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Isolation and Identification of Flavones Responsible for the Antibacterial Activities of *Tillandsia bergeri* Extracts

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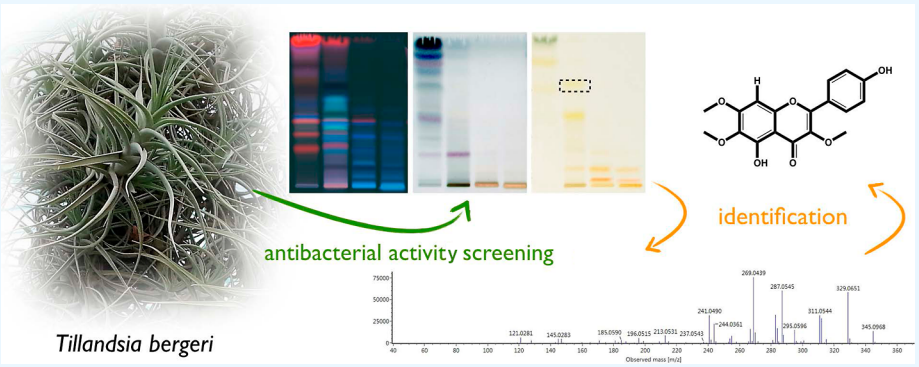

ABSTRACT: Plants are an everlasting inspiration source of biologically active compounds. Among these medicinal plants, the biological activity of extracts from some species of the *Tillandsia* genus has been studied, but the phytochemistry of the hardy species *Tillandsia bergeri* remains unknown. The aim of the present study was to perform the first phytochemical study of *T. bergeri* and to identify the compounds responsible for the antibacterial activity of *T. bergeri* extracts. Soxhlet extraction of predried and grinded leaves was first performed using four increasing polarity solvents. A bio-guided fractionation was performed using agar overlay bioautography as a screening method against 12 Gram-positive, Gram-negative, sensitive, and resistant bacterial strains. The results showed the inhibition of Gram-positive methicillin-sensitive *Staphylococcus aureus* ATCC 29213 (MSSA), methicillin-resistant *S. aureus* N-SARM-1 (MRSA), and *Staphylococcus caprae* ATCC 35538 by the dichloromethane fraction. A phytochemical investigation led to the isolation and identification by high-resolution mass spectrometry and nuclear magnetic resonance of the two flavones penduletin and viscosine, responsible for this antibacterial activity. For viscosine, the minimum inhibitory concentration (MIC) value is equal to 128 μg/mL against MSSA and is equal to 256 μg/mL against MRSA and *S. caprae*. The combination of these compounds with vancomycin and cloxacillin showed a decrease in MICs of the antibiotics. Penduletin showed synergistic activity when combined with vancomycin against MSSA (FICI < 0.258) and *S. caprae* (FICI < 0.5). Thus, unexplored *Tillandsia* species may represent a valuable source for potential antibiotics and adjuvants.

INTRODUCTION

Natural products from plants have long been known to inspire medicinal chemists for new drugs[1,2] in the fields of antiviral,[3] anticancer,[4,5] or antibacterial[6] agents among others.

Tillandsia is one of the 57 plant genera belonging to the Bromeliaceae family, a well-known family of monocotyledons endemic to the Neotropics.[7−10] *Tillandsia* is considered to be the most diverse genus in this family and comprises more than 700 species divided into 7 subgenera, of which the main are epiphytes.[7,11,12] The *Tillandsia* genus has shown some potentialities in pharmacy as a medicinal plant.[13,14]

More than 30 *Tillandsia* species have been mentioned in traditional Mexican medicine, and this has been reviewed recently by Estrella-Parra et al. in a comprehensive and pluridisciplinary approach including ethnobotany, chemistry, biological activity, and economic issues.[14] Uses in traditional medicine have led researchers to investigate the biological properties of compounds isolated from the extracts of *Tillandsia* species like *Tillandsia streptocarpa* (Baker), *Tillandsia recurvata*, *Tillandsia usneoides*, *Tillandsia aeranthos*, and *Tillandsia brachycaulos*, among others. This allowed to demonstrate the presence of several secondary metabolites, of which the major ones are triterpenoids and sterols (51%),

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flavonoids (45%), and cinnamic acids (4%), obtained from the organic extracts and presenting a wide range of biological activities such as antimicrobial, antiviral, antiherpetic, anti-inflammatory, antibacterial, hypoglycemic, and anticancer activities.[14]

Owing to the biological activities associated with secondary metabolites from the *Tillandsia* genus, investigation of the phytochemical composition of the *Tillandsia* species, especially those that are unstudied so far, can unveil bioactive compounds that have potential medical applications and highlight the economic importance of these plants.[14]

Tillandsia bergeri Mez is a caulescent species, belonging to subgenus *Anoplophytum*, endemic to Argentina[15−17] with a developed stem and dense clumps (Figure S1). It is a hardy species acclimated to the Mediterranean Riviera.[10,18] To our knowledge, although it is a well-known, cultivated, and commercially ornamental species easy to care for, the phytochemistry and biological applications of *T. bergeri* remain unstudied.

In this report, we describe the first phytochemical study of *T. bergeri* with antibacterial screening and identification of the compounds responsible for the activity.

Although possessing compounds of interest, the isolation of secondary metabolites from plants can be an obstacle due to the complexity of the chemical composition of plant extracts. However, thin layer chromatography (TLC) bioautography is an effective screening method to detect and isolate active compounds including antibacterial compounds from plant extracts.[19] This technique is known since 1946[20] and widely used for the evaluation of several biological activities such as antifungal, antitumor, antiestrogenic, antimutagenic, antioxidant, and antiprotozoal activities.[21] The principle relies on the ability of TLC to separate compounds within a complex matrix and in a relatively short time. This method performs the biological evaluation on the TLC plate to determine directly the biologically active compounds. The bioautography can also be combined with high-performance TLC, overpressured layer chromatography, and planar electrochromatography.[22,23] In order to evaluate antibacterial activities, several TLC bioautography techniques can be performed, such as contact bioautography, direct bioautography, and immersion or agar overlay bioautography.[24] Immersion/agar overlay bioautography is a technique considered to be a combination of contact and direct bioautography; in fact, for this third technique, the agar-bacteria suspension covers the TLC plate during the whole process from incubation to visualization of the inhibition zone.[25,26] For all these bioautography methods, the visualization is done after spraying a solution of tetrazolium salts such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or *p*-iodonitrotetrazolium (INT) which are transformed into formazan of purple or pink color, respectively, by the dehydrogenases of the living microorganisms.[27−29] The presence of antibacterial compounds is indicated by clear inhibition zones or a white halo on the colored background. TLC bioautography is a time-saving, simple, efficient, inexpensive, and widely used method for bio-guided fractionation of plant extracts.[23]

In order to identify biologically active compounds, they must first be purified and obtained in sufficient quantity. Following this, structural identification is performed using analytical techniques like nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS). Indeed, the combination of these two techniques allows a more efficient structural identification, the advantages of one overcoming the limitations of the other.[30]

The aim of this study is to perform a phytochemical analysis and identify the antibacterial compounds present in *T. bergeri*. Bio-guided fractionation will be performed using agar overlay bioautography as a screening method against several Gram-positive and Gram-negative bacterial strains as well as one resistant strain. Identification of antibacterial compounds will be done by NMR and HRMS. Minimum inhibitory concentrations (MICs) and synergistic effects of the compounds with conventional antibiotics will be determined.

