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Bioactive compounds and *in vitro* antibacterial activity of *Clinopodium bolivianum* (Benth) Kuntze

[Compuestos bioactivos y actividad antibacteriana *in vitro* de *Clinopodium bolivianum* (Benth) Kuntze]

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Abstract: *Clinopodium bolivianum* (Benth) Kuntze, known as Inca muña, is an aromatic plant traditionally used in the high Andean regions of Peru. This study evaluated the bioactive compounds and antibacterial activity of its essential oil and aqueous extract from fresh and dry leaves against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Bioactive compounds were identified using GC-MS and UPLC. Antibacterial activity was assessed by inhibition zone diameter (IZD), minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). The dry essential oil exhibited stronger antibacterial activity against *S. aureus* (IZD 23.30 ± 1.15 mm, MBC 5.49 g/L, MIC 2.74 g/L) than the fresh oil and aqueous extracts. These results suggest that the essential oil of dry *Clinopodium bolivianum* has potential therapeutic, antibacterial, and preservative applications, particularly against *S. aureus*.

Keywords: Inca muña; *Clinopodium bolivianum*; Essential oil; Aqueous extract; Antibacterial activity.

Resumen: *Clinopodium bolivianum* (Benth) Kuntze, conocido como Inca muña, es una planta aromática utilizada tradicionalmente en las regiones altoandinas de Perú. Este estudio evaluó los compuestos bioactivos y la actividad antibacteriana de su aceite esencial y extracto acuoso de hojas frescas y secas contra *Escherichia coli* ATCC 25922 y *Staphylococcus aureus* ATCC 25923. Se identificaron compuestos bioactivos mediante GC-MS y UPLC. La actividad antibacteriana se evaluó mediante el diámetro de la zona de inhibición (IZD), la concentración mínima inhibitoria (MIC) y la concentración mínima bactericida (MBC). El aceite esencial seco mostró una mayor actividad antibacteriana contra *S. aureus* (IZD 23.30 ± 1.15 mm, MBC 5.49 g/L, MIC 2.74 g/L) en comparación con el aceite fresco y los extractos acuosos. Estos resultados sugieren que el aceite esencial de *Clinopodium bolivianum* seco tiene aplicaciones terapéuticas, antibacterianas y conservantes potenciales, especialmente contra *S. aureus*.

Palabras clave: Inca muña; *Clinopodium bolivianum*; Aceite esencial; Extracto acuoso; Actividad antibacteriana.

INTRODUCTION

The inappropriate and disproportionate use of antibiotics contributes enormously to developing resistance in bacterial species and using natural products, or their combinations is necessary to inactivate pathogens in foods (Ghosh *et al*., 2019). The problem of antimicrobial resistance (AMR) requires urgent coordinated global action, AMR is estimated to have caused 4.95 million deaths worldwide in 2019, of which 1.27 million are related to Antibacterial Resistance (Ranjbar & Alam, 2024) *Staphylococcus aureus* is one of the most problematic bacteria in contemporary invasive medication that generates resistance (Shady *et al*., 2024).

Plant extracts have been used, for centuries, as medicines against microbial infections because they have low toxicity and are safe for therapeutic use (Pandey *et al*., 2024). Some plant extracts showed good anti-Quorum Sensing, antimotility, and antiaggregating activity, against diarrheal *E. coli* infections (Lebeloane *et al*., 2024). The main chemical compounds of the aqueous extracts are phenolic acids, mostly derived from hydroxycinnamic acid (Gopčević *et al*., 2022). The studies focus on herbal products as sources of antimicrobial compounds (Azuero *et al*., 2016) against gram-positive and gram-negative bacteria (Semeniuc *et al*., 2017). Combining herbal extracts (Saquib *et al*., 2019) and even herbal extracts with antibiotics could offer significant potential for developing new antimicrobial therapeutic agents and treating infections caused by resistant bacteria (Alam *et al*., 2022). Essential oils are volatile compounds from plants, they present biologically active constituents (Božović *et al*., 2017), mainly phenolics, terpenes, aliphatic alcohols, aldehydes, ketones, organic acids, saponins, thiosulfates and glucosinolates, which give them antimicrobial properties (Pisoschi *et al*., 2018) and can be used as antimicrobial agents with low risk of developing microbial resistance (Coello-Cedeño, 2021), some essential oils can have an effect equal to or greater than commercial antibiotics (Ez Zoubi *et al*., 2016). Combining cinnamon with oregano essential oil resulted in a synergetic effect on inhibiting intestinal pathogens but had no impact on the growth of probiotics (Wu *et al*., 2017).

