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Identification and Quantification of Bioactive Compounds in *Agave potatorum* Zucc. Leaves at Different Stages of Development and a Preliminary Biological Assay

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Abstract

The leaves of *Agave potatorum* Zucc. represent more than 50% of waste during the jima in the mezcal industry. To provide the basis for a knowledge-based integral use of these wastes, this research provides the identification and quantification of bioactive compounds of leaves at different stages of development, differentiated by the position of the leaves in the plant (basal, medium and apical), and dividing each leaf into apical and basal parts. Qualitative phytochemical analysis showed highly positive results for coumarins and tannins, and positive for cardiac glycosides and triterpenoids compounds, without an age-dependent or position of the leaves response of the plant. Quantitative analysis of phenolic compounds and flavonoids is not preferentially accumulated respect to the position of the leaf in the plant, only are higher in the apical parts, whereas for carbohydrates a positive gradient was evidenced through the leaves in the plant from apical to basal ones. The highest concentration of phenolics, flavonoids, fructose and fructans compounds were determined in 6-year leaves, with maximum observed values of 173.80 ± 9.36, 35.58 ± 6.41, 308.30 ± 3.62 mg/g d.w., and $37.23 \pm 4.5\%$, respectively. Interestingly, the ethanolic extract of the fresh leaves of *A. potatorum* showed an increase in the mycelial growth of *Botrytis cinerea*. The data showed that plant age is the most important factor influencing the content of bioactive compounds. In addition, a fungal growth enhancing effect was exhibited by the extract, suggesting a biotechnological advantage that can improve the growth of beneficial fungi in agricultural crops.

Graphic Abstract

Agave potatorum Zucc. leaves as potential source of bioactive compounds

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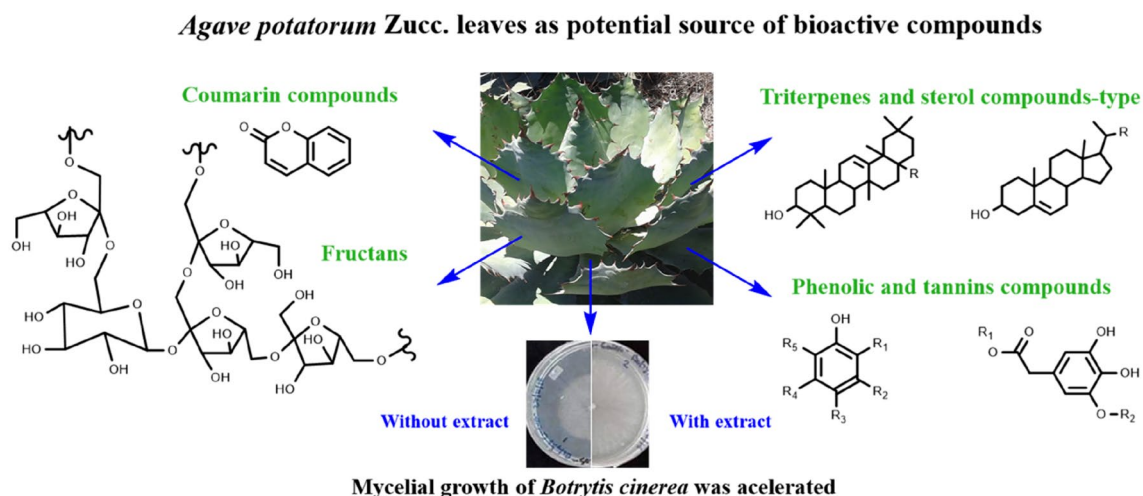
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Extended author information available on the last page of the article

Statement of Novelty

Agave potatorum leaves represent more than 50% of waste during the jima in the mezcal industry, and there are no previous works on their composition. To propose new uses of this plant and to expand its comprehensive use and benefits, it is necessary to characterize and to know the composition of these leaves. Our results showed the presence of secondary metabolites (coumarins, tannins, phenolic compounds, triterpenoid, and cardiac glycosides) that can be the base of new drugs; the low and high polymerization degree fructans in the leaves can be a functional food ingredient, and the ethanolic crude extract of leaves can improve the growth of beneficial fungi in agricultural crops, evidencing the biotechnological potential of *A. potatorum* leaves.

Introduction

México is the country of *Agave* since it shelters 160 species of the 210 in the world, representing 75% in its natural distribution area [1]. The most diverse center is in the limits of Puebla-Oaxaca where 29 species are found. *Agave* genus is well adapted to survive in conditions of extreme heat and drought due to a unique combination of morphological and physiological attributes. *Agave* stores carbohydrates in the form of fructans instead of starch, macromolecules highly water soluble that can act as membrane stabilizers [2], and to improve the resistance to stress from cold and drought of *Agave* [3].

