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# Bioactive Properties of *Pentacalia vaccinioides* (Kunth) Cuatrec. (Asteraceae) Essential Oils: Evaluation of Antimicrobial and Antioxidant Activities

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# Bioactive Properties of *Pentacalia vaccinioides* (Kunth) Cuatrec. (Asteraceae) Essential Oils: Evaluation of Antimicrobial and Antioxidant Activities

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**Abstract:** Essential oils (EOs) have unique properties, such as antibacterial, antioxidant, and antiviral activities, which are beneficial in various industries, including cosmetics, food, and pharmaceuticals. In this study, the antioxidant and antimicrobial activities of *Pentacalia vaccinioides* EOs obtained from leaves and flowers (fresh and dried plant material) were evaluated using hydrodistillation (HD), steam distillation (SD), simultaneous distillation-extraction (SDE), and solid-phase microextraction (SPME) techniques. Antimicrobial activity (minimum inhibitory concentration, MIC) and antioxidant capacity (half-maximal inhibitory concentration, $IC_{50}$) were determined. The identification and quantification of the compounds present in the EOs were conducted by gas chromatography coupled to mass spectrometry (GC-MS). The main secondary metabolites identified in most samples obtained by different extraction techniques included: phenol (~18%), 1S-α-pinene (~15%), β-phellandrene (~13%), β-pinene (~12%), 4-terpineol (~10%), γ-terpinene (~10%), trans-nerolidol (~8%), limonene (~8%), and β-thujene (~6%). EOs obtained by HD, SD and SDE exhibited antioxidant activity, with $IC_{50}$ values between 621.7 and 696.6 µg/mL. Additionally, the EOs demonstrated bactericidal activity against *Bacillus subtilis* and *Staphylococcus aureus*, with MIC values of 5.0 and 45 µg/mL, respectively. *Escherichia coli* and *Pseudomonas aeruginosa* did not show antimicrobial susceptibility to EOs. This study constitutes the first evaluation of *Pentacalia vaccinioides* EOs, demonstrating their bioactive potential and the relevance of the extraction method. The findings highlight this species as a promising source of natural compounds for therapeutic and preservative applications, depending on the type of plant material and extraction technique used.

**Keywords:** *Pentacalia vaccinioides*; antioxidant; antimicrobial; essential oil; volatile compounds; hydrodistillation; steam distillation; simultaneous distillation-extraction; solid-phase microextraction; Colombia

## 1. Introduction

Essential oils are volatile compounds present in various plant organs including flowers, leaves, bark, roots, and fruits. Their composition is complex, including terpenes, terpenoids, alcohols, phenols, aldehydes, ketones, esters, among others, and varies according to the plant species, the part used, the extraction method, and the environmental conditions where the plant grows [1,2]. EOs exhibit a range of biological activities, including insecticidal, antioxidant, and antibacterial properties, in addition to carminative, anti-inflammatory, antispasmodic, and analgesic effects [3–5]. EOs have diverse applications in the pharmaceutical, food, cosmetic, and fragrance industries, among others [6,7]. Furthermore, EOs used by these industries are recognized for their safety and health benefits by regulatory bodies such as the Food and Drug Administration (FDA) and the International Fragrance Association (IFRA) [8,9].

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In recent years, there has been a growing interest in the use of EOs as antioxidants and antimicrobials. Antioxidants are substances that help protect cells or molecules from free radical damage, and antimicrobials are substances that kill or inhibit the growth of microorganisms such as bacteria, fungi, and viruses. EOs, either in combination or alone, show antibacterial, antifungal, antiviral, and antioxidant activities. The effectiveness of these oils increases when combined, taking advantage of synergistic and additive effects [10,11]. Rosemary and sage EOs have antimicrobial and antioxidant activities, making them useful for food preservation and medical applications, offering a natural alternative to synthetic preservatives [12]. In other research, the EOs of thyme, oregano, lemongrass, mint, and rosemary have shown potential against multidrug-resistant food pathogens [13].

*Pentacalia vaccinioides* is a species of the Asteraceae family, endemic to the moorlands of Colombia and Venezuela, with no studies in pharmacognosy and phytochemistry. This plant species has unexplored potential in terms of its chemical composition and bioactive properties. In some departments of Colombia, this plant is known by different common names: "Chilquilla menuda, Hierba de páramo" in Cauca, "Tangue" in Santander, "Romerillo rusio", and "Panque" in Nevado del Cocuy —where it is popularly used to heal wounds that are difficult to heal—, and "Nabo" in Cundinamarca —where it is used to relieve stomach pains and as firewood by some local inhabitants [14]. Studies on other Pentacalia species have identified compounds such as nerolidol, humulene, farnesene, jacarone, methyl-jacarone and herzogole, among others, with relevant biological properties [15–17]. Pentacalia species have ethnobotanical uses focused on disinfection and healing of difficult-to-heal wounds. In the departments of Boyacá and Cundinamarca, farmers have traditionally used these plants to treat wounds, combat syphilis, cure persistent pimples and boils, as well as to relieve sore throats and treat ulcers [18].

The objective of this study was to characterize the chemical profile of the EOs of *Pentacalia vaccinioides* obtained by hydrodistillation (HD), simultaneous distillation-extraction (SDE), steam distillation (SD), and solid-phase microextraction (SPME) methods, as well as to evaluate their antioxidant and antimicrobial activity. It is expected that the results obtained will contribute to the scientific knowledge about this plant species and may be useful in the development of pharmaceutical, cosmetic, and agroindustrial applications based on its bioactive properties.

## 2. Materials and Methods

During the performance of the experiments, the laboratory's health and safety procedures were complied with, following the national and institutional regulations in force. In addition, the necessary precautions were taken to minimize the risks associated with the materials and substances used.

### *2.1. Reagents and Equipment*

The following Sigma-Aldrich reagents and standards were used: $C_7$-$C_{30}$ Saturated Alkanes Standard (1000 µg/mL each component in hexane, product number 49451-U), n-Tetradecane GC (≥99.5%, product number 87140) olefin-free, 6-hydroxy-2,5,78-tetramethylchroman-2-carboxylic acid (Trolox, product number 238813), 2,2-Azino-bis(3-ethylbenzothiazoline-6 sulfonic acid) (ABTS, product number A9941), sodium sulfate (Na2SO4, ≥99.0%, product number 238597), sodium chloride (NaCl, ≥99.0%, product number S9888), ethanol (EtOH, ≥99.5%, product number 459844), dimethyl sulfoxide (DMSO, ≥99.7%, product number 34869), and dicloromethane ($CH_2Cl_2$, ≥99.8%, product number 270997). The following extraction equipment was used: Clevenger-type hydrodistillation (HD, Labbox, reference 1531), steam distillation (SD, Kasalab, reference TE-2761), simultaneous distillation-extraction (SDE, Likens-Nickerson apparatus, Labxsci, reference Sku:La), solid-phase microextraction (SPME, Supelco Inc., Fiber Holder 57330-U, Fiber Assembly 57300-U, Vial Headspace SU860098, Magnetic Screw Cap SU860103, Septa PTFE/white silicone 27514) using a fused silica fiber of poly(dimethylsiloxane) (PDMS, 100 µm, 57300-U), rotary evaporator (Büchi, R-100), refractometer (Atago RX-i), and UV-VIS spectrophotometer (BMG LabTech, FluoStar Omega microplate reader).

### *2.2. Collection and Preparation of Plant Material*

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The plant material (leaves and flowers) was collected in the village San Francisco, municipality of Choachí (department of Cundinamarca, Colombia), between 3,000 and 3,500 meters above sea level. The material was selected taking into account that it should be free of fungi, physiologically intact, free of soil and dust, then dried at room temperature under shade (12°C) for 15 days. The fresh and dried leaves were crushed to obtain a greater contact surface with the solvent in the different extractive systems (Sharapin et al. 2000). The taxonomic identification was carried out by Botanist Carlos Parra-O. from the Colombian National Herbarium (COL), voucher COL 570482 - *Pentacalia vaccinioides* (Kunth) Cuatrec. This research has the authorization for access to genetic resources and their derived products, as well as for the collection of specimens, granted within the framework of the research project entitled "Phytochemical analysis of some plant species with potential antimicrobial, antioxidant, cytotoxic and remineralization activity, among others". The authorization was issued through Resolution 1141 of September 2, 2022, and the Contract for Access to Genetic Resources and their Derived Products No. 347 of 2022, issued by the Ministry of Environment and Sustainable Development of the Republic of Colombia (MinAmbiente, Colombia).

### *2.3. Obtaining the Essential Oil and Its Physicochemical Properties*

**Table 1** shows the notation of the extraction techniques, and the different experimental parameters used in each process. Fresh and dried leaves and flowers of *Pentacalia vaccinioides* were used to obtain the EOs. The extraction time for each process was 3 hours except for the SPME process, which was 30 minutes. The obtained EOs were dried over anhydrous sodium sulfate and stored in amber colored bottles at 2°C. Each extractive process and physicochemical test was performed in triplicate.

**Table 1.** Experimental parameters used in different extraction processes. *.

| No | Method | Acronym | Mass (g) | Extraction time (h) | Solvent |
|---|---|---|---|---|---|
| 1 | Hydrodistillation dry leaf | HD-DL | 150 | 3 | 1000 mL $H_2O$ |
| 2 | Hydrodistillation wet leaf | HD-WL | 250 | 3 | 1000 mL $H_2O$ |
| 3 | Steam distillation dry leaf | SD-DL | 150 | 3 | 1000 mL $H_2O$ |
| 4 | Steam distillation wet leaf | SD-WL | 250 | 3 | 1000 mL $H_2O$ |
| 5 | Simultaneous Distillation and Extraction flowers | SDE-WF | 250 | 3 | 100 mL $CH_2Cl_2$, 1000 mL $H_2O$ |
| 6 | Simultaneous Distillation and Extraction dry leaf | SDE-DL | 150 | 3 | 100 mL $CH_2Cl_2$, 1000 mL $H_2O$ |
| 7 | Simultaneous Distillation and Extraction wet leaf | SDE-WL | 250 | 3 | 100 mL $CH_2Cl_2$, 1000 mL $H_2O$ |
| 8 | Solid-Phase Microextraction flowers | SPME-WF | 10 | 0.5 | 25 mL $H_2O$ |
| 9 | Solid-Phase Microextraction dry leaf | SPME-DL | 10 | 0.5 | 25 mL $H_2O$ |
| 10 | Solid-Phase Microextraction wet leaf | SPME-WL | 10 | 0.5 | 25 mL $H_2O$ |

*Time selection was based on preliminary experiments and previous studies. Each extraction process was performed 3 times.