The *Clinopodium bolivianum (Benth)* Kuntze (Inca muña) is a wild aromatic herb that belongs to the Lamiaceae family, grows in the Peruvian Andes, in a wide range of altitudes and is a rich source of phenolic compounds (Chirinos *et al*., 2011). It has been used chiefly as a digestive infusion (Chirinos *et al*., 2013). Triterpene fractions obtained from this *Lamiaceae* family showed strong antiproliferative and anti-inflammatory activity (Jordamovc *et al*., 2023). Inca muña has been shown to have a protective role on bladder epithelial cells against uropathogenic *E. coli* infection by reducing bacterial adhesion, invasion, and biofilm formation (Mohanty *et al*., 2017). However, information on antibacterial activity against gram-positive and gram-negative bacteria is lacking. The significant findings could be used in future research to develop products with inca muña essential oils or extracts for therapeutic use (Divya *et al*., 2023), and for food preservation in the food industry (Pisoschi *et al*., 2018; Shi *et al*., 2018; Jugreet *et al*., 2020).

In this research, essential oil and aqueous extract of inca muña have been used, which resembles the natural consumption of herbs with a long history in traditional medicine and cultural practices (Sousa *et al*., 2024). Like alternatives to control bacterial infections and prevent AMR (Mohanty *et al*., 2017). Therefore, the objectives of this work were to determine the bioactive compounds of the essential oil and aqueous extract of the fresh and dried leaves with secondary stems of *Clinopodium bolivianum* (Benth) Kuntze (*C. bolivianum*) and determine the antibacterial activity of the essential oil and the aqueous extract against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923.

MATERIAL AND METHODS

Plant and extraction procedures

Clinopodium bolivianum (Benth) Kuntze, known as Inca muña, was identified by the laboratory of the Natural Museum of History at San Marcos National University. The plant material was collected from the "3 de Octubre" locality (3423 meters above sea level, 12° 50′ 34″ S, 74° 34′ 10″ W) in the Acobamba district, Huancavelica province, Peru, between January and March. Collection occurred at dawn (6:00 a.m.) when plant transpiration was minimal, during the vegetative and flowering stages to maximize its properties (Weksler *et al*., 2020).

The essential oil was extracted from fresh and dry Inca muña leaves with secondary stems using conventional hydrodistillation (HD) (Ambrosio *et al*., 2017) and stored in dark glass bottles at 4°C until analysis (Torrenegra *et al*., 2015). For the aqueous extract of fresh Inca muña, 1 g of crushed leaves and stems was mixed with 100 mL of distilled water at 20°C, 40°C, 60°C, and 89°C (boiling) with constant

stirring. After centrifugation at 4500 rpm for 30 minutes, the supernatant was filtered, concentrated using a rotary evaporator at 40°C for 35 minutes, and stored in amber flasks (Islam *et al*., 2012, with modifications). The aqueous extract of dry Inca muña was prepared similarly, using 1 g of powdered leaves and stems with a 3.88 fineness modulus, followed by infusion through filter papers at the same temperatures, centrifugation, filtration, and concentration (Duha & Yed, 1997).

Identification of Bioactive compounds

The bioactive compounds in the essential oil were identified using gas chromatography-mass spectrometry (GC-MS) following the methodology described by Torrenegra *et al*. (2015). The analysis was conducted with a SHIMADZU GC-2010 plus gas chromatograph, equipped with an AOC-6000 autosampler and a GCMS-QP 210 ultra mass spectrometry detector. A RESTEK RTX-5MS GC column (30 m x 0.25 mm ID x 0.25 µm) was used for separation (Serial: 1346249).

For the aqueous extract, bioactive compounds were identified following a purification process using SPE Strata C18-E columns (50 mg, 6 mL^-1). The column was initially activated with 5 mL of 80% methanol and subsequently washed with 5 mL of HPLC-grade water. One milliliter of the extract was added, followed by another wash with 2 mL of HPLC-grade water. To ensure complete recovery of polyphenols, 2 mL of 80% methanol was added. The elute (1.6 mL) was filtered through a 0.22 µm filter into a vial and subjected to HPLC analysis (Chirinos *et al*., 2008).

The HPLC analysis was conducted using Ultra Performance Liquid Chromatography (UPLC) with a SHIMADZU SPD-M3DA detector (cat. 228-45196-14, serial L2011551579 CD) and a CBN-20A NEXEIRA X2 DETECTOR, Japan. Separation was achieved using a SEA 18 Mediterranean column (5 µm, 25 x 0.46 cm). The elution was performed at a flow rate of 0.3 mL/min under gradient conditions with solvents A (6% acetic acid) and B (acetonitrile) at 30°C. The gradient elution program was as follows: 0-15% B in 40 minutes, 15-45% B in 40 minutes, and 45-100% B in 10 minutes, with an injection volume of 3 µL and detection at 280 nm.

Antibacterial activity

The antibacterial activity of the samples was assessed using *Escherichia coli* (ATCC® 25922) and *Staphylococcus aureus* (ATCC® 25923) as reference strains, obtained from Thermo Scientific (codes: R4607050 and R4607010, respectively). For bacterial growth, trypticase soy agar (Merck) was utilized to culture the bacteria.