Agave potatorum is an economically important crop in Oaxaca, México, because it is the only raw material (usually from 6 to 7 year-old plants) used to produce mezcal tobalá, an alcoholic beverage highly appreciated for its mild organoleptic qualities and its high content of volatile compounds [4]. *Agave potatorum* stores two fructan types, graminans and agavins (neoseries), being agavins the most abundant type [5], which makes this crop a potential source for various industrial purposes. However, of the entire plant, only the mezontle or stem of the plant is used, leaving the agave leaves (50% of the total plant weight) as waste because they are discarded during the jima (cutting of the leaves) of the stem or "piña" of the agave plants [6].

Most plants have an almost unlimited capacity to biosynthesize phytochemicals, which serve as a source of natural antioxidants that can act as defense mechanisms of plants [7]. In young leaves the epidermis is scarce in immature leaves, therefore, flavonoids could serve as protection against various environmental factors such as solar radiation [8]

and the attack of insects. Besides, phytochemicals play an important role in plant biology; thus, they respond to light and control auxin levels, which regulate the growth and differentiation in the plant [9–11].

Many species of the *Agave* genus constitute an important source of steroid sapogenins, mainly hecogenin [12]. In the pharmaceutical industry, these natural compounds are used for the semi-synthesis of medicinal steroids. In *agave* leaves the presence of triterpenes, steroids, tannins, coumarins, cardiotonic, and fructans [13], or such as saponins and phenolic compounds with levels between 0.5 and 4.5% as a function of agave species had been identified [14]. Additionally, these secondary metabolites possess different biological effects, as demonstrated by in vitro and in vivo tests [6]. Also, the extracts of agave leaves possess antioxidant, cytotoxic, and antimicrobial activities [15]. Previous studies set that phenolic compounds have potent antifungal effects via inhibition of cell wall formation, disruption of the cell membrane, and inhibition of mitochondrial function [6]. Likewise, different benefits for health care are attributed to them for their important antioxidant and anti-cancer activities [16]. Therefore, *agave* leaves byproducts constitute a potential source of bioactive compounds.

To our knowledge, there are no previous works on the phytochemical screening and bioactive compounds of *A. potatorum* leaves, for this reason, it is necessary to establish biotechnological techniques that allow to expand the comprehensive use and benefits of this plant. Therefore, the objective of this work was to study the *A. potatorum* Zucc leaves of 2, 4 and 6 years of growth with three different positions within the plant (basal, medium and apical), to determine if there is any tendency in terms of accumulation of secondary metabolites, and later to assess the effect of the ethanolic extract on *Botrytis cinerea* mycelial growth.

Materials and Methods

Biological Material

Agave potatorum leaves were obtained at random from 2, 4, and 6 years old (age corresponds to the time in the field, starting from the plantation of the seed) from Infiernillo, Zaachila Oaxaca, México. It is located at the Geographic coordinates, N 16° 89′, W 97° 19′, 1969 m above mean sea level, with a temperature from 10 to 26 °C with a semi-warm sub humid climate. Precipitation varies between 300 and 1200 mm. The plants were donated by the producer and identified according to morphological descriptors [17]. The leaves were cut off and grouped according to their

distribution in the plant, as basal, middle, and apical. Each leaf was divided into two parts, basal and apical, to determine if any tendency in terms of metabolites or soluble carbohydrates accumulation could be established. The leaves were collected of three independent plants and washed with abundant water and cut in squares of 10 cm by 10 cm; afterwards, they were stored at − 20 ℃ until their use.

The nomenclature used for each sample was established as **Xw**, where **X** corresponds to the distribution of the leaf in the plant, and could be: **B** (basal), **M** (middle), or **A** (apical), and **w** corresponds to the division of each leaf and could be: **b** (basal), or **a** (apical).

Phytochemical Screening and Quantifying of Phenolic and Flavonoid Content

Phytochemical analysis (coumarins, triterpenes, tannins and phenolic compounds, cardiac glucosides, saponins, and alkaloids) of all samples (leaves of 2, 4, and 6 years old, subdivided as described before) was carried out according to standard methods [18, 19]. The evaluation was made by direct observation and the relative concentration was determined as high (+++), medium (++), and low (+).

