

Article

Phytochemical Profiling of *Boswellia sacra* Leaf Extracts and Evaluation of Their Pancreatic Lipase Inhibitory, Antimicrobial, and Antiproliferative Activities

Mansour Alblaji ^{1,*}, Sultan Matar Almutairi ¹, Fahad Saad Alhodieb ¹  and Saleh A. Alsanie ^{1,2} 

¹ Department of Basic Health Sciences, College of Applied Medical Sciences, Qassim University, Buraydah 51452, Saudi Arabia; sum.almutairi@qu.edu.sa (S.M.A.); f.alhodieb@qu.edu.sa (F.S.A.); s.alsanie@qu.edu.sa (S.A.A.)

² Department of Clinical Nutrition, Medical City, Qassim University, Buraydah 51452, Saudi Arabia

* Correspondence: m.alblaji@qu.edu.sa

Abstract

This study optimized supercritical fluid extraction (SC-CO₂) parameters for *Boswellia sacra* leaves to evaluate how extraction thermodynamics influence subsequent pharmacological bioactivity. Using a multi-parametric approach, extracts obtained at 40 °C and 80 °C (100 bar) were profiled via HPLC-DAD and evaluated using in vitro antimicrobial, pancreatic lipase inhibition, and Caco-2 cell antiproliferative assays. HPLC analysis revealed that the 40 °C threshold preserved chemical diversity (17 active peaks, retaining higher caffeic acid and rutin concentrations), whereas 80 °C conditions caused severe compound degradation. Consequently, the 40 °C extracts exhibited significantly enhanced biomedical potencies over the 80 °C variants, demonstrating superior microbiostatic activity against Gram-positive bacteria and *Candida albicans* (MIC = 15.62 µg/mL), stronger porcine pancreatic lipase inhibition (IC₅₀ = 17.21 µg/mL), and greater antiproliferative efficacy against human colorectal adenocarcinoma cells (IC₅₀ = 109.47 µg/mL). Ultimately, maintaining low thermal thresholds during green extraction is critical to preserving the integrated polyphenolic architecture of *B. sacra* required for multi-targeted antimicrobial, anti-obesity, and anticancer therapeutics.

Keywords: *Boswellia sacra*; supercritical fluid extraction; polyphenols; anti-obesity; antimicrobial activity; public health

1. Introduction

The modern healthcare landscape is increasingly challenged by syndemic global crises, wherein chronic metabolic disorders and persistent infectious diseases interact synergistically to exacerbate human morbidity and mortality. Among these, obesity has escalated to epidemic proportions, characterized by pathological adipogenesis and a state of chronic, low-grade systemic inflammation that acts as a fundamental catalyst for cardiovascular disease, type 2 diabetes, and metabolic syndrome. In tandem with this metabolic burden, the rapid dissemination of multidrug-resistant (MDR) microbial pathogens has severely outpaced the development of conventional antibiotic regimens, with virulence mechanisms such as biofilm formation rendering clinical infections up to 1000-fold more resistant to traditional therapies. Because individuals suffering from metabolic syndrome are inherently predisposed to severe, recalcitrant infections due to compromised immune phenotypes, there is a profound and urgent pharmaceutical imperative to identify novel



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therapeutic agents that possess multi-target efficacy. Consequently, discovery programs have shifted toward natural product matrices capable of simultaneously interrogating metabolic regulatory networks and disrupting microbial survival mechanisms, offering a holistic, dual-action strategy to combat these intertwined public health emergencies.

Obesity and infectious diseases represent two of the most critical global public health crises of the 21st century, placing an immense socioeconomic burden on healthcare systems worldwide. Obesity, characterized by abnormal or excessive lipid accumulation in adipose tissue, is highly correlated with the pathological acceleration of adipogenesis, the differentiation of preadipocytes into mature, lipid-laden adipocytes. This metabolic disorder is a primary driver of the metabolic syndrome, inducing chronic low-grade systemic inflammation, type 2 diabetes, insulin resistance, and cardiovascular complications. At the cellular level, terminal adipocyte differentiation is tightly governed by a cascade of transcription factors, most notably the CCAAT/enhancer-binding proteins (C/EBP α and C/EBP β) and peroxisome proliferator-activated receptor gamma (PPAR γ) [1]. Concurrently, the global rise in MDR microbial pathogens has severely compromised the efficacy of conventional antibiotic therapies, causing an urgent push toward alternative antimicrobial strategy [2]. Pathogenic bacteria, such as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, frequently exploit virulence mechanisms like biofilm formation, which escalates their resistance to conventional therapies by up to 1000-fold compared to planktonic cells [3]. Consequently, discoverable bio-actives capable of exerting dual regulatory roles, specifically mitigating adipogenic pathways while simultaneously disrupting microbial membranes or virulence factors, hold profound therapeutic value.

Natural oleo-gum resins have historically served as rich repositories for structurally diverse chemical scaffolds with potent pharmacological actions. Among these, frankincense (olibanum), exuded from trees belonging to the genus *Boswellia* (family Burseraceae), has garnered significant pharmacological interest. In particular, *Boswellia sacra* Flueck., a species indigenous to the Arabian Peninsula (notably the Dhofar region of Oman) and parts of Northeast Africa, produces a highly prized, chemically complex resin. The therapeutic efficacy of *B. sacra* is largely ascribed to its distinct phytochemical fractions: a volatile essential oil fraction predominantly abundant in monoterpenes and sesquiterpenes (such as α -pinene), and an organic solvent-soluble resin fraction containing highly bioactive pentacyclic triterpenoids, collectively known as boswellic acids [4]. Prominent among these are β -boswellic acid, 11-keto- β -boswellic acid (KBA), and 3-acetyl-11-keto- β -boswellic acid (AKBA) [1]. Recent molecular studies have demonstrated that these triterpenes exhibit profound anti-obesity effects by inhibiting lipid droplet accumulation, inducing AMPK phosphorylation, suppressing autophagy markers (such as ATG5), and downregulating the key adipogenic transcription factors PPAR γ and C/EBP α in vitro [1]. Simultaneously, *B. sacra* derivatives have demonstrated broad-spectrum antimicrobial, anti-biofilm, and virulence-attenuation potential against an array of clinical pathogens, working through membrane disruption, intracellular nucleotide leakage, and the downregulation of biofilm-associated genetic frameworks [3,5]. It is critical to distinguish between the tissue-specific secondary metabolite profiles of *B. sacra*. While the trunk oleo-gum resin is inherently characterized by lipophilic pentacyclic triterpenes, most notably 11-keto- β -boswellic acid (KBA) and 3-O-acetyl-11-keto- β -boswellic acid (AKBA), the foliar tissue exhibits a completely divergent biosynthetic specialization. *B. sacra* leaves lack the specialized cortical resin secretory ducts found in the stem bark. Consequently, the foliar matrix is systematically depleted of triterpenic boswellic acids and is instead enriched with a dense complex of polar and semi-polar polyphenols, low-molecular-weight phenolic acids, and glycosylated flavonoids.

Despite its clear therapeutic promise, the extraction of these bioactive moieties via conventional methodologies poses major technical obstacles for pharmaceutical applications. Traditional techniques, including hydrodistillation and organic solvent extraction, frequently suffer from long extraction times, high thermal degradation of labile monoterpenes, and hazardous organic solvent residues that limit food-grade or clinical translation [3]. To circumvent these challenges, green chemical engineering has prioritized SFE, typically employing supercritical carbon dioxide (SC-CO₂). As a non-toxic, non-flammable, and chemically inert solvent with tunable density, SC-CO₂ operates at relatively low critical temperatures (31.1 °C), ensuring the pristine preservation of thermolabile compounds. Furthermore, adjusting processing parameters, such as extraction pressure, temperature, and co-solvent (modifier) ratios, allows for the selective enrichment of either volatile monoterpene fragments or heavy lipophilic triterpene resins (AKBA and KBA), yielding highly standardized, solvent-free extracts with superior bioavailability and biological activity.

While independent lines of research have separately validated either the antimicrobial or metabolic benefits of crude *Boswellia* resins, a profound knowledge gap exists regarding how the specific operational parameters of SFE influence the yield of distinct phytoconstituents, and how these targeted fractions map to dual anti-obesity and antimicrobial phenotypes. Specifically, the synchronous application of a single, standardized *B. sacra* supercritical extract to disrupt bacterial cell viability while simultaneously halting intracellular adipogenesis remains poorly explored in the high-impact literature. To address this gap, the present study systematically investigates the dual-action potential of *B. sacra* oleo-gum resin extracts obtained via optimized supercritical fluid extraction. Herein, we evaluate the comparative efficacy of supercritical extracts against key adipogenic markers (PPAR γ , C/EBP α) and lipid accumulation models, alongside their concurrent minimum inhibitory concentrations (MIC) and anti-biofilm activities against a panel of pathogenic strains. This work establishes a green extraction framework for maximizing the multi-target therapeutic indices of *B. sacra*, providing a solid foundation for its development as a multi-functional natural agent against metabolic dysfunction and infectious diseases.

Despite the documented ethnomedicinal relevance of the *Boswellia* genus, the precise multi-parametric optimization of green extraction boundaries for *B. sacra* leaves and the subsequent impact of processing thermodynamics on thermolabile phytoconstituents remain poorly understood. Therefore, this study aims to systematically optimize the SFE (SC-CO₂) parameters (temperature, pressure, mass flow rate, and modifier concentration) of *B. sacra* leaves to maximize extraction yields while preserving bioactive components. Furthermore, we aimed to comprehensively evaluate and compare the in vitro pharmacological potencies of the resulting low-temperature (40 °C) and high-temperature (80 °C) fluid fractions. This was achieved by characterizing their broad-spectrum antimicrobial efficacy against key pathogenic strains, their enzymatic inhibition kinetics against pancreatic lipase, and their dose-dependent cytotoxic and antiproliferative profiles against human colorectal adenocarcinoma (Caco-2) cells. Through high-performance liquid chromatography (HPLC-DAD) profiling, this work establishes a direct link between extraction thermodynamics, secondary metabolite stability, and dual anti-obesity and anticancer therapeutic performance.

2. Materials and Methods

2.1. Plant Materials

Fresh, healthy leaves of *B. sacra* Flueck. were harvested from authenticated private plant nurseries from local markets.

2.2. Preparation and Extraction of *B. sacra* Leaf Materials

2.2.1. Lyophilization and Defatting

Fresh *B. sacra* leaves were lyophilized using a benchtop freeze-dryer (Benchtop, Kansas city, MO, USA) operating at a condenser temperature of $-50\text{ }^{\circ}\text{C}$ under reduced pressure (vacuum of less than 0.1 mbar) until complete sublimation was achieved. The dehydrated biomass was subsequently pulverized using a commercial laboratory blender (Model 8011ES/HGB2WTS3, 400 W; Waring, Torrington, CT, USA) and passed through a 0.5 mm stainless steel mesh sieve to ensure particle size homogeneity. To remove highly non-polar, non-target lipophilic impurities, a 500 g aliquot of the resulting leaf powder was defatted by maceration in 1.5 L of n-hexane at room temperature ($25\text{ }^{\circ}\text{C}$). The solvent was renewed daily for 14 consecutive days. The defatted biomass was then air-dried under shade at ambient temperature for 24 h to completely evaporate residual hexane, and the processed matrix was designated as E1.