The disc diffusion method was employed following the guidelines of the Clinical and Laboratory Standards Institute (M2-A11, 2012). A sterile cotton swab was used to apply the 0.5 McFarland standardized inoculum suspension (approximately 1 to 2 x 10^8 CFU/mL) evenly across the surface of Mueller-Hinton agar plates. The plates were then left to rest for 5 minutes before applying sterile disks impregnated with the essential oils and aqueous extracts. These disks, sourced from OXOID (Cartridge x 50 discs, United Kingdom), were immersed in the samples, kept at room temperature in a dark bottle for 1 hour, and then placed at the center of the inoculated agar plates. The plates were incubated at 37°C for 18-24 hours. The inhibition zone diameter (IZD) was measured using a vernier, and the results were interpreted according to the CLSI M100S17 guidelines (Patel, 2017).

The percentage inhibition of bacterial growth by the samples was calculated based on the method described by (Njau *et al*., 2014). Standard antibiotics were used as controls: 10 µg of ampicillin for *E. coli* and 1 µg of oxacillin for *S. aureus*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the broth microdilution method, as per CLSI (2015). The bacterial suspension was prepared by adding 0.1 mL of standardized inoculum (0.5 MacFarland) to 9.9 mL of Mueller Hinton broth. Serial dilutions of the samples (from 50% to 0.3906%) were then prepared in sterilized, capped 96-well poly cuvettes, with 50 µL of bacterial suspension and 50 µL of each dilution added per well. Positive controls consisted of Mueller-Hinton broth with the bacterial suspension, while negative controls used only Mueller-Hinton broth. Incubation occurred at 37 ± 2°C, with readings taken every hour for 24 hours at 620 nm using a BIO TEK INSTRUMENTS microplate reader (Model: BIOTEK ELx 808IU, SN: 1905292, USA).

Statistical analysis

All tests were prepared in triplicate and reported as mean ± SD. The data were subjected to a two-way analysis of variance (ANOVA) and Tukey's multiple range test with a confidence level of $\alpha = 5\%$ using R-4.1.0 software for Windows and RStudio Desktop 1.4.1717.

RESULTS

Physical characteristics of essential oil and aqueous extract

The extraction yields obtained from *C. bolivianum* revealed significant differences between the various methods and conditions employed. As shown in Table No. 1, the fresh *C. bolivianum* aqueous extract exhibited the highest yield percentage at 68.151%, indicating its superior extraction efficiency under the conditions tested. Both the fresh and dry aqueous extracts demonstrated higher yield percentages compared to the essential oils, emphasizing the effectiveness of aqueous extraction methods in capturing the bioactive components of *C. bolivianum.*

Table No. 1
Physical characteristics of essential oil and aqueous extract of fresh and dry *C. bolivianum*

Physical characteristics	Essential oil		Aqueous extract	
	Fresh	Dry	Fresh	Dry
Yield (%w/w)	0.196 ± 0.011	0.450 ± 0.010	68.151 ± 0.552	16.016 ± 0.589
Density (g/mL)	0.881 ± 0.002	0.878 ± 0.020	1.069 ± 0.008	1.043 ± 0.013
pH (20°C)	4.950 ± 0.017	4.850 ± 0.026	6.270 ± 0.057	6.230 ± 0.057

Bioactive compounds of essential oil and aqueous extract

A total of 60 volatile compounds were identified in the fresh Inca muña essential oil, with the predominant components being cyclohexanone, 5-methyl-2-(1-methyl ethyl)- (2R-cis) (isomenthone) at 28.74%, linalool at 17.50%, and pulegone at 12.05%. In comparison, the dry Inca muña essential oil contained 107 volatile compounds, with the major constituents being 5-methyl-2-(1-methyl ethyl)- (2R-cis) at 28.65%, linalool at 15.80%, and pulegone at 15.32%, as illustrated in Figure No. 1.

