Chemical characterization, antioxidant and antibacterial activities of six Agave species from Sinaloa, Mexico

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ABSTRACT

Mexico has the greatest diversity of Agave species in the world and considering their uses in traditional medicine, these plants could be a rich source of bioactive compounds. In this research, we studied five wild Agave species from Sinaloa, México (A. rzedowskiana, A. impressa, A. ornithobroma, A. schidigera and A. angustifolia) and one cultivated (A. tequila). They were evaluated for antioxidant- and antibacterial activities and chemical composition. Statistical analysis consisted of a completely randomized design with one factor analysis of variance and the means were contrasted by the Tukey test ($p<0.05$). Agave tequila showed the highest antibacterial activity with a Minimal Inhibitory Concentration (MIC) of 5 mg/mL, while A. rzedowskiana showed the highest antioxidant capacity by the DPPH method; both activities were higher than those reported for other Agave species. Agave ornithobroma had a higher content of the evaluated phytochemicals, mainly triterpenes and steroids. An activity based separation was carried out with the hexane extract of A. rzedowskiana; chromatographic separation and analysis by gas chromatography–mass spectrometry (GC–MS) showed 2-(3,4-dimethoxyphenyl)-N-methyllethanamine, 9-octadecenoic acid and α-tocopherol (vitamin E) as the most abundant compounds. α-Tocopherol was clearly associated with the hexane extract antioxidant activity. This research showed a variety of phytochemicals in the studied Agave species and some of these species showed the highest antibacterial and antioxidant activities published up to date for this genus.

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1. Introduction

In recent years, infectious and chronic degenerative diseases have been the main mortality causes in the world (OMS, 2009). Antibiotics have been the most common agents against bacterial infections; however, public health concern is focused on bacterial resistance and the emergence of new pathogens. In Mexico, 4.7% of deaths in children younger than 5 y in 2004 were associated with diarrheas and 9.3% with pneumonia; in 2008, prevalence increased up to 6% and 13%, respectively (OMS, 2009; WHO, 2010).

Oxidative stress is an important mechanism in the development of chronic degenerative diseases such as cardiovascular diseases and cancer. Thus, the balance between pro-oxidant and antioxidant agents is essential for homeostasis and health (Eberhardt and Jeffery, 2006). Based on these facts, many researches have focused on the establishment of the antioxidant potential of plant compounds (e.g. phenolics, vitamins, terpenoids) as well as other human endogenous metabolites (Kaneria et al., 2009; Lim et al., 2007; Liu et al., 2009; Ou et al., 2002).

Drugs commonly used for the treatment of infectious and chronic degenerative diseases are costly and not 100% effective; therefore, new and better therapeutic alternatives are required. Since the origin of the human being, the knowledge of medical traditional uses of plants has been accumulated, whereas scientists look for the chemicals associated with such uses. Plants are big reservoirs of biologically active compounds (Chea et al., 2007; Vaghasiya and Chanda, 2007; Verastegui et al., 2008) but only 20–30% of the plants have been characterized for their secondary metabolites (Wink, 2010).

Sinaloa has a great diversity of plants with about 3000 out of the 25,000 vascular plants registered for Mexico (Semarnat, 2008; Vega-Aviña and Villaseñor-Ríos, 2008), and there is
evidence of more than 3500 species (Vega-Aviña and Villaseñor-Ríos, Unpublished results), many of them threatened mainly by deforestation (WWF, 2012; Zulueta Rodríguez et al., 2006). The conservation and rational use of this floristic richness requires of scientific based knowledge.

The Agave genus is endemic of the Americas and is distributed from the Southwest United States and South of Florida (US) to the tropical area of South America, including the Caribbean Islands (García-Mendoza, 2007; García Mendoza and Lott, 1994; Reveal and Hodgson, 2012). For North America, Gentry (1982) reported 197 taxa constituted by 136 species and 61 intraspecies.

