MMP2 expression in VSMCs. Notably, BJ substantially blunted this upregulation (Figure 7), suggesting that BJ functions as an inflammation modulator or remodeling inhibitor in VSMCs (45). This finding aligns with previous reports that IL-1 β potentiates MMP2 and MMP9 synthesis in VSMCs, promoting ECM degradation and cell migration (46). Increased MMP2 activity aggravates vascular wall remodeling, aneurysm formation, and neointimal hyperplasia. This observation, together with the phenotypic shift of VSMCs toward a migratory and synthetic state, supports our finding that IL-1 β stimulation increased MMP2 over 24 hours (47).

Our results demonstrate that BJ extract suppresses MMP2 expression in VSMCs, which is associated with its inhibitory effect on VSMC migration and neointimal formation. BJ contains bioactive compounds such as flavonoids, especially quercetin, known for their antioxidant and anti-inflammatory properties. These compounds have been shown to block MAPK pathways, which are central to gene expression (48). BJ's ability to maintain redox homeostasis and restrain pro-inflammatory cytokine release in various cell types supports the notion that its antioxidant and anti-inflammatory properties act synergistically to mitigate responses in VSMCs.

This is particularly relevant given the well-documented role of oxidative stress in upregulating MMPs during vascular injury (5).

Notably, IL-1 β sustained MMP2 expression over 24 hours, suggesting a long-term effect on the VSMC phenotype (46). BJ's ability to keep MMP2 levels near baseline throughout the assessment period demonstrates sustained control over inflammatory VSMCs and its capacity to counter the enduring drive of IL-1 β (Figure 7). This unique long-term regulatory effect suggests that BJ may act as a sustained modulator of the inflammatory VSMC response. Given that MMP2 is a key factor in ECM remodeling and vascular repair, these findings indicate the potential therapeutic application of BJ in preventing atherosclerosis, restenosis, or aneurysm formation (47). By reducing MMP2 levels, BJ may help preserve vascular integrity and is associated with reduced pathological remodeling following inflammatory injury (49). In summary, our findings show that 10 μ g/mL BJ extract suppresses MMP2 expression in VSMCs in a time-dependent manner (Figure 7), which is associated with its capacity to mitigate cytokine-induced vascular remodeling (25). Further studies should investigate whether this effect is mediated through inhibition of MAPK signaling pathways other than ERK1/2 and/or other oxidative stress-related pathways.

BJ extract mitigates endothelial denudation-induced intimal hyperplasia in mice

To evaluate the efficacy of BJ extract in reducing neointimal hyperplasia following endothelial denudation, we used a mouse femoral artery guide wire injury model (16). We selected a dose of 30 mg/kg/day based on previous studies showing BJ extract tolerability in mouse models without observable toxicity (50). Histomorphometric analysis revealed distinct structural differences between groups (Figure 8A). Uninjured arteries exhibited a thin, organized intimal layer and well-preserved lumen architecture. In contrast, injured control arteries displayed a hyperplastic response with marked intimal thickening and

luminal narrowing. Notably, BJ-treated mice showed a significant reduction in neointima formation, with thinner intimal layers and better-preserved vessel architecture (Figure 8A). Quantification of the I/M ratio confirmed that the injured + BJ group (0.313 ± 0.056) had a significantly lower I/M ratio than the untreated injured group (0.630 ± 0.089 , $p = 0.013$), indicating that BJ reduces injury-induced vascular remodeling (Figure 8A). Moreover, the IHC confirmed that BJ treatment downregulated MMP2 in the injured vasculature (1.342 ± 0.085 -fold compared with control) compared with the injured without BJ (1.687 ± 0.056 -fold compared with control, $p = 0.0071$) (Figure 8B).