#### 2.3.1. Hydrodistillation

The extraction of EOs was performed using a Clevenger-type hydrodistiller. The sample was deposited in a round-bottomed flask, then 1000 mL of deionized water containing 50 g of NaCl was added. The extraction was carried out for 3 hours [19,20].

#### 2.3.2. Steam Distillation

EOs were obtained by steam distillation using a Clevenger-type apparatus. Fresh and dried leaves, separately, were placed in the extraction system and steam was generated from a volume of 1000 mL of deionized water. The extraction was carried out for 3 hours [19–21].

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2.3.3. Simultaneous Distillation-Extraction

The homogenized sample with 1000 mL of distilled water was placed in a 2000 mL round bottom flask. Then, 100 mL of $CH_2Cl_2$ was placed in a 250 mL round-bottom flask. These two flasks were coupled to a Likens-Nickerson apparatus. The solvent and sample mixtures were heated to 60°C and boiling temperature, respectively. The temperature conditions were maintained for 3 hours. After cooling to room temperature, the dichloromethane extract was collected and dried with anhydrous sodium sulfate. The extract was then concentrated to 1.0 mL using a nitrogen flow evaporator/concentrator [19,20,22].

2.3.4. Headspace Solid-Phase Microextraction

The headspace mode solid-phase microextraction (HS-SPME) technique was carried out using a poly(dimethylsiloxane) (PDMS) fiber with a thickness of 100 µm, purchased from Supelco Inc. A total of 10.0 ± 0.1 grams of sample was deposited into 50 mL vials along with 25 mL of deionized water (containing 10% NaCl). Each vial was heated at 60°C for 10 min to reach thermal pre-equilibrium. Subsequently, the fiber was exposed to the headspace for 30 min, after which it was transferred to the GC injection port for 5 min [23–25]. These experimental conditions are based on preliminary and previous studies performed by our research group.

*2.4. Determination of Physical Properties*

The yield of the extractions was calculated as the ratio between the mass of EOs obtained and the mass of sample. The refractive index was measured using an ABBE Atago refractometer; 2 drops of the EOs were placed on the prism of the refractometer, and the reading was taken at 20°C. For the solubility of EOs in ethanol, 10 µL of EOs were added to 100 µL of ethanol (70%, v/v), the mixture was homogenized in a vortex for 5 min at 20 rpm. The density determination was performed taking into account the mass contained in a volume of 10 µL of the EOs. The methodology with pycnometer was not used since the yield obtained from the oils was less than 1.0 mL.

*2.5. Gas Chromatography-Mass Spectrometry (GC/MS)*

Ten mL of the EOs plus 0.2 mL of the internal standard (n-Tetradecane) was taken, and $CH_2Cl_2$ was added to a final volume of 1.0 mL. One microliter (1.0 µL) of this solution was analyzed by GC/MS. The analyses were performed on an Agilent 6890 gas chromatograph equipped with an Agilent 5975B VL mass selective detector (electron impact ionization, 70 eV), a split/splitless injector (1:50 split ratio) and Enhanced ChemStation MSD D.03.00.52 data system with Wiley and Nist spectral libraries. Two capillary columns were used: Agilent HP-5MS (5%-phenyl-poly(methylsiloxane), 60 m x 0.25 mm i.d, 0.25 µm film thickness), and Agilent HP-Innowax (100% cross-linked poly(ethylenglycol), 60 m x 0.25 mm i.d, 0.25 µm film thickness) capillary columns. The oven temperature was programmed from 60°C (2 min) to 250°C (2 min) at 50°C/min, then to 310°C (2 min) at 20°C/min and a post-run to 320°C (1 min). The temperatures of the injection port, ionization chamber and transfer line were set at 300, 185 and 285°C, respectively. Helium (99.999%) was used as carrier gas, with 85 kPa column head pressure and linear velocity at constant flow rate (1.0 mL/min). Mass spectra, total ionic currents (TIC) and extracted ion (EIC) were obtained with a quadrupole analyzer, by means of automatic radiofrequency scanning (full scan) in the mass range of m/z 30-500 (2.2 spectra/s). The tentative identification criteria were based on the analysis of mass spectra obtained by GC-MS and the linear retention indices in apolar and polar columns, calculated based on the homologous series of n-alkanes $C_7$-$C_{30}$ (Sigma-Aldrich) and compared with those from different mass spectral databases (Wiley 7n.1 and Nist 05a.L) and scientific literature data. Linear retention indices (LRI) were calculated using the following formula: $LRI = 100 \cdot n + 100 \cdot [(tRx - tRn)/(tRN - tRn)]$, where "n" is the number of carbon atoms in the n-paraffin eluting before the compound of interest (its retention time is tRx); tRn and tRN are retention times of n-paraffins with the numbers of carbon atoms n and N, respectively, eluting immediately before and after the analyte of interest [11,26–30].

*2.6. Antioxidant Activity Determination*

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Volumes of 2, 5, 8, 8, 11 and 14 µL of the EOs were diluted to a final volume of 10 mL with EtOH: $CH_2Cl_2$ (9:1) from Sigma-Aldrich. Antioxidant activity assays were performed following the ABTS method proposed by Sequeda et al., 2021 [31].

*2.7. Antimicrobial Activity Determination*

*Bacillus subtilis* (ATCC 6638, CMPUJ 75), *Staphylococcus aureus* (ATCC 6538, CMPUJ 80), *Escherichia coli* (ATCC 8739, CMPUJ 76), and *Pseudomonas aeruginosa* (ATCC 9721, CMPUJ 55) bacterial strains obtained from Pontificia Universidad Javeriana Microorganism Collection. The antimicrobial activity assays were performed following the well and disc diffusion method proposed by Ortiz-Ardila et al., 2017 and Sequeda-Castañeda et. al., 2019, with some modifications: 20 µL of the EOs at concentrations between 1215, 435, 135, 45, 15, 15, 5.0, and 1.67 µg/mL were dosed into each well and disc using $CH_2Cl_2$: DMSO (9:1) as solvent. 20 µL of $CH_2Cl_2$, DSMO, and $H_2O$ were used as negative controls, and 20 µL of Gentamicin (30 µg/µL) as a positive control [32,33].

2.7.1. Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism under defined conditions. For this purpose, the agar diffusion method was used [34–37]. The concentrations of the EOs used ranged from 1.67 to 1215 µg/mL. A volume of 20 µL of the EOs solutions was deposited in 6 mm diameter wells on Mueller Hinton (MH) agar plates, previously inoculated with each microorganism [32,33].

*2.8. Statistical Analysis*

Results are representative of six independent replicates and are expressed as mean ± SD. To determine which extraction method identified more compounds, the maximum likelihood method given by Hogg et al. (2019) for proportions was used. It corresponds to a success-failure method applied to the extraction of each component: a value of 1 is given if the extraction method was successful, and the compound is present in the EOs. On the other hand, a failure is considered when the compound is not found in the EOs and in this case a value of 0 is assigned. With the estimation of the proportions, the method that allows the extraction of the highest number of compounds, i.e. the highest number of successes (presence of compounds in the essential oil), is determined. According to the maximum likelihood method, this ratio is estimated with the following formula: p = (# of identified compounds)/(# of total compounds) [38].

## 3. Results

*3.1. Obtaining EOs by Different Extraction Techniques and Physicochemical Parameters*

EOs obtained by various extraction methods, including hydrodistillation (HD), steam distillation (SD), simultaneous distillation-extraction (SDE), and solid-phase microextraction (SPME), were analyzed. The parameters evaluated were yield (% m/m), density (ρ), refractive index (η), color, odor, and ethanol solubility (**Table 2**).

**Table 2.** Extraction yields and physicochemical parameters. *.

| No | Acronym | % (m/m) | r (g/mL) | h | Color | Smell |
|---|---|---|---|---|---|---|
| 1 | HD-DL | 0.0014 ± 0.002 | 0.8666 ± 0.0030 | 1.611 ± 0.002 | Yellow | It is penetrating, somewhat spicy, turpentine notes. |
| 2 | HD-WL | 0.0016 ± 0.002 | | | | |
| 3 | SD-DL | 0.0017 ± 0.003 | 0.8666 ± 0.0030 | 1.611 ± 0.002 | | |
| 4 | SD-WL | 0.0019 ± 0.003 | | | | |
| 5 | SDE-WF | 0.0012 ± 0.002 | | | | |
| 6 | SDE-DL | 0.0145 ± 0.006 | 0.8667 ± 0.0030 | 1.612 ± 0.001 | | |
| 7 | SDE-WL | 0.0156 ± 0.007 | | | | |
| 8 | SPME-WF | NA | NA | NA | | |

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| | |
|---|---|
| 9 | SPME-WL |
| 10 | SPME-WL |

*Average ± standard deviation, n=3. NA = It was not possible to calculate this value due to the difference in weight of the fibril before and after the extraction process. HD-DL: Hydrodistillation dry leaf. HD-WL: Hydrodistillation wet leaf. SD-DL: Steam distillation dry leaf. SD-WL: Steam distillation wet leaf. SDE-WF: Simultaneous Distillation and Extraction flowers. SDE-DL: Simultaneous Distillation and Extraction dry leaf. SDE-WL: Simultaneous Distillation and Extraction wet leaf. SPME-WF: Solid-Phase Microextraction flowers. SPME-DL: Solid-Phase Microextraction dry leaf. SPME-WL: Solid-Phase Microextraction wet leaf.