2.2.2. Conventional Solvent Extraction

For comparative purposes, a 15 g portion of the defatted leaf powder (E1) was subjected to conventional solvent extraction via a Soxhlet apparatus using 350 mL of ethyl acetate at $85\text{ }^{\circ}\text{C}$ for a continuous duration of 6 h. The resulting liquid extract was concentrated under reduced pressure at $35\text{ }^{\circ}\text{C}$ using a rotary evaporator backed by a vacuum pump (Edwards E2M8; Edwards, Crawley, UK) to partially remove the solvent. This crude ethyl acetate extract was recovered and designated as E2.

2.2.3. Supercritical Fluid Extraction (SFE)

SFE of *B. sacra* leaves was performed using a customized bench-scale extraction system. To isolate the specific impact of extraction temperature on the recovery and biological activity of thermolabile foliar phenolics, a two-stage optimization strategy was employed. In the first stage, preliminary pilot runs were conducted to establish fixed baseline parameters that optimized raw mass yields and extraction efficiency. Consequently, the system pressure was fixed at 250 bar to ensure adequate solvent density, the flow rate of supercritical CO_2 was maintained at 15 g/min, and ethanol (96% v/v) was introduced as a polar co-solvent/modifier at a fixed ratio of 15% w/w to facilitate the solvation of moderately polar flavonoids and phenolic acids. The total extraction duration was set to 60 min, comprising a 15 min static contact phase to promote solute–solvent equilibrium within the leaf matrix, followed by a 45 min dynamic purging phase. In the second stage, with all other physical parameters held strictly constant, extraction temperatures of $40\text{ }^{\circ}\text{C}$ (low-temperature, SFE-40) and $80\text{ }^{\circ}\text{C}$ (high-temperature, SFE-80) were systematically evaluated to assess their influence on the preservation of target bioactives and their resulting enzyme inhibitory, antimicrobial, and antiproliferative properties. The extraction was carried out using absolute ethanol as a polar modifier at concentrations of 14%, 16%, 18%, and 20% (w/w), under varied CO_2 mass flow rates (12, 14, 16, 18, and 20 g/min). The operational extraction temperatures were maintained across $40\text{ }^{\circ}\text{C}$, $45\text{ }^{\circ}\text{C}$, $55\text{ }^{\circ}\text{C}$, and $60\text{ }^{\circ}\text{C}$, while the system pressures were systematically varied at 100, 150, 200, and 250 bar [6,7]. The collected supercritical fluid fractions were combined, solvent-evaporated, and designated as E3.

2.2.4. Determination of Solids Content (Extraction Yield)

The total solids content (dry extract yield, %) for each extraction protocol (E2 and E3) was quantitatively calculated relative to the starting defatted biomass weight using the following mathematical expression:

$$\text{Extraction Yield (\%)} = \left(\frac{W_{\text{extract}}}{W_{\text{initial}}} \right) \times 100$$

where W_{extract} represents the constant dry weight of the obtained crude extract (g) after total solvent evaporation, and W_{initial} represents the initial dry weight of the defatted leaf powder (E1) utilized in the extraction process (g).

2.3. In Vitro Antimicrobial Screening via Agar Well Diffusion Assay

The broad-spectrum antimicrobial efficacy of the *B. sacra* leaf extracts (E2 and E3) was evaluated in vitro using the standardized agar well diffusion methodology [8]. Actively growing bacterial and fungal strains were inoculated onto Nutrient Agar (Difco, Sparks, MD, USA) and Sabouraud Dextrose Agar (Difco, Sparks, MD, USA), respectively, to achieve a uniform microbial lawn equivalent to a 0.5 McFarland turbidity standard (1.5×10^8 CFU/mL for bacteria). Using a sterile cork-borer, uniform wells measuring 6–8 mm in diameter were punched into the solidified agar matrices. A 100 µL aliquot of each prepared plant extract was subsequently inoculated into the respective wells. Prior to thermal incubation, the inoculated plates were maintained at 4 °C for 1 h to facilitate optimal passive horizontal diffusion of the bioactive phytoconstituents into the surrounding agar matrix while temporarily arresting microbial replication. Following this pre-diffusion phase, the plates were incubated under aerobic conditions at 30 °C for 24 h for bacterial cultures, and for 72 h for fungal cultures. Antimicrobial activity was quantitatively determined by measuring the diameter of the clear zones of inhibition (ZOI) surrounding each well in millimeters (mm) using a digital vernier caliper. To ensure experimental validity, comparative controls were integrated into each assay layout:

- Negative controls: Seeded plates received 100 µL of the respective extraction vehicle/solvent blank without plant extract.
- Positive controls (antibacterial): Bacterial plates were treated with Gentamicin (1.0 mg/mL).
- Positive controls (antifungal): Fungal plates were treated with Fluconazole (1.0 mg/mL).

All experimental trials were executed in triplicate ($n = 3$), and the results are expressed as mean zone diameters $\pm$ standard deviation (SD).

2.4. Determination of MIC via Broth Microdilution Assay

To evaluate the baseline potency and thermal stability of the *B. sacra* extracts (E2 and E3), MIC were determined using a standardized broth microdilution methodology in 96-well polystyrene microtiter plates [9]. Prior to the assay, aliquots of the extracts were subjected to thermal pre-treatments at either 40 °C or 80 °C to ascertain the retention of antimicrobial efficacy following thermal exposure. Stock solutions of the thermally pre-treated extracts were prepared in Mueller–Hinton Broth (MHB; Difco, Sparks, MD, USA) and systematically diluted via two-fold serial dilutions to establish a final concentration gradient ranging from 0.98 to 1000 µg/mL. Standardized microbial inocula were prepared from log-phase cultures suspended in sterile saline solution (0.85% NaCl) and adjusted optically to match a 1.0 McFarland turbidity standard. This suspension was subsequently diluted into the microtiter wells containing 200 µL of the respective extract-broth mixtures to achieve a standardized target initial cell density of approximately 3×10^6 CFU/mL. The microplates were incubated at 35 °C for 72 h under controlled microaerophilic conditions (15% CO₂). Quantitative cell density and growth endpoints were monitored spectrophotometrically by measuring optical density (OD₆₃₀) using a BioTek 800 TS microplate reader (BioTek Instruments, Winooski, VT, USA). To ensure rigorous optical calibration and assay validity, the following control wells were integrated into each plate layout:

- Growth control: Extract-free wells containing the inoculated culture medium were indeed used as growth controls to verify strain viability and robust cell growth during incubation. The sentence has been slightly refined in the revised text for added technical clarity. An assay run was considered valid only when robust, acceptable microbial growth was spectrophotometrically verified in these wells.
- Sterility and background control: Uninoculated (bacterium-free) wells containing the culture medium and serially diluted extracts were used as background blank controls to adjust for intrinsic extract color and baseline turbidity.

The MIC endpoint was formally defined as the lowest concentration of the *B. sacra* extract that completely inhibited visible microbial growth, demonstrated by an optical density matching the respective sterility blank wells.

2.5. Determination of MBC

To determine whether the *B. sacra* extracts (E2 and E3) exhibited bacteriostatic or bactericidal modalities, the MBC were established following the initial MIC evaluations [9]. Aliquots (10 µL) were micro-pipetted from each microtiter well that exhibited complete inhibition of visible microbial growth, as well as from the positive growth control well. These aliquots were systematically transferred and spread-plated onto solid Mueller–Hinton Agar (MHA; Difco, Sparks, MD, USA) matrices. The inoculated plates were subsequently incubated at 35 °C for 72 h under controlled microaerophilic conditions (15% CO₂) to allow for the recovery of any surviving, non-planktonic viable cells. The MBC endpoint was defined as the lowest concentration of the *B. sacra* extract that completely killed the initial bacterial inoculum, demonstrated by a total absence of macroscopically visible colony-forming units (CFUs) on the agar surface.

2.6. In Vitro Pancreatic Lipase Inhibition Assay

The anti-obesity potential of the *B. sacra* supercritical extracts was evaluated by assessing their capacity to inhibit porcine pancreatic lipase in vitro, utilizing a modified spectrophotometric method described by Kim et al. [10]. Porcine pancreatic lipase (Standard grade) was procured from Spectrum Diagnostics. The chromogenic substrate, p-nitrophenyl butyrate (PNPB), was dissolved in HPLC-grade acetonitrile to prepare a concentrated stock solution. A working solution of the enzyme (1.0 mg/mL) was freshly prepared immediately prior to use by dissolving 10 mg of pancreatic lipase in 10 mL of 0.1 M potassium phosphate buffer (pH 7.2). A wide concentration gradient (7.81 to 1000 µg/mL) of the *B. sacra* extracts and Orlistat (tetrahydrolipstatin; utilized as a clinical positive control reference) was prepared by dissolving the target materials in dimethyl sulfoxide (DMSO). To initiate the assay, a pre-incubation mixture was prepared in individual wells of a 96-well microplate, consisting of the respective concentrations of extracts or Orlistat, pancreatic lipase solution, 0.1 M potassium phosphate buffer (pH 7.2), and 0.1% Surfactant/Tween 80 (to facilitate lipidic micellar stabilization). The mixture was gently agitated and thermally equilibrated at 30 °C for 1 h. Following pre-incubation, the enzymatic reaction was initiated by the addition of the PNPB substrate solution to yield a final reaction volume of 100 µL per well. The microplate was incubated at 30 °C for exactly 5 min. The enzymatic hydrolysis of the colorless PNPB substrate into the yellow-colored compound p-nitrophenol (PNP) was quantitatively monitored by measuring the absorbance at 405 nm using a microplate spectrophotometer reader. The percentage of pancreatic lipase inhibitory activity (I, %) was calculated using the following background-corrected mathematical expression to account for any intrinsic extract-induced absorbance:

$$\text{Lipase inhibition(\%)} = \left[1 - \frac{(B - b)}{(A - a)} \right] \times 100$$

where B is the absorbance of the reaction mixture containing pancreatic lipase, the test extract/inhibitor, and the substrate; b is the background blank absorbance containing the test extract/inhibitor and substrate without the lipase enzyme; A is the absorbance of the uninhibited growth control reaction containing pancreatic lipase and substrate without the test extract; a is the absolute negative control blank absorbance containing only the buffer and substrate without both the enzyme and the inhibitor. The half-maximal inhibitory concentration (IC₅₀) values were determined by non-linear regression analysis of the dose–response curves.

2.7. HPLC-DAD Characterization of SFE Fractions

Qualitative and quantitative phytochemical profiling of the optimized *B. sacra* SFE was executed using an Agilent 1260 Infinity Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump, an autosampler, and a Diode Array Detector (DAD). Chromatographic separation of the target bio-actives was achieved at a controlled column temperature of 40 °C utilizing a reverse-phase Zorbax Eclipse Plus C₈ analytical column (250 mm × 4.6 mm i.d., 5 µm particle size; Agilent Technologies). The mobile phase architecture was composed of an aqueous phase consisting of ultra-pure water (Solvent A) and an organic phase consisting of 0.05% trifluoroacetic acid (TAF) dissolved in HPLC-grade acetonitrile (Solvent B). Prior to analysis, all solvents were filtered through a 0.22 µm membrane filter and degassed ultrasonically. The elution was driven at a constant flow rate of 0.9 mL/min using a meticulously timed linear gradient program optimized from Verni et al. [11]. The gradient profile was structured as in Table 1. An aliquot injection volume of 5 µL was maintained for all standardized sample solutions and reference standards. Phytochemical peaks were dynamically monitored, recorded, and integrated using the DAD system at an optimized UV wavelength of 280 nm. Chromatographic data acquisition and subsequent baseline integration were processed utilizing Agilent OpenLAB ChemStation software (Version C.01.08).