EXPERIMENTAL SECTION

Plant Material and Chemicals. The *T. bergeri* plants used for this study were harvested in March 2019 from the *Tillandsia* PROD plant nursery located in Le Cailar (Occitanie, France, 43°41′31.98″N, 4°14′34.85″E) (see Figure S1 in the Supporting Information).

All analytical grade solvents and liquid chromatography (LC)−MS grade solvents used in this report were ordered from VWR (Radnor, Pennsylvania, USA). High-performance LC (HPLC) grade ultrapure water was prepared with a PURELAB Classic water purification system (Elga, Veolia), and analytical grade pure formic acid was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All Si60 F_{254} aluminum TLC plates (0.25 mm thickness) and preparative SIL G-200 glass TLC plates (2 mm thickness) were ordered from Merck (Darmstadt, Germany). For chemical TLC development, vanillin, 2-aminoethyl diphenylborinate, polyethylene glycol-4000 (PEG), and *p*-anisaldehyde were ordered from Sigma-Aldrich, and glacial acetic acid and sulfuric acid were purchased from VWR.

For TLC bioautography, the Mueller−Hinton (MH) broth, MH agar, and Ringer's solution were ordered from Fisher Scientific (Hampton, New Hampshire, USA), and INT was ordered from Sigma-Aldrich. A standard of penduletin (purity >95%) was ordered from PhytoLab (PhytoLab GmbH & Co., Vestenbergsgreuth, Germany) and that of viscosin (purity >90%) was ordered from Sigma-Aldrich (St. Louis, Missouri, USA). Vancomycin was purchased from Sigma-Aldrich, and cloxacillin was obtained from the microbiology department of the University Hospital of Nîmes.

Extraction. *T. bergeri* leaves were dried in an incubator at 50 °C until the constant point was reached. This was obtained after 6−7 days of drying. Once dried, they were stored in amber glass bottles in a dry and shaded place. Grinding was performed using a SPEX planetary ball grinder with 50 mL bowls containing 1 mm diameter zirconium beads. In this case, a 4 min cycle was performed for a satisfactory powder size. After grinding, four successive extractions were performed in Soxhlet with solvents of increasing polarity, first with hexane, then with dichloromethane, followed by methanol, and finally with methanol/water (50/50), in order to extract the maximum of compounds. A quantity of 30 g of the dried *T. bergeri* leaf powder was placed in the Soxhlet cartridge, and each extraction was performed for 5 h corresponding to 25 cycles in 350 mL of the appropriate solvent. In total, four large extractions were performed with a total amount of powder of 120 g which allowed us to obtain, after complete dryness, the following fractions noted F1 (3.0 g), F2 (1.3 g), F3 (5.6 g), and F4 (4.2 g) corresponding to the fractions obtained after extraction with hexane, dichloromethane, methanol, and

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methanol/water (50/50), respectively. The dry extracts were kept cold (−20 °C) until analysis.

Phytochemical Screening. Fractions F1, F2, F3, and F4 were prepared at a concentration of 5 mg/mL and deposited on a silica gel 60 F_{254} TLC plate (20 × 10 cm) using the Linomat 5 automatic depositor (CAMAG, Muttenz, Switzerland). A deposit of 20 μL was thus carried out in four series for each fraction. The migration was performed with the eluent system chloroform/methanol (90/10) over a distance of 55 mm. The developed TLC plates were visualized under UV 254 and 366 nm using the CAMAG TLC Visualizer. Specific chemical developers were also prepared to visualize the presence of certain chemical families or functions:

Sulfuric vanillin: a solution consisting of 1 g of vanillin, 2 mL of sulfuric acid, and 100 mL of ethanol (95%). The solution was sprayed on the TLC plate which was then heated at 110 °C for 5 min. Several colorations appear depending on the compound.

Anisaldehyde: a solution consisting of 50 mL of glacial acetic acid, 1 mL of sulfuric acid, and 0.5 mL of *p*-anisaldehyde. The solution thus prepared was sprayed onto the TLC plate, which was subsequently heated to 110 °C for 5 min.

Neu's reagent (polyphenols): two solutions were prepared, one consisting of 1 g of 2-aminoethyldiphenylboric acid and 100 mL of methanol and the second solution consisting of 5 g of PEG 4000 in 100 mL of ethanol. A mixture of 50 mL of the first solution and 40 mL of the second solution was sprayed onto the TLC plate. The plate was subsequently heated at 110 °C for 2 min. The compounds colored orange, yellow, or green are flavonoids. The TLC plates were then visualized with the CAMAG TLC Visualizer, and pictures were taken with the Vision CATS software.

Bacterial Strains. The antibacterial effect of crude extracts of *T. bergeri* was first evaluated by agar overlay bioautography against the following bacterial strains: seven Gram-positive bacteria methicillin-sensitive, *Staphylococcus aureus* ATCC 29213 (MSSA), methicillin-resistant *S. aureus* N-SARM-1 (MRSA) (clinical strain), *Staphylococcus caprae* ATCC 35538, *Streptococcus agalactiae* ATCC 13813, *Enterococcus faecalis* ATCC 51299, *Enterococcus faecium* CIP 103226T, *Bacillus subtilis* subtilis ATCC 6051, and five Gram-negative bacteria, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* PAO1 (clinical strain), *Proteus mirabilis* ATCC 35659, *Klebsiella pneumoniae* ATCC 700603, and *Salmonella enterica* indica ATCC 43976. The bacterial strains were obtained from the collection of the Pasteur Institute (Paris, France) except for MSSA, MRSA (clinical strain), and *P. aeruginosa* PAO1 (clinical strain) which were obtained from the collection of the microbiology department of the University Hospital of Nimes.

TLC Agar Overlay Bioautography. Bacterial suspensions: two colonies of each bacterial strain, listed above, were collected and placed in tubes containing 4 mL of the MH broth; then, the tubes were placed in the shaker incubator at 37 °C and 200 rpm overnight. After the bacteria were grown, twofold dilutions were performed for each bacterial strain (1 mL of the bacterial suspension in 9 mL of Ringer's liquid) to obtain a concentration of 10^6 CFU/mL. At the end, 0.50 mL of the bacterial suspension (dilution 2) was then added to 15 mL of MH agar (kept liquid at 45 °C).