### 3.2. *Chemical Composition of the Essential Oil Obtained by Different Extraction Techniques*

EOs of *Pentacalia vaccinioides* was extracted by various techniques: HD, SD, SDE, and SPME. The chemical composition was analyzed by GC-MS using apolar and polar columns to obtain linear retention indices (LRI) and their mass spectra for compound identification (**Table 3,** and Supplementary material **Figure 1**). According to the results obtained, between 18 and 33 compounds were identified in the EOs, depending on the extraction method and the plant part analyzed. The most abundant compounds (> 1.0%) include: phenol, 1S-$\alpha$-pinene, β-phellandrene, β-pinene, 4-terpineol, γ-terpinene, trans-nerolidol, limonene, and β-thujene. These compounds exhibit a wide range of chemical properties, from monoterpenes and sesquiterpenes to alcohols and ketones, which determine the olfactory and functional properties of the EOs. The plant part used (fresh leaves, dried leaves, and flowers) also affected the composition of the EOs. Monoterpene hydrocarbons were more abundant in flowers and leaves (especially in SPME), whereas phenols and oxygenated sesquiterpenes were more common in dried leaves. This result suggests that plant part selection is crucial to obtain EOs with specific chemical profiles.

**Table 3.** Relative amount (> 1.0%) and identification of the main components of *Pentacalia vaccinioides* EOs obtained by different techniques. [a.]

| No. | Compund | Type | LRI | | HD | | SD | | SDE | | | SPME | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Leaf | | Leaf | | Leaf | | Flower | Leaf | | Flower |
| | | | Apola | Polar | Fresh | Dry | Fresh | Dry | Fresh | Dry | Fresh | Fresh | Dry | Fresh |
| **1** | 2-Butanone, 3-hydroxy- | * | 775 | - | 1.6 | 2.9 | 3.7 | - | 3.2 | 3.7 | 3.3 | - | - | - |
| **2** | 1-Butanol, 3-methyl- | * | 780 | - | - | - | 4.8 | - | 1.5 | 1.8 | 1.0 | - | - | - |
| **3** | 1-Octene | * | 786 | - | - | - | - | - | - | - | - | 1.4 | 1.5 | 1.5 |
| **4** | Hexanal | * | 802 | 815 | 1.8 | 5.5 | 1.0 | 1.3 | 1.4 | 2.5 | 2.7 | - | - | - |
| **5** | Furfural | * | 831 | 1078 | - | - | 1.1 | 1.1 | - | - | - | - | - | - |
| **6** | 3-Hexen-1-ol, (Z)- | * | 852 | - | 1.6 | 1.7 | 3.2 | 3.4 | 1.7 | 2.0 | 2.2 | - | - | - |
| **7** | 2-Hexenal, (E)- | * | 851 | 1249 | 2.3 | 3.4 | 2.0 | 1.3 | 1.2 | 1.2 | 1.4 | - | - | - |
| **8** | 1-Hexanol | * | 863 | - | 1.3 | 1.5 | 1.5 | 1.6 | 2.3 | 2.7 | 3.0 | 3.0 | 4.3 | 4.9 |
| **9** | Phenol | ** | 883 | 1537 | 11.4 | 9.4 | 19.2 | 18.0 | 2.9 | 3.7 | 1.0 | 3.1 | 7.1 | 3.1 |
| **10** | Cyclopropane, 1-methyl-2-pentyl- | * | 890 | 1612 | 1.2 | 1.4 | 1.4 | 1.5 | 1.8 | 1.6 | 2.0 | 2.3 | 2.1 | 2.7 |
| **11** | Propanal, 3-(methylthio)- | * | 907 | - | - | - | 1.3 | 1.4 | - | - | - | - | - | - |
| **12** | 2(5H)-Furanone | * | 915 | 1060 | 3.0 | 1.7 | 6.0 | 5.3 | - | - | - | - | - | - |
| **13** | Bicyclo [3.1.0]hexane, 4-methyl-1-(1- methylethyl)-, didehydro deriv | * | 927 | 1175 | 1.8 | 1.2 | - | - | 3.1 | 1.7 | 3.2 | 3.7 | 1.6 | 4.2 |
| **14** | 1S-.alpha.-Pinene | M | 934 | 1245 | 7.4 | 3.8 | 2.0 | 2.6 | 14.6 | 13.0 | 14.4 | 22.8 | 24.3 | 22.3 |
| **15** | Cyclohexanone, 4-methylidene- | * | 949 | 1386 | 4.4 | 2.6 | 3.1 | 2.3 | 1.2 | 1.6 | 1.8 | - | - | - |
| **16** | Benzaldehyde | * | 961 | - | 1.8 | 1.6 | 1.9 | 1.1 | 1.8 | 1.7 | 1.8 | 2.1 | 2.5 | 3.1 |
| **17** | beta.-Phellandrene | M | 974 | 1042 | 6.6 | 4.7 | 1.4 | 2.6 | 11.3 | 9.3 | 12.5 | 7.9 | 8.8 | 8.3 |
| **18** | beta.-Pinene | M | 978 | 1683 | 5.3 | 4.2 | 1.3 | 2.1 | 10.9 | 10.1 | 11.9 | 7.4 | 5.8 | 8.6 |
| **19** | beta.-Tujene | M | 991 | 1808 | 3.8 | 4.2 | 1.0 | 1.9 | 4.3 | 4.3 | 5.1 | 5.7 | 2.6 | 5.9 |
| **20** | alpha.-Phellandrene | M | 1006 | 1069 | 2.9 | 2.3 | 1.8 | 1.5 | 3.0 | 2.4 | 3.1 | 3.9 | 2.1 | 3.3 |
| **21** | o-Cymene | M | 1025 | - | 1.2 | 2.1 | 1.5 | 2.1 | 1.9 | 2.3 | 3.0 | 3.2 | 3.2 | 4.0 |
| **22** | Limonene | M | 1029 | 1323 | 6.2 | 6.3 | 3.2 | 3.5 | 7.9 | 5.4 | 6.2 | 8.3 | 8.1 | 8.4 |
| **23** | 2,4-Heptadienal, (E,E)- | * | - | - | 1.0 | 1.3 | - | - | - | - | - | - | - | - |

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| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **24** | Undecane | * | 1045 | 1484 | 1.4 | 1.5 | 1.9 | 1.0 | - | - | - | - | - | - |
| **25** | Gamma Terpinene | M | 1059 | 1646 | 1.7 | 2.0 | - | 1.3 | 1.7 | 1.4 | 1.8 | 9.9 | 9.0 | 8.4 |
| **26** | 1-Octanol | * | 1069 | 1760 | 1.5 | 1.6 | - | - | - | - | - | - | - | - |
| **27** | 2-Furanmethanol, 5-ethenyltetrahydro-.alpha.,.alpha.,5-trimethyl-, Cis | * | 1073 | - | - | - | - | 1.1 | - | - | - | - | - | - |
| **28** | Terpinolene | M | 1089 | 1982 | 1.5 | 1.3 | 2.2 | 1.1 | 1.2 | 1.2 | 1.0 | 2.1 | 1.1 | 1.2 |
| **29** | Linalool | MO | 1100 | 2095 | 2.6 | 2.0 | 2.8 | 2.2 | 1.2 | 1.9 | 2.2 | 2.0 | 2.4 | 3.0 |
| **30** | Nonanal | * | 1104 | 1048 | 2.4 | 2.2 | 1.2 | 1.3 | 1.4 | 2.0 | - | - | - | - |
| **31** | Phenylethyl Alcohol | * | 1114 | - | - | - | 1.6 | 1.6 | - | - | - | - | - | - |
| **32** | 2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis- | MO | 1123 | - | 1.2 | 1.4 | 4.7 | 6.2 | - | - | - | - | - | - |
| **33** | 4-Terpineol | MO | 1180 | 2049 | 9.4 | 6.9 | 7.4 | 8.0 | 3.8 | 2.5 | 3.0 | 6.2 | 4.0 | 4.9 |
| **34** | Alpha Terpineol | MO | 1192 | 2200 | - | - | 1.5 | 1.7 | 2.0 | 2.5 | 2.7 | - | - | - |
| **35** | Di-epi-.alpha.-cedrene-(I) | * | 1481 | 2614 | - | - | 1.7 | 1.1 | - | - | - | - | - | - |
| **36** | 1-Pentadecene | * | 1491 | 2765 | - | - | 1.0 | 1.2 | - | - | - | - | - | - |
| **37** | 2-Fluorobenzyl alcohol | * | 1532 | - | 3.3 | 1.8 | 2.3 | 3.0 | 2.3 | 2.7 | 2.9 | - | - | - |
| **38** | Trans- Nerolidol | SO | 1539 | 2560 | 7.2 | 8.0 | 6.0 | 7.1 | 5.1 | 5.4 | 5.5 | 1.0 | 1.1 | 1.1 |
| **39** | 4-(2,3,4,6-Tetramethylphenyl)-3-buten-2- one | * | 1653 | - | - | - | 1.9 | 2.0 | - | - | - | - | - | - |
| **Total compounds** | | | | | **29** | **29** | **33** | **33** | **26** | **26** | **25** | **18** | **18** | **18** |
| Total identified (%) | | | | | 98.8 | 90.5 | 98.4 | 94.5 | 94.6 | 90.5 | 98.6 | 96.1 | 91.5 | 98.7 |
| **Compound family** | | | | | **Relative quantity (%)** | | | | | | | | | |
| Monoterpene hydrocarbons (M) | | | | | 36.6 | 31.0 | 14.4 | 18.7 | 56.7 | 49.3 | 59.0 | 71.1 | 64.9 | 70.1 |
| Oxygenated monoterpenes (MO) | | | | | 13.2 | 10.3 | 16.3 | 18.1 | 7.1 | 6.9 | 7.9 | 8.2 | 6.4 | 7.9 |
| Sesquiterpene hydrocarbons (S) | | | | | - | - | - | - | - | - | - | - | - | - |
| Oxygenated sesquiterpenes (SO) | | | | | 7.2 | 8.0 | 6.0 | 7.1 | 5.1 | 5.4 | 5.5 | 1.0 | 1.1 | 1.1 |
| Phenol ** | | | | | 11.4 | 9.4 | 19.2 | 18.0 | 2.9 | 3.7 | 1.0 | 3.1 | 7.1 | 3.1 |
| Other compounds * | | | | | 30.4 | 31.8 | 42.5 | 32.6 | 22.8 | 25.2 | 25.3 | 12.6 | 12.0 | 16.4 |

[a] Identification made by: 1) Linear retention indices determined experimentally and compared with databases. 2) Experimental mass spectra (EI, 70 eV), fragmentation pattern analysis and comparison with database mass spectra (Nist 05a.L and Wiley 7n.1). Apolar: Agilent HP-5MS column (5%-phenyl-poly(methylsiloxane). Polar: Agilent HP-Innowax column (100% cross-linked poly(ethylenglycol). EOs was obtained using hydrodistillation (HD), steam distillation (SD), simultaneous distillation- extraction (SDE), and solid-phase microextraction (SPME).