Mexico is the center of origin for the Agave species with 186 taxa (150 species and 36 intraspecies) (García-Mendoza, 2007), but 39 of them are in danger of extinction (Conabio, 1998). Oaxaca has the highest number of registered species with 37 and Sinaloa has 21 (García-Mendoza, 2007).

Agave species have many traditional uses around the world and some of these are supported by scientific information. In ethnopharmacology, Agave species have been used for the treatment of diseases of bacterial etiology (e.g. gastrointestinal and wound infections, urologic disorders, dysentery) and against those associated with oxidative stress (e.g. cancer, diabetes and hypertension) (Cornara et al., 2009; Instituto Nacional Indigenista, 2009; Montesano et al., 2012; Semenya et al., 2012). On the other hand, anti-inflammatory (Da Silva et al., 2002), antihypertensive (Duncan et al., 1999), immunomodulatory (Chen et al., 2009), antiparasitary (Orestes Guerra et al., 2008), and antifungal (Verastegui et al., 2008) activities have been demonstrated for Agave species.

Our research group is interested in the chemical- and biological characterization of wild plant species from Sinaloa to establish their potential utility. We are involved in the generation of scientific information to support strategies for conservation and rational use of the regional/national floristic biodiversity. Thus, the objective of this research was the chemical characterization and evaluation of the antioxidant and antibacterial properties of five wild Agave species and one cultivated from Sinaloa, Mexico.

2. Materials and methods

2.1. Materials

A minimum of five adult plants were sampled for each of the wild Agave species from Sinaloa, Mexico during September 2010; samples were representative of the Agave species in the geographical area of collection. Two or more leaves were collected depending on the size of the plant, taking special care to avoid plant damage. The following description of each Agave species includes in parentheses the coordinates, the name of the collector, and assigned number in the herbarium of the Agronomy Faculty of the Autonomous University of Sinaloa (UAS). Agave impressa Gentry is distributed in the states of Jalisco, Nayarit and Sinaloa, it was collected in the low mountain range area of Tecualailla, Escuinapa, Sinaloa (300 meters above sea level, masl, 22 45'36" N, 105 38'24" O; Vega-Aviña R., 10076); Agave ornithobroma Gentry is distributed in Nayarit and Sinaloa, and it was collected from the Copala region, Concordia, Sinaloa (200 masl, 23'20'24" N, 105 56'06" O; Vega-Aviña R., 10750); Agave rzedowskiana P. Carrillo, R. Vega & R. Delgadillo is found in Jalisco and Sinaloa, and collected near “La Petaca” town, Concordia, Sinaloa (1700 masl, 23'23'52" N, 105 48'37" O; Vega-Aviña R., 11671); Agave schidigera Lem. is widely distributed in the states of the central and north regions of Mexico (Gentry, 1982), and it was collected from the low mountain range of the “Baila” town, Culiacan, Sinaloa (120 masl, 24'11'30" N, 106 58'54" O; Vega-Aviña R., 7814); Agave angustifolia Hawis distributed in the States of the coast, central and north regions of Mexico, and in Central America (Gentry, 1982), it was collected in the municipality of Elota, Sinaloa (74 masl, 23'51'22" N, 106°47'29" O; Vega-Aviña R., 10142); Agave tequilana Weber grows wild from Jalisco to Oaxaca and Puebla (Gentry, 1982), and currently, it is found along all the states of the Pacific Coast of Mexico (McVaugh, 1989), it was collected from a commercial plantation in the municipality of Elota, Sinaloa (60 masl, 23°51'42.18" N, 106°50'44.7" O; Pío-León J.F., without number).

The bacteriological culture media used were tryptic soy agar (LSA) and Muller Hinton broth (Becton Dickinson). The reagents were analytical grade: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2’-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid (Trelax, 3,4,5-trihydroxybenzoic acid (gallic acid), 2,2’-azo-bis(2-amidino propane) dihydrochloride (APAH), 3,6-dihydroxypropiro [isobenzofuran-1[3H],9'[H]-xanthen]-3-one (fluorescein), linoleic acid, β-carotene, Tween 80, Tween 40 and butylated hydroxytoluene (BHT) (Sigma-Aldrich).