TLC plates: a first screening was performed with fractions F1, F2, F3, and F4 of *T. bergeri* to see which fraction presented antibacterial compounds. For this, each crude extract was prepared at a concentration of 100 mg/mL and deposited, without migration, on the TLC plates. In addition, three antibiotics (cefotaxime, cloxacillin, and ciprofloxacin), used in the treatment of infections caused by these bacteria, and a blank sample (methanol) were applied. When the fraction showed antibacterial activity, TLC separation was performed to determine the compound responsible for the activity. In our case, the F2 fraction of *T. bergeri* showed activity against MSSA as well as MRSA and *S. caprae* ATCC 35538 and was manually deposited using a microsyringe Hamilton (Bonaduz, Switzerland). Then, TLC plates were developed with a chloroform/methanol (90/10) eluent at a distance of 55 mm. After separation, the TLC plates were dried to completely remove the solvent. They were then sterilized under UV light and placed in Petri dishes.

15.5 mL of the agar−bacteria suspension was added to the Petri dish containing the TLC plate. After solidification of the medium, incubation was performed for 24 h at 37 °C.

After incubation, a 2 mg/mL solution of INT was sprayed on the surface of the agar−bacteria suspension and incubated again for 4 h. Inhibition is indicated by the presence of clear zones.

Isolation of Antibacterial Compounds. To isolate the antibacterial compounds, two purification methods were used as follows:

Preparative TLC. First, several preparative TLC was performed from the F2 fraction. For this, for each preparative TLC, 100 mg of F2, solubilized in dichloromethane, was deposited on a 2 mm SIL G-200 glass plate using a micropipette. The plate was then placed in a migration chamber containing 100 mL of the chloroform/methanol (90/10) eluent to allow separation of the compounds. The migration was performed twice and was carried out over a distance of 17 cm. After elution, the plate was well dried and then revealed by UV at different wavelengths (254 and 365 nm). The obtained subfractions were scraped, crushed to obtain a powder, and then collected in glass pillboxes before being dissolved in methanol and shaken for 30 min. The methanol/silica mixtures were then filtered to recover the filtrate. The solvent was evaporated to dryness, and the subfractions were weighed. A total of 10 preparative TLCs were performed from F2, allowing 72.4 mg of the subfraction F2.3 and 114.2 mg of F2.5.

Preparative HPLC. The subfractions F2.3 and F2.5 thus obtained were repurified by preparative HPLC to obtain compounds **1** and **2**, respectively. They were solubilized in a methanol/ultrapure water mixture (50/50) and then filtered on polytetrafluoroethylene filters (13 mm; 0.22 μm) (Cloup, Champigny-sur-Marne, France). Preparative HPLC was performed with an LC-20AD system (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SIL-10AP autosampler. The separation column was a SUPELCOSIL ABZ+Plus column (25 cm × 22.1 cm; 5 μm) (Sigma, St. Louis, Missouri, USA). Elution was performed with a mobile phase flow rate of 17 mL/min consisting of a mixture of ultrapure water containing 0.05% formic acid (A) and methanol with 0.05% formic acid (B); the program started at t = 0 min, ratio (A/B) 50:50; at t = 20 min, 30:70; at t = 45 min, 5:95; at t = 50 min, 95:5; at t = 55 min, 50:50; and t = 70 min, 50:50. After purification and drying, 4.3 mg of compound **1** and 3.6 mg of compound **2** were obtained.

High-Performance Liquid Chromatography-Ultraviolet. The purity of the active compounds was verified by HPLC-

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UV on an LC-2010HT system (Shimadzu, Kyoto, Japan). A SUPELCOSIL ABZ+PLUS column (25 cm × 4.6 mm; 5 μm) (Sigma, St. Louis, Missouri, USA) was used to check the purity, with ultrapure water containing 0.05% formic acid and methanol with 0.05% formic acid at a flow rate of 0.8 mL/min. The mobile phase gradient was the same as that used for preparative HPLC.

Identification of Antibacterial Compounds. *Ultra-Performance Liquid Chromatography−High-Resolution Mass Spectrometry.* The ultra-performance LC coupled to HRMS (UPLC−HRMS) analysis was performed on an ACQUITY UPLC I-class chain coupled to the Vion IMS Quadrupole time-of-flight high-resolution mass spectrometer, equipped with an electrospray ionization (ESI) (Waters, Manchester, UK) source (Z-spray) and an additional spray for the reference compound (Lock Spray). The ionization was performed in positive and negative modes. A double detection was performed by ESI mass spectrometry (range 50−2000 Da) and using a photodiode array (PDA) detector (UV detection between 190 and 500 nm). Elution was performed with a mobile phase flow rate of 0.5 mL/min consisting of a mixture of ultrapure water containing 0.01% formic acid (A) and methanol (B). The program starts at t = 0, ratio (A/B) 80:20; at t = 0.5 min, 80:20; at t = 5 min, 40:60; at t = 6 min, 10:90; at t = 7 min, 10:90; at t = 7.5 min, 80:20; and at t = 10 min, 80:20. 1 μL of the sample was injected into a Kinetex Biphenyl column (2.1 × 100 mm; 1.7 μm) (Phenomenex, Torrance, California, USA) heated to 55 °C. Mass spectra were acquired in the MS^E mode including a low-energy function (CE 6 eV) as well as a second high-energy function (CE 25 to 50 eV) for both positive and negative mode ionizations to obtain information on fragmentation. Tandem MS (MS/MS) experiments were also carried out, and argon was used as collision gas, and the collision energy value was optimized to reach a relative intensity between 10 and 20% for each selected precursor ion. Parameters were adapted from the previous method as described by Tchoumtchoua et al.[75] The spectra obtained were processed using software UNIFI (version 1.9.2, Waters).

Nuclear Magnetic Resonance. Each sample, after evaporation, was solubilized in 650 μL of deuterated methanol and transferred to 5 mm 509-Up NMR tubes (Eurisotop, Cambridge Isotope Laboratories, Cambridge, UK). The one-dimensional (1D) (^{1}H and ^{13}C DEPTQ) and two-dimensional (2D) [^{1}H J-resolved (JRES), correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond coherence (HMBC)] NMR spectra were recorded using a BRUKER AVANCE III 600 MHz instrument (Bruker, Billerica, Massachusetts, USA) equipped with a probe (BBFO 5 mm tube) and an automatic tube changer. For the classical 1D proton, a 90° flip angle was performed, and the spectra were acquired using eight scans of 128 K data points with a spectral window of 14.02 ppm. The 2D ^{1}H JRES NMR spectra were acquired using four scans per 64 increments which were collected in 64 K data points, with a spectral window of 14.02 ppm for F2 and 0.08 ppm for F1. The 1D DEPTQ spectra were acquired using 3 K scans using spectral widths of 251 ppm. The 2D COSY spectra were acquired using two scans per 512 increments collected at 4 K data points, with a spectral window of 14 ppm for F2 and 14 ppm for F1. The 2D HSQC spectra were acquired using 16 scans per 512 increments which were collected in 2 K data points, using a spectral window of 14 ppm for F2 and 200 ppm for F1. The 2D HMBC spectra were acquired using 16 scans per 512 increments which were collected in 2 K data points, using a spectral window of 14 ppm for F2 and 250 ppm for F1. All spectra were processed using Topspin software (version 3.5pl6, Bruker).