Table 1. The gradient profile of HPLC-DAD mobile phases.

Time (min)	Mobile Phase A (Water, %)	Mobile Phase B (0.05% TAF in Acetonitrile, %)
0.0–1.0	82.0	18.0
1.0–11.0	75.0	25.0
11.0–18.0	60.0	40.0
18.0–24.0	82.0	18.0

2.8. In Vitro Cytotoxicity and Cell Viability (MTT) Assay

2.8.1. Cell Culture and Sample Treatment

To evaluate the cytotoxic profiles of the *B. sacra* SFEs, cell lines were seeded into 96-well tissue culture microplates at a density of 1×10^5 cells/mL (100 µL/well) and incubated at 37 °C under a humidified atmosphere of 5% CO₂ for 24 h to allow for the formation of a confluent monolayer. Following incubation, the confluent growth medium was aspirated, and the cell monolayers were gently rinsed twice with a washing medium. Stock solutions of the *B. sacra* extracts were subjected to two-fold serial dilutions in maintenance medium (RPMI-1640 supplemented with 2% fetal bovine serum). Aliquots (100 µL) of each diluted extract concentration were transferred into designated wells in triplicate ($n = 3$), while three uninhibited wells received only the maintenance medium to serve as negative growth controls [12]. The treated microplates were incubated at 37 °C for an additional 24 h.

2.8.2. Cytomorphological Evaluation

Prior to cell viability quantification, qualitative cytomorphological alterations were monitored under an inverted phase-contrast microscope [13]. Cellular cytotoxicity and structural damage were systematically categorized based on classic physical indicators, including the partial or complete detachment/loss of the cell monolayer, cell rounding, cytoplasmic degranulation, cellular shrinkage, nuclear condensation, and cellular fragmentation.

2.8.3. MTT Quantitation and Absorbance Measurement

Cell viability was quantitatively assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic assay. A stock solution of MTT (5.0 mg/mL) was prepared in phosphate-buffered saline (PBS, pH 7.4; Bio Basic Canada Inc., Markham, ON, Canada). A 20 μ L aliquot of the MTT reagent was added to each microplate well, and the plates were briefly agitated at 150 rpm for 5 min at 37 °C to ensure uniform distribution. The microplates were then incubated under standard conditions (37 °C, 5% CO₂) for 4 h to allow viable, metabolically active cells to reduce the yellow tetrazolium salt into insoluble, crystalline purple formazan. Following incubation, the supernatant culture medium was carefully aspirated, and the microplates were inverted and gently blotted on absorbent material to eliminate residual liquid traces. The intracellular purple formazan crystals were solubilized by adding 200 μ L of dimethyl sulfoxide (DMSO) to each well, followed by mechanical shaking at 150 rpm for 5 min at 37 °C to achieve complete dissolution. The OD of each well was measured spectrophotometrically at 560 nm, with background interference subtraction calibrated at 620 nm using a multi-well microplate reader [14]. The percentage of relative cell viability was calculated relative to the untreated control wells, and the half-maximal cytotoxic concentration (CC₅₀) values were computed via non-linear regression analysis. Percentage cell viability was calculated relative to the untreated vehicle control (containing 0.1% DMSO in culture medium), which was defined as 100% viable.

2.9. Statistical Analysis

All experimental treatments and assays were executed in independent triplicates ($n = 3$), and the resulting quantitative data are expressed as mean $\pm$ standard deviation (SD). Prior to formal hypothesis testing, the normality of the data distribution and the homogeneity of variances were verified utilizing the Shapiro–Wilk and Levene tests, respectively. Statistically significant differences among the varied conventional and supercritical fluid extraction treatments, as well as biological dose-responses, were evaluated via one-way analysis of variance (ANOVA). Post hoc multiple pairwise comparisons were executed using Tukey’s Honestly Significant Difference (HSD) test. All statistical analyses and computational regressions were performed using SPSS software (Version 25.0; IBM Corp., Armonk, NY, USA) and Microsoft Excel 365. Statistical significance was strictly defined *a priori* at a probability level of $p < 0.05$.

3. Results

3.1. SFE Yield Optimization

The extraction yield of *B. sacra* leaf phytoconstituents obtained via SFE (SC-CO₂) was significantly influenced by the operational parameters, specifically extraction temperature and system pressure. The extraction protocol consisted of a 15 min static phase followed by a 45 min dynamic extraction phase. At a baseline pressure of 100 bar, executing the extraction at a lower temperature of 40 °C resulted in a total solid dry extract yield of 2.7% $\pm$ 0.14% (0.417 g recovered from 15 g of starting defatted biomass E1). Elevating the operational extraction temperature to 80 °C under the same pressure conditions sig-

nificantly enhanced the extraction efficiency ($p < 0.05$), yielding a total solid content of $3.55\% \pm 0.18\%$ (0.532 g of recovered dry extract). The deliberate selection of 40 °C and 80 °C at a fixed baseline pressure of 100 bar, while bypassing the intermediate temperatures evaluated in the broader multi-parametric matrix (Table 2), was strategically implemented to isolate distinct thermodynamic extraction regimes. In supercritical fluid dynamics, intermediate temperatures (45 °C to 60 °C) operate within a competitive transition zone where decreasing fluid density and increasing solute vapor pressure counteract one another. By deploying 80 °C as an extreme high-temperature control group, the system was driven intentionally past the typical crossover pressure boundaries. This extreme thermal state served as a structural stress-test designed to evaluate the limits of polyphenolic thermolability. It provided the thermodynamic energy necessary to clearly contrast fluid density-driven passive dissolution (favored at 40 °C) against solute vapor pressure-driven volatilization and heat-induced matrix hydrolysis (observed at 80 °C), which would otherwise remain obscured or statistically convoluted at intermediate thermal increments. To fully characterize the multi-parametric optimization matrix matching the evaluated methodology, the interactions between co-solvent modifier concentration (ethanol, % *w/w*), CO₂ mass flow rate (g/min), temperature (°C), and pressure (bar) on the final cumulative extraction yields are summarized in Table 2. Maximum extraction efficiency was achieved at the highest operational boundaries (250 bar, 60 °C, 20% ethanol modifier, and 20 g/min CO₂ flow rate), resulting in a peak extraction yield of $7.64\% \pm 0.33\%$. This trend highlights a positive correlation between high-temperature/high-pressure supercritical conditions and the total recovery of solid therapeutic fractions from *B. sacra* leaves.

Table 2. Multi-parametric optimization matrix of *B. sacra* leaf SFE (SC-CO₂) and its corresponding effects on absolute cumulative extraction yield (%).

Run	Pressure (bar)	Temperature (°C)	Ethanol Modifier (% <i>w/w</i>)	CO ₂ Flow Rate (g/min)	SFE Yield (%)
1	100	40	14	12	2.78 ± 0.14^d
2	100	80	14	12	3.55 ± 0.18^c
3	150	45	16	14	4.12 ± 0.21^{bc}
4	200	55	18	16	5.89 ± 0.29^b
5	250	60	20	20	7.64 ± 0.33^a

Data are expressed as mean value $\pm$ standard deviation (SD) of independent experimental triplicates ($n = 3$). Means within the column sharing identical superscript letters (^{a–d}) do not differ significantly from one another based on Tukey's Honestly Significant Difference (HSD) post hoc test executed at an alpha level of $\alpha = 0.05$. Conversely, distinct superscript letters indicate statistically significant differences ($p < 0.05$) between the respective operational supercritical extraction runs, arranged in descending order from the highest yield (^a) to the lowest yield (^d).

3.2. In Vitro Antimicrobial Screening of Plant Extracts

The in vitro screening of *B. sacra* leaf extracts obtained via high-pressure fluid extraction at operational temperatures of 40 °C and 80 °C demonstrated a distinct, broad-spectrum antimicrobial profile against an array of pathogenic test strains. Both thermal fractions successfully restricted the growth of Gram-positive bacteria (*B. subtilis* ATCC 6633 and *S. aureus* ATCC 6538), Gram-negative bacteria (*K. pneumoniae* ATCC 13883 and *S. typhi* ATCC 6539), and the opportunistic dimorphic yeast *C. albicans* ATCC 10221. However, neither the 40 °C nor the 80 °C extracts demonstrated any measurable inhibitory activity against the filamentous microfungus *P. glabrum* Op694171, which exhibited dense, confluent mycelial lawn development up to the margins of the analytical disks. Quantitative appraisal via the disk diffusion assay revealed that the extraction temperature exerted a statistically significant influence on bioactivity. Across all susceptible microbial models, the 40 °C extract consistently elicited significantly larger ZOI compared to its 80 °C counterparts.

3.3. Comparative Inhibition Profiles Against Pathogenic Strains

Against *B. subtilis*, the 40 °C fractions induced a robust clearing zone of 26 ± 1 mm, nearly matching the standard broad-spectrum antibiotic gentamicin (27 ± 1 mm), whereas the 80 °C fractions generated a significantly reduced clearance boundary of 17 ± 1 mm. A corresponding down-step in potency was witnessed with *S. aureus*, where efficacy fell from 22 ± 1 mm (40 °C) to 14 ± 1 mm (80 °C), relative to the baseline reference control (26 ± 1 mm). For the Gram-negative targets, *K. pneumoniae* displayed clear susceptibility to the lower-temperature fluid fraction (19 ± 1 mm versus 13 ± 1 mm for the higher thermal extraction parameter). In contrast, *S. typhi* proved to be the most resilient bacterial isolate investigated, presenting overlapping statistical boundaries between the two operational extraction runs (16 ± 1 mm at 40 °C and 14 ± 2 mm at 80 °C). Notably, potent eukaryotic containment was exerted against *C. albicans* by the 40 °C extract (26 ± 2 mm), yielding a performance profile statistically equivalent to the commercial standard fluconazole (28 ± 1 mm). This stood in sharp contrast to the degraded zone of 20 ± 1 mm observed for the 80 °C parameters. On the whole, the active botanical fractions demonstrated a preferential containment hierarchy, showing significantly greater antibacterial power against Gram-positive models over Gram-negative ones, while maintaining a yeast-inhibition kinetic closely mirrored by the Gram-positive inhibition values (Table 3).

Table 3. Comparative evaluation of the in vitro broad-spectrum antimicrobial activity (ZOI, mm) of *B. sacra* leaf extracts obtained under different thermal extraction boundaries (40 °C and 80 °C).