### *3.3. Antimicrobial Activity and Minimum Inhibitory Concentration*

Antimicrobial activity was evaluated using the agar diffusion method using well and disc, and the minimum inhibitory concentration was determined. The agar diffusion method with disc did not show positive results, while the well method was effective. This difference in effectiveness may be due to several factors related to the amount of essential oil used, the diffusion of the active compounds and the nature of the essential oil such as its polarity, size, and molecular shape, among others. Antimicrobial activity was determined in terms of the relative percentage of inhibition, and variability in biological activity was observed depending on the microorganism and the concentration of the oil, but not as a function of the plant organs from which the oils were obtained (**Table 4a**).

The EOs obtained by SD produced the highest antibacterial activity, with 90.7% inhibition of *Bacillus subtilis* at 1215 µg/mL, decreasing to 10.1% at 5.0 µg/mL. In contrast, the EOs obtained by HD and SDE showed lower effectiveness, especially at low concentrations. For example, in HD the inhibition of *Bacillus subtilis* reached 28.5% at 1215 µg/mL and was non-existent at lower concentrations. These results suggest that the extraction technique directly influences the antimicrobial efficacy, probably due to the ability of each method to extract different bioactive compounds, such as monoterpenes and phenols, known for their antimicrobial properties. On the other hand, a concentration dependence was observed, with higher activity at higher concentrations, regardless of the extraction method. For example, in the case of *Staphylococcus aureus*, both in HD and in SD and SDE, inhibitions higher than 30% were observed only at high concentrations (1215 µg/mL and 405 µg/mL), indicating that the antibacterial effectiveness of EOs is dose-dependent. In summary,

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it is highlighted that, although there was no variation in antimicrobial activity related to the plant part (leaves or flowers), the extraction method and concentration are critical factors that determine the efficacy of the EOs against gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*. These results are in agreement with previous studies highlighting the influence of the extraction method on the composition and activity of EOs (Burt 2004; Nazzaro et al. 2013).

**Table 4.a.** Antimicrobial from *Pentacalia vacciniodes* essential oils (µg/mL) obtained by different extraction techniques. Agar diffusion method. *.

| **Relative Percentage of Inhibition (RI, %)** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Microorganism** | **HD-DL, HD-WL - µg/mL** | | | | | | |
| | **1215** | **405** | **135** | **45** | **15** | **5.0** | **1.67** |
| *B. subtilis* | 28.5 ± 4.4 | 21.4 ± 2.2 | - | - | - | - | - |
| *S. aureus* | 34.0 ± 2.2 | 26.2 ± 2.5 | 17.5 ± 2.7 | - | - | - | - |
| *E. coli* | - | - | - | - | - | - | - |
| *P. aeruginosa* | - | - | - | - | - | - | - |
| **Microorganism** | **SD-DL, SD-WL - µg/mL** | | | | | | |
| | **1215** | **405** | **135** | **45** | **15** | **5.0** | **1.67** |
| *B. subtilis* | 90.7 ± 3.8 | 75.2 ± 5.4 | 59.0 ± 7.7 | 37.3 ± 5.4 | 20.9 ± 2.1 | 10.1 ± 1.6 | - |
| *S. aureus* | 35.7 ± 4.1 | 24.5 ± 3.5 | 15.3 ± 2.8 | - | - | - | - |
| *E. coli* | - | - | - | - | - | - | - |
| *P. aeruginosa* | - | - | - | - | - | - | - |
| **Microorganism** | **SDE-WF, SDE-DL, SDE-WL - µg/mL** | | | | | | |
| | **1215** | **405** | **135** | **45** | **15** | **5.0** | **1.67** |
| *B. subtilis* | 73.7 ± 5.2 | 69.9 ± 7.6 | 53.5 ± 6.6 | 37.1 ± 5.4 | - | - | - |
| *S. aureus* | 49.0 ± 2.4 | 39.8 ± 3.7 | 33.6 ± 2.6 | 32.7 ± 2.1 | - | - | - |
| *E. coli* | - | - | - | - | - | - | - |
| *P. aeruginosa* | - | - | - | - | - | - | - |

* When evaluating the antibacterial activity of EOs obtained from different organs of *Pentacalia vaccinioides* (leaves and flowers), no significant variation was observed depending on the part of the plant used. Antimicrobial activity was only observed when a well diffusion method was used. Average ± standard deviation, *n*=6. HD-DL: Hydrodistillation dry leaf. HD-WL: Hydrodistillation wet leaf. SD-DL: Steam distillation dry leaf. SD-WL: Steam distillation wet leaf. SDE-WF: Simultaneous Distillation and Extraction flowers. SDE-DL: Simultaneous Distillation and Extraction dry leaf. SDE-WL: Simultaneous Distillation and Extraction wet leaf. Positive control: Gentamicin (30 µg/mL). Negative control: dichloromethane, dimethylsuffoxide and water, 20 uL of each in well.

The Minimum Inhibitory Concentration (**Table 4b**) of *Pentacalia vaccinioides* EOs shows a variation in antimicrobial efficacy according to the extraction method and the microorganism evaluated. In the case of *Bacillus subtilis* and *Staphylococcus aureus*, low MIC values are observed in the EOs obtained by SD, with a minimum effective concentration of 5.0 µg/mL for *Bacillus subtilis* and 45 µg/mL for *Staphylococcus aureus* in the case of SDE. However, both *Escherichia coli* and *Pseudomonas aeruginosa* showed resistance, with MIC values higher than 1215 µg/mL for all extraction techniques, indicating a low effectiveness of EOs against these gram-negative bacteria. This behavior may be related to the cell membrane structure of gram-negative bacteria, which acts as an effective barrier against many antimicrobial compounds. In particular, it has been shown that the outer membrane of these bacteria prevents the penetration of certain lipophilic compounds present in EOs [39,40]. In addition, *Pseudomonas aeruginosa* is known for its ability to form biofilms and use efflux pumps that expel antimicrobial compounds, which contributes to its resistance [41,42].

**Table 4.b.** The Minimum Inhibitory Concentration (µg/mL).

| **Microorganism** | **HD-DL** | **HD-WL** | **SD-DL** | **SD-WL** | **SDE-WF** | **SDE-DL** | **SDE-WL** |
|---|---|---|---|---|---|---|---|

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *B. subtilis* | 405 | 405 | 5.0 | 5.0 | 45 | 45 | 45 |
| *S. aureus* | 135 | 135 | 135 | 135 | 45 | 45 | 45 |
| *E. coli* | >1215 | >1215 | >1215 | >1215 | >1215 | >1215 | >1215 |
| *P. aeruginosa* | >1215 | >1215 | >1215 | >1215 | >1215 | >1215 | >1215 |

The Minimum Inhibitory Concentration Bactericidal: Average ± standard deviation, n = 6 determinations. HD-DL: Hydrodistillation dry leaf. HD-WL: Hydrodistillation wet leaf. SD-DL: Steam distillation dry leaf. SD-WL: Steam distillation wet leaf. SDE-WF: Simultaneous Distillation and Extraction flowers. SDE-DL: Simultaneous Distillation and Extraction dry leaf. SDE-WL: Simultaneous Distillation and Extraction wet leaf. .

### *3.4. Antioxidant Activity*

The antioxidant activity of the EOs obtained from *Pentacalia vaccinioides* was evaluated by different extraction techniques using the ABTS method, which measures the ability of the compounds to neutralize free radicals. The 50% inhibition concentration ($IC_{50}$) reflects the concentration required to reduce 50% of the ABTS radicals, indicating the antioxidant efficacy of each essential oil. The results obtained are presented in Table 5, where it can be seen that the $IC_{50}$ values vary according to the extraction method and the type of plant organ used.

**Table 5.** Antioxidant activity from *Pentacalia vacciniodes* essential oils (µg/mL) obtained by different extraction techniques – ABTS method. *.

| **Inhibition Concentration - $IC_{50}$ (µg/mL)** | | | | | | |
|---|---|---|---|---|---|---|
| **HD-DL** | **HD-WL** | **SD-DL** | **SD-WL** | **SDE-WF** | **SDE-DL** | **SDE-WL** |
| 633.82 ± 20.98 | 621.62 ± 23.55 | 668.83 ± 21.28 | 658.24 ± 20.42 | 673.39 ± 26.21 | 696.59 ± 25.50 | 682.54 ± 30.03 |

*Average ± standard deviation, n=6. Positive control: Trolox (154.08 ± 3.91 µg/mL), BHT (248.47 ± 6.8 µg/mL), Vitamin E (160.45 ± 5.7 µg/mL); Vitamin C (128.74 ± 4.21 µg/mL). HD-DL: Hydrodistillation dry leaf. HD-WL: Hydrodistillation wet leaf. SD-DL: Steam distillation dry leaf. SD-WL: Steam distillation wet leaf. SDE-WF: Simultaneous Distillation and Extraction flowers. SDE-DL: Simultaneous Distillation-Extraction dry leaf. SDE-WL: Simultaneous Distillation and Extraction wet leaf. .

HD of both dried leaves (HD-DL) and wet leaves (HD-WL) showed outstanding results in terms of antioxidant activity, with HD-WL being the most efficient, with an $IC_{50}$ of 621.62 µg/mL. This value suggests that fresh leaves provide a higher concentration of antioxidant compounds, possibly because the technique better preserves bioactive compounds such as oxygenated terpenes and phenols. HD-DL, with an $IC_{50}$ of 633.82 µg/mL, also showed good activity, although slightly lower. This could be due to the loss of some volatile compounds during leaf drying. HD seems to be a suitable method to maximize the extraction of antioxidant compounds. SD showed a similar trend to HD, although with slightly higher $IC_{50}$ values, indicating lower antioxidant activity. SD-DL presented an $IC_{50}$ of 668.83 µg/mL, while SD-WL was more efficient, with an $IC_{50}$ of 658.24 µg/mL. The difference between fresh and dried leaves in this method suggests that the presence of moisture could help to preserve or extract antioxidant compounds more efficiently. However, compared to HD, SD is less efficient, which could be related to differences in heat transfer and volatilization of certain compounds. The technique, which combines SDE, presented the highest IC50 values, indicating a lower efficiency in the extraction of antioxidant compounds. Both leaves and flowers showed similar results, with $IC_{50}$ of 673.39 µg/mL for fresh flowers (SDE-WF) and 696.59 µg/mL for dried leaves (SDE-DL). Wet leaves (SDE-WL) also presented a relatively high $IC_{50}$ of 682.54 µg/mL. These values indicate that, although this technique is useful for extracting EOs, it is not the most efficient for obtaining antioxidant compounds compared to the other methods. This could be due to the fact that the combination of SDE does not favor the preservation of antioxidant compounds as much. Grouping the results by technique, HD proved to be the most effective technique for extracting antioxidant compounds, especially when using fresh leaves.