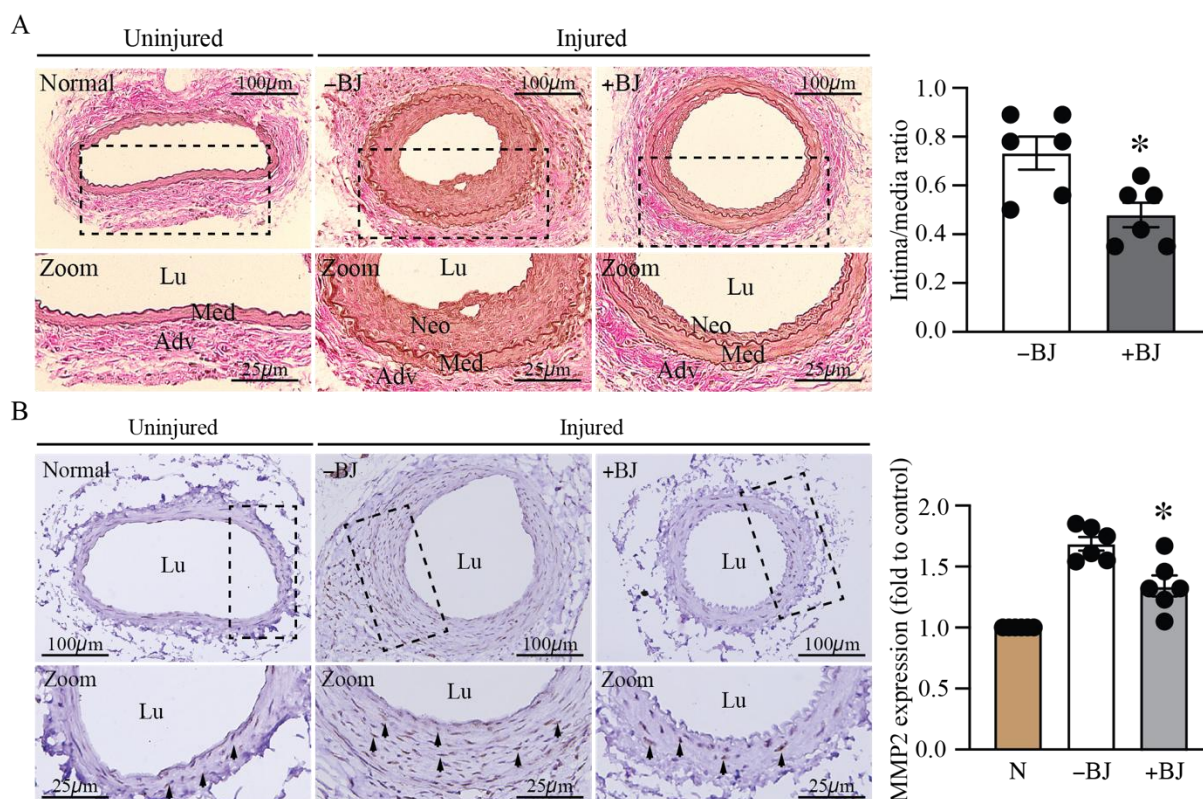


Figure 8.

Effect of BJ extract on neointimal hyperplasia and MMP2 expression following endothelial denudation-induced vascular injury in mice. (A) Representative elastin-stained femoral artery sections and quantitative analysis of the intima/media ratio. (B) Representative immunohistochemical staining and quantitative analysis of MMP2 expression in uninjured (Normal) and injured mice treated with vehicle (–BJ) or BJ extract (+BJ). Data are presented as mean ± SEM from $n = 6$, and significance was determined using GraphPad Prism version 10.4.1 software via t-test and considered $*p < 0.05$ versus the –BJ group.

BJ's ability to diminish neointimal hyperplasia demonstrates its vascular protective effects *in vivo*. Neointima formation primarily results from VSMC proliferation, migration, and ECM remodeling (51). Thus, the *in vivo* results are consistent with our *in vitro* findings that BJ reduces VSMC proliferation, migration, and MMP2 expression in VSMCs (Figures 4 and 7). Because MMP2 promotes ECM degradation and VSMC infiltration into the intima, its reduction is associated with the decrease in neointimal

growth observed in BJ-treated animals (52). The flavonoids, phenolic acids, and quassinoid metabolites in BJ possess well-documented antioxidant and anti-inflammatory properties, which may interact with the vascular microenvironment to alleviate oxidative stress and inflammatory cytokine cascades while preserving endothelial integrity (53). This upstream action likely reduces MMP2 activity, thereby inhibiting VSMC migration, proliferation, and neointimal formation (6, 40).

Although this study demonstrated the potency of BJ extract in mitigating endothelial denudation-induced neointimal hyperplasia, which correlates with MMP2 downregulation, further validation is required. Specifically, the effect of BJ on the intimal hyperplasia mouse model was tested at a single dose of 30 mg/kg BW; testing a wider range of doses would be valuable for future studies. Furthermore, because the Western blot results were obtained using only 10 µg/mL of BJ, evaluating other concentrations is required to fully determine its effect on MMP2.

Conclusions

This investigation demonstrates that BJ extract effectively inhibits pathological VSMC activation by mitigating proliferation, migration, and lamellipodia formation. Mechanistically, BJ reduces MMP2 expression in VSMCs and injured arteries, eventually reducing the I/M ratio of endothelial denudation-induced neointimal hyperplasia in mice. Collectively, these findings position BJ as a potential regulator of vascular remodeling correlated with MMP2.

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Availability of data and materials

All original data generated and analyzed during this study, including characterization, *in vitro* assays (MTT assay, proliferation, migration, western blot analyses, immunocytochemistry, lamellipodia protrusion), and *in vivo* (injury-induced intimal hyperplasia, vascular elastin staining, and immunohistochemistry), are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The Ethics Committee of the Faculty of Medicine and Health Sciences, University of Mataram approved the *in vivo* study design under No. 122/UN18.F8/ETIK/2025.

AI use disclosure

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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