MIC Evaluation. Compounds **1** and **2** that showed an antibacterial potential were then evaluated for MICs on MSSA, MRSA, and *S. caprae*. Indeed, the MIC is the minimum concentration at which the compound inhibits bacterial growth. The method of dilution in the liquid medium was thus carried out, for which the bacterial suspensions were prepared in the same way as for the agar overlay bioautography method. The compounds were first solubilized in dimethylsulfoxide to a concentration of 2 mg/mL. Subsequently, dilutions were made in the MH medium to have 10 solutions with concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 μg/mL. Microplates of 96 wells were used; 100 μL of the bacterial suspension and 100 μL of diluted solutions were added in wells 1−10 (the most concentrated solution in well 1 and the least concentrated solution in well 10), and well 11 represents the control of sterility of the medium, so 200 μL of MH was added to it, and well 12 represents the control of culture of the bacteria. The plate was then incubated at 37 °C for 24 h. After incubation, the lowest concentration for which no turbidity is observed corresponds to the MIC.

Fractional Inhibitory Concentration Index. Antimicrobial checkerboard assays were performed using the bacterial strains MSSA, MRSA, and *S. caprae* to determine the synergistic effects of the isolated compounds with the antibiotics cloxacillin and vancomycin. The compounds were evaluated using the concentration range of 2−128 μg/mL on all three bacteria; the concentration range of cloxacillin was from 3.75 × 10^{-3} to 0.25 μg/mL on MSSA and *S. caprae* and from 6.25 × 10^{-2} to 4 μg/mL on MRSA; the concentration range of vancomycin was from 3.12 × 10^{-2} to 2 μg/mL on all three bacteria. The effect of the combination of compounds and antibiotics was evaluated by calculating the fractional inhibitory concentration index (FICI) according to the following formula

$$\mathrm{FICI} = \frac{\mathrm{MIC\ (antibiotic\ combined\ with\ the\ compound)}}{\mathrm{MIC\ of\ the\ antibiotic\ alone}} + \frac{\mathrm{MIC\ (the\ compound\ combined\ with\ the\ antibiotic)}}{\mathrm{MIC\ of\ the\ compound\ alone}}$$

"Synergy" effects were defined when the FICI was less than or equal to 0.5. "Additive" effects were defined when the FICI was greater than 0.5 and less than or equal to 1. An "indifference" effect was defined when the FICI was greater than 1 and less than or equal to 2.0. Antagonistic effects were observed when the FICI was greater than 2.0.

RESULTS

Phytochemical and Antibacterial Screening. After drying and grinding the leaves of *T. bergeri*, the compounds were extracted with a Soxhlet apparatus using solvents of increasing polarity leading to four fractions F1−F4 (see Extraction in the Experimental Section). The agar overlay bioautography method was used to verify the antibacterial properties of *T. bergeri* plant extracts. Biological evaluations were performed with a concentration of 100 mg/mL of *T. bergeri* fractions F1, F2, F3, and F4 on 12 bacterial strains including reference and clinical strains. This first screening

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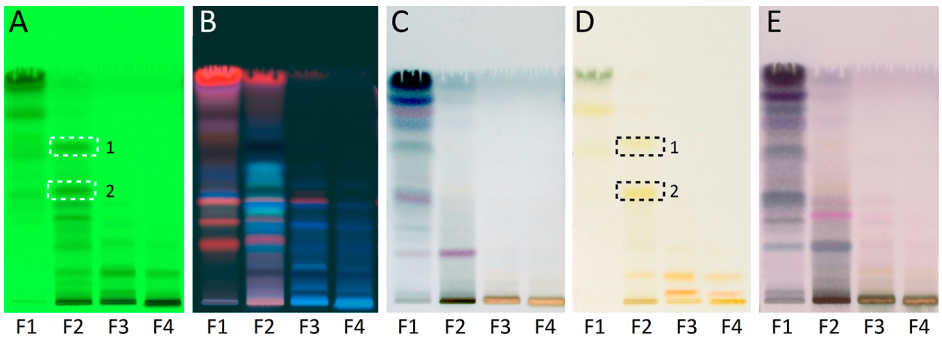

Figure 1. TLC plates of the four fractions obtained from *T. bergeri*. Mobile phase: chloroform/methanol (90/10). (A) UV 254 nm. (B) UV 365 nm. (C) Sulfuric vanillin. (D) Neu's reagent. (E) Anisaldehyde. **1** and **2** represent compounds **1** and **2**, respectively.