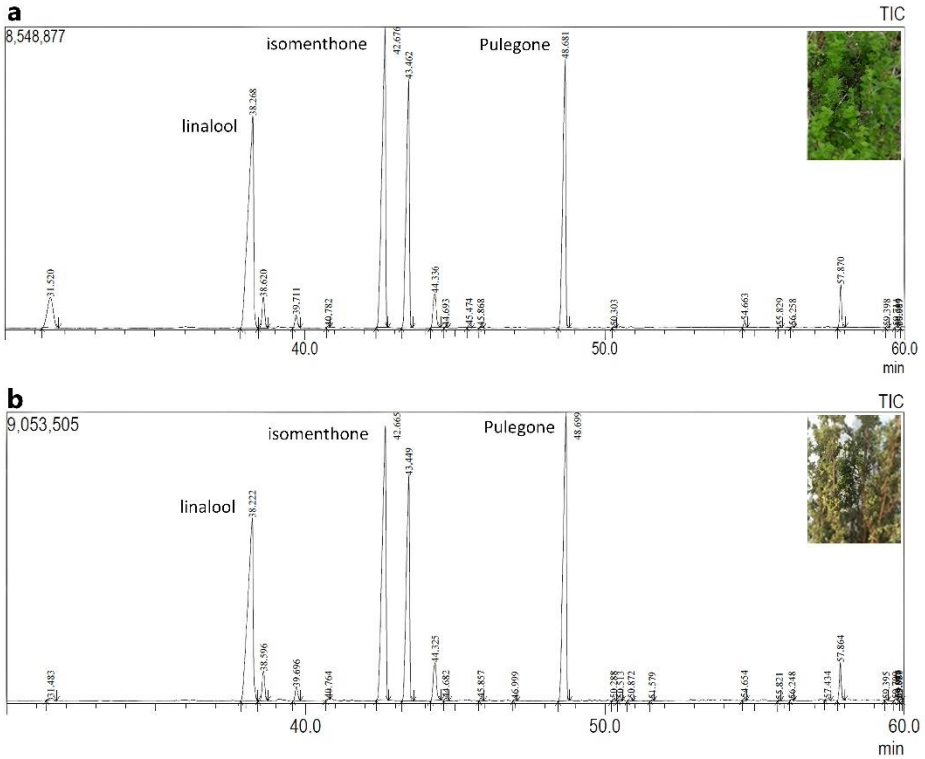

Figure No. 1
Bioactive compounds of the essential oil of fresh (a) and dry (b) *C. bolivianum*

In the *C. bolivianum* extracts, eight phenolic compounds were tentatively identified and quantified as gallic acid equivalents. The fresh Inca muña extract contained 11.45 mg/mL, while the dry extract contained 2.48 mg/mL. The most prominent peaks observed corresponded to ρ-coumaric derivatives (peak 2) and hydroxycinnamic acid derivatives (peaks 1, 3, 4, 5, 7, and 8), as shown in Figure No. 2.

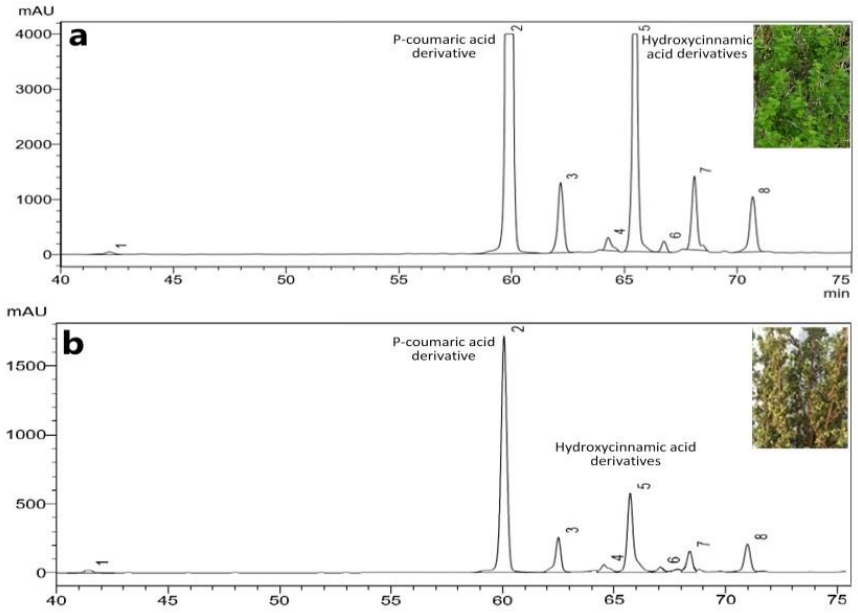

Figure No. 2
Bioactive compounds of the aqueous extract of fresh (a) and dry (b) *C. bolivianum*

Antibacterial activity of essential oil and aqueous extract

The aqueous extracts of fresh and dry *C. bolivianum* at 89°C (boiling temperature) showed a more significant IZD against *E. coli* and *S. aureus* (see Figure No. 3), Therefore, MIC, MBC and sensitivity were performed for these aqueous extracts (red line).