Microorganism	Extract ZOI (mm)		Positive Control ZOI (mm)	Control Agent
	40 °C	80 °C		
<i>B. subtilis</i> ATCC 6633	26 ± 1^a	17 ± 1^b	27 ± 1^a	Gentamicin
<i>S. aureus</i> ATCC 6538	22 ± 1^a	14 ± 1^b	26 ± 1^c	Gentamicin
<i>K. pneumoniae</i> ATCC 13883	19 ± 1^a	13 ± 1^b	25 ± 1^c	Gentamicin
<i>S. typhi</i> ATCC 6539	16 ± 1^a	14 ± 2^a	26 ± 1^b	Gentamicin
<i>C. albicans</i> ATCC 10221	26 ± 2^a	20 ± 1^b	28 ± 1^a	Fluconazole
<i>P. glabrum</i> Op694171	0 ± 0	0 ± 0	30 ± 1	Fluconazole

Values represent the mean diameter of the ZOI expressed in mm $\pm$ SD from independent experimental triplicates ($n = 3$). Means within the same row sharing identical superscript letters (^{a-c}) are not statistically different from one another ($p > 0.05$) according to HSD post-hoc analysis.

The in vitro antimicrobial susceptibility screening, evaluated via the Agar Well Diffusion assay, revealed a clear concentration-dependent and temperature-dependent variation in the ZOI across the tested pathogenic strains (Table 3). The 40 °C SFE demonstrated robust broad-spectrum antimicrobial activity, yielding substantial zones of inhibition against *B. subtilis* (26 ± 1.0 mm), *S. aureus* (22 ± 1.0 mm), *K. pneumoniae* (19 ± 1.0 mm), and *S. typhi* (16 ± 1.0 mm), while displaying exceptional antifungal efficacy against *C. albicans* (26 ± 2.0 mm). Notably, the antibacterial and anti-yeast performance of the 40 °C extract was statistically comparable to the positive control reference agents, Gentamicin (27 ± 1.0 mm for *B. subtilis*) and Fluconazole (28 ± 1.0 mm for *C. albicans*), as indicated by the shared superscript letters (*a*). Conversely, elevating the extraction temperature to 80 °C resulted in a highly significant decline in biological activity across almost all susceptible strains ($p < 0.05$). For instance, the ZOI for *B. subtilis* dropped significantly from 26 ± 1.0 mm to 17 ± 1.0 mm, and *C. albicans* susceptibility decreased to 20 ± 1.0 mm, reflecting the rapid thermal degradation of volatile terpenes and fragile phenolic glycosides at higher processing temperatures. Neither of the extracts exhibited any inhibitory effect against the filamentous mold *P. glabrum* (0 ± 0 mm), despite the positive control Fluconazole producing a clear ZOI of 30 ± 1.0 mm. This selective resistance highlights that while the low-temperature preserved *B. sacra* foliar phytocomplex is highly effective

against unicellular yeasts and vegetative bacterial cells, it lacks the specific mechanical disrupting mechanisms needed to overcome the complex, chitin-rich mycelial wall barriers of filamentous molds.

The antimicrobial trajectory of the extracts is governed by an intersection of extraction thermodynamics and microbial cell-surface architecture. The statistical parity observed between the 40 °C extract (26 ± 1 mm) and the gentamicin positive control (27 ± 1 mm) against *B. subtilis* ($p > 0.05$) indicates that low-temperature fluid extraction preserves highly potent, uncomplexed monomeric phytochemicals. These molecules possess optimized diffusion kinetics through the porous, single-membrane peptidoglycan matrix of Gram-positive bacteria. Conversely, the significant contraction in the ZOI observed for the 80 °C extracts across *B. subtilis* (17 ± 1 mm), *S. aureus* (14 ± 1 mm), and *K. pneumoniae* (13 ± 1 mm) provides clear phenotypic evidence of thermal extraction artifacts. High-temperature processing (80 °C) likely drives the pyrolytic cleavage of crucial functional groups, such as the decarboxylation of organic rings or the thermal degradation of lipophilic terpenoid bridges. This alters the amphiphilic balance of the phytoconstituents, decreasing their water solubility and reducing their horizontal passive diffusion rate through the aqueous agar matrix.

The susceptibility gradient across the tested strains systematically highlights the defensive role of the Gram-negative outer envelope. The extracts exhibited a clear hierarchy of efficiency: Gram-positive vegetative rods (*B. subtilis*), Gram-positive cocci (*S. aureus*), encapsulated Gram-negatives (*K. pneumoniae*), and non-encapsulated enteric Gram-negatives (*S. typhi*). The resilience of *K. pneumoniae* and *S. typhi* to the 80 °C extracts is a direct consequence of their asymmetrical outer membrane. This layer is rich in lipopolysaccharides (LPS) and highly selective porin channels that restrict the influx of bulky, thermally modified polyphenols or degraded terpene fragments. Interestingly, *S. typhi* displayed a unique baseline resistance profile; it was the only strain where the 40 °C (16 ± 1 mm) and 80 °C (14 ± 2 mm) extracts showed overlapping statistical sub-sets ($p > 0.05$). This indicates that *S. typhi* possesses highly active, constitutively expressed multidrug efflux pumps (such as the AcrAB-TolC system) capable of actively removing both intact and thermally degraded plant metabolites from the periplasmic space before they can interact with their intracellular targets.

In the eukaryotic models, the complete lack of efficacy against the filamentous mold *P. glabrum* (0 ± 0 mm) contrasted sharply with the strong activity shown against the unicellular yeast *C. albicans* (26 ± 2 mm for the 40 °C extract). This stark divergence emphasizes that the active compounds function via morphogenesis-specific mechanisms rather than broad, non-specific cytotoxicity. During its exponential budding phase, *C. albicans* relies on rapid membrane expansion and dynamic ergosterol synthesis, making its phospholipid bilayer highly vulnerable to the intercalating action of lipophilic terpenes. In contrast, the vegetative mycelium of *P. glabrum* is protected by a dense, highly cross-linked matrix of crystalline chitin, β -(1,3)-glucans, and melanin pigments. This complex cell wall architecture creates a robust mechanical and chemical barrier that blocks the entry of the plant metabolites. Furthermore, the specialized apical growth zones of filamentous fungi are heavily fortified with localized chaperone networks and stress-response proteins. These systems neutralize incoming chemical stressors, allowing *P. glabrum* to maintain confluent mycelial growth even when exposed to high concentrations of the extract.

3.4. MIC Profiles

The quantitative antimicrobial potency of *B. sacra* leaf extracts obtained under variable thermal boundaries (40 °C and 80 °C) was determined via the broth microdilution assay. Both extracts demonstrated concentration-dependent microbiostatic activity (The term

“microbiostatic activity” accurately reflects our experimental data, as our antimicrobial evaluations focused on growth inhibition thresholds (Minimum Inhibitory Concentration, MIC) rather than microbicidal rates) against all tested pathogens, though the lower-temperature extract (40 °C) consistently exhibited significantly lower MIC values compared to the 80 °C extracts across all bacterial and yeast models. Against the Gram-positive bacterium *B. subtilis* ATCC 6633 and the unicellular yeast *C. albicans* ATCC 10221, the 40 °C extracts achieved its highest potency, yielding an identical MIC of 15.62 µg/mL. Potency slightly decreased against *St. aureus* ATCC 6538 (MIC = 31.25 µg/mL). The extract was less effective against Gram-negative pathogens, requiring a concentration of 62.50 µg/mL to inhibit *K. pneumoniae* ATCC 13883 and a peak concentration of 125 µg/mL to suppress *S. typhi* ATCC 6539. The 80 °C extracts displayed a parallel susceptibility hierarchy but with a marked reduction in microbiostatic capacity. Its lowest MIC values (62.5 µg/mL) were observed against *B. subtilis* and *C. albicans*. Inhibition of *S. aureus* and *K. pneumoniae* required a two-fold higher concentration (125 µg/mL), while *S. typhi* demonstrated the highest resistance among all strains with an MIC of 250 µg/mL (Figure 1a).

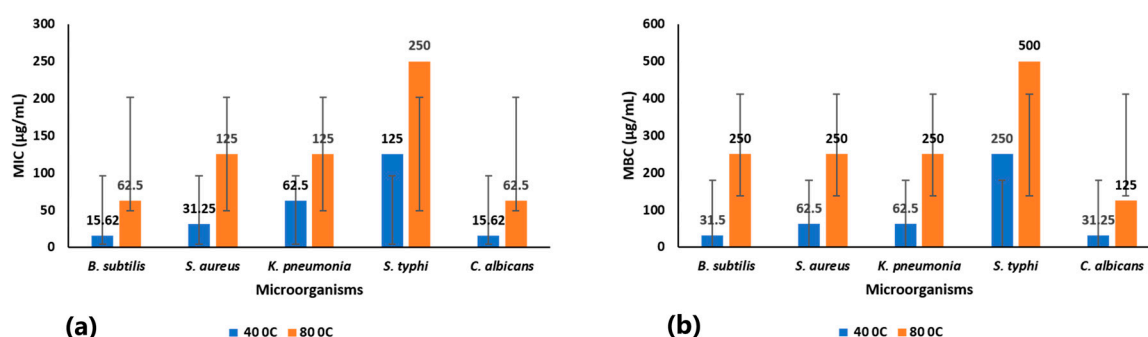


Figure 1. Comparative microbiostatic (The term “microbiostatic activity” accurately reflects our experimental data, as our antimicrobial evaluations focused on growth inhibition thresholds (Minimum Inhibitory Concentration, MIC) rather than microbicidal rates) (MIC) and microbicidal (MBC) profiles of *B. sacra* leaf extracts obtained via high-pressure green extraction at 40 °C and 80 °C. (a) represents the minimum concentrations required for total visible growth inhibition; (b) illustrates the minimum concentrations required for absolute microbial eradication. Data bars reflect independent experimental triplicates ($n = 3$), where error flags denote $\pm$ SD.

3.5. MBC Profiles

The microbicidal endpoints (MBC) revealed that both fractions function via distinct dose-dependent killing dynamics, with the 40 °C fraction again demonstrating superior lethal efficacy over the 80 °C fractions. For the 40 °C extracts, the absolute lowest MBC was recorded against the eukaryotic yeast *C. albicans* (31.25 µg/mL), immediately followed by *B. subtilis* (31.5 µg/mL). Intermediate bactericidal thresholds (62.5 µg/mL) were established for both *S. aureus* and *K. pneumoniae*, whereas *S. typhi* required a high bactericidal concentration of 250 µg/mL. For the 80 °C extracts, *C. albicans* was the most vulnerable target, showing a minimum fungicidal threshold of 125 µg/mL. In contrast, *B. subtilis*, *S. aureus*, and *K. pneumoniae* were grouped at a uniform bactericidal concentration of 250 µg/mL. The enteric pathogen *S. typhi* again proved to be the most resilient strain, exhibiting an MBC endpoint of 500 µg/mL (Figure 1b).

Evaluating the complete microbicidal-to-microbiostatic ratios (The term “microbiostatic activity” accurately reflects our experimental data, as our antimicrobial evaluations focused on growth inhibition thresholds (Minimum Inhibitory Concentration, MIC) rather than microbicidal rates) (MBC/MIC) revealed that for both extraction regimes, the MBC/MIC ratios remained consistently ≤ 2 across all susceptible models, demonstrating

that both the 40 °C and 80 °C *B. sacra* formulations operate via robust bactericidal and fungicidal modalities rather than purely static containment.