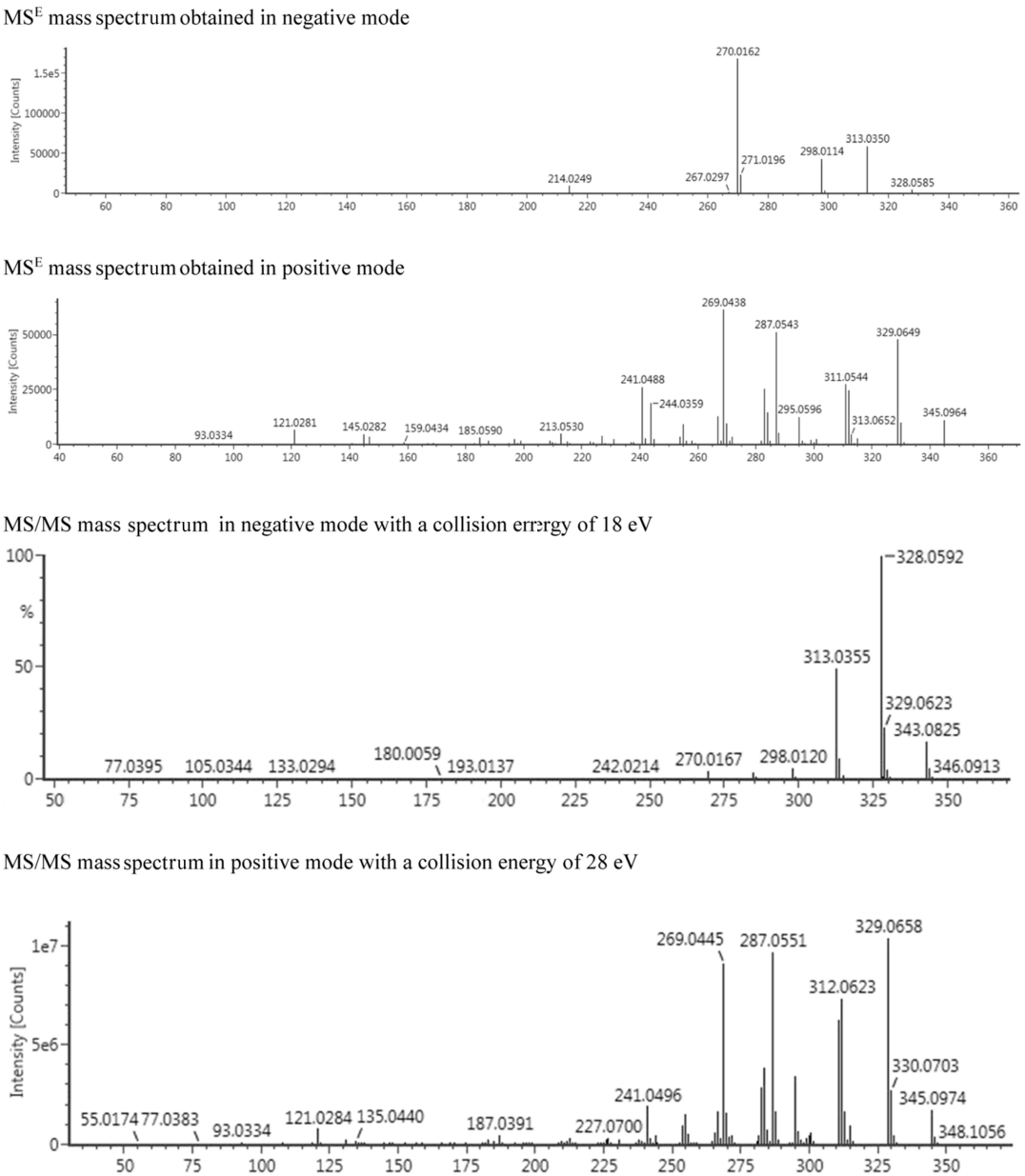

Figure 2. MS^E (high-energy) and MS/MS spectra of compound **1** isolated from the dichloromethane fraction (F2) of *T. bergeri*. (A) MS^E mass spectrum obtained in the negative mode. (B) MS^E mass spectrum obtained in the positive mode. (C) MS/MS spectrum in the negative mode with a collision energy of 18 eV. (D) MS/MS spectrum in the positive mode with a collision energy of 28 eV.

showed antibacterial activity of the hexanic and dichloromethane fractions (namely, F1 and F2) on methicillin-sensitive *S. aureus* ATCC 29213 (MSSA) and methicillin-resistant *S. aureus* N-SARM-1 (MRSA) and antibacterial activity of F2 on

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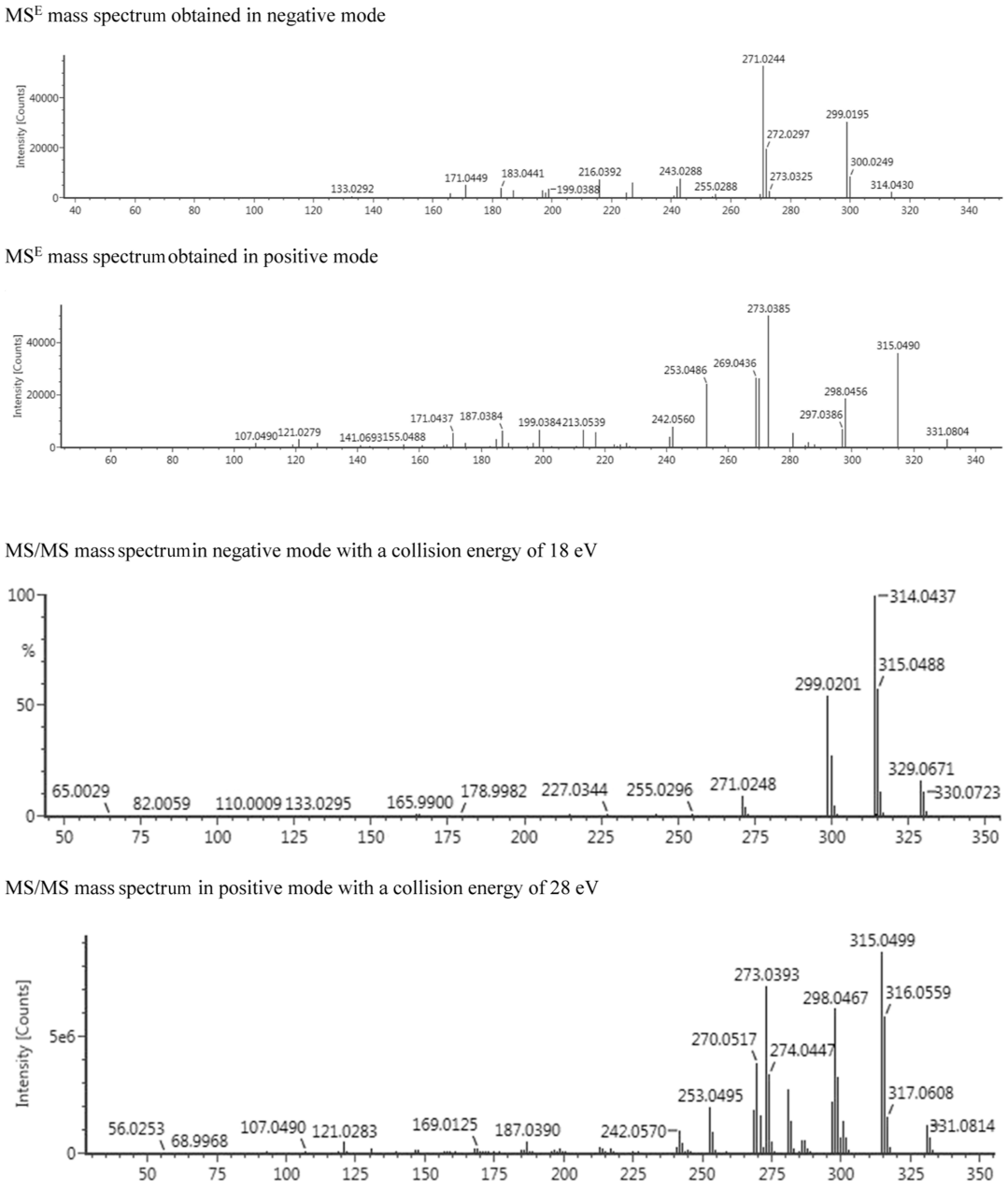

Figure 3. MS^E (high-energy) and MS/MS spectra of compound **2** isolated from the dichloromethane fraction (F2) of *T. bergeri*. (A) MS^E mass spectrum obtained in the negative mode. (B) MS^E mass spectrum obtained in the positive mode. (C) MS/MS mass spectrum in the negative mode with a collision energy of 18 eV. (D) MS/MS mass spectrum in the positive mode with a collision energy of 28 eV.