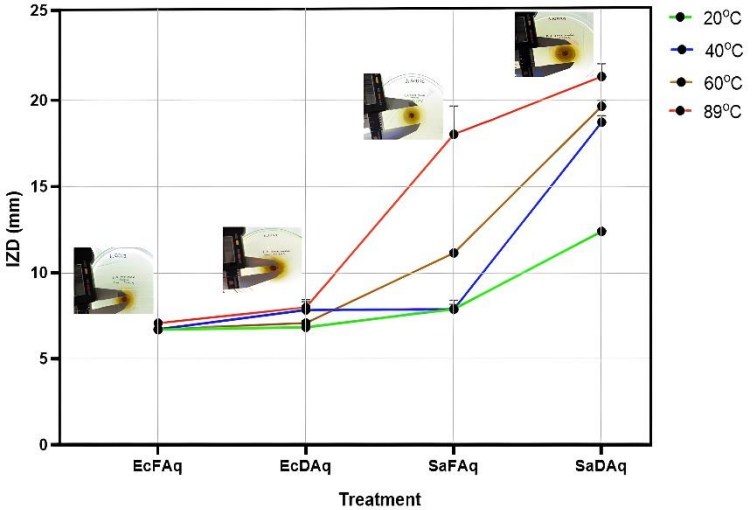

Figure No. 3
Inhibition zone diameter of *C. bolivianum* aqueous extracts at different temperatures
EcFAq: Fresh *C. bolivianum* aqueous extract against *E. coli*; EcDAq: Dry *C. bolivianum* aqueous extract against *E. coli*; SaFAq: Fresh *C. bolivianum* aqueous extract against *S. aureus*; SaDAq: Dry *C. bolivianum* aqueous extract against *S. aureus*

Table No. 2 illustrates the antimicrobial sensitivity of *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 25923 to the tested essential oils and aqueous extracts of *C. bolivianum*. Both bacterial

strains demonstrated sensitivity to the essential oils; however, *S. aureus* was notably more susceptible, particularly to the *C. bolivianum* aqueous extract, which did not exhibit similar efficacy against *E. coli*. Among all the treatments evaluated, the dry Inca muña essential oil displayed the most significant inhibition zone diameter (IZD) against *S. aureus*. This potent antibacterial activity can be attributed to its high concentrations of linalool, pulegone, and isomenthone, as detailed in Figure No. 1.

Table No. 2
Antimicrobial activity of essential oil and aqueous extract of *C. bolivianum*

Indicator	Treatment	IZD (mm)	S	% I	MIC mg mL^{-1} (%D)	MB mg mL^{-1} (%D)
E. coli ATCC 25922	FEo	21.25 ± 0.84^{b}	S	135.69 ± 5.35^{cd}	5.51 (12.50)	11.01 (25.00)
	DEo	22.72 ± 1.42^{ab}	S	145.10 ± 9.04^{bc}	5.49 (12.50)	10.97 (25.00)
	FAq T4	7.07 ± 0.02^{d}	R	45.14 ± 0.14^{e}	NI	NI
	DAq T4	7.99 ± 0.45^{d}	R	51.03 ± 2.84^{e}	13.04 (25.00)	26.07 (50.00)
S. aureus ATCC 25923	FEo	22.26 ± .41^{ab}	S	157.66 ± 2.88^{ab}	2.75 (6.25)	5.51 (12.50)
	DEo	23.30 ± 1.15^{a}	S	165.04 ± 8.15^{a}	2.74 (6.25)	5.49 (12.50)
	FAq T4	18.03 ± 1.63^{c}	S	127.74 ± 11.56^{d}	13.10 (25.00)	26.20 (50.00)
	DAq T4	21.39 ± 0.74^{ab}	S	151.49 ± 5.27^{ab}	6.52 (12.50)	13.04 (25.00)

S: sensitivity against *E. coli* (Sensitive ≥ 17, Intermediate 14-16, Resistant ≤ 13) (CLSI-M100)
S: sensitivity against *S. aureus* (Sensitive ≥ 13, Intermediate 11-12, Resistant ≤ 10) (CLSI-M100)
%I: Percentage inhibition (Positive control for *E. coli* was 10 µg Ampicillin (IZD=15.66 ± 0.25), and for *S. aureus* was 1 µg Oxacillin (IZD=14.12 ± 0.04))
%D: Percentage dilution
T4: 89ºC (boiling temperature)
NI: No inhibition
Lowercase letters represent significant statistical differences in vertical according to the Tukey test at a 95% confidence level

Figure No. 4 illustrates the bacterial growth dynamics over 24 hours for both essential oils and aqueous extracts, with measurements taken every hour and comparisons made against negative and positive controls. The curves C1, C2, and C3 in Figures No. 4c and No. 4d, which closely align with the negative control at the baseline, indicate the most effective minimum bactericidal concentration (MBC) values observed in this study, with inhibition reaching up to 12.5% for the essential oils against *S. aureus*. Additionally, curve C2 in Figure No. 4g shows the MBC effectiveness at a 25% dilution, although weaker, it was the most effective among the extracts tested. The minimum inhibitory concentration (MIC) results further highlight the efficacy of the essential oils, with the best outcomes achieved at a 6.25% dilution, as seen in curves C4 of Figures No. 4c and No. 4d, specifically against *S. aureus*. Notably, the fresh Inca muña aqueous extract exhibited no inhibitory effect against *E. coli*, as depicted in Figure No. 4f.