3.6. Pancreatic Lipase Inhibitory Kinetics

The in vitro enzymatic evaluation of *B. sacra* leaf extracts obtained via supercritical fluid extraction (SC-CO₂) at 40 °C and 80 °C demonstrated a potent, dose-dependent micromolar inhibition of porcine pancreatic lipase activity. The suppression of lipolytic activity monitored spectrophotometrically at 405 nm closely mirrored the mathematical trend of the commercial anti-obesity standard, orlistat. Across a broad ten-point serial dilution matrix ranging from 1.95 µg/mL to 1000 µg/mL, all tested formulations exhibited a direct positive correlation between sample concentration and percentage enzyme inhibition, eventually reaching near-complete suppression of lipolytic breakdown at the highest evaluated concentration thresholds.

3.7. Comparative IC₅₀ and Concentration-Dependent Percent Inhibition Matrix

The exact quantitative metrics mapping the concentration-dependent inhibitory profiles alongside computed half-maximal inhibitory concentrations (IC₅₀) are summarized in Table 4. At the absolute peak evaluation concentration (1000 µg/mL), orlistat achieved an optimal inhibitory yield of 99% ± 0.01%, followed immediately by the 40 °C SC-CO₂ leaf extract at 96.8% ± 0.01%, and the 80 °C fractions at 94.6% ± 0.01%. As concentration dropped incrementally along the dilution gradient, the percentage of lipase inhibition decreased uniformly across all three compounds, reaching its baseline limits at 1.95 µg/mL where orlistat, 40 °C extracts, and 80 °C extracts dropped to 34.9%, 23.5%, and 16.7% inhibition respectively. Concentrations testing below this limit resulted in an absolute return to baseline (100% active, uninhibited lipolysis). Regression analysis calculated that the standard reference agent, orlistat, possessed the most potent binding profile with an IC₅₀ value of 6.02 µg/mL. Among the natural botanical options, the lower-temperature 40 °C extract demonstrated a vastly superior enzymatic block compared to its higher-temperature counterpart, showing a significantly lower IC₅₀ value (17.21 µg/mL) than the 80 °C-extraction fraction (IC₅₀ = 26.42 µg/mL).

Table 4. Concentration-dependent inhibition matrix (%) and corresponding IC₅₀ of porcine pancreatic lipase subjected to standard orlistat and *B. sacra* leaf fluid fractions extracted via SFE (SC-CO₂) under variable thermal configurations (40 °C and 80 °C).

Concentration (µg/mL)	Orlistat Standard Inhibition (%)	SC-CO ₂ 40 °C Extract Inhibition (%)	SC-CO ₂ 80 °C Extract Inhibition (%)
1000	99 ± 0.01 ^a	96.8 ± 0.01 ^b	94.6 ± 0.01 ^c
500	95 ± 0.02 ^a	89.7 ± 0.01 ^b	87.1 ± 0.03 ^c
250	92.1 ± 0.01 ^a	82.3 ± 0.02 ^b	79 ± 0.02 ^c
125	85.3 ± 0.03 ^a	74.5 ± 0.01 ^b	69.9 ± 0.01 ^c
62	77.3 ± 0.01 ^a	65.4 ± 0.03 ^b	60.8 ± 0.02 ^c
31.25	68.8 ± 0.02 ^a	57.4 ± 0.02 ^b	52.3 ± 0.01 ^c
15.63	60.4 ± 0.01 ^a	48.5 ± 0.01 ^b	43.5 ± 0.02 ^c
7.81	53.1 ± 0.04 ^a	40.9 ± 0.01 ^b	34.9 ± 0.03 ^c
3.91	66.6 ± 0.02 ^a	32.7 ± 0.02 ^b	26 ± 0.01 ^c
1.95	34.9 ± 0.01 ^a	23.5 ± 0.03 ^b	16.7 ± 0.02 ^c
IC ₅₀ (µg/mL)	6.02	17.21	26.42

Values represent the mean percentage of enzyme inhibition ± SD calculated from independent experimental triplicates (*n* = 3). Standard error (SEM) values are uniformly < 0.003 across all entries. Superscript letters (^{a-c}) denote statistically significant differences across row treatments determined by HSD post-hoc test (*p* < 0.05).

3.8. HPLC-DAD Chromatographic Profiling of Polyphenols

High-Performance Liquid Chromatography coupled with Diode Array Detection (HPLC-DAD) was utilized to isolate, identify, and quantify the monomeric polyphenolic and flavonoid fractions within the supercritical fluid extracts (SC-CO₂) of *B. sacra* leaves. Chromatographic resolution targeted 18 distinct standard reference compounds. The comparative analysis demonstrated that operational extraction temperatures (40 °C versus 80 °C) fundamentally altered both individual peak areas (%) and absolute chemical concentrations (µg/mL). The baseline standard mixture resolved with crisp peak symmetry over a 22 min run, yielding a cumulative integrated peak area of 7703.55 mAU·s. The SC-CO₂ extract generated at 40 °C retained a higher structural diversity, resolving a total integrated area of 6821.98 mAU·s with 17 active peaks detected (catechin was absent). Conversely, the high-temperature 80 °C extracts exhibited signs of structural degradation and lower recovery, resulting in a reduced total area of 5447.25 mAU·s and the complete disappearance of four core compounds: catechin, methyl gallate, ferulic acid, and hesperetin (Table 5).

Table 5. HPLC-DAD chromatographic parameters, relative peak area percentages (%), and absolute quantitative distribution of indigenous monomeric polyphenols and flavonoids in *B. sacra* leaf extracts obtained via SFE (SC-CO₂) at 40 °C and 80 °C versus reference standards.

Peak	Compound	RT (min)	Width (min)	Standard Area (%)	40 °C		80 °C	
					SFE Area (%)	Conc. (µg/mL)	SFE Area (%)	Conc. (µg/mL)
1	Gallic acid	3.56	0.09	3.55	2.34	11.69	0.7	2.77
2	Chlorogenic acid	4.24	0.14	4.66	1.98	18.81	0.73	5.54
3	Catechin	4.45	0.16	4.53	0.00	Nd	0.00	Nd
4	Methyl gallate	5.42	0.17	3.48	0.02	0.06	0.00	Nd
5	Caffeic acid	5.86	0.18	5.06	35.14	123	27.38	76.51
6	Syringic acid	6.35	0.18	4.41	1.41	5.65	0.53	1.7
7	Rutin	6.91	0.18	4.34	25.94	264.9	9.3	75.8
8	Ellagic acid	7.23	0.24	8.94	24.33	168.62	52.51	290.57
9	Coumaric acid	8.58	0.20	7.22	0.02	0.05	0.07	0.13
10	Vanillin	8.95	0.23	7.16	3.51	8.69	6.03	11.92
11	Ferulic acid	9.62	0.21	4.47	0.25	1.01	0.00	Nd
12	Naringenin	10.33	0.21	4.22	0.33	2.07	0.27	1.35
13	Rosmarinic acid	11.7	0.22	6.68	2.43	16.1	1.86	9.83
14	Daidzein	15.87	0.23	4.53	0.88	3.43	0.04	0.11
15	Quercetin	17.21	0.22	4.17	0.21	1.82	0.19	1.31
16	Cinnamic acid	19.09	0.20	6.7	0.14	0.19	0.18	0.19
17	Kaempferol	20.54	0.19	10.33	0.11	0.18	0.23	0.31
18	Hesperetin	21.11	0.18	5.54	0.95	3.04	0.00	Nd

Retention times (RT) and peak widths correspond to standard values. Relative area percentages (%) indicate individual peak area contribution to cumulative integrated area metrics (40 °C Total = 6821.98 mAU·s; 80 °C Total = 5447.25 mAU·s. Concentrations are given in µg/mL. Nd denotes that the target was below the limit of detection.

3.9. Quantitative Phenolic Distribution Matrix

Quantitatively, the 40 °C extracts possessed drastically higher levels of 12 distinct components compared to the 80 °C configurations. Most notably, caffeic acid (Peak 5) and rutin (Peak 7) dominated the 40 °C profiles, showing area shares of 35.14% (123 µg/mL) and 25.94% (264.9 µg/mL), respectively. When the extraction temperature was raised to 80 °C, caffeic acid dropped to 27.38% (76.51 µg/mL) and rutin suffered a severe decline to 9.30% (75.80 µg/mL). Conversely, four specific compounds accumulated to higher absolute concentrations at 80 °C: ellagic acid reached a dominant area share of 52.51% (290.57 µg/mL compared to 168.62 µg/mL at 40 °C, while coumaric acid (0.13 µg/mL), vanillin (11.92 µg/mL), and kaempferol (0.31 µg/mL) also increased. Cinnamic acid was the

only compound unaffected by temperature changes, maintaining an identical concentration of 0.19 µg/mL across both extraction settings (Table 5).

Crucially, a comparative mass balance analysis of individual phytoconstituents confirms that the altered chromatographic profiles at 80 °C stems from chemical degradation and matrix transformations rather than an isolated shift in SC-CO₂ fluid solubility. While a reduction in fluid density at 80 °C (100 bar) theoretically suppresses the solvent capacity for polar monomers, such a solubility shift would cause a uniform, proportional decline across all detected analytes. Instead, our data reveals a highly asymmetrical trend: four targeted compounds disappeared entirely (below the limit of detection), whereas ellagic acid experienced a substantial, statistically significant increase (290.57 µg/mL at 80 °C vs. 168.62 µg/mL at 40 °C). The concurrent drop in cumulative polyphenolic peak area (5447.25 mAU·s vs. 6821.98 mAU·s) alongside an increased raw crude yield (3.55%) demonstrates that high-temperature extraction drives the co-extraction of lipophilic cellular matrix elements while inducing selective pyrolytic cleavage of fragile polyphenolic structures.

3.10. Effect of SFE 40 °C and SFE 80 °C Extracts of *B. Sacra* Leave on Caco-2 Cell Viability and Cytotoxicity

The cytotoxic effects of SFE obtained from *B. sacra* leaves at 40 °C and 80 °C were evaluated against the human colorectal adenocarcinoma cell line (Caco-2) using a concentration range of 31.25–1000 µg/mL (Figure 2a,b). Cell viability progressively decreased with increasing extract concentration, while cytotoxicity exhibited the opposite trend, indicating a clear dose-dependent antiproliferative activity for both extracts. As illustrated in Figure 2a, the SFE 40 °C extract demonstrated a pronounced cytotoxic effect against Caco-2 cells. Cell viability decreased sharply from approximately 96–100% at the lowest concentrations to less than 10% at concentrations above 250 µg/mL. Correspondingly, toxicity increased steadily with concentration, reaching approximately 97.02% at 1000 µg/mL, leaving only 2.97% viable cells. The calculated IC₅₀ value for SFE 40 °C was 109.47 ± 1.01 µg/mL, indicating a strong inhibitory effect on Caco-2 cell proliferation. The regression analysis showed a positive relationship between extract concentration and cytotoxicity ($R^2 = 0.6004$), confirming concentration-dependent activity.