S. caprae ATCC 35538 (*S. caprae*). However, the *T. bergeri* fractions did not show any bacterial activity against the Gram-negative bacteria studied in this report and against the other Gram-positive bacteria mentioned in the Experimental Section (see Table S1 in the Supporting Information). Therefore, we first focused on the F2 fraction in order to isolate the compounds responsible for the antibacterial activity against MSSA, MRSA, and *S. caprae* bacteria. For this purpose, the compounds present in the F2 fraction were separated by TLC using a chloroform/methanol (90/10) solvent system. After the development of the TLC plates, the biological evaluation was carried out this time only on MSSA, MRSA, and *S. caprae* bacteria. The results showed the inhibition zone of MSSA, MRSA, and *S. caprae* bacteria at the level of the subfractions noted F2.2, F2.3, F2.4, and F2.5 (Figure S2 in the Supporting Information). Based on these results, a bio-guided fractionation was performed. Indeed, the subfractions were isolated from F2 by preparative TLC, and new agar overlay bioautographies against MSSA, MRSA, and *S. caprae* bacteria were performed by depositing only the above-mentioned subfractions on TLC plates. The results showed that only the subfractions F2.3 and F2.5 have an antibacterial activity against these three bacteria, and they comprise two distinct compounds named compounds **1** and **2**, respectively. A chemical derivatization on TLC plates was therefore performed in order to know the chemical family of these two compounds using specific visualizing reagents (Figure 1). Indeed in TLC, chemical derivatization is used to detect colorless compounds that cannot be visualized by UV light or fluorescence. Most often universal or polyvalent reagents such

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Table 1. UPLC−HRMS Data of Compounds 1 and 2

#	compound name	*RT* (minutes)	$[M + H]^+$, *m*/*z*	$[M - H]^-$, *m*/*z*	molecular mass	molecular formula
1	penduletin	6.12	345.0973	343.0819	344	$C_{18}H_{16}O_7$
2	viscosine	5.74a	331.0820	329.0657	330	$C_{17}H_{14}O_7$

Table 2. NMR Data of Compounds 1 and 2

	1				2			
C/H	δ_H (ppm)	*J* (Hz)	DEPT	δ_C (ppm)	δ_H (ppm)	*J* (Hz)	DEPT	δ_C (ppm)
2			C	158.6			C	158.1
3			C	139.4			C	138.9
4			C	180.4			C	180.0
5			C				C	
6			C	133.5			C	132.7
7			C	160.5			C	159.0
8	6.76, s		CH	92.1	6.52, s		CH	95.0
9			C	154.1			C	153.7
10			C	107.1			C	106.3
1′			C	122.5			C	122.4
2′	8.02, d	8.7	CH	131.5	7.98, d	8.3	CH	131.4
3′	6.95, d	8.7	CH	116.7	6.94, d	8.3	CH	116.6
4′			C	161.7			C	161.6
5′	6.95, d	8.7	CH	116.7	6.94, d	8.3	CH	116.6
6′	8.02, d	8.7	CH	131.5	7.98, d	8.3	CH	131.4
3-OMe	3.78, s		CH_3	60.7	3.77, s		CH_3	60.5
6-OMe	3.83, s		CH_3	61.3	3.87, s		CH_3	60.9
7-OMe	3.96, s		CH_3	57.1				

as vanillin sulfuric acid or *p*-anisaldehyde sulfuric acid are used to locate most classes of organic compounds. However, selective reagents can be used as derivatization agents such as Neu's reagent (2-aminoethyldiphenylboric acid + PEG) for the identification of polyphenols. Derivatization showed a positive reaction of compound **1** (R_f = 0.56) and compound **2** (R_f = 0.41) with Neu's reagent, suggesting the presence of polyphenols.

Identification of Antibacterial Compounds. The F2.3 and F2.5 subfractions were analyzed by HPLC-UV for their chromatographic profiles (Figure S3); then, they were subsequently repurified by preparative HPLC to remove small impurities that were present, and the purity of the compounds was controlled by HPLC-UV. The compounds **1** and **2** obtained were then identified by HRMS and NMR.

In order to have preliminary information on the identity of the compounds, low-energy MS^E and MS/MS spectra were acquired in positive and negative modes (Figures 2 and 3). Compound **1** with a retention time of 6.12 min has an $[M + H]^+$ ion with *m*/*z* = 345.0973 and an $[M - H]^-$ ion with *m*/*z* = 343.0819; the exact masses were used to generate the crude formula $C_{18}H_{16}O_7$. Regarding compound **2**, it has a retention time of 5.74 min and has an $[M + H]^+$ ion with *m*/*z* = 331.0820 and an $[M - H]^-$ ion with *m*/*z* = 329.0657; these exact masses generated the crude formula $C_{17}H_{14}O_7$ (Table 1).

After obtaining the masses and the elemental compositions of the compounds, the NMR data allow a more complete identification. For compound **1**, 6 proton signals and 18 carbon signals were observed in the 1D ^1H and ^{13}C DEPTQ NMR spectra, respectively (Table 2). The three singlets at 3.78 ppm (s, 3H), 3.83 ppm (s, 3H), and 3.96 ppm (s, 3H), correlated in HSQC to the carbons at 60.7, 61.3, and 57.1 ppm, respectively, indicate the presence of three methoxylated groups. In HMBC, these methoxylated groups are related to carbons C-3 (139.4 ppm), C-6 (133.5 ppm), and C-7 (160.5 ppm). The singlet at 6.76 ppm (s, 1H) is connected in HSQC with the C-8 carbon at 92.1 ppm. HMBC correlations between this proton and carbons C-6 (133.5 ppm), C-7 (160.5 ppm), C-9 (154.1 ppm), C-10 (107.1 ppm), and C-4 (180.4 ppm) show the presence of two aromatic rings constituting rings A and C. The proton signals at 8.02 ppm (d, 2H, 8.77 Hz) and 6.95 ppm (d, 2H, 8.77 Hz) indicate the presence of four aromatic protons which are H-2′/6′ and H-3′/5′, respectively. The 2D COSY spectrum and the coupling constants show that these protons are ortho correlated. The HMBC correlations between the H-3′/5′ protons with the C-1′ carbon (122.5 ppm) and the H-2′/6′ protons with the C-2 carbon (158.6 ppm) show that the B ring is attached to the C ring in position 2. These chemical shifts and correlations are consistent with those in the literature and identify this compound **1** as 5,4′-dihydroxy-3,6,7-trimethoxyflavone, also called penduletin (Figure 4A).

For compound **2**, NMR spectra were quite similar to those of compound **1** except that the NMR signals of a methoxy group were missing. Indeed, 5 proton signals and 17 carbon signals were observed in the 1D ^1H and ^{13}C DEPTQ spectra, respectively (Table 2). The singlet at 6.52 ppm (s, 1H)

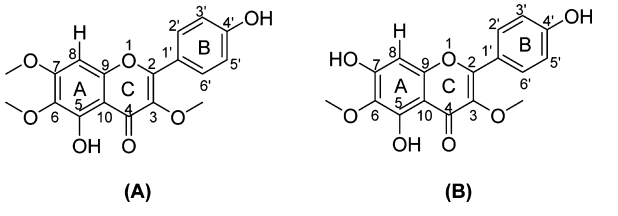

Figure 4. Chemical structures of (A) penduletin (compound **1**) and (B) viscosine (compound **2**).