2.2. Preparation of the methanol extracts of Agave species

Leaves were washed, cut in small parts and freeze dried (The Virtis Company, New York, USA). Dried samples were milled in a waring blender (Osterizer, México) to obtain meals which pass through a number 40-mesh sieve. Then, Agave meal (1 g) was mixed with methanol (10 ml), sonicated (15 min) (Sonicator FS30H Fisher Scientific, USA), centrifuged (28,620 × g/15 min/20°C) and the supernatant was recovered. Extraction was carried out one more time with the residual meal. The recovered supernatants were mixed and the solvent was eliminated under vacuum (40°C) with a rotary evaporator (BÜCHI Labortechnick AG, Switzerland), followed by removal of any residual solvent in a vacuum oven at 40°C. Three methanol extracts were prepared for each Agave species and stored at 4°C in darkness until their use.

2.3. Preparation of the methanol and hexane extracts of Agave rzedowskiana

A. rzedowskiana was chosen for the antioxidant bioguided strategy of plant compound purification; i.e., in the chromatographic separations only the fractions with the highest antioxidant activities were selected for the next step of purification/analysis. Agave meal extracts were obtained by Soxhlet extraction using a 1:21 (p/v) meal-solvent ratio; extraction temperature was 67–72°C; meal was first extracted with hexane (4 h) and then with methanol (12 h). The hexane and methanol extracts were obtained by elimination of the extraction solvents under vacuum (40°C); both extracts were stored at 4°C in darkness until their use.

2.4. Phytochemical analyses

Secondary metabolites (i.e. terpenes/stereoids, flavonoids, tan-nins, sapoines, volatile coumarins, free anthracenic derivatives, alkaloids, reducing sugars and cardiotonics) were determined by tube and thin layer chromatography (TLC) tests as reported by Harborne (1973). The evaluation was done by direct visual observation and the content was estimated based on a relative scale of high, medium, low and trace.

2.5. Determination of the antibacterial activity

Antibacterial activity was evaluated against Gram negative and Gram positive human pathogenic bacteria, four ATCC control strains (Staphylococcus aureus 29213, Enterococcus faecalis 29212, Escherichia coli 25922 and Pseudomonas aeruginosa 27853) and five strains isolated from clinical samples (Streptococcus group A-4,

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S. aureus, E. coli, Salmonella enterica, Typhi and Shigella dysenteriae), which were donated by the Laboratory of Bacteriology of the National Institute of Pediatrics, D.F., Mexico.

Evaluation was carried out by the broth microdilution method as described by the CLSI (Clinical and Laboratory Standards Institute, 2009). Bacteria was incubated at 37 °C for 18–20 h; an inoculum suspension (1 × 10^8 UFC/mL) was prepared in saline solution (0.85%v/v) and Mueller Hinton broth was added to get 1x10^6 UFC/mL. U-bottom 96 microwell plates were used, 50 μL of inoculum, 50 μL of Mueller Hinton broth, and 50 μL of the extracts to be evaluated were be dissolved in Tween 80 (10%v/v); the same mixture without the extract was used as negative control, while the positive control contained gentamicin (0.25–16 μg/mL) instead of the extract. Microwell plate was incubated (37 °C/18–20 h) and the Minimal Inhibitory Concentration (MIC) was determined by visual examination (absence of turbidity or button of growth at the bottom of the well). The Minimal Bactericidal Concentration (MBC) was determined based on the results of the MIC assay, TSA plates were inoculated with aliquots of every well of the MIC assay where bacterial growth was not observed, including that of the MIC value, and incubated (18–20 h/37 °C). The MBC value corresponded to the well with the minimal extract concentration that prevented bacterial growth in the TSA plates. Assays were carried out by triplicate.

