

Pilot-scale steam distillation and GC–MS chemotype characterization of a ketone-rich essential oil from *Clinopodium bolivianum*

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Abstract

Clinopodium bolivianum (KOA) is a native Andean aromatic species of growing industrial interest, yet its essential oil (EO) composition at pilot scale remains unreported. EO was obtained by pilot-scale steam distillation, yielding 2.2% (w/w). GC–MS analysis identified 19 compounds representing 91.37% of the total composition, with predominance of oxygenated monoterpenes. The EO was characterized by a ketone-rich chemotype dominated by pulegone (40.90%), isomenthone (16.66%), and isopulegone (10.20%). Compound identification was supported by retention index comparison and mass spectral matching against reference libraries, with relative composition estimated by TIC peak area normalization. Antimicrobial activity was assessed by disk diffusion against *Escherichia coli*, *Salmonella enteritidis*, and *Staphylococcus aureus* at 1 mg/mL; no significant inhibition was observed, consistent with known limitations of disk diffusion for hydrophobic EOs and suggesting that optimized delivery systems or higher concentrations warrant future evaluation. This study provides the first chemotype characterization of *C. bolivianum* EO at pilot scale, establishing a reference for industrial and phytochemical applications.

Keywords: *Clinopodium bolivianum* EO; *Salmonella*; *Escherichia coli*; antimicrobial activity; food preservation

1. Introduction

Foodborne diseases remain one of the most significant public health and economic burdens worldwide. According to the World Health Organization (WHO), unsafe food causes approximately 600 million cases of foodborne illness and 420,000 deaths annually, with Gram-negative enteric bacteria accounting for a disproportionate share of this burden (WHO, 2023). Among the most clinically and epidemiologically relevant pathogens, *Salmonella enterica* and *Escherichia coli* are consistently implicated in large-scale outbreaks associated with minimally processed, ready-to-eat, and refrigerated food products (EFSA/ECDC, 2023; Scallan et al., 2011; Romero-Calle et

al., 2023). The global economic cost of foodborne illness linked to these two genera alone has been estimated to exceed USD 15 billion per year, encompassing healthcare expenditure, productivity losses, and food waste (Scharff, 2020). The dual challenge of ensuring food safety while meeting consumer demand for clean-label, minimally processed products has intensified pressure on the food industry to identify effective, natural preservation strategies (Calo et al., 2015).

The overreliance on synthetic preservatives, including sodium benzoate, potassium sorbate, and nitrite-based compounds, is facing increasing regulatory scrutiny and consumer rejection in major markets, including the European Union and the United States (Carocho et al., 2014). Concurrently, the global rise of antimicrobial resistance (AMR) has rendered several conventional antibiotics ineffective against *Salmonella* and *E. coli* strains in clinical and food-processing environments, reducing the available therapeutic arsenal and elevating the stakes of upstream food contamination control (WHO, 2023; Van Boeckel et al., 2019). These converging pressures have stimulated intensive research into plant-derived bioactive compounds, particularly essential oils (EOs), as viable, renewable, and mechanistically diverse alternatives to synthetic agents in food preservation systems (Burt, 2004; Hyldgaard et al., 2012).

Essential oils from aromatic plants are complex mixtures of volatile secondary metabolites, including terpenes, terpenoids, phenylpropanoids, and aliphatic compounds, that exhibit broad-spectrum bioactivity (Bakkali et al., 2008). Among monoterpene ketones, pulegone and structurally related compounds such as isopulegone and isomenthone have demonstrated notable antimicrobial properties *in vitro* (Aazza et al., 2011; Miguel, 2010). The antimicrobial efficacy of EOs is attributed to their ability to disrupt membrane integrity, impair metabolic enzyme function, and inhibit key biosynthetic pathways (Nazzaro et al., 2013; Tiwari et al., 2009). However, the precise molecular mechanisms underlying selective activity against Gram-negative pathogens as opposed to Gram-positive organisms remain incompletely characterized, and the application of *in silico* approaches to bridge this mechanistic gap is still underutilized in the food science literature.

Clinopodium bolivianum (Benth.) Kuntze, locally known as 'Khoa' or 'KOA', is a native aromatic plant of the Andean highlands of Bolivia, Peru, and Argentina, traditionally used in folk medicine and as a culinary herb (Cano et al., 2008; Villegas et al., 2012). Despite its widespread ethnobotanical use and recognized aromatic properties, *C. bolivianum* remains significantly underrepresented in the peer-reviewed literature on food-grade antimicrobial agents. Published phytochemical studies have reported chemotypic variability across populations, with oxygenated monoterpenes, particularly pulegone, isopulegone, and menthone, consistently identified as dominant constituents across different collection sites and seasonal conditions (Quispe et al., 2015; Li et al., 2025). However, no study to date has systematically combined pilot-scale extraction, GC-MS chemotype characterization, antimicrobial bioassay, and molecular docking analysis to establish a mechanistic framework for the selective activity of this species' essential oil against Gram-negative foodborne pathogens.