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Table 3. Antibacterial Activities of Compounds 1 and 2

strains	agents	MIC (μg/mL) alone	MIC (μg/mL) combination	FIC	FICI
methicillin-sensitive *Staphylococcus aureus* ATCC 29213 (MSSA)	penduletin (1)	>256	2	<0.008	<0.258
	vancomycin	1	0.25	0.25	
	penduletin (1)	>256	64	<0.25	<0.75
	cloxacillin	0.25	0.125	0.5	
	viscosine (2)	128	32	0.25	0.75
	vancomycin	1	0.5	0.5	
	viscosine (2)	128	4	0.0312	0.531
	cloxacillin	0.25	0.125	0.5	
methicillin-resistant *Staphylococcus aureus* N-SARM-1 (MRSA)	penduletin (1)	>256	64	<0.25	<0.75
	vancomycin	1	0.5	0.5	
	penduletin (1)	>256	8	<0.0312	<0.531
	cloxacillin	4	2	0.5	
	viscosine (2)	256	64	0.25	0.75
	vancomycin	1	0.5	0.5	
	viscosine (2)	256	16	0.0625	0.562
	cloxacillin	4	2	0.5	
Staphylococcus caprae ATCC 35538 (*S. caprae*)	penduletin (1)	>256	64	<0.25	<0.5
	vancomycin	1	0.25	0.25	
	penduletin (1)	>256	4	<0.016	<0.516
	cloxacillin	0.25	0.125	0.5	
	viscosine (2)	256	64	0.25	0.75
	vancomycin	1	0.5	0.5	
	viscosine (2)	256	8	0.0312	0.531
	cloxacillin	0.25	0.125	0.5	

correlates in HSQC with C-8 (95.0 ppm) and in HMBC with carbons C-4 (180.0 ppm), C-6 (132.7 ppm), C-7 (159.0 ppm), C-9 (153.7 ppm), and C-10 (106.3 ppm), indicating the presence of both rings A and C. The two singlets at 3.77 ppm (s, 3H) and 3.87 ppm (s, 3H), respectively, correlated in HSQC to the carbons at 60.5 and 60.9 ppm, indicate the presence of two methoxylated groups.

These are linked in HMBC to the C-3 (138.9 ppm) and C-6 (132.7 ppm) carbons, respectively. The protons H-2′/6′ at 7.98 ppm (d, 2H, 8.3 Hz) and H-3′/5′ at 6.94 ppm (d, 2H, 8.3 Hz) are ortho-coupled and are linked in HSQC to the carbons C-2′/6′ (131.4 ppm) and C-3′/5′ (116.7 ppm), respectively. The HMBC correlations of the H-2′/6′ protons at carbon C-2 (158.1 ppm) and H-3′/5′ at carbon C-1′ (122.4 ppm) show that ring B is attached to ring C at position 2. All this information allows us to identify compound **2** as 4′,5,7-trihydroxy-3,6-dimethoxyflavone, also called viscosine (Figure 4B). The identification of these two compounds was also confirmed by analysis of the standards which showed similar retention times and the same behaviors toward fragmentation by MS/MS or high-energy MS^E with identical fragment intensities (see MS^E and MS/MS mass spectra in Supporting Information, Figures S4, S5, S6, and S7).

Evaluation of Antibacterial Activity. Having demonstrated the growth inhibition of MSSA, MRSA, and *S. caprae* bacteria using agar overlay bioautography, the MICs of compounds **1** and **2** were determined using the broth microdilution method. The results obtained show a MIC value greater than 256 μg/mL for penduletin against the three bacterial strains. For viscosine, the MIC value is equal to 128 μg/mL against MSSA and is equal to 256 μg/mL against MRSA and *S. caprae* (Table 3). These MIC values were high compared to those of the antibiotics, so the compounds were combined with vancomycin and cloxacillin to investigate their synergistic antibacterial effects. The results obtained show that when the compounds were combined with antibiotics, they were able to reduce the MIC values of the latter. With FICI values between 0.5 and 1, viscosine showed additive effects when combined with both antibiotics against all three bacterial strains. However, it is interesting to note that penduletin showed synergistic effects with vancomycin against MSSA and *S. caprae* strains with FICI values inferior to 0.258 and 0.5, respectively.

DISCUSSION

Plants produce secondary metabolites with interesting biological properties, particularly antibacterial properties, which gives them an important place in the search for new drugs. For this reason, research studies ranging from ethnobotanical and ethnopharmacological studies of plants to the isolation and optimization of key plant compounds are common. They represent an important part of the research for new antibacterial compounds motivated by the need to solve the problem of bacterial resistance to antibiotics. Being used for a long time in the American continent as medicinal plants, the species of the genus *Tillandsia* have aroused the interest of researchers who have studied the biological properties of their constituents. These studies have demonstrated the presence of compounds with antiangiogenic,[31] antiviral,[32] antitumor,[33] hypoglycemic,[34] and anticancer[35−39] activities. Regarding antibacterial activity, it was reported in 1945 that the crude extract of *T. usneoides* is effective against Gram-positive bacteria.[40] This was later confirmed by the isolation of a glycosylated flavonol with antibacterial activity against *S. aureus*.[41] Subsequently, in 1995, aqueous extracts of *T. recurvata*, *T. aeranthos,* and *T. usneoides* showed antimicrobial activity against *E. coli*, *S. aureus*, *P. aeruginosa*, *B. subtilis,* and *Micrococcus luteus*. Furthermore, in the same study, the

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aqueous extracts of *T. aeranthos* and *T. recurvata* showed strong activity against *P. aeruginosa* which is a Gram-negative bacterium. This is an interesting fact as Gram-negative bacteria are generally more resistant than Gram-positive bacteria.[42] Indeed, the problems of bacterial resistance and the spread of resistance are currently more related to Gram-negative bacteria such as *P. aeruginosa* and enterobacteria. More recently in 2017, a study was conducted by Faller and his team to explore the antibacterial efficacy of *T. usneoides* against skin infections in wound healing. The results showed a high inhibition of the growth of *S. aureus*, *P. aeruginosa,* and *Staphylococcus epidermis* bacteria by methanolic and ethanolic extracts of *T. usneoides*.[43] For all these studies, the alcoholic or aqueous extracts are responsible for the antibacterial activity. In our study, the methanolic and methanol/water extracts did not show any inhibition of the different bacteria used. This could be explained by a low selectivity of the compounds present in these extracts against the bacteria chosen for this study, by a very low concentration of the active compounds in these extracts, or by a disruption of the synergy between the active compounds caused by TLC.

The studies mentioned just above confirm the usefulness of *Tillandsia* species as sources of antibacterial agents; however, none of these studies have carried out the structural identification of the compounds responsible for the antibacterial activity within the different species studied. Moreover, no study has focused on the antibacterial properties of *T. bergeri* species. Our present study is the first to perform the structural identification of antibacterial compounds from *T. bergeri* extracts. In order to identify antibacterial compounds, agar overlay bioautography was used as a screening method. It is a very popular screening method because of its facility of realization, low cost, time saving, and ability to perform simultaneously the biological evaluation of a large number of samples.[44] This bioautographic method is very suitable for broad spectrum microorganisms such as yeasts and bacteria.[45] It guided us to the isolation and identification of two compounds belonging to the flavonoid family. This identification was performed using NMR and HRMS. These two characterization techniques, which are both very efficient, can only reveal their full efficiency when they are skillfully combined. This complementarity allows one to solve most of the structural elucidation cases encountered. With HRMS, the crude formulas and fragments of the different compounds helped us obtain information about structures of the molecules. Also, thanks to NMR, the results obtained in HRMS could be confirmed while establishing a precise structural characterization.