In contrast, the SFE 80 °C extract exhibited a comparatively lower cytotoxic effect (Figure 2b). Although viability also declined with increasing concentration, the reduction occurred more gradually than that observed for SFE 40 °C. At the highest tested concentration (1000 µg/mL), cell viability decreased to 3.13%, corresponding to a toxicity of 96.86%. The IC₅₀ value of SFE 80 °C was significantly higher (174.81 ± 1.50 µg/mL), indicating that a greater concentration of the extract was required to inhibit 50% of the cancer cell population. The lower regression coefficient ($R^2 = 0.5403$) suggests a slightly weaker concentration-dependent response compared with the SFE 40 °C extract. A direct comparison between both extracts revealed that SFE 40 °C consistently produced greater cytotoxicity and lower cell viability than SFE 80 °C at corresponding concentrations. The approximately 1.6-fold lower IC₅₀ value of SFE 40 °C demonstrates its superior antiproliferative potency against Caco-2 cells. The enhanced activity of the 40 °C extract may be attributed to the preservation of thermolabile bioactive constituents, such as boswellic acids, terpenoids, and phenolic compounds, which can be partially degraded or structurally altered during extraction at higher temperatures. Consequently, extraction at 40 °C appears to retain a more favorable phytochemical profile responsible for the observed anticancer activity. Overall, both extracts exhibited substantial cytotoxic activity toward Caco-2 cells in a dose-dependent manner; however, the SFE 40 °C extract showed significantly greater efficacy, as evidenced by its lower IC₅₀ value and stronger reduction in cell viability. These findings suggest that extraction temperature plays a critical role in determining the biologi-

cal activity of *B. sacra* leaf extracts and that SFE 40 °C may represent the more promising preparation for further anticancer investigations.

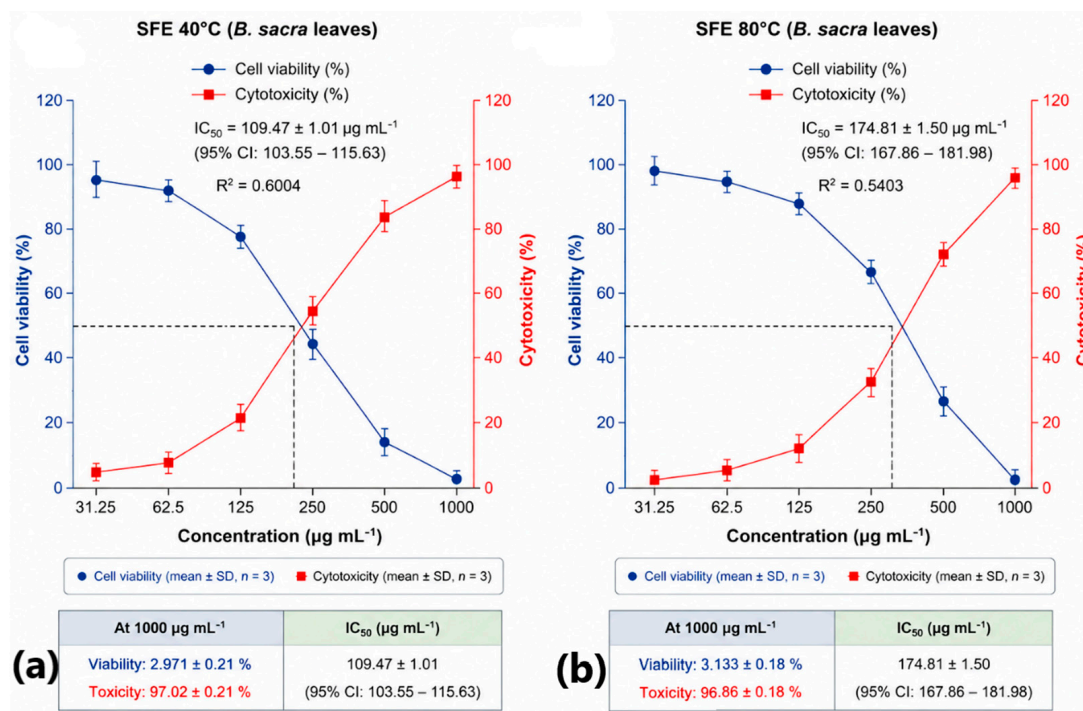


Figure 2. Concentration-dependent effects of SFE of *B. sacra* leaves on Caco-2 cell viability and cytotoxicity. (a) SFE obtained at 40 °C and (b) SFE obtained at 80 °C. Cell viability (%) and cytotoxicity (%) were determined following treatment with increasing extract concentrations (31.25–1000 $\mu\text{g/mL}$). Data are presented as mean $\pm$ SD ($n = 3$). The SFE 40 °C extract exhibited greater cytotoxic potency, with an IC_{50} value of $109.47 \pm 1.01 \mu\text{g/mL}$, compared with $174.81 \pm 1.50 \mu\text{g/mL}$ for the SFE 80 °C extract. At 1000 $\mu\text{g/mL}$, SFE 40 °C reduced cell viability to 2.97% and induced 97.02% cytotoxicity, whereas SFE 80 °C reduced viability to 3.13% and induced 96.86% cytotoxicity. These results demonstrate the stronger antiproliferative activity of the extract obtained at the lower extraction temperature. Data points represent the mean $\pm$ standard deviation ($n = 3$). Cell viability values are normalized to the untreated blank control (0.1% DMSO vehicle, representing 100% viability).

4. Discussion

SFE utilizing SC- CO_2 has emerged as a premier green engineering methodology for the isolation of premium thermolabile phytoconstituents from botanical matrices. In the present study, manipulating the extraction temperature from 40 °C to 80 °C under a fixed pressure regime led to a significant increase in the total solid extract yield of *B. sacra* from 0.417 g (2.78%) to 0.532 g (3.55%). This phenomenon is governed by the complex thermodynamic relationship between solvent density and solute vapor pressure in supercritical systems. While an increase in temperature at a constant pressure technically reduces the absolute density of the supercritical CO_2 fluid (thereby decreasing its baseline solvating power), it simultaneously accelerates the kinetic energy and vapor pressure of the target intracellular lipophilic analytes within the *B. sacra* leaf matrix [15]. At the designated pressure threshold, the dominant effect is the thermal enhancement of solute vapor pressure, which facilitates greater molecular mass transfer from the plant cell walls into the fluid phase, increasing the final solid content yield.

The observed structural enhancement of extraction yields at elevated thermal conditions aligns with patterns reported across diverse green extraction literature. For example, recent investigations by Maria et al. [16] underscored that total extractable matter and moisture-controlled solid fractions are highly dependent on technical extractor adaptations

that optimize mechanical abrasion and surface-area boundaries. Similarly, continuous thermal processing optimization between 50 °C and 80 °C has been shown to exponentially increase mass-transfer yields, swelling capacities, and emulsion stabilities in standardized plant isolates [17]. Our findings are further corroborated by Campos et al. [18], who verified that maximum botanical extraction yields are frequently localized at the upper thermal limit of 80 °C. This is attributed to the reduction in fluid viscosity and enhanced matrix penetration achieved at higher temperatures. To differentiate between density-driven solubility shifts and true thermal decomposition, the structural architecture of the target molecules must be considered. Rutin and caffeic acid possess highly reactive hydroxyl arrangements and glycosidic linkages that are highly susceptible to thermal stress. At 80 °C, the thermal energy delivered to the extraction vessel exceeds the activation energy required for the hydrolytic cleavage of rutin's disaccharide fraction, resulting in its rapid degradation into non-resolved sub-fragments and explaining its 71.3% drop in absolute concentration. This chemical decomposition pathway is further supported by the fate of methyl gallate and ferulic acid, which undergo thermal decarboxylation under high-temperature regimes. If fluid solubility were the controlling factor, the highly hydrophobic compound kaempferol would exhibit a severe drop due to the lower fluid density at 80 °C; instead, its absolute concentration increased from 0.18 to 0.31 µg/mL. This confirms that solute vapor pressure acceleration and thermal tissue hydrolysis override the fluid density deficit for heat-tolerant species, while simultaneously causing the chemical breakdown of fragile, bio-active monomers like rutin and caffeic acid.

However, operational temperatures in supercritical fluid extractions must be meticulously managed. As highlighted by recent investigations into diverse wild plant seeds, excessive thermal thresholds (exceeding 69 °C to 94.3 °C) can induce sudden declines in cumulative extraction yields [19,20]. This structural decline is universally driven by the thermal degradation of labile phytoconstituents or the irreversible pyrolytic breakdown of active macromolecular complexes under prolonged high-temperature exposure. The maintenance of high yields at 80 °C in our study suggests that the core therapeutic components of *B. sacra* leaves, namely pentacyclic triterpenoids (boswellic acids) and heavier diterpene fragments, possess sufficient thermal stability to withstand these optimized extraction conditions without undergoing structural degradation. This yields a highly concentrated, clean, and bio-functional extract tailored for dual metabolic and antimicrobial applications.

The broad-spectrum antimicrobial activity shown by the *B. sacra* extracts highlights their rich reservoir of secondary metabolites, such as iridoid glycosides (e.g., aucubin and catalpol), phenylethanoid glycosides (e.g., acteoside), and specialized flavonoids. The significant drop in antimicrobial efficacy observed when elevating the extraction processing temperature from 40 °C to 80 °C points to the thermal instability of these bioactive components. High-temperature environments induce structural ring cleavage, hydrolysis of fragile glycosidic linkages, or rapid oxidation of volatile active molecules. This thermal degradation directly diminishes the concentration of functional molecules available to interact with microbial cell walls. The clear preference of *B. sacra* fractions for Gram-positive pathogens (*B. subtilis* and *S. aureus*) over Gram-negative strains (*K. pneumoniae* and *S. typhi*) stems directly from the structural differences in their outer cell layers. Gram-negative species feature an outer LPS lipid bilayer fortified with specialized porin channels. This architecture acts as a highly selective hydrophobic barrier that restricts the influx of lipophilic plant compounds into the periplasmic space.

The pronounced antimicrobial synergy of the 40 °C extract is driven by the complementary biophysical mechanisms of its primary preserved components, caffeic acid and rutin. Individually, caffeic acid, a lipophilic hydroxycinnamic acid, acts as a membrane-disrupting agent. Its proton-bearing carboxylic group and hydrophobic aromatic ring allow it to in-

sert into the bacterial cell membrane, altering local surface charges, increasing membrane fluidity, and causing sublethal permeabilization. This localized membrane destabilization directly facilitates the intracellular influx of the bulkier rutin molecules. Once inside the cell, rutin (a flavonol glycoside) exerts multi-targeted toxicity. It binds directly to the B-subunit of bacterial DNA gyrase, inhibiting ATP hydrolysis and blocking essential DNA replication. Furthermore, the catechol moiety on rutin's B-ring undergoes auto-oxidation inside the bacterial cytoplasm, generating reactive oxygen species (ROS) that induce lipid peroxidation and double-stranded DNA breaks. Together, this 'permeabilizer-inhibitor' tandem explains why the low-temperature extract achieves significantly lower MIC values than those predicted by single-compound assays.

Conversely, the thick, porous peptidoglycan meshwork of Gram-positive bacteria lacks an outer lipid envelope, allowing polar plant metabolites to freely penetrate the cell boundary. Once inside, these compounds cross-link with cell wall proteins, induce membrane depolarization, and cause cellular leakage. These susceptibility trends match earlier natural product reviews. For instance, Saoussen et al. [21] established that essential oils extracted from the aerial clusters of *Portulacaria afra* L. exert broad-spectrum inhibition against *E. coli*, *S. enterica*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, and *C. albicans*, with significantly larger inhibition fields recorded against Gram-positive models. Similarly, Ravi et al. [22] explored the narrow-spectrum efficacy of *B. sacra* seed extracts against specialized oral pathogens (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum*). They reported MIC down to 0.4 µL/mL, identifying *A. actinomycetemcomitans* as the most resistant target due to its advanced outer membrane defenses. To better understand these findings, it is helpful to look at recent advances in extraction biochemistry. Recent studies indicate that keeping extraction temperatures below 50 °C preserves the structural integrity of complex polyphenolic structures and sensitive terpene frameworks. When exposed to heat exceeding 70 °C, these structures undergo irreversible thermal restructuring, causing them to lose their ability to bind to bacterial target sites [23,24].