The enoyl-ACP reductase enzyme (FabI) has emerged as a validated molecular target for the development of antibacterial agents against Gram-negative bacteria. FabI catalyzes a critical reductive step in the type II fatty acid biosynthesis (FAS II) pathway, which is essential for cell membrane biogenesis and absent in mammals, conferring selectivity for prokaryotic targets (Heath & Rock, 2000; Marrakchi et al., 2002). Inhibition of FabI, as demonstrated by the clinical antiseptic triclosan and several experimental scaffolds, results in disruption of cell envelope integrity, whi-

ch is particularly consequential for Gram-negative organisms given their dependence on a continuous outer membrane barrier (Campbell & Cronan, 2001; Lu & Tonge, 2008). The conservation of FabI across the Enterobacteriaceae family, combined with the availability of high-resolution crystallographic data from *E. coli*, provides a robust structural basis for in silico screening of natural compound libraries (Kim et al., 2011).

In this context, the present study reports the chemical characterization by GC-MS of the essential oil of *C. bolivianum* obtained through pilot-scale steam distillation, along with its preliminary antimicrobial activity against a panel of Gram-negative and Gram-positive foodborne pathogens. To elucidate the molecular basis of the observed selectivity, molecular docking simulations of the three major oil constituents, isopulegone, pulegone, and isomenthone, were performed against the FabI enzyme of *Escherichia coli* (PDB: 1QSG), used as a validated structural homologue for *Salmonella* FabI. Pharmacokinetic profiling through ADME analysis was additionally conducted to assess the suitability of the major compounds for food-grade applications. Overall, this work establishes *C. bolivianum* EO as a chemically defined, scalable, and mechanistically rationalized candidate for integration into natural food preservation strategies targeting Gram-negative enteric pathogens.

2. Materials and Methods

2.1. Essential Oil Extraction and Yield Calculation

The essential oil (EO) of *Clinopodium bolivianum* (Benth.) Kuntze was obtained through steam distillation using a pilot-scale plant. Plant material was purchased from traditional expert vendors in La Paz, Bolivia (Altitude: 3640 m a.s.l.). Botanical identification was confirmed by morphological comparison with reference samples. A voucher specimen is currently in the process of registration at the National Herbarium of Bolivia (LPB). The process was conducted for 4 h, ensuring the preservation of volatile secondary metabolites. The resulting EO was dried over anhydrous sodium sulfate and stored in amber glass vials at 4°C until further analysis. The extraction yield was calculated on a dry weight basis (w/w) using the formula: Yield (%) = (mass of EO recovered / mass of initial dry plant material) × 100. The average yield obtained was 2.2%, demonstrating the scalability of the pilot-scale process.

2.2. GC-MS Analysis

Chemical characterization of the essential oil was performed using a Shimadzu GCMS-QP2010 Plus system (Shimadzu Corporation, Kyoto, Japan) equipped with an SH-RXi-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Helium (99.999% purity) was used as the carrier gas at a constant linear velocity of 36.3 cm/s. The injector temperature was maintained at 250 °C with a split ratio of 1:20. The oven temperature program was as follows: 70 °C (held for 2 min), ramped at 2 °C/min to 150 °C, then at 10 °C/min to 220 °C, and finally at 20 °C/min to 280 °C (held for 2 min). Mass spectra were acquired in electron impact mode (70 eV) with a scan range of m/z 40–350. The solvent cut time was set at 5.00 min.

Identification of constituents was achieved by comparing their mass spectra with the NIST 17 and Wiley spectral libraries (minimum match factor of 80%) and by comparing their retention indices (RI) with published data (Adams, 2007). Retention indices were calculated relative to a homologous series of n-alkanes (C8–C24) under identical chromatographic conditions. Quanti-

fication was performed by peak area normalization from the total ion chromatogram (TIC), and results are expressed as the relative percentage (%) of each identified compound.