DISCUSSION

Physical characteristics of essential oil and aqueous extract

The density of *Minthostachys mollis* (muña), reported as 0.9640 g/mL by Peña & Gutierrez (2017), and 0.90 g/mL at 20°C by Torrenegra-Alarcón *et al*. (2016), was found to be slightly higher than the density of *Clinopodium bolivianum* (Benth) Kuntze (Inca muña) reported in this study. Both herbs belong to the Lamiaceae family and are aromatic plants native to the Andes of Peru. The essential oil yield of Inca muña in this study, ranging from 0.196% to 0.450%, falls within the reported range for muña essential oil as described by Cano *et al*. (2006). Additionally, the minimum inhibitory concentration (MIC) of *M. armillaris* essential oil required to inhibit *Staphylococcus aureus* ATCC 29213 was 25 µL/mL at pH 7.4 and 6.5, but this value decreased by half at pH 5.0, indicating enhanced antibacterial activity at lower pH levels (Buldain *et al*., 2021). In contrast, while pH was found to influence the antibacterial activity of essential oils, it did not affect the antibacterial activity of Inca muña extracts in this study.

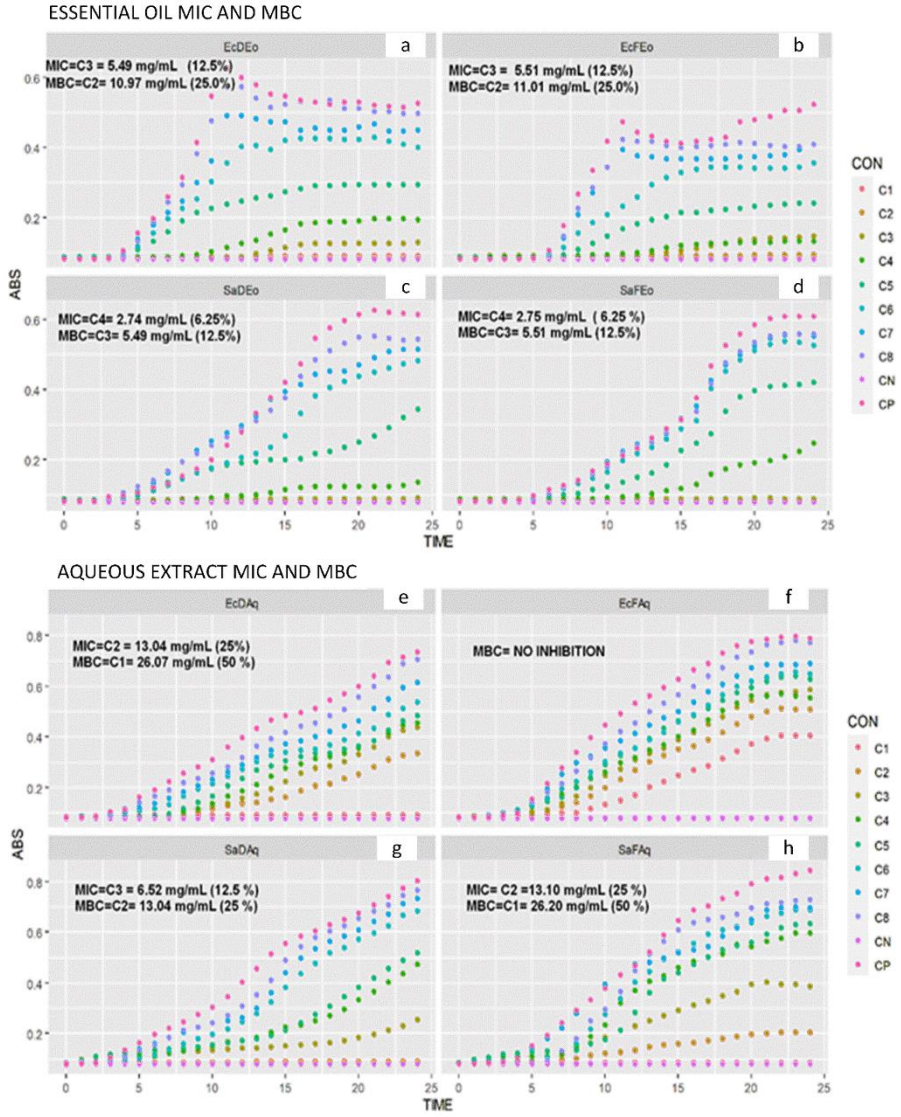

Figure No. 4
MIC y MBC of essential oil and aqueous extract by bacterial growth during 24-hour-incubation
C1=50%, C2=25%, C3=12.5%, C4=6.25%, C5=3.13%, C6=1.56%, C7=0.78%, C8=0.39%, CN=Negative control, CP=Positive control
EcDEo: Dry *C. bolivianum* essential oil against *E. coli*, *EcFEo*: Fresh *C. bolivianum* essential oil against *E. coli*
SaDEo: Dry *C. bolivianum* essential oil against *S. aureus* SaFEo: Fresh *C. bolivianum* essential oil against *S. aureus*