Penduletin is the first compound identified from the dichloromethane extract of *T. bergeri*. It was first isolated in 1958, in the glucosylated form, from the ethanolic extract of the species *Brickellia pendula* obtained in Contreras near Mexico City.[46] *B. pendula* is thus native to Mexico and belongs to the Asteraceae family.[47] Later, penduletin was isolated from several other species including *Cleome amblyocarpa*, *Cleome brachycharpa*, *Cleome chrysantha*,[48] *Rabdosia rubescens*,[49] *Tanacetum artemisioides*,[50] *Sideritis caesarea*,[51] *Digitalis thapsi*,[52] *Artemisia annua*,[53] and *Dodonaea viscosa*.[54] A derivative of penduletin, penduletin-4′-*O*-methylether was isolated from the methanolic extract of *Tillandsia pupurea*.[55] Penduletin is a flavone in the form of an amorphous yellow powder with anti-inflammatory and cytotoxicity activities,[49] butyrylcholinesterase inhibitor activity,[51,54] antioxidant properties,[56] and strong *in vitro* activity against enterovirus 71 (EV71) with low cytotoxicity. Regarding antibacterial properties, penduletin showed weak antibacterial activity (32−64 μg/mL) against *Neisseria gonorrhoeae* which is a Gram-negative bacterium responsible for gonococcal disease[57] as well as low antibacterial activity against *S. aureus* with a MIC value >128 μg/mL.[58] It has also been recently considered as inactive against *S. aureus*, *Bacillus cereus*, *S. enteritidis,* and *C. albicans*.[59] These results correlate with our results for the MIC value of penduletin on MSSA.

The second compound identified from the dichloromethane extract of *T. bergeri* is viscosine. It is a flavone that has been isolated many times from the medicinal plant *D. viscosa* which belongs to the Sapindaceae family.[60−63] It has also been isolated from the flowers of *Centaurea jacea*[64] and from a strain of actinobacteria derived from marine sediments.[65] It is a compound that has demonstrated a significant inhibitory effect on lipoxygenase.[66] It has antioxidant, anticholinergic, and antiepileptic properties.[67] Viscosine has also shown significant activity against a human colon carcinoma cell line and a hepatocellular carcinoma cell line.[65] As for penduletin, viscosine was also studied for its antibacterial properties. Indeed, the study carried out by Teffo et al. showed a moderate antibacterial activity of viscosine against Gram-positive bacteria *S. aureus* and *E. faecalis* with respective MIC values of 125 and 32 μg/mL and against Gram-negative bacteria *E. coli* and *P. aeruginosa* with respective MIC values of 26 and 32 μg/mL.[62] However, a more recent study showed no inhibition of *P. aeruginosa* by viscosine but a moderate activity of the latter against Gram-positive bacteria *S. aureus* and *B. cereus*.[65] In our study, penduletin and viscosine showed moderate antibacterial activity against MSSA, MRSA, and *S. caprae,* and no inhibition of Gram-negative bacteria was observed by the crude extract containing these two compounds. The contradictory results sometimes observed on the antibacterial activity of flavonoids can be attributed to the methods used to evaluate the antibacterial activity which are different from one study to another. These inconsistencies can also be attributed to variations in each antibacterial evaluation method including differences in the amount of the bacterial strain, differences in the types or volumes of the broth and agar, or differences in the incubation period. Another factor is related to the problem of solubilization of flavonoids in organic solvents, leading to precipitation of the latter which can affect the biological evaluation by decreasing the contact between the bacterial cells and the flavonoid molecules and lead to false negative reports of the antibacterial activity.[68]

Since both antibacterial compounds isolated from the dichloromethane extract of *T. bergeri* are flavonoids, the mechanism of action of flavonoids is mainly based on the disruption of the bacterial plasma membrane which can trigger a multitude of effects such as pore formation, leakage, polarity modification, increase in permeability, fluidity modification, delocalization of membrane proteins, and other phenomena responsible for the antibacterial activity. Although little information is available on the structure−function relationship of antibacterial flavonoids, conjugation with sugar moieties at certain positions of the aromatic ring could increase the antibacterial activity of these compounds.[69] Flavonoids are also inhibitors of bacterial efflux pumps.[70]

Although phytochemicals generally have higher MIC values than traditional antibiotics, they may have different mechanisms and target sites compared to those of antibiotics. For this

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reason, their combination with conventional antibiotics has been proposed to provide superior efficacy in suppressing resistance development. This was observed in our study with higher MIC values of penduletin and viscosine than those of vancomycin and cloxacillin. These compounds were therefore combined with these antibiotics to increase the antibacterial activity of the latter. The results of their combinations showed not only a decrease in MICs of the antibiotics but also a synergistic effect of penduletin when combined with vancomycin against MSSA and *S. caprae* bacteria. Indeed, synergistic effects between various flavonoids and antibacterial agents are known,[68,71−74] and a study on the synergistic antibacterial effects of flavonoids from *A. rupestris* against *S. aureus* had shown a synergistic effect of penduletin when combined with norfloxacin, NorA efflux pump being affected.[58]

CONCLUSIONS

Bio-guided fractionation of *T. bergeri* extracts using agar overlay bioautography as a screening method allowed us to demonstrate the inhibition of Gram-positive MSSA, MRSA, and *S. caprae* bacteria by a dichloromethane fraction. The phytochemical investigation of this fraction led to the isolation and identification of two flavones: penduletin and viscosine. These two compounds were isolated for the first time from *T. bergeri* species. The combination of these compounds with vancomycin and cloxacillin showed not only a decrease in MICs of the antibiotics but also a synergistic effect of penduletin when combined with vancomycin against MSSA and *S. caprae* bacteria.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04195.

Photograph of a *T. bergeri* individual; results of antibacterial screening of hexanic and dichloromethane fractions of *T. bergeri* methanol and methanol/water fractions of *T. bergeri* against a panel of Gram-positive and Gram-negative pathogens; TLC agar overlay bioautography of the dichloromethane fraction against MSSA, MRSA, and *S. caprae*; HPLC chromatograms of subfractions A F2.3 and B F2.5; MS^E mass spectra of compound **1** isolated from the dichloromethane fraction of *T. bergeri* and those of the standard of penduletin; MS^E mass spectra of compound **2** isolated from the dichloromethane fraction of *T. bergeri* and those of the standard of viscosine; MS/MS spectra of compound **1** isolated from the dichloromethane fraction of *T. bergeri* and those of the standard of penduletin; and MS/MS spectra of compound **2** isolated from the dichloromethane fraction of *T. bergeri* and those of the standard of viscosine (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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