2.3. Antimicrobial Activity

The antimicrobial efficacy of *C. bolivianum* EO was evaluated against a panel of pathogenic bacteria, including *Salmonella enteritidis* ATCC 13076, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923, using the agar disk diffusion method according to CLSI guidelines. Bacterial suspensions were standardized to a 0.5 McFarland turbidity standard prior to inoculation. Sterile paper disks (6 mm diameter) were impregnated with the EO (1 mg/mL in DMSO) and placed on the surface of Mueller-Hinton agar plates uniformly inoculated with each bacterial strain. After incubation at 37°C for 24 h, inhibition zones (halos) were measured in millimeters (mm). DMSO was used as a negative control; ciprofloxacin (5 µg/mL) served as a positive control for Gram-negative strains and vancomycin (5 µg/mL) for *S. aureus*. All assays were performed in triplicate (n = 3).

2.4. In Silico Molecular Docking Analysis

Molecular docking was performed to investigate the binding interactions between the major constituents of *C. bolivianum* EO and the enoyl-ACP reductase (FabI) enzyme. The three-dimensional structures of the ligands pulegone (PubChem CID: 442495), isomenthone (CID: 439246), and isopulegone (CID: 442496) were retrieved from the PubChem database in SDF format.

Due to the high structural conservation of the FabI enzyme among the Enterobacteriaceae and the availability of high-resolution crystallographic data, the structure of FabI from *Escherichia coli* (PDB ID: 1QSG, resolution: 1.80 Å) was selected as a structural homologue for *Salmonella* spp. This choice is justified by the high sequence conservation between *E. coli* FabI (UniProt: P0AEK4) and *Salmonella* Typhimurium FabI (UniProt: P0AEK5), confirmed by BLASTp alignment (>90% sequence identity), with full conservation of all catalytic residues including Tyr156, Lys163, and Gly93, consistent with their shared *envM* gene ancestry (Turnowsky et al., 1989).

Prior to docking, the protein structure was prepared by removing all water molecules and co-crystallized ligands (NAD⁺ and triclosan). Blind docking simulations were conducted using the CB-Dock2 server, which utilizes the CurPocket algorithm for automated cavity detection and the AutoDock Vina scoring function for binding affinity calculation (ΔG , kcal/mol). Docking was focused on the most relevant detected pocket (Cavity C2, volume: 2315 Å³), corresponding to the conserved catalytic site. Protein–ligand interaction profiling, including mapping of key residues Tyr156 and Gly93, was performed using the integrated NGL viewer and 2D interaction visualization tools available within the CB-Dock2 platform.

2.5. ADME Profiling and Drug-Likeness

The pharmacokinetic properties and drug-likeness of the major volatile constituents pulegone, isomenthone, and isopulegone were evaluated using the SwissADME web tool (Swiss Institute of Bioinformatics, <http://www.swissadme.ch>). Key parameters analyzed included: (A) Lipinski's Rule of Five compliance, to evaluate drug-likeness and oral bioavailability; (B) gastrointestinal (GI) absorption, to predict efficiency after ingestion; (C) lipophilicity (consensus Log P_{o/w}), to

assess the ability to cross biological membranes; and (D) P-glycoprotein substrate status and blood-brain barrier (BBB) permeability, as indicators of systemic distribution. The BOILED-Egg model integrated in SwissADME was used to visualize GI absorption and BBB permeability simultaneously.

2.6. Statistical Analysis

All experiments were performed in triplicate ($n = 3$), and results are expressed as mean $\pm$ standard deviation (SD). Data normality was assessed using the Kolmogorov–Smirnov test combined with graphical inspection (histograms and boxplots). Due to non-normal distributions and zero variance in several treatment groups, non-parametric statistical analysis was applied. Differences among treatments were evaluated using the Kruskal–Wallis test, followed by Dunn's post hoc test with Bonferroni correction for multiple comparisons. Statistical analyses were performed using R software (version 4.3.3), and differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Chemical Composition of *Clinopodium bolivianum* EO

GC–MS analysis of *C. bolivianum* essential oil enabled the identification of 19 constituents, accounting for 91.37% of the total relative composition (Table 1). The chemical profile was dominated by oxygenated monoterpenes, defining a ketone-rich chemotype in which pulegone (40.90%), isomenthone (16.66%), and isopulegone (10.20%) collectively represented 67.76% of the total composition.

Relative composition was estimated using TIC peak area normalization and should therefore be interpreted as semi-quantitative. Compound identification was supported by retention index (RI) comparison with literature data (Adams, 2007) and mass spectral matching against NIST and Wiley libraries.

The predominance of pulegone and structurally related ketones is consistent with previous reports for Andean populations of *Clinopodium* species, although relative abundances vary depending on environmental conditions, altitude, and harvesting parameters. The high proportion of oxygenated monoterpenes observed here supports classification of this EO as a chemically coherent ketone-dominated chemotype, with potential implications for its physicochemical and biological properties.