SD is also effective, but less than hydrodistillation, while SDE showed the highest $IC_{50}$ values, indicating a lower antioxidant capacity of the oils obtained by this method. These results highlight

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the importance of properly selecting the extraction technique according to the specific objective of the product, as different methods could affect the composition and efficacy of the EOs obtained.

## 4. Discussion

### *4.1. Performance and Physical Parameters*

The yield of EOs, expressed as the mass percentage with respect to the original sample, presented low values in all extractions (**Table 2**). Yields ranged from 0.0012 to 0.0156%, with the highest value recorded for the SDE-WL method (0.0156 ± 0.007%). This behavior reflects the nature of EOs, which are obtained in small quantities due to their volatile character and high concentration of aromatic compounds. The SDE method produced the highest yields compared to HD and SD, being notably more efficient in the extraction of EO's from dry and wet leaves. On the other hand, the SPME method does not provide a sufficient amount of oil to calculate yield values. The density (ρ) of essential oils was relatively uniform in the samples, with values close to 0.8666 ± 0.0030 g/mL. This data is consistent with the literature, as essential oils usually have lower densities than water due to their lipophilic nature and high proportion of terpenes, key components in these oils. The homogeneity in the density values suggests that, despite the different extraction methods used, the volumetric properties of the essential oils are similar.

The refractive index (η), measured at 20°C, showed values close to 1.611 ± 0.002 in the HD, SD and SDE samples. The refractive index is a key property that can be related to the composition and purity of the EOs, providing an indication of its content of terpenes and other volatile aromatic compounds. The similarity in h values indicates that the oils extracted by these methods have comparable optical characteristics. The color in all samples presented a yellow hue. This color may be related to the presence of certain compounds such as carotenoids or oxygenated sesquiterpenes, which contribute shades to the essential oils. The odor of the essential oil in the samples was described as penetrating, with pungent notes and reminiscent of turpentine. These olfactory characteristics are typical of essential oils with high contents of monoterpenes and sesquiterpenes, compounds responsible for the strong and spicy aromas usually found in oils obtained from leaves and resins.

Salt (usually NaCl) is added to water in EOs hydrodistillation processes for several reasons (and SPME): 1) To increase the ionic strength - The addition of salts increases the ionic strength of the aqueous solution, which decreases the solubility of the less polar organic compounds (EOs) in water. This favors their transfer from the aqueous phase to the vapor phase. 2) Saline effect - The presence of saline ions alters the hydrophobic interactions between water and non-polar organic compounds, weakening these interactions and making it easier for essential oils to separate from water. 3) Decrease surface tension - Salts reduce the surface tension of water, which facilitates the formation of vapor bubbles and improves the mass transfer of volatile organic compounds from the sample to the vapor. 4) Increase boiling point - The addition of salts slightly increases the boiling point of the aqueous solution, which allows HD to be carried out at higher temperatures, favoring the recovery of less volatile compounds. 5) Prevent hydrolysis - Some salts, such as NaCl, can inhibit hydrolysis reactions that could degrade sensitive compounds in essential oils during the distillation process. In general, the addition of salts improves the yield and extraction efficiency of essential oils during hydrodistillation by altering the physicochemical properties of the aqueous solution and favoring the transfer of volatile organic compounds to the vapor phase. [43,44].

All the EOs samples evaluated were soluble in 70% (v/v) ethanol. This behavior is characteristic of essential oils, which are a mixture of volatile organic compounds, such as terpenes and their derivatives, which are usually soluble in alcohols. The solubility test is an important indicator of the interaction between the essential oil and polar solvents, which has several implications such as: 1) The solubility of essential oils in ethanol is essential for their use in commercial products, such as perfumes, cosmetics and foods, where ethanol is a common solvent. This ensures that essential oils can mix homogeneously in these matrices without separating or generating turbidity. 2) The fact that all essential oils are soluble in ethanol allows this solvent to be used for dilution in analytical studies or in the preparation of extracts for therapeutic or industrial purposes. Ethanol acts as a good extraction and dissolution medium for volatile compounds. 3) Solubility in ethanol suggests the

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presence of low molecular weight apolar compounds in essential oils, such as monoterpenes and sesquiterpenes, which have good affinity for alcohol. This is relevant in the characterization of oils, as it may give a clue to the nature of the major components present in the samples [45–47].

In summary, the results obtained show that the SDE technique provides the highest yields of EOs, especially in dry and wet leaves. Physicochemical parameters, such as density and refractive index, were consistent in the samples, suggesting a similar chemical composition among the extracted oils. Sensory analysis of odor and color reinforces the presence of characteristic volatile compounds, responsible for the penetrating aromas.

*4.2. Analysis of the Main Bioactive Compounds and Their Industrial and Pharmaceutical Applications*

The following is an analysis of the main compounds identified, highlighting their bioactive properties of both industrial and pharmaceutical interest. These compounds, commonly used in the fragrance, cosmetics, and cleaning products industries, have also demonstrated antimicrobial, antioxidant, and anti-inflammatory properties, which position them as potential candidates for the development of natural therapies and conservation products.

**Phenol** and/or phenols in essential oils are known for their antimicrobial and antioxidant properties. They are found in plants such as oregano, thyme, and cloves, and are used in food preservation, cosmetics, and medicinal treatments for their ability to inhibit the growth of bacteria and fungi. At the industrial level, phenol is key in the manufacture of plastics, resins, and pharmaceuticals, as well as being an effective disinfectant. However, its use involves risks, as it can cause irritation and burns, requiring careful handling. Essential oils rich in phenols are also appreciated in aromatherapy and respiratory treatments for their immunostimulant properties, although they should be used with caution to avoid adverse effects such as irritation or drug interactions. Thus, phenols are valuable in both industry and medicine, with applications ranging from preservation to natural therapies [48–50].

**1S-α-pinene** is a monoterpene present in essential oils from conifers such as pine trees and plants such as rosemary. Recognized for its fresh and resinous aroma, it has been used in fragrances, disinfectants, and cleaning products due to its antimicrobial properties. In industry, it is used as a chemical precursor for the synthesis of compounds such as linalool and camphene, used in cosmetics and food. In medicine, α-pinene has demonstrated anti-inflammatory, antioxidant, and anticancer properties, being investigated for respiratory treatments and as a modulator of the nervous system. Although generally safe in low concentrations, it can cause skin and eye irritation, and in large quantities affects the respiratory system. It is flammable and toxic to aquatic organisms, which requires precautions in its industrial handling. These characteristics make it widely used, but always with special care due to its toxicity and volatile properties [51–53].

**β-phellandrene** is a cyclic monoterpene found in essential oils of various plants such as eucalyptus, lavender, peppermint, and pine. Historically, it has been used primarily in products requiring fresh scents, such as fragrances, cleaning products, and some cosmetics because of its characteristic odor that blends woody and minty notes. In traditional medicine, it has been used in oriental medicine to treat digestive disorders and respiratory problems. It has also been found in small amounts in some types of cannabis and medicinal plants, where therapeutic properties are attributed to it, such as anti-inflammatory, analgesic, and possible anti-cancer effects. Recent studies indicate that it could inhibit the growth of tumor cells in the liver, although more research is needed to confirm these results. At the industrial level, it is used with caution due to its toxicity in large concentrations. It can cause skin and eye irritation, and prolonged inhalation may affect the respiratory system. It is also toxic to aquatic life, so it is important to handle it properly in its production and industrial use [54–56].

**β-pinene** is a monoterpene present in essential oils of many plants, especially in conifers such as pines. Discovered by Adolf von Baeyer in 1896, it is essential for the natural defense of plants against herbivores and pathogens. Its industrial applications include the production of fragrances, flavors, and cleaning products due to its characteristic pine aroma. It is also a key precursor in the manufacture of derivative products such as myrcene and nopol, used in cosmetics and flavoring. In

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the pharmaceutical field, β-pinene stands out for its antimicrobial, anti-inflammatory, antioxidant, and anticancer properties, showing promise for the development of natural therapies, especially against antibiotic-resistant bacteria (Staphylococcus aureus MRSA). It has also shown neuroprotective potential, in combination with compounds such as THC and limonene, to treat cognitive disorders such as Alzheimer's disease. However, in high concentrations it can be toxic, causing irritation and respiratory risks, as well as being dangerous for the environment [52,53,57].

**4-terpineol** is a monoterpene and a major component of tea tree oil (*Melaleuca alternifolia*). It has been investigated for its broad medicinal properties and industrial applications. It is known for its antimicrobial capacity, especially against antibiotic-resistant bacteria such as Staphylococcus aureus and Pseudomonas aeruginosa, making it a valuable component in pharmaceuticals and cosmetics. In addition, it has been shown to be effective against fungi and other microorganisms, with applications in skin products such as antiseptic creams and in treatments for eye infections. This compound is also used in cosmetic products, such as soaps and perfumes, due to its ability to eliminate bacteria without being irritating at low concentrations. However, at high concentrations it can be toxic, causing skin and eye irritation, so it is important to handle it properly [58–60].