2.6. Determination of the antioxidant activity of A. rzedowskiana

The Agave species with the highest antioxidant activity (AA) was chosen based on the results obtained using the DPPH method. Hexane and methanol extracts were obtained from the selected Agave species and the AA was evaluated by the DPPH (2,2-diphenyl-1-pycrilhydrizyl), ABTS (2,2′-azinobis(3-ethylbenzothiazolin)-6-sulfonic acid), ORAC (Oxygen Radical Absorbance Capacity), and β-carotene bleaching (BCBM) methods; the results were expressed as TEAC (Trolox Equivalent Antioxidant Capacity; μM Trolox equivalents/g d.w.) for the first three methods and as percentage of antioxidant activity (%AA) relative to the control used for the last one. To determine the TEAC values, a calibration curve was prepared using Trolox as antioxidant (0–800 μM) and following the procedure established for the corresponding method of AA.

2.6.1. DPPH method

It was determined as described by Brand-Williams et al. (1995). Briefly, 0.2 mL of sample, or methanol for blank, were mixed with 1.8 mL of 150 μM DPPH in a tube; the mixture was allowed to stand for 20 min in darkness and the absorbance was measured at 515 nm (Spectronic 20 Genesis, Spectronic Instruments, USA). The calibration curve for quantitation as TEAC was \( y = 0.2444x + 1.2819 \) (\( r^2 = 0.9912 \)), where “y” is the % of inhibition of the radical inactivation and “x” is the Trolox concentration.

2.6.2. ABTS method

It was carried out as described by Re et al. (1999). The sample (0.05 mL) was mixed with 1.95 mL of ABTS* radical (5 mL of 7 mM ABTS and 88 μL of 140 mM sodium persulfate), allowed to stand (10 min/37 °C) and the absorbance was measured at 734 nm. The calibration curve for quantitation as TEAC was \( y = 0.1364x - 0.6415 \) (\( r^2 = 0.9989 \)), where “y” is the % of inhibition of the radical inactivation and “x” is the Trolox concentration.

2.6.3. ORAC method

The methanol and hexane extracts were dissolved in methanol and DMSO, respectively. The wells of a microwell plate were added with 25 μL of the sample to be evaluated, 25 μL of phosphate buffer for the blank or 25 μL of Trolox (6.25–100 μM) to construct the standard curve. The microwell plate was placed in the fluorescence equipment (Synergy 2 SL, BioTek Instruments, USA), where 200 μL of fluorescein and 75 μL of AAPH were automatically added. The fluorescence intensity (485 nm (ex)/525 nm (em)) was measured for 75 min (37 °C) with 1 min intervals (Huang et al., 2002).

The calibration curve for quantitation as TEAC was \( y = x - 0.0003 \) (\( r^2 = 0.9912 \)), where “y” is the net area under the curve and “x” is the Trolox concentration.

2.6.4. β-Carotene bleaching method

Determination was in the method described by Velioğlu et al. (1998). The reaction mixture was prepared with 50 mg of Tween 40, 6.25 μL of linoleic acid, 500 μL of β-carotene (2 mg/mL in CH₂Cl₂); solvent was eliminated under N₂(g) and 25 mL of H₂O₂ were added. A blank mixture was prepared with the same procedure but without β-carotene. Using 96 microwell plates, a blank was prepared by mixing 50 μL of DMSO and 250 μL of the blank mixture; 50 μL of the sample and 250 μL of the reaction mixture were used for sample evaluation; negative and positive controls contained 50 μL of DMSO or BHT (at the same concentrations used for the samples), respectively. Plates were incubated at 50 °C and the absorbance was measured (Multiskan Bichromatic, Fisher Scientific, USA) at 492 nm every 15 min during 2 h.

The rate of the β-carotene bleaching was calculated as \( R = [\text{Ina/b}]/t \) and the antioxidant activity as % Antioxidant Activity = [\( R_{\text{control}} - R_{\text{sample}} \)]/\( R_{\text{control}} \) × 100; where “a” is the absorbance at time 0 and “b” at time t for \( t = 15, 30, 45, 60, 75, 90, 105 \) and 120 min.