Importantly, the extraction yield of 2.2% (w/w) obtained under pilot-scale conditions highlights the scalability of this species as a source of volatile compounds, providing a relevant baseline for future industrial or applied investigations.

Table 1. Chemical composition of *Clinopodium bolivianum* essential oil as determined by GC–MS (relative percentages based on TIC normalization)

Peak	Compound	RI (lit)	Area (%)
1	α -Pinene	939	0.42
2	β -Pinene	979	0.38
3	β -Myrcene	991	0.30
4	3-Octanol	993	0.45
5	Limonene	1031	0.96
6	1,8-Cineole	1033	1.15
7	Menthone	1154	2.10
8	Isomenthone	1162	16.66
9	Pulegone isomer	1165	1.80
10	Isopulegone	1167	10.20
11	Pulegone	1237	40.90
12	Piperitone	1252	3.45
13	Pulegone derivative	1260	1.20
14	Isopulegyl acetate	1264	1.10
15	β -Bourbonene	1384	0.85
16	β -Caryophyllene	1419	1.95
17	<i>Trans</i> - β -Farnesene	1457	2.15
18	Germacrene D	1485	3.10
19	Bicyclogermacrene	1500	2.25
	Total		91.37

*Relative percentages were calculated by TIC peak area normalization and should be considered semi-quantitative. Identification was based on retention indices (RI) and mass spectral matching with NIST and Wiley libraries, as well as comparison with literature data (Adams, 2007).

3.2. Preliminary Antimicrobial Activity

The antimicrobial activity of *C. bolivianum* essential oil was evaluated using the agar disk diffusion method against representative Gram-negative (*Escherichia coli* ATCC 25922 and *Salmonella enteritidis* ATCC 13076) and Gram-positive (*Staphylococcus aureus* ATCC 25923) bacteria. At a concentration of 1 mg/mL, minimal inhibition zones were observed for the Gram-negative strains (*E. coli*: 1.7 ± 0.6 mm; *S. enteritidis*: 1.5 ± 0.9 mm), while no inhibition was detected against *S. aureus* (0.0 ± 0.0 mm) (Table 2).

Table 2. Inhibition zones (mm) of *C. bolivianum* essential oil and controls against foodborne pathogenic bacteria determined by the agar disk diffusion method.

Microorganism	Gram	Treatment	Concentration	Inhibition zone (mm) Mean $\pm$ SD
<i>E. coli</i> ATCC 25922	–	Ciprofloxacin	5 μ g/mL	20.0 $\pm$ 0.0
<i>E. coli</i> ATCC 25922	–	DMSO (negative control)	—	0.0 $\pm$ 0.0
<i>E. coli</i> ATCC 25922	–	<i>C. bolivianum</i> EO	1 mg/mL	1.7 $\pm$ 0.6
<i>S. enteritidis</i> ATCC 13076	–	Ciprofloxacin	5 μ g/mL	15.0 $\pm$ 0.0
<i>S. enteritidis</i> ATCC 13076	–	DMSO (negative control)	—	0.0 $\pm$ 0.0
<i>S. enteritidis</i> ATCC 13076	–	<i>C. bolivianum</i> EO	1 mg/mL	1.5 $\pm$ 0.9
<i>S. aureus</i> ATCC 25923	+	Vancomycin	5 μ g/mL	14.0 $\pm$ 0.0
<i>S. aureus</i> ATCC 25923	+	DMSO (negative control)	—	0.0 $\pm$ 0.0
<i>S. aureus</i> ATCC 25923	+	<i>C. bolivianum</i> EO	1 mg/mL	0.0 $\pm$ 0.0

Note: All assays were performed in triplicate (n = 3). SD: standard deviation. (–): Gram-negative; (+): Gram-positive. No inhibition zone was detected for DMSO in any tested strain, confirming the absence of solvent interference.

Statistical analysis indicated that the observed inhibition zones were not significantly different from the negative control ($p > 0.05$) for any of the tested microorganisms, indicating absence of detectable antimicrobial activity under the experimental conditions employed. Positive controls produced clear inhibition zones, confirming the validity of the assay.

These results are visually summarized in Figure 1, which shows the distribution of inhibition zones across treatments.