The collected *A. potatorum* leaves were conditioned to room temperature, washed again with water, and cut into small pieces of 1 by 1 cm. For each sample, a total of 18 g of leaves were extracted in 20 mL of ethanol into an Ultrasonic bath (Branson, Mod. 3800, BGJ101625780B, USA) at 40 kHz for 60 min maintaining the water temperature between 25 and 27 °C, an effective extraction method to extract bioactive compounds from agave bagasse [16, 20]. Afterwards, the solvent was decanted and centrifuged at 3000 rpm for 5 min. The supernatant was stored in closed tubes covered with aluminum at 4 °C for phytochemical screening. The quantification of bioactive compounds was determined in an extract of 10 g of leaves in 5 mL of 90% ethanol/water. The mixture was sonicated for 30 min and passed through Whatman filter paper number 3.

Coumarins

Two methodologies were carried out, sodium hydroxide and fluorescence test. A curcuma solution was used as a positive control in both tests. For sodium hydroxide, 300 μL of ethanolic extract of each sample was taken into a test tube and 300 μL of 10% NaOH solution was added; the appearance of a yellow-colored precipitate, which after the addition of some drops of diluted H_2SO_4 disappears, was considered as positive. For fluorescence test, on a silica thin layer a spot of 10 μL of ethanolic extract in subsequent applications was placed. Once the sample was completely dried, 5 μL of 10% $CoCl_2$ solution was applied over the thin layer chromatography (TLC). After dryness, TLC was revealed at 365 nm

with a handled UV light lamp. The appearance of a blue fluorescent halo around the samples was considered positive for the presence of coumarins. A curcuma solution was used as a positive control.

Triterpenes

Liebermann–Burchard's test was carried out. 500 μL of the extract was treated with a few drops of acetic anhydride, boiled, and cooled. H_2SO_4 concentrated was added from the sides of the test tube which showed a brown ring at the junction of two layers. Color from yellow to deep red indicated the presence of triterpenoids, including triterpenoid saponins.

Tannins and Phenolic Compounds

The ferric chloride test was developed. A $FeCl_3$ solution was prepared, 1.27 g of $FeCl_3$ was dissolved in 25 mL of water and gauged to 50 mL with methanol in a volumetric flask. For the tests, 250 μL of the extract were placed into a test tube and 100 μL of the $FeCl_3$ solution was added. It was left to rest for 15 min. The appearance of bluish black precipitate indicated the presence of tannins and phenolic compounds. Red wine was used as a positive control.

Cardiac Glycosides

For this determination, a Legal's test for lactones, and Keller–Kiliani reagent for deoxysugars were developed [21]. For lactones, 2–3 drops of pyridine were added to 250 μL of ethanolic extract. A drop of fresh 5% sodium nitroprusside solution and 1–3 drops of 2 N NaOH were added. The intense red color indicated the presence of lactones. Digoxin was used as a positive control. For deoxysugars, 100 μL of glacial acetic acid was added to 300 μL of ethanolic extract, a drop of 5% $FeCl_3$ and a drop of H_2SO_4 was added slowly from the sides of the test tube. A brown ring formed between the layers was considered as positive. Digoxin was used as a positive control.

Saponins

The foam test was carried out to identify the presence of saponins. 300 μL of the extract was taken into a test tube with 400 μL of water and shaken vigorously. The appearance of foam persisting for 10 min indicated the presence of saponins.

Alkaloids

To 200 μL of ethanolic extract, 2 drops of the corresponding reagent were added (Meyer or Draguendorff). For the

Bouchardart test, 1 drop of HCl was added previously to 4 drops of Bouchardart reagent. The appearance of a white, orange-red, or reddish-brown precipitate indicated the presence of alkaloids for Meyer, Draguendorff, or Bouchardart test, respectively. Caffeine was used as a positive control.

Total Phenolic Content

1 mL of 0.5% Na_2CO_3 aqueous solution (w/v) and 1 mL of Folin–Ciocalteu 2 mol/L reagent (Sigma-Aldrich, Co. Ltd., City, México) were diluted 10 -times in water and added to the extracts (1 mL in triplicate). Samples were protected from light and vortexed before being left for 1 h at 25 °C. Absorbance was measured at 750 nm using a UV–visible (model UV-1800, Shimadzu, Japan) spectrophotometer. The results were compared with a calibration curve ($r^2 = 0.976$), using a gallic acid (Sigma-Aldrich, Co. Ltd.) standard (0–500 ppm). The content of total phenols is expressed as gallic acid equivalents (GAE) (mg/kg dry weight basis).

Total Flavonoids Content

150 μL of 5% $NaNO_2$ was added to the extracts (2 mL in triplicate) and left to rest for 5 min. Then, 150 μL of 10% $AlCl_3 6H_2O$ was supplement and, after 6 min, 1 mL of NaOH 1 mol/L. Absorbance was measured at 510 nm in a UV–visible (model UV-1800, Shimadzu, Japan) spectrophotometer. The results were compared with a calibration curve ($r^2 = 0.996$) using quercetin (Sigma-Aldrich, Co. Ltd.) (0–100 ppm) and expressed as mg quercetin equivalents (QE) (mg/kg dry weight basis).