**γ-terpinene** is a monoterpene present in plants such as tea tree, oregano, thyme, and citrus. It is part of the essential oils of several aromatic plants and is known for its bioactive properties. It is used in the food and cosmetics industry for its fresh and citrus aroma, and in the production of soaps and cleaning products. In the pharmaceutical industry, it is valued for its antioxidant, anti-inflammatory, and antimicrobial properties, making it a promising compound for medicinal and natural products. Studies have shown that it has potent antioxidant activity, helping to prevent free radical damage and reducing inflammation. It has also demonstrated the ability to inhibit the growth of bacteria and fungi. Although generally safe in low concentrations, prolonged exposure can cause irritation and is toxic to aquatic life, requiring careful handling [61□63].

**trans-Nerolidol** is a sesquiterpene alcohol present in essential oils from plants such as tea tree, ginger, lavender, and cannabis. This compound stands out for its floral and fruity aroma, which makes it useful in the fragrance and cosmetics industry. It is also used as a food additive due to its ability to enhance flavors and as a component in skin care products, aiding the penetration of topical agents. From a medical point of view, it has shown a number of therapeutic properties. Antimicrobial, antifungal, neuroprotective and anticancer effects have been attributed to it. For example, studies have shown its ability to induce cell death in liver and colon cancer lines, as well as to enhance the effect of anticancer drugs such as doxorubicin. Its possible use in the treatment of neurological disorders and as a natural sedative has also been investigated, showing anxiolytic effects in animal models. In terms of toxicity, it is relatively safe at low concentrations, although further clinical studies are still required to determine long-term effects and its interaction with other drugs [64–66].

**Limonene** is a cyclic monoterpene found mainly in the peels of citrus fruits, such as lemon and orange, and is responsible for their characteristic fresh, citrus aroma. It is one of the most abundant terpenes in nature and has been widely used in various industries. Limonene is highly valued in the food and cosmetics industry for its aromatic properties and is used as an additive in food, beverages, and cleaning products. In the pharmaceutical field, it has been studied for its antioxidant and anti-inflammatory properties, and as a chemopreventive agent, showing efficacy in cancer prevention in preliminary studies. It is also used in environmentally friendly cleaning products due to its ability as a natural solvent. Although limonene is considered safe for human consumption and is classified as GRAS (Generally Recognized as Safe) by the FDA, it can cause dermal and eye irritation at high concentrations. It is also a skin sensitizer, which means that repeated use on the skin can lead to allergic reactions in some people. In addition, it is known that, in prolonged contact with light and air, limonene can oxidize and form compounds that are more irritating. In terms of environmental impact, limonene is slightly toxic to aquatic organisms, so it should be handled with caution in large quantities. However, its overall low toxicity makes it a favorable choice as an alternative to synthetic solvents in industrial applications [67–70].

**β-thujene** is a bicyclic monoterpene found in essential oils of various plants, such as conifers and oregano. Although less common than its isomer *α*-tujene, it has important applications in several

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industries due to its chemical structure and properties. At the industrial level is used as a precursor in the synthesis of fragrances and aromatic compounds, taking advantage of its fresh, woody, and penetrating aroma. In addition, it is also found in certain cleaning products and natural flavorings. In terms of its biological properties, has shown antimicrobial potential, suggesting its usefulness in skin care products and disinfectants. However, studies on its therapeutic effects are still limited. Its toxicity is not fully determined, but like many monoterpenes, it can be irritating to the skin and respiratory system at high concentrations. In addition, its impact on aquatic organisms requires proper handling in industrial environments [71–73].

*4.3. Comparison of Extraction Techniques*

The extraction techniques used had a significant influence on the composition and concentration of the compounds (**Table 3**). Significant differences were observed in the number of compounds identified and in the relative abundance of chemical groups. By **HD**, between 29 and 33 compounds were identified, with a predominant profile of monoterpene hydrocarbons (36.6% in fresh leaves, and 31.0% in dry leaves). This method showed high concentrations of compounds such as α-pinene (7.4% in fresh leaves) and phenol (11.4% in fresh leaves), highlighting the ability of HD to extract monoterpenes and phenols. Using **SD** (similar to HD) up to 33 compounds were identified, but with a higher concentration of phenols (19.2% in dry leaves). A decrease in the amount of monoterpene hydrocarbons (14.4%) was observed compared to HD, suggesting that steam may be less efficient in the extraction of these compounds in certain cases. **SDE** was the most efficient technique in the extraction of monoterpene hydrocarbons (56.7% in fresh leaves and 59.0% in flowers). It stands out for the high concentration of α-pinene (14.6% in fresh leaves), β-pinene (10.9% in fresh leaves), and β-Phellandrene (12.5% in fresh flowers) indicating that SDE is particularly effective in extracting volatile terpenes. However, it presented a lower amount of phenols (2.9% in fresh leaves). **SPME** showed a more specific profile, with a lower number of compounds identified (up to 18), and a high concentration of monoterpene hydrocarbons (71.1% in fresh flowers). This method proved to be the most selective for volatile compounds such as α-pinene (22.8% in fresh leaves) and γ-Terpinene (9.9% in fresh leaves), but less efficient for extracting oxygenated compounds and sesquiterpenes.

Among the advantages and disadvantages of the extraction techniques used to obtain EOs are: **1) HD** consists of distilling plant material submerged in water and then collecting the essential oil. Advantages: a) It is a traditional and relatively simple technique that does not require sophisticated equipment; b) It is economical and can be performed on a small scale, suitable for commercial applications or research; c) The water acts as a barrier that protects the volatile components from overheating. Disadvantages: a) As observed in the data (**Table 2**), the yields (% m/m) of EOs are low, especially when this method is employed, which reduces its efficiency; b) Although water partially protects, some heat-sensitive compounds may decompose during prolonged distillation. **2) SD** uses water vapor to carry away volatile compounds from the plant matrix without immersing the plant in water. Advantages: a) The use of steam minimizes the contact of the material with water, which helps to better preserve some volatile compounds that are soluble in water; b) It is more efficient in large-scale operations, which makes it suitable for industrial applications. Disadvantages: a) Tighter control of temperature and steam flow is necessary to avoid degradation of volatile compounds; b) Requires more sophisticated equipment than hydrodistillation, which can increase operating costs. **3) SDE** combines solvent extraction and distillation to obtain EOs. Advantages: a) As observed in the results, this method tends to generate higher yields than HD and SD, making it more efficient in the extraction of essential oils; b) The combination of solvent and distillation reduces the loss of volatile compounds, ensuring higher recovery of aromatic components. Disadvantages: a) The need for solvents can increase costs and also raises concerns about residues and purity of the final product; b) This method is more complex than the previous ones, requiring specialized equipment and trained personnel. **4) SPME** involves adsorption of volatile compounds onto a solid fiber, followed by desorption and analysis. Advantages: a) It allows detection of compounds at very low concentrations, which makes it ideal for trace analysis of volatile compounds; b) Unlike SDE, no solvents are needed, which reduces the risk of contamination of the essential oil and facilitates the preparation of the extract; c)

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It is faster than others and can be performed with very small amounts of sample. Disadvantages: a) As observed in the data, this method does not provide sufficient quantity of EOs for physicochemical measurements, which limits it mainly to analytical purposes, rather than for the collection of large quantities of oil; b) It requires specific equipment for handling and desorption of the fibers, which may increase operating costs; c) It requires specific equipment for handling and desorption of the fibers, which may increase operating costs; d) It does not provide sufficient quantity of essential oil for physicochemical measurements, which limits it mainly to analytical purposes, rather than for the collection of large quantities of oil.

The distribution of chemical groups within the essential oil of *Pentacalia vaccinioides* provides key insights into its bioactive and therapeutic profile. The high proportion of monoterpene hydrocarbons, particularly in SPME and SDE techniques, highlights the importance of these compounds in the antimicrobial and antioxidant properties of the oil. Oxygenated monoterpenes, which play critical roles in anti-inflammatory and relaxant actions, present significant variations among extraction methods, suggesting that some techniques, such as HD and SD, are more efficient in extracting these compounds. On the other hand, the presence of oxygenated sesquiterpenes and phenols, although in smaller proportion, adds important therapeutic value, particularly due to their antiseptic and antioxidant properties. These results suggest that the choice of extraction method has a direct impact not only on the chemical composition, but also on the functionality and potential application of the essential oil in different medicinal and cosmetic contexts.

In summary, each extraction method has specific advantages and disadvantages that make it suitable for different objectives. If the objective is to obtain large quantities of EOs, SDE is the most efficient technique, although it is more complex and requires the use of solvents. On the other hand, if simplicity and low cost are sought, HD remains a viable option, although with lower yield. For analytical purposes, SPME is highly effective, although it does not produce enough material for large-scale studies. Finally, SD balances efficiency and protection of volatile compounds, being a preferred option in industrial applications. The extraction method and the plant part used are determining factors in the chemical composition of *Pentacalia vaccinioides* EOs. HD and SD provide a more balanced extraction of monoterpene and oxygenated compounds, while SDE is more efficient for the extraction of monoterpene hydrocarbons. SPME, on the other hand, specializes in the extraction of volatile compounds. These results provide valuable information for the selection of the most suitable extraction method, depending on the desired chemical profile and the application of the essential oil in the cosmetic, pharmaceutical, or food industry.

### *4.4. Antimicrobial Activity*

The evaluation of the antimicrobial activity of substances against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* is performed because they represent a diverse group of microorganisms with different biological characteristics, allowing a broader understanding of the effectiveness of antimicrobial compounds [74]. *Bacillus subtilis* is a gram-positive bacterium that is commonly used as a model in antimicrobial studies due to its sensitivity to a wide variety of agents. Evaluating its inhibition provides insight into the efficacy of compounds against sporulating gram-positive bacteria, which are relevant in environmental and food contamination [75]. *Staphylococcus aureus*, also gram-positive, is an important pathogen in human infections, especially due to the emergence of antibiotic-resistant strains, such as MRSA (methicillin-resistant *S. aureus*). Proving effectiveness against S. aureus is crucial for the development of treatments against skin, systemic, and drug-resistant infections [76]. *Escherichia coli* is a gram-negative bacterium that is part of the intestinal flora, but some strains can cause serious infections, such as diarrhea or urinary tract infections. Since gram-negative bacteria have a more complex cell membrane, it is more difficult for antimicrobial agents to penetrate it, so it is important to evaluate the effectiveness of compounds against this type of pathogen [77]. *Pseudomonas aeruginosa* is a gram-negative pathogen notorious for its resistance to antibiotics and its ability to form biofilms. It is an opportunistic organism, often responsible for infections in immunocompromised individuals or in hospital settings. Assessing its susceptibility is essential to develop effective agents against hospital-acquired and multidrug-resistant infections [78].