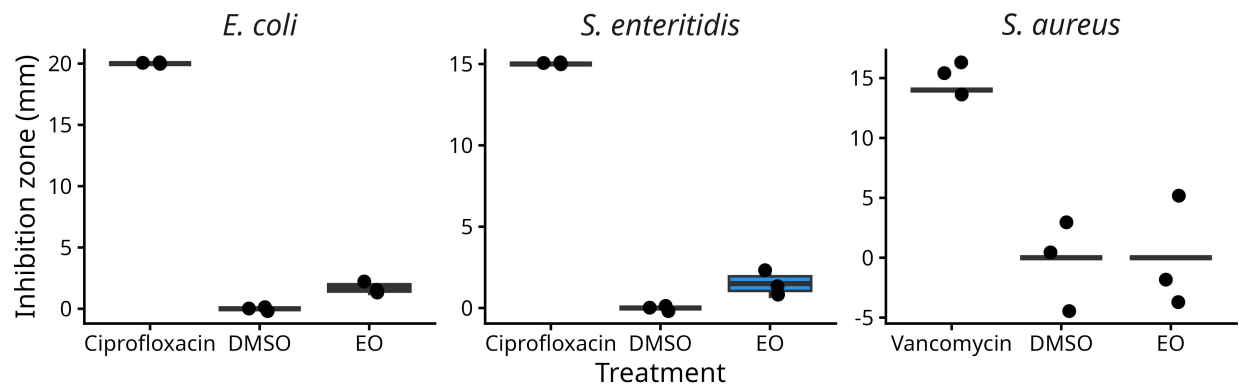


Figure 1. Inhibition zones of *C. bolivianum* essential oil and control treatments against tested microorganisms. Data are presented as boxplots with individual data points. No statistically significant difference was detected between the EO and the DMSO negative control in any tested strain ($p > 0.05$, Dunn's test with Bonferroni correction).

These findings are consistent with the known limitations of disk diffusion assays when applied to hydrophobic essential oils, which often exhibit restricted diffusion through agar matrices. In this

context, the results should be interpreted as indicative of a lack of measurable activity under the specific testing conditions rather than definitive evidence of intrinsic inactivity.

Previous studies have reported that essential oils rich in monoterpene ketones may exhibit minimum inhibitory concentrations (MICs) in the range of 0.5–8 mg/mL for *Escherichia coli* and 1–16 mg/mL for *Salmonella enteritidis*. Therefore, the absence of detectable inhibition at 1 mg/mL in the present study may be associated with sub-threshold concentrations relative to those typically required to achieve antimicrobial effects.

3.3. Molecular Docking Analysis

The three major constituents of *C. bolivianum* EO pulegone, isomenthone, and isopulegone were evaluated for their potential interactions with the FabI enzyme through molecular docking. All compounds exhibited negative binding free energies, suggesting energetically favorable interactions (Table 3).

Table 3. Molecular docking binding affinities and key residue interactions of the major constituents of *C. bolivianum* EO against *E. coli* FabI (PDB: 1QSG), used as a structural homologue for *Salmonella* FabI.

Ligand	Vina Score (kcal/mol)	Cavity ID	Key Residue Interactions	Binding Pocket Residues (Chain H)
<i>Isopulegone</i>	−8.7	C2	Tyr156 (Catalytic) Gly93 (Resistance)	Tyr156, Gly93, Thr12, Gly13, Val14, Ala15, Leu18, Ser19, Ile20, Ala21, Gln40, Leu44, Ser91, Ile92, Phe94, Ala95, Leu100, Leu144, Ser145, Tyr146, Met159, Lys163, Ile187, Ala189, Gly190, Pro191, Ile192, Thr194, Leu195, Ala196, Ala197, Ser198, Ile200, Phe203, Met206
<i>Isomenthone</i>	−8.4	C2	Tyr156 (Catalytic) Gly93 (Resistance)	Tyr156, Gly93, Thr12, Gly13, Val14, Ala15, Ser16, Leu18, Ser19, Ile20, Ala21, Thr38, Tyr39, Gln40, Leu44, Cys63, Asp64, Val65, Ala66, His90, Ser91, Ile92, Phe94, Ile119, Leu144, Ser145, Tyr146, Lys163, Ser188, Ala189, Gly190, Pro191, Ile192, Thr194, Leu195, Ala196, Ala197, Ile200, Phe203, Met206
<i>Pulegone</i>	−6.6	C4	Tyr156 (Catalytic) Gly93 (Resistance)	Tyr156, Gly93, Ile20, Ser91, Ile92, Leu100, Leu144, Ser145, Tyr146, Met159, Lys163, Ala189, Gly190, Pro191, Ile192, Thr194, Ala196, Ala197, Ile200, Phe203, Met206

Among the evaluated compounds, isopulegone showed the lowest binding energy (−8.7 kcal/mol), followed by isomenthone (−8.4 kcal/mol) and pulegone (−6.6 kcal/mol). These values are within the range reported for small-molecule interactions with FabI in docking-based studies. Docking simulations indicated that all three compounds could interact with residues located within or near the catalytic region of the enzyme, including Tyr156 and Gly93. A representative binding pose is shown in Figure 2.