Bioactive compounds of essential oil and aqueous extract

In the essential oil of *Clinopodium bolivianum*, the primary bioactive compounds vary depending on the region. For instance, the essential oil from Cusco, Peru, contains iso-menthone (20.8%), thymol (16.1%), menthone (14.8%), pulegone (6.7%), and linalool (2.8%) (Solís-Quispe *et al*., 2018). In contrast, the oil from La Paz, Bolivia, is characterized by pulegone (24.65%) and limonene or γ-terpineol (29.54%) (Cumara & Choquehuanca, 2015). This study, focusing on the essential oil from Huancavelica, Peru, identified iso-menthone (28.74%), linalool (17.50%), and pulegone (12.05%) as the main components. The variation in the concentration of these compounds across different studies is likely due to factors such as climate, altitude, and harvest period (Rezouki *et al*., 2021). Notably, linalool, although considered a limited antibacterial agent, has been shown to be effective when combined synergistically with vitamin C and copper (Ghosh *et al*., 2019).

The aqueous extract of Inca muña tentatively revealed the presence of several flavonoid derivatives, including flavanone derivatives (quantified as eriodyctiol), flavone derivatives

(quantified as luteolin and apigenin), and hydroxycinnamic acid derivatives (quantified as caffeic acid) (Chirinos *et al*., 2011). Additionally, hydroxycinnamic acid derivatives identified in this study align with those reported by Campos *et al*. (2022). Specifically, *p*-coumaric acid was found at concentrations up to 10.43 ± 2.63 mg/L, and caffeic acid at 7.94 ± 3.46 mg/L, consistent with the findings of De Magalhães & Dos Santos (2020), in most herbal infusions, which also detected chlorogenic acid and rutin. The presence of these compounds in *C. bolivianum* extract further supports its potential as a bioactive agent.

Phytochemical studies according to H NMR spectra, shows bicyclofermacrene, alpha cadinol, 1,8 cineole, alpha cymene as volatile oils, caffeic acid 3-glucoside, caffeic acid 3-rutinoside as phenolic acids, apigenin.7-0-apioglucoside, kaempferol-7-0-ramnoside, eriodyctiol-7-glucoside, eriodyctiol-7-rutinoside as flavonoids. The presence of diterpenes and polyphenols in dichloromethane/methanol extracts of *C. bolivianum* exhibit significant anti-inflammatory and antibacterial properties (Apaza Ticona *et al*., 2024).

Antibacterial activity of essential oil and aqueous extract

There are no previous studies of the antibacterial activity of C. *bolivianum* essential oil against *S. aureus* and *E. coli*. However, significant larvicidal activity (Solís-Quispe *et al*., 2018) and a protective role on bladder epithelial cells against UPEC infection by decreasing bacterial adhesion, invasion, and biofilm formation have been demonstrated (Mohanty *et al*., 2017). *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were sensitive to the essential oil of *Minthostachys mollis* (muña), an herb from the same *Lamiaceae* family (Torrenegra *et al*., 2015). Ambrosio *et al*. (2017), reported inhibition zone diameters (IZD) of 28 different essential oils and their mixtures against *E. coli* ranging from 13.4 to 27.1 mm and against *S. aureus* from 12.1 to 28.4 mm, which demonstrates a broad range, though not exceeding the values obtained in this study. The antibacterial activity of *Rosmarinus officinalis* (rosemary) essential oil against 11 pathogenic bacteria, including *E. coli* and *S. aureus*, showed MIC > 6.4 mg/mL (Nieto *et al*., 2018), very similar to the MIC > 5 mg/mL for *E. coli* but different from the MIC > 2.7 mg/mL for *S. aureus* in Inca muña essential oil found in this study.

C. bolivianum essential oils show greater inhibitory activity against *S. aureus*, aligning with Argote-Vega *et al*. (2017), who evaluated the inhibitory capacity of herb essential oils from Ecuador and demonstrated that *S. aureus* was the most susceptible bacterium, while *E. coli* showed greater resistance. The essential oils of wild herbs generally have a greater inhibitory effect against Gram-positive bacteria than Gram-negative bacteria (Chaib *et al*., 2017) because the outer membrane of Gram-negative bacteria is primarily composed of lipopolysaccharides, which have an affinity for hydrophobic terpenes and form a permeability barrier with highly hydrophilic surfaces. In contrast, Gram-positive bacteria lack an outer membrane, and their highly permeable cell wall allows the penetration of terpenes toward the cytoplasmic membrane with lipophilic ends (Coello-Cedeño, 2021).