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In general, the use of substances with proven antimicrobial activity improves consumer safety and product effectiveness.

In this study, the results obtained using the well diffusion method showed clear effectiveness in inhibiting bacterial growth, while the disc method showed no detectable antimicrobial activity. This difference may be attributed to the fact that the well method allows a larger volume of EOs to be applied directly, whereas paper disks generally absorb a much smaller amount of oil, which may require repeated application to achieve the desired volume. In the well method, the active compounds of the essential oil can penetrate deeper into the agar, creating a more effective diffusion gradient. In contrast, in the disk method, the active compounds may not diffuse efficiently from the disk into the culture medium. The volatile compounds of the EOs may evaporate more quickly from the paper disk due to its greater exposure to air, whereas the well method allows a greater amount of compounds to remain in direct contact with the agar, favoring their diffusion into the medium. In addition, some components of the EOs could form intermolecular interactions, such as hydrogen bridges or dipole-dipole interactions with the cellulose molecules (the main component of the paper discs), which could delay or prevent the diffusion of the compounds from the disc to the agar; in contrast, in the well method, the contact with the agar is more rapid [33,79].

Antimicrobial activity was determined in terms of the relative percentage of inhibition (**Table 4**), and variability in efficacy was observed depending on the microorganism and the concentration of the oil. As for the microorganisms, *Bacillus subtilis* showed sensitivity to the EOs obtained with all extraction techniques, presenting the highest inhibitory activity with the SD technique, followed by SDE and HD. *Staphylococcus aureus* was also inhibited by the EOs, especially noticeable in those obtained by SDE. *Escherichia coli* and *Pseudomonas aeruginosa* did not show antimicrobial susceptibility to EOs at any of the concentrations tested for these bacteria. This behavior can be explained by several characteristics of gram-negative bacteria: 1) They have an additional outer membrane that acts as a protective barrier, limiting the penetration of antimicrobial compounds such as EOs. This membrane contains lipopolysaccharides, which makes it difficult for the more lipophilic compounds, typical of the EOs, to pass through this layer and reach the bacterial cell to exert their effect. This barrier is one of the main reasons why essential oils tend to be more effective against gram-positive bacteria lacking this additional external structure [80]. 2) They express efficient efflux pumps, such as the AcrAB-TolC system in Escherichia coli, which expel antimicrobial compounds from the cell before they can reach toxic concentrations. These pumps allow bacteria to rapidly eliminate EOs or their compounds before they succeed in inhibiting their growth [41,81]. 3) They possess adaptive mechanisms that allow them to survive in the presence of antimicrobial agents. *Pseudomonas aeruginosa*, for example, can form biofilms, structures that protect bacteria within them from antimicrobial attack. It has been observed that some EOs may have difficulty penetrating these biofilms, which decreases their efficacy [82].

Gentamicin was used as a positive control. This molecule is an aminoglycoside antibiotic that exerts its bactericidal action mainly by interfering with protein synthesis in bacteria. Its specific mechanism of action corresponds to: 1) It irreversibly binds to the 30S subunit of the bacterial ribosome, which interferes with the reading of messenger RNA (mRNA). 2) It causes incorrect translation of the mRNA, leading to the incorporation of incorrect amino acids into the polypeptide chain, generating defective or non-functional proteins. This process alters the vital cellular functions of the bacterium. 3) It inhibits the synthesis of proteins necessary for bacterial survival, causing cell death, thus exerting its bactericidal effect. 4) It can alter the permeability of the bacterial cell membrane, allowing the entry of more gentamicin and other antibiotics, which enhances its destructive effect on bacterial cells. Gentamicin is effective mainly against gram-negative bacteria such as *Escherichia coli*, *Klebsiella*, *Pseudomonas aeruginosa*, and some gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* [83–86].

Interest in the antimicrobial properties of EOs has increased due to the possibility of finding an alternative to respond to consumer demands in the use of natural additives in food, cosmetics, and pharmaceuticals and to the resistance that microorganisms have presented to antibiotics. A wide variety of EOs possess antimicrobial properties and in many cases this activity is due to the presence of active constituents, such as monoterpenes, sesquiterpenes and alcohols, other hydrocarbons, and

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some phenols. The lipophilic character of the hydrocabide skeleton and the hydrophilic character of their functional groups are their main importance in the antimicrobial action. Thus, the following range of antimicrobial activities has been proposed: phenols > aldehydes > ketones > alcohols > esters > hydrocarbons [87,88]. The antimicrobial activity is recognized in EOs such as oregano, lemongrass, thyme, sage, rosemary, clove, coriander garlic, and onion among others and in their individual compounds such as carvacrol, thymol, citral, eugenol, 1-8 cineol, limonene, pinene, linalool and other precursors [89,90]. Lemongrass EOs possesses considerable amounts of citral, citronellal, citronellal, linalool and geraniol which have been shown to possess antimicrobial activity against *Escherichia coli*, *Bacilluis subtilis* and *Staphylococcus aureus* [91,92]. Another example is the EOs of *Melaleuca alternifolia* (Tea tree) which is composed, among other things of terpineol, linalool and terpinen-4-ol and has been shown to have high antimicrobial effectiveness [93,94]. The effectiveness of the compounds isolated from the EOs has been tested, however, it has been evident that the EOs have more biocidal strength when found with their minority components, as these provide a synergistic effect in activity [89]. The combination of citral with vanillin, thymol, carvacrol or eugenol presented synergistic effects to inhibit the growth of *Zygosaccharomyces bailii* [95]. Synergistic activity between carvacrol and thymol and carvacrol and cymene have also been reported [87,96–98]. Some investigations of the two major chemical constituents of *Osmitopsis asteriscoides*, (1,8-cineole and camphor), both independently and in combination showed that the synergy between them has a high antimicrobial effect on *Candida albicans* than when tested independently [99].

The mechanism of action of natural preservatives in antimicrobial activity is not fully explained, but it is proposed that their main site of action is the cell membrane. Terpenoids, lipophilic compounds, disrupt the integrity and permeability of the cell membrane [100,101]. Phenolic terpenoids, such as carvacrol and thymol, have been shown to cause loss of membrane integrity, leading to ion leakage, which affects both gram-positive and gram-negative bacteria. $K^+$ ion leakage is a signal of cell damage, followed by migration of cytoplasmic constituents. For example, terpinene-4-ol inhibits oxidative respiration and causes membrane swelling, increasing its permeability [102,103]. Several studies have pointed out that terpenoids and phenylpropanoids, such as carvone and carvacrol, induce leakage of ions and other cellular components, which decreases bacterial viability and generates membrane alterations [104]. Compounds such as thymol, eugenol and carvacrol not only disrupt the cell membrane, but also inhibit ATPase activity, releasing intracellular ATP. Cinnamaldehyde, on the other hand, increases membrane permeability, causing leakage from the cytoplasm and inhibiting key enzymes in amino acid biosynthesis. In addition, thymol binds to membrane proteins hydrophobically, altering their permeability, while terpenes accumulate in the membrane, causing loss of integrity and dissipation of proton motive force [105]. The effectiveness of EOs and their active compounds in inhibiting microorganisms depends on factors such as pH, chemical structure, concentration, compound activity, type of microorganism, and storage conditions. At low pH, the hydrophobicity of the compounds increases, favoring their antimicrobial action. The concentration thresholds of the EOs required for inhibition vary according to the antimicrobial substance and the mechanisms involved, which include the cell wall and membrane, metabolic enzymes, and genetic systems. The use of combinations of antimicrobial agents is often more effective, as some microorganisms are not susceptible to common doses of a single antimicrobial [105].

### *4.5. Antioxidant Activity*

Assessing the antioxidant activity of EOs is of great importance in various fields such as the food, pharmaceutical, cosmetic, and medical industries, due to their ability to neutralize free radicals and prevent cell damage [106]. Antioxidants are essential because they help protect cells from oxidative stress, which is linked to premature aging and various chronic diseases, including cardiovascular disease, cancer, diabetes, and neurodegenerative disorders such as Alzheimer's disease [107].

In preventing cell damage, EOs may contain compounds such as phenols, terpenes, and flavonoids that act as natural antioxidants. These compounds can neutralize free radicals, thus preventing damage to cell membranes, proteins, and DNA. This is key for the prevention of

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degenerative diseases and cellular aging [108]. In shelf-life extension of products in the food industry, natural antioxidants, such as essential oils, are used to extend the shelf life of foods by preventing oxidation of fats and oils, which delays rancidity. This is also relevant in cosmetic products, where antioxidants help stabilize formulations and prevent degradation of active ingredients [109]. In the pharmaceutical industry, EOs with antioxidant properties can be used as therapeutic agents to treat diseases related to oxidative stress. Evaluating antioxidant activity allows the identification of substances with potential for the development of new drugs or supplements [110]. In cosmetics and natural medicine, antioxidant-rich EOs are valued for their protective properties for the skin, preventing damage caused by UV radiation and pollution. Assessing antioxidant activity is key to selecting the most effective ingredients in the formulation of cosmetic and dermatological products [111].

Antioxidant activity is evaluated by methods such as ABTS, DPPH and FRAP, among others, which measure the capacity of one or several compounds to neutralize free radicals or reduce metals. ABTS is used to evaluate the antioxidant capacity of EOs, due to its versatility and applicability in both hydrophilic and lipophilic media. This is relevant for EOs, as they contain both polar and apolar compounds. Unlike other methods, such as DPPH, which is more selective for compounds soluble in organic solvents, ABTS can be used in a wide range of solvents, making it more flexible for evaluating EOs containing complex mixtures of terpenes, flavonoids, and phenols. In addition, the ABTS method is more sensitive, less time-consuming, less expensive, and offers better solubility in both polar and non-polar media, which is a great advantage for the evaluation of hydrophilic and fat-soluble antioxidants [31].