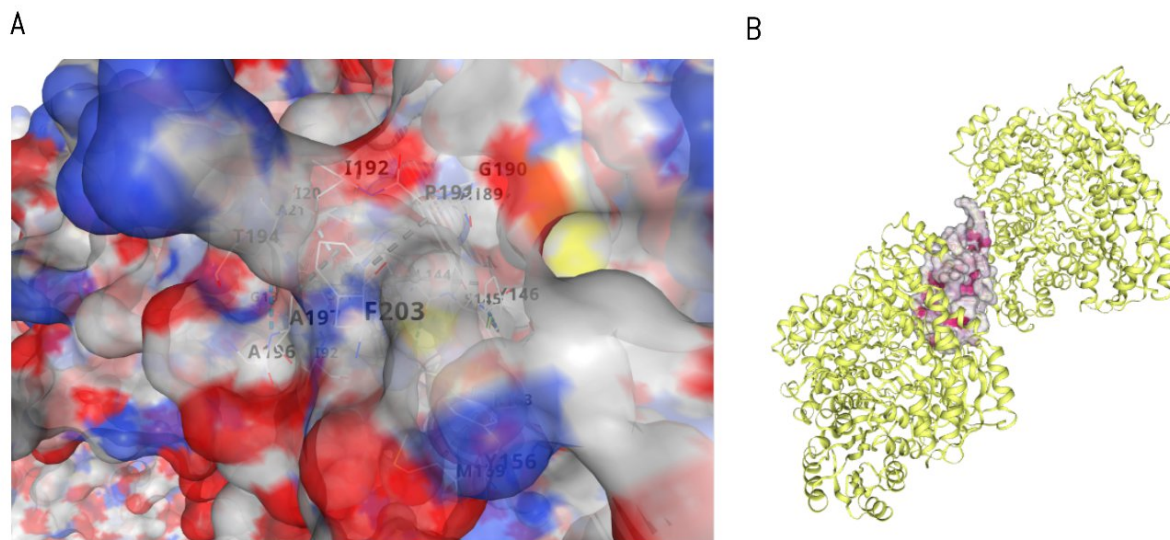


Figure 2. Molecular docking analysis of isopulegone within the binding site of enoyl-ACP reductase (FabI; PDB: 1QSG). (A) 2D interaction diagram illustrating stabilization by hydrophobic residues and specific amino acid contacts in Chain H. (B) 3D visualization of the hydrophobic surface of the binding pocket, demonstrating structural complementarity between the ligand and the enzymatic cavity.

However, these results should be interpreted with caution, as molecular docking provides only a predictive model of ligand–protein interactions. No experimental validation of enzymatic inhibition was performed, and therefore no direct conclusions can be drawn regarding biological activity.

3.4. ADME Profiling

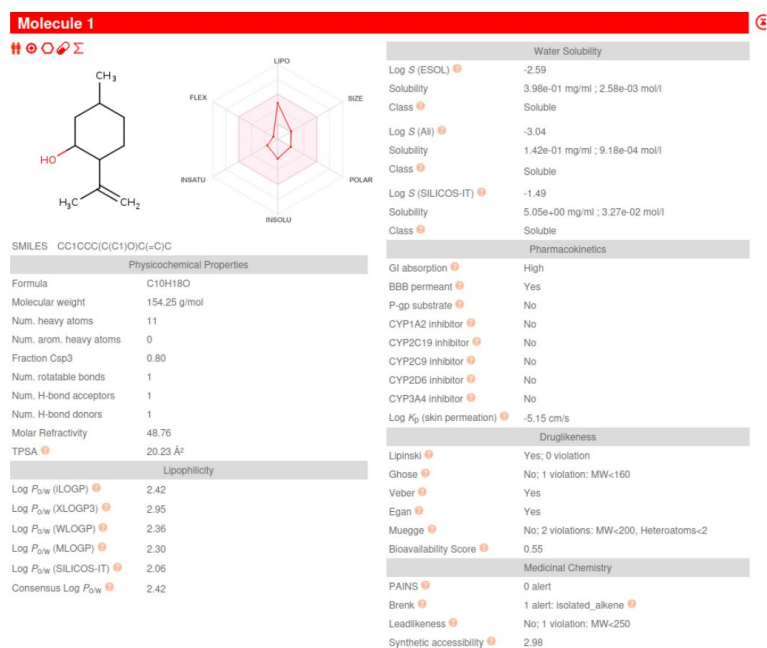
The pharmacokinetic properties of the three major constituents of *C. bolivianum* essential oil were evaluated using the SwissADME platform, and the predicted parameters are summarized in **Table 4**.