2.7. Determination of the total phenolics content

The total phenolics content (TPC) was determined by the Folin Ciocalteu method as described by Singleton and Rossi (1965) with slight modifications. Briefly, 0.2 mL of the sample to be evaluated were mixed with 1.58 mL of distilled water and 0.1 mL of the Folin-Ciocalteu reagent; the components were mixed (2 min/40 °C) in darkness and 0.3 mL of a sodium carbonate saturated solution was added; the mixture was allowed to stand for 30 min/40 °C and the absorbance was read at 765 nm (Spectronic 20 Genesis, Spectronic Instruments, USA); water was used as blank. Quantification was carried out by using a gallic acid standard curve (0–500 μg/mL), which was prepared by the procedure described for the samples. TPC was expressed as mg of gallic acid equivalents/g d.w. (mg GAE/g d.w.)

2.8. Fractionation of the A. rzedowskiana hexane extract

The A. rzedowskiana hexane extract (282 mg) was separated by thin layer radial chromatography (Chromatotron 7924T, T-Squared Technology, Inc. USA). The thin layer plate in the equipment (630 rpm) was prepared by passing 100 mL of hexane; hexane extract was injected and elution started with hexane (100 mL), followed by hexane: ethyl acetate mixtures of increasing concentration v/v in the second component (98:2, 600 mL; 97:3, 200 mL; 95:5, 100 mL; 90:10, 500 mL; 85:15, 1250 mL; 80:20, 150 mL). Fractions (5 mL each) were collected and analyzed by TLC on aluminum silica covered plates (Silica gel 60 GF254 Merck) developed with hexane: ethyl acetate (9:1 v/v); similar chromatographic fractions were mixed.

2.9. Analysis by Gas Chromatography–Mass Spectrometry (GC–MS) of the A. rzedowskiana fraction with the highest antioxidant activity

The fraction with the highest antioxidant activity (5 mg/mL) was filtered (0.45 μm PVDF, Titan, USA) and injected (5 μL) to a GC–MS HP 6890 equipment (Agilent Technologies, USA). Separation was
Table 1

Phytochemical analysis of Agave species.

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<th>Agave species</th>
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* The content was estimated as: +, medium; +, low; +, trace; and –, absence. An empty cell corresponds with non determined value. All the evaluated Agave species/extracts were negative for free anthracenes and alkaloids.

b) Extracts: M, methanol; E, ethanol; A, aqueous; and C, chloroform.

carried out in a QUADREX 007 column (30 m × 0.25 mm i.d. × 0.25 μm) (Quadrex Corp., Woodbridge, CT, USA) using helium as the carrier gas; the flow started at 0.6 mL/min and then increased up to 0.9 mL/min with increments of 0.2 mL/min. The temperatures used were: injector, 250°C; oven temperature, 60°C; gradient of 5°C/min up to 200°C and 10°C/min up to 275°C, constant temperature until the end of the run (60.5 min). Mass detector was operated in the electron impact mode with 70 eV of energy. Temperatures of detector and quadrupole were maintained at 245 and 150°C, respectively (Fiorentino et al., 2009).

2.10. Purification of the main compound of the antioxidant fraction of A. rzedowskiana

The A. rzedowskiana hexane extract (620 mg) was fractionated by preparative thin layer chromatography (20 × 20 cm Preparative TLC Glass Plates S 600 F 254 2 mm) (155 mg/plate) using hexane: ethyl acetate (9:1 v/v) as mobile phase. The main band of the hexane extract was recovered, extracted with hexane, concentrated and analyzed by GC–MS as described above. The identity of α-tocopherol was corroborated by co-chromatography with a commercial pure standard.

2.11. Quantification of tocopherol

A standard curve was prepared with α-tocopherol acetate in hexane. GC–MS analysis was performed as described for the analysis of the chosen fraction from A. rzedowskiana hexane extract (2.5 mg/mL).

2.12. Statistical analysis

A completely randomized design with a single factor analysis of variance was used. For the first part of the study, the factor was the Agave species with six levels (A. rzedowskiana, A. impressa, A. ornithobroma, A. schidigera, A. angustifolia and A. tequilana). For the second part, the factor was the type of extract for A. rzedowskiana with two levels (methanol or hexane). Significant differences were established by contrast of means using the Tukey HDS test (p < 0.05). All statistical analyses were carried out with the software STATGRAPHICS Centurion XVI (Statpoint Inc., Warrenton, VA, USA).