In a study by Sharopov et al. (2015), the antioxidant activity of 18 components of EOs was evaluated using the ABTS method, among others. The results showed that phenolic compounds such as carvacrol, thymol and eugenol presented the highest antioxidant activity, which underlines the importance of these compounds in neutralizing free radicals and preventing oxidative damage [112]. On the other hand, Wang et al. (2020) performed a comparison of the antioxidant activity of 45 common essential oils, finding that cinnamon leaf and clove bud oils, rich in eugenol, were the most effective in terms of antioxidant capacity, with more than 96% ABTS radical scavenging. This study confirmed that oils high in phenolic compounds, such as eugenol, are particularly effective in the fight against oxidation [113]. Finally, Noriega et al. (2023) focused their research on essential oils from Ecuadorian medicinal plants, highlighting those from *Mollinedia mollis* and *Alnus glutinosa*, which showed high antioxidant capacity in the ABTS assay. In this case, thymol and carvacrol were identified as the main responsible for this antioxidant activity, reinforcing the findings of previous studies on the efficacy of these phenolic compounds [114]. These studies together highlight the importance of phenolic compounds present in essential oils for antioxidant activity and their potential application in various industries such as food, pharmaceuticals, and cosmetics. [112–114].

In our work, when comparing the results of the antioxidant activity with the chemical composition of the EOs of *Pentacalia vaccinioides*, a relationship was observed between the presence of certain groups of compounds and the antioxidant capacity of the EOs. The essential oils evaluated showed an antioxidant capacity measured by $IC_{50}$, which varied according to the extraction technique and the plant organs used. However, this behavior can be explained in part by the variation in chemical composition observed in these oils. Monoterpenes, such as $\alpha$-pinene, $\beta$-pinene, and limonene, constituted the largest proportion of the essential oils in most extraction techniques, especially in SDE extraction. These compounds are known for their moderate antioxidant capacity [115]. However, their antioxidant efficacy is generally lower compared to phenolic compounds and oxygenated terpenes, which may explain the higher $IC_{50}$ values (i.e., lower antioxidant activity) observed in samples extracted by SDE. The $IC_{50}$ values in these methods ranged from 673.39 to 696.59 µg/mL, indicating a lower capacity to neutralize free radicals. EOs with a higher proportion of phenolic compounds and oxygenated terpenes, such as linalool and 4-terpineol, showed higher antioxidant activity. These compounds are potent antioxidants due to their ability to donate electrons and neutralize free radicals [116]. The presence of phenols, which was especially notable in the EOs obtained by hydrodistillation (HD-DL and HD-WL), correlates with the lowest $IC_{50}$ values (633.82

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μg/mL for HD-DL and 621.62 μg/mL for HD-WL). These results indicate that HD techniques are more effective in extracting compounds with high antioxidant capacity. Although in smaller amounts, oxygenated sesquiterpenes, such as trans-nerolidol, also play a role in the antioxidant activity of essential oils. These compounds were found in higher concentrations in the samples extracted by SD, where the antioxidant activity was moderate, with $IC_{50}$ of 668.83 μg/mL (dry leaves) and 658.24 μg/mL (wet leaves). The contribution of oxygenated sesquiterpenes could slightly improve the antioxidant capacity, although their effect is less pronounced compared to oxygenated phenolic compounds and terpenes [117]. In summary, the chemical composition of *Pentacalia vaccinioides* EOs directly influences their antioxidant capacity. EOs with a higher concentration of phenolic compounds and oxygenated terpenes, such as those obtained by HD, have a higher antioxidant activity. On the other hand, oils rich in monoterpenes, extracted mainly by SDE, have a lower antioxidant capacity, which is reflected in their higher $IC_{50s}$. This highlights the importance of selecting the appropriate extraction method to optimize the antioxidant activity of EOs according to their chemical composition.

Some studies on EOs have elucidated the activity of their different components. For example, γ-terpinene retards the peroxidation of linoleic acid, while sabinene has shown a strong ability to scavenge free radicals. The α-pinene and limonene, on the other hand, showed low antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test. In contrast, terpinene and terpinolene exhibited high hydrogen-donating capacity against the DPPH radical. In addition, citronellal evidenced a strong protective activity in lipid peroxidation processes, highlighting its effect on free radical scavenging. These antiradical effects have also been described for neral and geranial [87,118]. In many cases, the antioxidant activity of EOs cannot be attributed exclusively to the majority compounds, as minority compounds can play a significant role. Similarly, synergistic effects have been reported. For example, in the EOs of the species *Melaleuca teretifolia*, containing 1,8-cineole (34%) and terpinen-4-ol (19%), the latter showed higher antioxidant activity than those oils with a high content of methyleugenol (97%) or 1,8-cineole (64.30%) [87,119].

Currently the most widely used antioxidants to prevent oxidation and prolong the stability of chemical, cosmetic, pharmaceutical, and food products are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tertiary butyl hydroquinone (TBHQ), tocopherols ($\delta > \gamma > \beta > \alpha$), ascorbic acid, lipoic acid, resveratrol, niacinamide (vitamin B3), superoxide dismutase (SOD), glutathione, N-acetylcysteine (NAC), Coenzyme Q10, flavonoids, phenolic acids, among others [120–122]. Some of these compounds are under debate about their safety, as some research shows evidence of liver damage, tumorogenic potential, carcinogenic, cytotoxic, and endocrine disrupting effects, which is why the demand for natural antioxidants, especially of plant origin, has increased in recent years [123–126]. Natural antioxidants have the following advantages: 1) They are accepted by consumers, 2) They are considered safe, 3) They do not require safety testing because they are already found in foods, 4) They have functional properties, and 5) They are sensorially accepted [127]. As a natural source of antioxidants, EOs have gained recognition as potentially safe additives to preserve, prolong shelf life, and improve the quality of food products, today it is possible to find them commercially in the category DMC Natural base (mixture of extracts rich in polyphenols, flavonoids, organic acids, and other natural products, with antioxidant properties, for example: 50% EOs of rosemary, sage, and citrus and 50% glycerol) [128]. Although their use has increased, they have been limited by altering the organoleptic properties of food products; however, recently, encapsulation techniques have been found that use various surfactants to overcome such problems [129], as well as the use of deodorization processes.

It has been demonstrated [130–132] that natural plant extracts with high contents of polyphenolic compounds have an antioxidant potential comparable, and in some cases superior to that of synthetic preservatives, which is why there is currently a growing trade of this type of products in the cosmetic and food industry. Regardless of the origin of antioxidants, for their incorporation into final products, some basic requirements must be observed [109,121,133,134]: 1) They must be effective to provide sufficient degree of protection at low concentration levels; 2) They must be compatible with the ingredients and components of the final formulations; 3) They must be

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easily soluble or dispersible in the medium, and easy to apply; 4) They must be stable to pH variations or changes under processing conditions. This stability must be maintained during the shelf life of the products; 5) They must be free of objectionable or unacceptable colors and odors; 6) They must not be toxic or irritating, and their oxidation products must not be harmful, nor have physiological activity; 7) They must not constitute a risk for consumers, and must have a recognized toxicological support for their use in products for human use; 8) They must be low cost and available.

### *4.6. Correlation Between Antioxidant and Antimicrobial Activities of Essential Oils: Significance and Applications*

The relationship between the antioxidant and antimicrobial activities of *Pentacalia vaccinioides* EOs highlights their potential as multifunctional natural agents. Both types of activity rely heavily on the presence of bioactive compounds such as oxygenated phenols and terpenes, which not only neutralize free radicals, but also disrupt bacterial cell membranes, resulting in inhibition of microbial growth. This duality is especially useful in the food, cosmetic, and pharmaceutical industries, where protection against oxidation and microbial control are essential for product safety and effectiveness. The use of EOs with these properties improves food safety by preventing oxidation and limiting the growth of pathogens, while in cosmetics, they protect the skin from oxidative damage and reduce the risk of skin infections. Likewise, in pharmaceutical applications, the discovery of compounds that combine both activities is crucial in the fight against antimicrobial resistance, opening up new possibilities in the development of natural and effective therapies.

### *4.7. Strengths and Weaknesses of this Study*

The present work has several strengths that make it stand out; it is the first study to investigate the antioxidant and antimicrobial activities of *Pentacalia vaccinioides* EOs. This provides new knowledge on a plant species that had not previously been evaluated for these properties, thus expanding the potential use of this natural resource. In addition, the methodology employed is robust, using different extraction techniques to obtain essential oils, allowing comparison of how bioactive properties vary depending on the method and part of the plant used. The use of widely recognized methods for measuring antioxidant (ABTS) and antimicrobial (well and disk diffusion) activity ensures the reliability and reproducibility of the results. However, this work also presents some weaknesses. One of the main limitations is that an analysis of the molecular mechanisms behind the observed antioxidant and antimicrobial activities was not performed. Although the presence of certain bioactive compounds in the EOs is identified, more detailed studies would be beneficial to identify how these compounds act at the cellular level. In addition, the evaluation of antimicrobial activity was limited to a small number of microorganisms, which restricts the generalizability of the results. Including a greater diversity of bacteria, fungi, and yeasts could provide a more complete picture of the antimicrobial potential of essential oils. In summary, this study lays a foundation for future research on *Pentacalia vaccinioides*, but also points to areas that require further investigation, such as the study of molecular mechanisms and the expansion of the spectrum of microorganisms evaluated.

## 5. Conclusions

This study represents the first work on *Pentacalia vaccinioides* in which its EOs are evaluated in terms of biological activities as antioxidants and antimicrobials. The results show that the method of extraction and the state of the plant organs (fresh or dried) significantly influence the chemical composition and thus the biological effectiveness of the oils. EOs obtained by HD, especially from fresh leaves, showed higher antioxidant activity, reflected in lower $IC_{50}$ values. This is due to the higher concentration of oxygenated phenols and terpenes in these samples. On the other hand, SD and SDE were less efficient in the extraction of antioxidant compounds, reflected in their higher $IC_{50}$s. As for antimicrobial activity, EOs obtained by HD and SD were more effective against gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*. However, both *Escherichia coli* and *Pseudomonas aeruginosa*, gram-negative bacteria, showed significant resistance to the evaluated oils,

which may be due to the structure of their cell membranes, which act as a protective barrier against antimicrobial compounds.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure 1: Chromatogram (TIC) of the essential oil of *Pentacalia vacciniodes* (Asteraceae). A) HP-5MS column (60 m x 0.25 mm, 0.25 mm). B) HP-Innowax column (60 m x 0.25 mm, 0.25 mm). Split 1:50.

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