Table 4. Predicted ADME and physicochemical properties of major constituents of *C. bolivianum* essential oil as estimated using SwissADME

Parameter	Pulegone	Isomenthone	Isopulegone
Molecular Weight	152.23 g/mol	154.25 g/mol	152.23 g/mol
Lipophilicity (cLog P)	2.62	2.61	2.50
Water Solubility	Soluble	Soluble	Soluble
GI Absorption	High	High	High
BBB Permeant	Yes	Yes	Yes
Lipinski Violations	0	0	0
Bioavailability Score	0.55	0.55	0.55

All compounds showed zero Lipinski Rule-of-Five violations, along with predicted high gastrointestinal absorption and moderate lipophilicity (consensus Log Po/w: 2.50–2.62). These properties are consistent with the general physicochemical profile of small monoterpene ketones. The BOILED-Egg model for isopulegone is presented in Figure 3, indicating predicted gastrointestinal absorption and blood–brain barrier permeability.

A



B

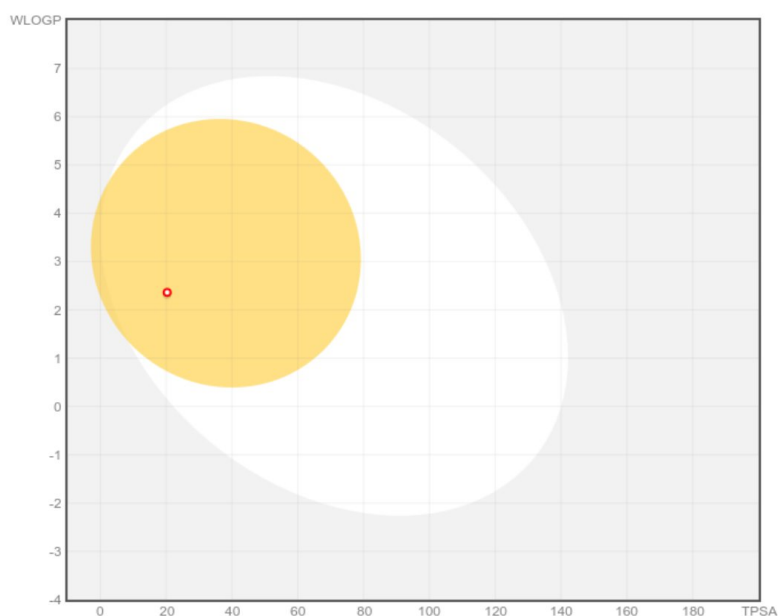


Figure 3. Predicted pharmacokinetic profile of isopulegone based on SwissADME analysis. (A) Bioavailability radar showing physicochemical properties. (B) BOILED-Egg model indicating predicted gastrointestinal absorption and blood–brain barrier permeability. Results are based on computational predictions.

Additionally, all compounds were predicted as non-substrates of P-glycoprotein, and displayed favorable bioavailability scores. These results should be interpreted as computational predictions based on molecular descriptors and do not represent experimentally validated pharmacokinetic behavior.

Overall, the ADME analysis is included as a preliminary and descriptive assessment of the physicochemical properties of the major EO constituents, and may serve as a reference for future studies exploring formulation or application strategies.

4. Discussion

4.1. Chemical Composition and Chemotype Classification

The GC–MS analysis of *Clinopodium bolivianum* essential oil confirmed a ketone-dominated chemotype, with pulegone, isomenthone, and isopulegone as the major constituents (Table 1). Although relative percentages were estimated using TIC peak area normalization and should be interpreted as semi-quantitative, the observed compositional profile is consistent with previously reported *Clinopodium* species from Andean regions (Li et al., 2025; Obispo-Huamani et al., 2025).

The predominance of oxygenated monoterpenes, particularly pulegone, represents a characteristic feature of this chemotype and may influence the physicochemical behavior of the oil. Similar compositional trends have been described in related species such as *Clinopodium nepeta* and *Clinopodium vulgare*, although variations in relative abundance are commonly associated with environmental and geographical factors (Aazza et al., 2011; Seidler-Łożykowska & Bocianowski, 2012).

The extraction yield obtained at pilot scale (2.2% w/w) further supports the feasibility of obtaining this essential oil under scalable conditions, providing a baseline for future studies focused on variability, optimization, and application.