3. Results and discussion

3.1. Phytochemical analysis

The Agave species showed different compound families (Table 1). The results were similar to those reported for Agave intermíxta, Agave vera and Agave sisalana (Garcia et al., 1999; Hammuel et al., 2011; Vaghasiya and Chanda, 2007). Chemical compounds belonging to the phytochemicals registered for the Agave species used in this research have been characterized and quantitated for other Agave species: free reducing sugars (Arrizón et al., 2010), terpenes (Peña-Alvarez et al., 2004), tannins (Castillo et al., 2010), flavonoids (Morales-Serna et al., 2010) and saponins (Eskander et al., 2010). The variety of compounds in the studied Agave spp. could be associated with biological activities (e.g. antibacterial, antioxidant) previously established for compounds of every group of the phytochemicals determined.

3.2. Antibacterial activity

Antibacterial activity was observed against five of the nine evaluated bacteria. The MIC values obtained for four out of the six studied plants (Table 2) were similar to those reported for other Agave species. Remarkably, the antibacterial activities of A. tequilana and A. schidigera (MIC 5 mg/mL) were higher than those reported previously for Agave species, e.g. A. picta and A. sisalana showed MIC values in the range of 6 to 20 mg/mL, depending of the bacteria (Ade-Ajayi et al., 2011; Hammuel et al., 2011; Verastegui et al., 2008). The MIC values of ATCC strains to Gentamicin (Table 2) corresponded with published data (Lennette et al., 1987); thus, the antibacterial activities of the studied Agave species could be
better contrasted with those generated in future studies with other plants using the same controls (ATCC strains/gentamicin). As it can be observed, the MIC values for the Agave species extracts, mixtures of compounds, were at least three orders of magnitude higher than that for pure gentamicin. Vaghasiya and Chanda (2007) studied the antibacterial activity of the methanol extract of A. vera leaves; their extract was not active against S. aureus ATCC 25923, P. aeruginosa ATCC 27853 and E. coli ATCC 25922, while in our study the same P. aeruginosa and E. coli ATCC strains were inhibited by the methanol extracts of A. tequilana and A. schidigera (Table 2). Considering the bactericidal activities (Table 2), the studied Agave species showed higher activity than that reported for A. sisalana (Ade-Ajayi et al., 2011; Hammuel et al., 2011); the results obtained with A. schidigera against S. enterica Typhi were very remarkable (Table 2).

The antibacterial activity of Agave species has been associated with the presence of tannins, alkaloids, flavonoids and saponins (Ade-Ajayi et al., 2011; Hammuel et al., 2011; Vaghasiya and Chanda, 2007). In this research, the phytochemical composition of the methanol extracts of the Agave species with the higher and lower antibacterial activities did not show great differences, except for the tannins content (Table 1); thus, further studies are required to identify the antibacterial compounds.

The antibacterial activity of the Agave species used in this study (Table 2) was the highest reported up to date; however, this activity could be considered as weak (MIC > 1.6 mg/mL) (Aliqianis et al., 2001) and it was not considered for the bioguided fractionation.

### 3.3. Antioxidant activity of the studied Agave species and their total phenolics content

The methanol extract of A. rzedowskiana showed the highest AA by the DPHH method (Table 3) and it was chosen to carry on the bioguided fractionation. The methanol extract of A. ornithobroma had the highest TPC (Table 3), which was similar to the value reported by Wu et al. (2004) for a non-defined Agave sp. Taking into consideration the phytochemical analysis (Table 1), the TPC differences found among the studied Agave species could be associated with differences in the tannins content.

The AA and TPC did not show significant correlation ($r = 0.74$, $P \geq 0.05$). This result could be explained by the presence of phenolics with higher AA or more reactive in the evaluated system, even though their TPC contents were lower than other samples; another explanation could be the participation of non-phenolic antioxidants. Scientific reports about the AA-TPC correlation have showed contrasting results, being positive, negative and in other cases null.