Soluble Solids, Moisture and Ash

The total soluble solids (TSS) content was obtained with a HANNA HI 96,801 refractometer. For the determination of humidity and ash, the AOAC 2000 techniques [22] were used. The humidity was determined by drying at 105 °C until constant weight. The ashes of the residue were determined by calcination at 550 °C for 8 h. All determinations were made in triplicate.

Water Soluble Carbohydrates

Due to the highest sensibility of the techniques to quantify water soluble carbohydrates, and to avoid interference, the sample was prepared as described. The leaves of *A. potatorum* of each age were dried in a convection oven at 65 °C. Samples were then pulverized in a mill (GM 200) for 30 seconds at 10,000 rpm. 100 g of the sample was taken for the determination of soluble sugars and quantification of fructans. Total carbohydrates were extracted from 1 g of dehydrated leaves and 50 mL of 80% ethanolic solution. The

suspension was stirred for 30 min at 80 °C, filtered with Whatman No. 1 paper, and the solid residue was extracted twice more in an aqueous medium for 15 min. The three supernatants were pooled and gauged to a final volume of 100 mL.

Carbohydrates

25 μL of the above-described solution were taken up to 1 mL with deionized water and were used for the determination of total carbohydrates [23]. The calibration curve was determined by glucose solutions in a range concentration from 2 to 100 μg/mL. To 1 mL of the sample contained in test tubes, 1 mL of 5% phenol solution was added, subsequently, 5 mL of H_2SO_4 were added. The tubes stood for 10 min at room temperature; they were shaken and placed for 20 min in a water bath at 20 °C. Absorbance was read on a Shimadzu UV–visible spectrophotometer within the next 30 min at 490 nm.

Direct reducing carbohydrates were also performed according to the method of Somogyi (1945). Fructose determination was done following the ATS (anthrone–tryptophan–sulfuric acid) method, established by Somani et al. [24] in the concentrations range of 1 to 200 μg/mL.

Enzymatic Determination of Fructans

Fructan quantification was performed with the commercial kit "Fructans" analytical test kit (Megazyme International Ireland, Ltd., Wicklow, Ireland), according to the instructions of the manufacturer.

Qualitative Analysis of Fructans in Leaves of *A. potatorum* by Thin-Layer Chromatography (TLC)

The analysis of fructans was performed by TLC following the indications described by Mellado-Mojica and López [25]. A total of 1 μL of *A. potatorum* fructan solution (25 mg/mL) was applied to a silica gel TLC plate. The TLC plate was eluted in a system of butanol/propanol/water [26] and sprayed with the reagent aniline/diphenylamine/phosphoric acid in acetone for carbohydrates visualization [27].

Effect of the *Agave* Leaves Extracts on *Botrytis cinerea* Mycelia Growth

Sample Preparation

The use of the ultrasonic bath was not feasible at this stage due to the scale in the extraction processes, and the aqua-ethanolic extract was not suitable to obtain a dry extract. Therefore, fresh agave leaves of 2, 4, and 6 years old were washed with abundant water and cut in strips.

Independently, each sample was poured in ethanol anhydrous in a relation of 250 g of leaves: 1.5 L of ethanol, and stood in maceration for 48 h. Afterwards, the solvent was filtered off and removed by rotaevaporation at 40 °C until approximately 15 mL, these were brought to a Petri dish and the remnant solvent was removed by air flux during 2 h. Finally, the concentrated extract was desiccated on anhydrous silica until the water was removed (until constant weight). The extracts were stored at 4 °C prior to use.

Biological Activity

The biological effect of the ethanolic extract on the mycelial growth of a pathogenic strain of *B. cinerea*, was assessed. *B. cinerea* was isolated from blueberry fruits (*Vaccinium corymbosum* L.) and morphologically characterized. The bioassays were carried out according to the technique of deposition of mycelium on a solid substrate. In this study, five treatments were established by varying the concentration of ethanolic extract in potato-dextrose-agar culture medium (PDA, BD Bioxon®, USA). The evaluated concentrations were of 1000, 5000, 10,000, 20000, and 30000 ppm. Control without extract was also included. The PDA with the extract was emptied into Petri dishes (100 × 15 mm) and allowed to solidify for 24 h. Then, an 8-day-old *B. cinerea* mycelium disk (0.8 cm Ø) was placed in the center of each box with PDA culture medium and the extract, and these boxes were incubated at 25 ± 2 °C in darkness during 120 h. The effect of treatments was evaluated by measuring fungal radial growth (cm) with a digital calibrator every 24 h versus the radial growth of the control.