The dichloromethane extract showed greater antibacterial activity (14.8 mm IZD) than the hydroalcoholic and aqueous extracts of *Clinopodium bolivianum* from La Paz, Bolivia, against *Helicobacter pylori* (Claros *et al*., 2007). Compared to the aqueous extract, the hydroethanolic and methyl extracts have a better inhibitory effect, though they are toxic compounds that promote secondary effects (Ponzilacqua *et al*., 2018). One of the extracts with the best MIC against *S. aureus* MRSA is the methanolic extract of *P. officinalis* root, reaching up to 0.00047 mg/mL (Shady *et al*., 2024). Methanolic extracts showed greater antibacterial activity (MIC > 1 mg/mL) compared to aqueous extracts (MIC > 5 mg/mL) in most herbs studied against *S. aureus*, because polar components often do not dissolve non-polar ones, causing aqueous extracts to lose some of their antibacterial activity (Ibrahim & Kebede, 2020). However, a very limited antibacterial activity (DZI < 8 mm) has been observed in the ethanolic extract of *Cyclamen persicum* (Al-Rimawi *et al*., 2024). The aqueous extract of fresh *C. bolivianum* presented MIC of 13.10 mg/mL and dry *C. bolivianum* MIC of 6.52 mg/mL against *S. aureus*, demonstrating that the inhibitory effect is limited but reflective of the traditional medicinal consumption of infusions in the high Andean region of Peru.

The hydroxycinnamic acid derivatives in Inca muña extracts are important biological and structural components of the plant cell wall, presenting potential antimicrobial and anti-inflammatory activities due to their ability to eliminate free radicals (El-Seedi *et al*., 2017). A decrease in double bonds within hydroxycinnamic acid significantly reduces its antibacterial activity, which can alter cell membranes and cause cytoplasmic leakage (Kumar & Goel, 2019). The antimicrobial activity of chitosan film

improved after adding caffeic acid (derived from hydroxycinnamic acid) with DZI 10-11 mm for E. coli and 11-12 mm for *S. aureus* (Yong *et al*., 2021). Phenolic and hydroxycinnamic acids are potentially immunostimulating compounds and are found in many herbs (Lorigooini *et al*., 2020).

p-Coumaric acid has demonstrated stronger inhibition against *S. aureus* with an MIC of 20 mg/mL and less intense inhibition against *E. coli* with an MIC of 80 mg/mL due to a dual mechanism of bactericidal activity: altering bacterial cell membranes and binding to the phosphate anion in the DNA double helix, which could affect replication, transcription, and expression, leading to cell death (Lou *et al*., 2012). These findings are consistent with Inca muña extract MIC values > 6 mg/mL against *S. aureus* and the absence of inhibition against *E. coli.* p-Coumaric and hydroxycinnamic acids inhibit S. aureus due to esters and amides that possess bulky aromatic groups (8-hydroxy quinoline and naphthylamine, respectively), which increase antibacterial potential (Khatkar *et al*., 2017).

Essential oils have a more significant inhibitory effect than aqueous extracts (Table No. 2). These values align with MIC > 1 mg/mL for essential oils and MIC > 4 mg/mL for aqueous extracts against *E. coli*, as well as MIC > 1 mg/mL for essential oils and MIC > 2 mg/mL for aqueous extracts against *S. aureus* from different herbs (Mapeka *et al*., 2024). The essential oil and the aqueous extract of dried Inca muña showed good antimicrobial activity, as dried herbs tend to concentrate more bioactive compounds. The influence of the drying method on antibacterial activity indicates that lower temperatures result in lower MIC and IC50 values (Chua *et al*., 2019). Inca muña essential oil was more effective than Inca muña extract in this study, like *Matricaria chamomilla* essential oil (MIC = 0.11 µg/mL), which was more effective than the aqueous and ethanolic extracts in 91 strains of E. coli resistant to multiple drugs (MDR) from patients with urinary tract infections (Jafarzadeh *et al*., 2020).

The concentrated extract could be used in films for antibacterial packaging (Yong *et al*., 2021), the essential oil as a beef preservative (Nieto *et al*., 2018), and infusions as functional food to reduce the effects of elevated blood glucose levels, as well as anti-inflammatory activity through collagenase inhibition due to polyphenols (Studzińska-Sroka *et al*., 2021).

CONCLUSIONS

The major bioactive compounds iso-menthone, linalool, and pulegone in the essential oils, along with hydroxycinnamic acids in the aqueous extracts were identified in *Clinopodium bolivianum*, all demonstrating inhibitory effects against *Staphylococcus aureus*. The antibacterial activity followed the order: dry *C. bolivianum* essential oil > fresh *C. bolivianum* essential oil > dry *C. bolivianum* aqueous extract > fresh *C. bolivianum* aqueous extract, with a particular efficacy against *S. aureus*. These findings align with the traditional use of *C. bolivianum* infusions for relieving stomach infections and inflammation. Given their pleasant odor, mild intensity, and easy accessibility, these extracts and oils hold promise for application in food preservation without compromising sensory acceptability.

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