### Table 2

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Agave species</th>
<th>MIC</th>
<th>MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus group A-4</td>
<td>A. impressa</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Salmonella enterica Typhi</td>
<td>A. ornithobroma</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>A. rzedowskiana</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Escherichia coli 2922</td>
<td>A. tequilana</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa 27853</td>
<td>A. schidigera</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Enterococcus faecalis 29212</td>
<td>G.A. angustifolia</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$a$ Evaluated extract concentrations were 15, 10, 5 and 2.5 mg/mL; extracts were dissolved in 10% Tween 80 (v/v).

$b$ The “-” character stands for no activity up to the maximal tested concentration (15 mg/mL).

$^c$ G stands for gentamicin and evaluated concentrations were in the range 0.25–16 μg/mL.

### Table 3

<table>
<thead>
<tr>
<th>Agave species</th>
<th>Antioxidant activity (μM TE/g d.w.)</th>
<th>Total phenolics content (TPC)(mg GAE/g d.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. tequilana</td>
<td>9.86 ± 1.51</td>
<td>5.22 ± 0.81</td>
</tr>
<tr>
<td>A. ornithobroma</td>
<td>19.93 ± 0.49</td>
<td>12.37 ± 2.35</td>
</tr>
<tr>
<td>A. impressa</td>
<td>8.00 ± 1.32</td>
<td>3.57 ± 0.49</td>
</tr>
<tr>
<td>A. rzedowskiana</td>
<td>27.41 ± 3.35</td>
<td>7.72 ± 0.59</td>
</tr>
<tr>
<td>A. schidigera</td>
<td>15.79 ± 3.72</td>
<td>4.04 ± 1.22</td>
</tr>
<tr>
<td>A. angustifolia</td>
<td>6.46 ± 1.24</td>
<td>2.06 ± 0.25</td>
</tr>
</tbody>
</table>

Results were expressed as:

$a$ μM of Trolox Equivalents/g d.w. (μM TE/g d.w.).

$^b$ mg of Gallic Acid Equivalents/g d.w. (mg GAE/g d.w.). Values were the mean of three independent measurements ± standard deviation. Different letters in the same column (w, x, y, z) showed significant differences ($p \leq 0.05$).

### 3.4. Antioxidant activity of the hydrophobic and hydrophilic components of A. rzedowskiana

The hexane and methanol extracts of A. rzedowskiana were obtained as the first stage of chromatographic separation of components with AA. The methanol extract showed the highest AA but in the BCBM it was a pro-oxidant (Table 4). The hexane extract showed the highest inhibition of DPHH and ABTS radicals; however, when the results were expressed as μM TE/g d.w., the values were higher for the methanol extract, which was associated with its higher extraction yield. The Agave AA has been scarcely studied; it was reported that an aqueous extract of a non-specified Agave

### Table 4

<table>
<thead>
<tr>
<th>Evaluation method</th>
<th>Extract [Concentration, mg/mL]</th>
<th>Antioxidant activity (μM TE/g d.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH</td>
<td>Hexane [1]</td>
<td>7.76 ± 2.18$^a$</td>
</tr>
<tr>
<td>ABTS</td>
<td>Methanol [4]</td>
<td>15.63 ± 4.31$^b$</td>
</tr>
<tr>
<td>ORAC</td>
<td>Hexane [1]</td>
<td>212.12 ± 1.65$^y$</td>
</tr>
<tr>
<td>β-carotene bleaching</td>
<td>Methanol [4]</td>
<td>862.62 ± 18.75$^x$</td>
</tr>
</tbody>
</table>

Different letters in the column (x, y) showed significant differences among extracts for each method.

$^a$ Antioxidant activity was the mean ± standard deviation of three independent measurements. Values are expressed as μM of Trolox Equivalents/g d.w. (μM TE/g d.w.) but for the β-carotene bleaching method as percentage of antioxidant activity.
sp., as well as extracts of phenolics and flavonoids of *A. americana*, showed inhibition of the DPPH radical (Ben Hamissa et al., 2012; Rodríguez-Hernández et al., 2000).