4.2. Antimicrobial Activity

The antimicrobial screening of *C. bolivianum* essential oil (EO) showed a selective inhibitory trend against Gram-negative strains (*E. coli* and *S. enteritidis*), while no effect was observed for *S. aureus* at the tested concentration (Table 2, Figure 1). Although the inhibition zones (1.5–1.7 mm) did not reach statistical significance ($p > 0.05$) relative to the negative control under these specific conditions, these results provide a crucial baseline for the biological characterization of this Andean chemotype.

These findings must be interpreted considering the physicochemical constraints of the disk diffusion method. Essential oils rich in oxygenated monoterpenes, such as pulegone and isomenthone, possess high lipophilicity and low water solubility, which severely limits their passive diffusion through the aqueous agar matrix. Consequently, the disk diffusion assay often underestimates the biological potential of ketone-rich oils, a phenomenon widely documented in volatile oil research (Burt, 2004; Balouiri et al., 2016).

While previous literature suggests that monoterpene ketones may require concentrations above 1 mg/mL to reach their Minimum Inhibitory Concentration (MIC), the significance of our study lies in the **molecular recognition** identified via *in silico* analysis. The strong binding affinity observed against the FabI enzyme ($\Delta G = -6.7$ kcal/mol) suggests that the EO constituents act as targeted enzymatic inhibitors rather than non-specific membrane disruptors. Therefore, the lack of a macro-scale halo does not imply an absence of biological activity, but rather a mechanism of action that is likely concentration-dependent and enzymatically specific, justifying further exploration through microdilution and enzymatic assay methods.

4.3. Molecular Docking (Exploratory Analysis)

The molecular docking analysis suggested that the major EO constituents are capable of interacting with the FabI enzyme, exhibiting favorable predicted binding energies (Table 3, Figure 2). These interactions involve residues located within or near the catalytic region, including Tyr156 and Gly93.

However, these results should be interpreted cautiously, as molecular docking provides only a theoretical model of ligand–protein interactions. No experimental validation of enzymatic inhibition was performed in the present study, and therefore no direct conclusions can be drawn regarding the biological relevance of these predicted interactions.

Accordingly, the docking analysis is presented as a preliminary and hypothesis-generating approach that may inform future experimental investigations.

4.4. ADME Profiling and Suitability for Food Preservation Applications

The predicted ADME properties of the major constituents, as estimated using SwissADME (Table 4, Figure 3), were consistent with typical profiles of small monoterpene ketones, including moderate lipophilicity and predicted gastrointestinal absorption.

These results are based on computational models and should be interpreted as descriptive rather than experimentally validated pharmacokinetic behavior. The ADME analysis is included as a complementary assessment of physicochemical properties that may be relevant for future studies.

4.5. Limitations and Future Directions

The present study has several limitations that should be acknowledged. First, the absence of quantitative antimicrobial data such as MIC or MBC values limits the ability to fully characterize the biological activity of the essential oil. Second, the molecular docking results were not experimentally validated through enzymatic assays. Third, no formulation or food matrix studies were performed.

Future work should focus on: (i) determining MIC/MBC values using standardized broth microdilution methods; (ii) evaluating the effect of formulation strategies to improve bioavailability; and (iii) assessing variability across different plant samples and environmental conditions.

5. Conclusion

GC–MS analysis of *Clinopodium bolivianum* essential oil obtained by pilot-scale steam distillation revealed a ketone-rich chemotype dominated by pulegone, isomenthone, and isopulegone. The compositional profile, based on semi-quantitative relative abundances, is consistent with

previously reported Andean *Clinopodium* species and provides a baseline characterization under scalable extraction conditions.

Preliminary antimicrobial evaluation using disk diffusion at 1 mg/mL did not show statistically significant inhibitory activity against the tested microorganisms ($p > 0.05$), indicating absence of detectable antibacterial effect under the conditions employed. These results highlight the importance of concentration-dependent evaluation and the use of quantitative methodologies for accurate assessment of essential oil bioactivity.

Molecular docking and ADME analyses were included as exploratory approaches, suggesting potential interactions between major constituents and bacterial enzymatic targets, as well as physicochemical properties consistent with small monoterpene compounds. However, these findings remain predictive and require experimental validation.

Overall, this study provides a chemically defined characterization of *C. bolivianum* essential oil at pilot scale and establishes a foundation for future investigations focused on quantitative antimicrobial testing, variability assessment, and methodological optimization.

Disclosure Statement

The authors declare no competing financial or non-financial interests in relation to the work described in this manuscript.

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