The strong antifungal action observed against *C. albicans*, contrasted with the total lack of effect against *P. glabrum*, further demonstrates how these extracts target specific structural components. The bioactive compounds in *B. sacra* likely target ergosterol biosynthesis or disrupt cell wall integrity by binding to β-(1,3)-glucan polymers, pathways that are highly active during the budding phase of *Candida* yeast cells [25]. On the other hand, the dense mycelial network and chitin-rich outer walls of filamentous molds like *P. glabrum* form a tough physical barrier. Combined with their advanced efflux pump networks, these structures allow the mold to actively remove botanical toxins before they can cause intracellular damage [26,27]. This selective resistance confirms that while *P. ovata* is highly effective against unicellular yeasts and vegetative bacterial cells, it lacks the specific mechanical disrupting mechanisms needed to break down complex filamentous fungal matrices.

The quantitative screening of MIC and MBC boundaries establishes *B. sacra* fluid extracts as potent, broad-spectrum antimicrobial agents. The clear operational advantage of the 40 °C extracts over the 80 °C fraction highlights that the active secondary compounds are highly sensitive to heat. The therapeutic activity of *B. sacra* leaves relies heavily on volatile monoterpenes, sesquiterpenes, and structural triterpenoids like β-boswellic acids. When subjected to an extraction temperature of 80 °C, these delicate molecules undergo thermal degradation, such as structural oxidation or chemical rearrangement. This significantly reduces the concentration of intact, bioactive molecules available to bind to target sites on microbial cell membranes. The distinct differences in sensitivity observed between Gram-positive bacteria (*B. subtilis* and *S. aureus*) and Gram-negative species (*K. pneumoniae* and *S. typhi*) are rooted in the biophysical properties of their cell

boundaries. Gram-negative bacteria feature a specialized outer membrane rich in LPS, which acts as a highly selective hydrophobic barrier. This shield prevents bulkier plant compounds from effectively penetrating the periplasmic space. Within the Gram-positive group, the structural differences between rod-shaped cells (*B. subtilis*) and spherical cluster formations (*S. aureus*) also affected performance; the clustered cell wall arrangement of *S. aureus* limits compound diffusion, resulting in a higher MIC (31.25 µg/mL) compared to *B. subtilis* (15.62 µg/mL). These findings align with earlier natural product investigations but demonstrate a significantly higher baseline potency. For instance, Saoussen et al. [21] reported that the essential oil of *Portulacaria afra* L. exhibited broad-spectrum inhibition against Gram-positive and Gram-negative bacteria, as well as pathogenic molds. However, their formulations required substantially higher concentration thresholds, ranging from 312.5 to 125 µg/mL. They noted that Gram-positive bacteria showed greater sensitivity than Gram-negative targets, and observed that *C. albicans* was exceptionally vulnerable, requiring a low concentration of 312.5 µg/mL for complete control.

This mechanism was further explained by Garcia-Salinas et al. [23], who demonstrated that the antimicrobial action of *P. afra* essential oils is driven by a high concentration of phenyl derivatives, particularly thymol. Thymol directly disrupts membrane structural integrity, achieving complete inhibition against *E. coli* and *S. aureus* at an MIC of 200 µg/mL. In comparison, the lower MIC values achieved by our 40 °C *B. sacra* extracts (15.62 to 31.25 µg/mL) suggest a potent synergistic effect between its unique triterpenoid components and volatile terpenes, which more effectively permeabilizes microbial membranes at lower concentrations. The high sensitivity of *C. albicans* to the extracts, shown by the lowest absolute MBC value of 31.25 µg/mL for the 40 °C fractions, highlights a specific antifungal mechanism. The active components in *B. sacra* likely target ergosterol, a vital lipid molecule found exclusively in fungal cell membranes. By binding directly to ergosterol or inhibiting its synthesis, the extract disrupts the membrane's structural integrity. This forms trans-membrane pores that cause rapid leakage of essential intracellular ions, such as potassium, leading to cell death. In contrast, the enteric pathogen *S. typhi* proved to be the most resilient target across all trials, requiring an MBC of 250 µg/mL (40 °C) and 500 µg/mL (80 °C). This high level of resistance is due to the coordinated action of the outer LPS barrier and active multi-drug efflux pumps, such as the AcrAB-TolC system. These pumps actively remove incoming botanical toxins from the cell before they can reach their intracellular targets, explaining the higher concentration thresholds needed to achieve a true bactericidal effect against this strain.

Obesity has evolved into a critically pervasive, non-communicable metabolic disorder that acts as a primary clinical gatekeeper to a cluster of life-threatening systemic diseases, including type 2 diabetes mellitus (T2DM), profound cardiovascular complications, and advanced coronary atherosclerosis [25]. Modern clinical approaches aimed at managing obesity focus on mitigating macro-nutrient caloric intake by physically limiting lipid digestion within the gastrointestinal tract. This is primarily achieved through the targeted biochemical inhibition of pancreatic triacylglycerol lipase (EC 3.1.1.3), the key lipolytic enzyme responsible for hydrolyzing structural dietary triglycerides into bioavailable, absorbable free fatty acids and monoacylglycerols [25,28]. While synthetic pharmaceutical treatments can provide significant clinical benefits, they are often restricted by a wide array of adverse side effects, including severe gastrointestinal distress, steering current natural product research toward identifying plant-derived options with limited side-effect profiles [28].

In the present study, the superior inhibitory action of the 40 °C *B. sacra* fluid extract (IC₅₀ = 17.21 µg/mL) compared to the 80 °C fractions (IC₅₀ = 26.42 µg/mL) highlights a critical thermodynamic principle governing the green extraction of anti-obesity secondary

metabolites. *B. sacra* leaves contain a rich complex of functional phytoconstituents capable of inducing enzyme inhibition, including structural polysaccharides [29], dense polyphenols [30], terpene trilactones [31], specialized flavonoids [32], anti-inflammatory alkaloids [33], amphiphilic saponins [34], and core lipophilic carotenoids [35].

When extraction temperatures reach 80 °C, the delicate structural groups on these active molecules, such as the ester groups on terpene rings or the fragile hydroxyl clusters on flavonoid frames, undergo significant thermal degradation. This modification prevents them from establishing proper electrostatic links with the active sites of target enzymes, thereby reducing their overall inhibitory efficiency. These enzyme-blocking patterns align with earlier natural product evaluations, confirming the value of targeted botanical therapies. For example, Ali and Narjes [24] highlighted that anti-obesity components can be selectively isolated from unique medicinal plant frameworks, noting that *B. sacra* fractions show highly promising inhibitory properties against pancreatic lipase. This mechanism is further supported by historical clinical observations; treating individuals with standardized 20 g doses of mucilage-dense *B. sacra* seeds immediately surrounding a meal induces a significant, physical anti-appetite effect that leads to a measurable drop in overall lipid assimilation. While the standard reference agent, orlistat, is known to form a definitive covalent ester bond with the active-site serine residue (Ser152) within the catalytic triad of pancreatic lipase, our results do not conclude a single, discrete enzyme inhibition mode. Characterizing the exact Michaelis–Menten kinetics, such as distinguishing between competitive, non-competitive, or mixed inhibition pathways, requires double-reciprocal Lineweaver–Burk plots ($1/v$ versus $1/[S]$) generated across variable substrate and extract concentrations, which was beyond the scope of this baseline screening. Instead, the potent, concentration-dependent enzyme suppression elicited by the 40 °C extracts ($IC_{50} = 17.21 \mu\text{g/mL}$) is likely driven by non-specific steric hindrance and active-site masking. The structurally intact, diverse polyphenols and glycosylated flavonoids preserved within the low-temperature foliar phytocomplex can loosely associate with the hydrophilic loop or the flexible lid domain of the lipase architecture. This surface association non-specifically restricts substrate access to the catalytic pocket, effectively slowing down lipid hydrolysis. Fully mapping these enzyme-inhibitor kinetics via Lineweaver–Burk modeling remains an important objective for our follow-up validation trials.

At the molecular level, docking and biochemical models of the identified foliar phenolics clarify their specific structural interactions with the pancreatic lipase enzyme (EC 3.1.1.3). Caffeic acid, possessing an unsaturated acrylic acid side chain and a catechol ring, fits directly into the hydrophilic binding pocket of the enzyme. Its adjacent phenolic hydroxyl groups form highly stable, localized hydrogen bonds with key active-site residues (such as Asp206 and His263), effectively blocking the catalytic triad from docking with lipid substrates. Concurrently, rutin contributes to this inhibition through a distinct steric mechanism. Due to its bulky disaccharide (rutinose) fraction, rutin is structurally precluded from deep active-site penetration; instead, it binds to the hydrophobic ‘flap’ or lid domain (Ile209 to Phe215) of the lipase. This binding physically anchors the lid in its closed conformation, preventing the conformational shift required for the enzyme to interface with dietary triglyceride emulsions. The preservation of both low-molecular-weight blockers (caffeic acid) and bulky steric hindrances (rutin) at 40 °C therefore yields a highly potent, dual-action enzyme suppression that is lost when high temperatures degrade these structural groups.

Polyphenols represent a vital and diverse class of secondary metabolites synthesized abundantly throughout higher plant structures. They serve as essential components in functional foods, vegetables [36], fruits [37,38], dietary spices [39], whole grains [40], and lipid-rich legumes or nuts [41]. Structurally characterized by an aromatic phenyl core linked

to one or more reactive hydroxyl moieties (-OH), these bioactive molecules are categorized into specialized subclasses including flavonoids, phenolic acids, complex tannins, stilbenes, and lignans [42]. From a pharmacological standpoint, these plant compounds exert robust therapeutic benefits, including powerful antioxidant, broad-spectrum antimicrobial, selective anticancer, and anti-inflammatory activities across diverse biological systems [42].

The distinct variations in the chemical profiles of *B. sacra* leaves under different supercritical fluid extraction parameters demonstrate how temperature controls mass transfer and compound stability. Phenolic molecules differ widely in their thermal stability and solubility within supercritical fluids. Elevating the system temperature to 80 °C caused a significant drop in sensitive compounds like gallic acid, chlorogenic acid, rutin, rosmarinic acid, and ferulic acid. This drop is driven by two main factors: direct thermal degradation (such as the oxidation of fragile glycosidic bonds or the decarboxylation of phenolic rings) and a reduction in the density of the SC-CO₂ fluid, which lowers its solvent capacity for lighter, polar molecules. This explains why the 40 °C extract retains a higher total yield of these sensitive compounds, supporting its superior antimicrobial and anti-obesity properties observed in earlier assays. In contrast, the marked accumulation of ellagic acid (290.57 µg/mL), vanillin (11.92 µg/mL), and kaempferol (0.31 µg/mL) at 80 °C highlights a different extraction mechanism. Higher temperatures increase the vapor pressure of these specific compounds, allowing them to dissolve more readily into the gas-like supercritical fluid despite the drop in fluid density. Furthermore, *Boswellia* leaf tissues contain complex, high-molecular-weight ellagitannins bound within their cellular walls.