Based on the results with the ORAC method, lipophilic components (hexane extract) showed lower AA values than those of higher polarity (methanol extract) (Table 4), a pattern similar to that reported by Wu et al. (2004). The differences registered between extracts and AA methods could be associated with differences in the composition of each extract, antioxidant compounds whose reactivity depends on the environment of the reaction mixture, as well as with the type of radical used; e.g. it has been reported that some antioxidants react fast with peroxy radicals but they react slow, or are inactive against the DPPH radical, probably associated with steric hindrance (Prior et al., 2005).

Our results suggested that the hexane extract of *A. rzedowskiana* had components with higher AA than those in the methanol extract, although these components are in low concentration in the meal; thus, the bioguided fractionation was carried out with the hexane extract of *A. rzedowskiana*.

### 3.5. Fractionation of the hexane extract of *A. rzedowskiana* and analysis of the fraction with the highest antioxidant activity

Seventeen fractions were collected from the radial chromatography of the *A. rzedowskiana* hexane extract; their antioxidant activity (DPPH method) showed radical inhibition in the range of 0.22% to 60.34%; fraction 5 of the hexane extract (HE-F5) was the best and it represented about 3.8% (w/w) of the methanol extract.

GC–MS analysis of HE-F5 (Fig. 1A) showed the presence of 2-(3,4-dimethoxyphenyl)-N-methylmethylaniline, 9-octadecanoic acid and α-tocopherol as the compounds with the highest concentration. Peaks not numbered correspond to fatty acids, amines, carboxylic acids and sterols, ordered from low to high retention times (Table 5); taking into consideration the TLC and GC–MS analysis, these are in lower concentrations. The established composition was similar to that reported for other Agave species (Abdel-Gawad et al., 1999; Gutiérrez et al., 2008; Peña-Alvarez et al., 2004).

### 3.6. Isolation and identification of the main compound in the antioxidant fraction of *A. rzedowskiana*

Preparative TLC of the *A. rzedowskiana* hexane extract was used to obtain the main separated band. It was analyzed by GC–MS (Fig. 1B) and demonstrated the presence of α-tocopherol; moreover, 2-(3,4-dimethoxyphenyl)-N-methylmethylaniline was also identified after the purification process; this compound has been previously reported as a plant natural product, particularly, it has been isolated from peyote (*Lophophora williamsii*), a cactaceae with the same type of metabolism of the Agave species (Batis and Rojas-Aréchiga, 2002).

### 3.7. α-Tocopherol analysis

The α-tocopherol concentration of the band recovered by preparative TLC of the *A. rzedowskiana* hexane extract was 413.63 μg/mL. The antioxidant activity of the purified fraction was contrasted with a solution of commercially-pure α-tocopherol (413.63 μg/mL); the antioxidant activities by the DPPH method of these two samples did not show significant differences (p < 0.05), showing values of 93.7% and 91.60%, respectively. Consequently, it was confirmed that antioxidant activity of the studied *A. rzedowskiana* fraction was mainly associated with its α-tocopherol content. The α-tocopherol is a well-known natural antioxidant, it is a liposoluble molecule that can inactivate free radicals, property probably associated with its structural phenolic group (Asensi-Fabado and Munné-Bosch, 2010).

### 4. Conclusions

This paper had showed that the antioxidant activity of *A. rzedowskiana* and the antibacterial activity of *A. tequilana* were higher than those previously reported for other Agave species. Moreover, *A. ornithobroma* showed a more diverse range of phytochemicals among the studied Agave species. The bioguided characterization showed that the *A. rzedowskiana* antioxidant activity was...
mainly associated with non-polar compounds (hexane extract) and specifically with the content of α-tocopherol. This information provides the basis for a knowledge-based preservation strategy of the studied Agave species, which have very restricted geographic distribution and are endangered.

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