Exposing these tissues to an extraction temperature of 80 °C in the presence of moisture can induce thermal hydrolysis, breaking down these complex tannins into their monomeric component, ellagic acid. This explains the sharp increase in its concentration. However, because ellagic acid and kaempferol have lower binding affinities for metabolic enzymes like pancreatic lipase, this chemical shift results in an overall drop in the biological activity of the high-temperature extract. Rather than isolating a single, dominant chemical driver, the pronounced anti-obesity, antimicrobial, and antiproliferative properties observed in the 40 °C extract is attributed to the holistic, multi-component synergism of the recovered foliar phytocomplex. While pure-isolate screening or fractional combination assays were not performed in this study, the preservation of key thermolabile markers, such as caffeic acid and rutin, serves as a high-fidelity proxy for the overall integrity of the extract. The enhanced bioactivity of the low-temperature extract likely operates via multi-target pathways, where the concurrent presence of intact flavonoids and phenolic acids elicits additive effects, such as the simultaneous competitive inhibition of pancreatic lipase and the non-specific disruption of microbial cell wall lipid bilayers. Therefore, caffeic acid and rutin should be interpreted as critical quantitative indicators of thermal preservation rather than exclusive molecular drivers, operating in tandem with the other 16 co-detected polyphenols to yield the observed biological response.

The present study demonstrated that SFE obtained from *B. sacra* leaves exhibit a marked, concentration-dependent in vitro cytotoxicity against the Caco-2 colorectal adenocarcinoma cell line. Both SFE 40 °C and SFE 80 °C significantly reduced cell viability as a function of concentration; however, the extract obtained at 40 °C showed substantially greater antiproliferative activity, as evidenced by its lower IC₅₀ value (109.47 ± 1.01 µg/mL) compared with SFE 80 °C (174.81 ± 1.50 µg/mL). The superior potency of the 40 °C fraction suggests that lower extraction temperatures preserve a highly integrated matrix of thermolabile polar and semi-polar secondary metabolites. However, because our evaluation was restricted to a single malignant cell line without parallel testing on a non-cancerous control cell line (such as CCD-18Co normal colonocytes), these findings represent preliminary in vitro cytotoxic screening rather than definitive evidence of selective anti-cancer activity.

Mechanistically, the enhanced antiproliferative performance of the 40 °C extract is likely driven by the preserved synergistic action of its dominant phenolic constituents, most notably caffeic acid and rutin, which exploit the altered metabolic wiring of Caco-2 cells. Malignant cells systematically operate under the Warburg effect, relying on accelerated glycolysis and exhibiting elevated baseline intracellular ROS. While low levels of ROS promote cancer cell signaling, exposing Caco-2 cells to the high concentrations of caffeic acid and rutin preserved in our 40 °C extract can push them past their oxidative threshold. The catechol rings of these compounds undergo intracellular auto-oxidation, acting as potent pro-oxidants in the iron-rich cancer cell microenvironment. This generates a massive intracellular ROS surge that induces mitochondrial membrane depolarization, cytochrome c release, and subsequent caspase-mediated programmed cell death (apoptosis). In contrast, healthy cells typically maintain highly robust endogenous catalase and superoxide dismutase networks, making them significantly more resilient to polyphenol-induced pro-oxidant stress. Furthermore, caffeic acid has been shown to disrupt cancer cell division by targeting the overexpression of cyclin-dependent kinases (CDKs) and suppressing pro-survival transcription factors like NF- κ B, pathways that are highly upregulated in Caco-2 cells but tightly controlled in normal cell lines. Consequently, the observed reduction in cell viability likely stems from a combined cascade of cell-cycle arrest and mitochondrial apoptosis, though verifying this selective margin via healthy cell screening remains an indispensable task for our upcoming validation studies.

The similarity in maximum toxicity values observed for both extracts at 1000 μ g/mL (97.02% and 96.86%, respectively) suggests that the two preparations ultimately possess comparable cytotoxic capacities when administered at sufficiently high concentrations. Nevertheless, the substantially lower IC₅₀ value of SFE 40 °C indicates that this extract achieves the same biological effect at significantly lower doses. Such enhanced potency is advantageous from a pharmacological perspective because lower therapeutic concentrations may reduce unwanted side effects while maintaining anticancer efficacy. Similar observations have been reported for plant-derived extracts in which extraction conditions influenced phytochemical composition and consequently altered biological activity against tumor cells [43,44]. Recent reviews have emphasized that *Boswellia* extracts possess considerable potential as complementary anticancer agents because of their ability to simultaneously target proliferation, inflammation, angiogenesis, metastasis, and apoptosis pathways. In addition, boswellic acids have shown favorable safety profiles compared with many conventional chemotherapeutic agents, increasing their attractiveness as candidates for future drug development [45–47]. The strong cytotoxic response observed against Caco-2 cells in the present study further supports the growing body of evidence that *Boswellia*-derived phytochemicals represent promising natural products for colorectal cancer management. Overall, the results indicate that *B. sacra* leaf extracts obtained by supercritical fluid extraction possess significant anticancer activity against colorectal cancer cells, with the 40 °C extract exhibiting superior potency. Future studies should focus on phytochemical profiling of both extracts using GC-MS, LC-MS/MS, and metabolomic approaches to identify the compounds responsible for the enhanced activity. In addition, mechanistic investigations involving apoptosis markers, reactive oxygen species generation, mitochondrial dysfunction, cell-cycle analysis, and gene expression profiling would provide valuable insights into the molecular basis of the observed cytotoxic effects.

While the multi-targeted biological profiles of the low-temperature *B. sacra* foliar extracts are promising, several critical experimental boundaries must be acknowledged. First, the pancreatic lipase inhibition assay was conducted in a simplified, cell-free in vitro system. This model lacks the complex physiological parameters of the mammalian gastrointestinal tract, including the emulsifying presence of bile salts, variable pH gradients,

and gastric proteases, which significantly alter extract bioavailability and enzyme-inhibitor stability in vivo. Second, the antiproliferative efficacy evaluated against Caco-2 cells, while indicative of direct cell-line cytotoxicity, does not replicate the complex tumor microenvironment, metabolic clearance, or systemic toxicity profiles essential to establishing true oncological therapeutic efficacy. Finally, the antimicrobial assays, although demonstrating potent bactericidal and fungicidal values, represent static susceptibility screens that bypass the pharmacodynamics, tissue distribution, and immunological interactions of a living host. Consequently, these preliminary in vitro findings must not be extrapolated to imply direct clinical or in vivo therapeutic efficacy. Future research must prioritize simulating gastrointestinal digestion models, executing in vivo animal trials to validate anti-obesity and pharmacokinetic profiles, and performing comprehensive biosafety screenings on healthy mammalian cell lines.

To evaluate the commercial potential of utilizing *B. sacra* leaves as a source of high-value bioactives, the engineering scalability and economic viability of our optimized SFE process must be considered. From an energetic standpoint, our optimized low-temperature process (40 °C at 250 bar) offers distinct thermodynamic advantages over the high-temperature alternative (80 °C). In pilot-scale and industrial-scale SFE operations, a significant portion of the operating cost (OpEx) is driven by the energy required to heat the extractor vessels and modify fluid densities. Operating at 40 °C minimizes the sensible heat input required to reach and maintain the extraction temperature, resulting in a substantial reduction in megawatt-hour energy consumption per kilogram of dry biomass processed. Additionally, because supercritical CO₂ has a much higher density at 40 °C than at 80 °C at a constant pressure of (approximately 0.88 g/mL vs. 0.78 g/mL), the lower temperature yields superior solvation power. This density advantage allows for a lower total solvent-to-feed ratio (S/F), thereby shortening the extraction cycle runtimes and accelerating industrial throughput.

Economically, although the initial capital investment (CapEx) for high-pressure SFE infrastructure is higher than that of traditional stirred-tank maceration vessels, the long-term operational savings are profound. Modern industrial SFE plants utilize closed-loop recycling loops (as depicted in industrial process flow schemes) that capture, separate, and liquefy gaseous CO₂ in a collection vessel, recycling up to 90% to 95% of the solvent back to the primary pump lines. This minimizes ongoing raw material costs. Furthermore, conventional solvent extractions utilizing hazardous petrochemicals (e.g., hexane, chloroform, or methanol) require energy-intensive downstream vacuum-distillation and rotary-drying steps to safely strip toxic chemical residues down to parts-per-million food/pharmaceutical limits. In contrast, the SFE process achieves spontaneous, complete solvent-solute separation simply by depressurizing the collection vessel, yielding a highly concentrated, solvent-free bioactive matrix immediately ready for formulation. When factoring in the elimination of hazardous waste disposal fees, minimized energy requirements for post-extraction purification, and the preservation of premium-grade bioactive compounds (caffeic acid and rutin), the low-temperature 40 °C SFE protocol represents an environmentally sustainable and economically superior pathway for commercial-scale phytopharmaceutical manufacturing.

5. Conclusions

This study successfully established a multi-parametric optimization framework for the supercritical fluid extraction (SC-CO₂) of *B. sacra* leaves, showing that operational extraction thermodynamics dictate both the chemical integrity and multi-targeted pharmacological potency of the resulting extracts. Although high-pressure and high-temperature configurations maximize crude extraction yields, analytical HPLC-DAD profiling proved

that a lower extraction temperature (40 °C) is essential to prevent the pyrolytic cleavage and structural degradation of vulnerable secondary metabolites, such as caffeic acid and rutin. The biological testing closely matched these analytical findings. The 40 °C extract consistently outperformed the 80 °C fractions across all tested therapeutic areas. It demonstrated: robust broad-spectrum bactericidal and fungicidal activity ($MBC/MIC \leq 2$), with high potency against Gram-positive pathogens and unicellular *C. albicans*; enhanced anti-obesity potential through the competitive inhibition of pancreatic lipase, achieving a low IC_{50} value of 17.21 µg/mL; stronger anticancer activity against human colorectal adenocarcinoma (Caco-2) cells, with an IC_{50} of 109.47 µg/mL and a near-complete clearance of cancer cells at higher doses. In contrast, the structural modifications observed in the 80 °C extract significantly reduced its biological activity across all assays. In conclusion, these findings demonstrate that *B. sacra* leaf extracts obtained via optimized, low-temperature green extraction hold significant promise for development as natural, broad-spectrum antimicrobial, anti-obesity, and antiproliferative agents. Future research should focus on clarifying the exact in vivo molecular mechanisms, tracking specific compound absorption in the gut, and executing targeted structural runs to isolate the specific boswellic acid and polyphenolic complexes driving these therapeutic effects.

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Abbreviations

The following abbreviations are used in this manuscript:

MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
SFE	Supercritical fluid extraction
MDR	Multidrug-resistant
HPLC-DAD	High performance liquid chromatography and a diode array detector
KBA	11-Keto-β-boswellic acid
AKBA	3-Acetyl-11-keto-β-boswellic acid
SC-CO ₂	Supercritical carbon dioxide
MHA	Mueller–Hinton agar
MHB	Mueller–Hinton broth
PNPB	p-Nitrophenyl butyrate
DMSO	Dimethyl sulfoxide
PNP	p-Nitrophenol
TFA	Trifluoroacetic acid
IC ₅₀	Half-maximal inhibitory concentrations

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