Statistical Analysis

From the quantification of secondary metabolites, all determinations were made in triplicate. In the biological effect, the experiment was established under a completely randomized design with seven replications. The data were analyzed using an analysis of variance (ANOVA) and Tukey test with $p < 0.05$ in order to find significant differences, and $p < 0.001$ to find highly significant differences, using Sigma Plot program version 11.

Results and Discussion

Phytochemical Screening and Quantifying of Phenolic and Flavonoid Content

From Table 1, the ethanolic extracts of *A. potaturum* leaves, divided by the position of the leaf in the plant, separated into basal (B), medium (M), and apical (A), and each leaf divided into basal (b) and apical (a), contain coumarins, according to the determination with NaOH and fluorescence; however, the presence of these metabolites is independent of the position of the leaf in the *Agave* or as a function of the age. The same is observed in terms of tannins and phenolic compounds, tested by $FeCl_3$, although these were positive in all the samples, no selective accumulation is observed, neither according to the age nor to the position of the leaf. In addition, triterpenoids are present in all leaves independently of the age or position in the leaf. The presence of cardiac glycosides, rehearsed with the Keller–Kiliani reagent for deoxysugars and the Legal reagent for lactones, could be considered as positive only in ethanolic extracts of 6-year-old agave at medium (**Mb**, **Ma**) and apical (**Ab**, **Aa**) leaves.

Table 1 Qualitative phytochemical analysis of ethanolic extract of *Agave potatorum* Zucc. leaves of 2, 4, and 6 years old

Sample	Secondary metabolites														
	Coumarins [NaOH (fluorescence)]			Tannins [FeCl$_3$]			Saponins [foam]			Cardiac glycosides [Legal (Keller–Kiliani)^a]			Triterpenoid [Liebermann–Burchard]		
Age of plant (years): Sample	2	4	6	2	4	6	2	4	6	2	4	6	2	4	6
	++ (+)	++ (+)	++ (+)	+	+	++	−	−	−	+ (+, −)	+ (+, −)	+ (+, −)	+	+	+
Ba	+++ (+)	+++ (+)	+ (+)	+	+	++	−	−	−	+ (+, −)	+ (+, −)	+ (+, −)	+	+	+
Mb	+++ (+)	++ (+)	++ (+)	++	++	+	−	−	−	+ (+, −)	+ (+, −)	+ (+, +)	+	+	+
Ma	++ (+)	+++ (+)	++ (+)	++	+	++	−	−	−	+ (+, −)	+ (+, −)	+ (+, +)	+	+	+
Ab	++ (+)	++(+)	++ (+)	++	++	+	−	−	−	+ (+, −)	+ (+, −)	+ (+, +)	+	+	+
Aa	+++ (+)	+++ (+)	+++ (+)	++	+	++	−	−	−	+ (+, −)	+ (+, −)	+ (+, +)	+	+	+

The method test is indicated in [ ]. ^{a}The reagent of Keller–Kiliani was assessed with plants harvested in different years, and for the first was positive while in the next were mostly negative. +++: strong positive test, ++: moderate positive test, +: low positive test, − − −: negative test. Leaves localization: apical (A), basal (B), medium (M); Leaf region: apical (a), basal (b), medium (m)

Also, the presence of saponins was not decisive in the foam test since the foam was not stable for more than 3 min. Although the presence of triterpenic saponins could be considered positive according to Liebermann–Burchard test, it is necessary to bear in mind that other steroids, triterpenoids, and cardiac glycosides could be testing positive in this assay. Regarding alkaloids, tested with Meyer, Draguendorff, and Bouchardat reagents, these were not found in ethanolic extract of the leaves.

The secondary metabolites identified in ethanolic extract of leaves [coumarins, tannins, and phenolic compounds, triterpenoids and cardiac glycosides (only in 6-year-old leaves)] are similar to those reported by Ahumada-Santos et al. [15] for *A. rzedowskiana*, *A. impressa*, *A. ornithobroma*, *A. schidigera*, *A. angustifolia* and *A. tequilana* cultivated, as well as for *A. sisalana* [28], and *A. intermixta* [29]. Except for the saponins, for which the tests were not conclusive, but, as in the report of Ahumada-Santos, et al. [15] in the ethanolic and methanolic extracts, the tests for saponins were negative, and positive in aqueous media.

Therefore, it is concluded that the leaves of *A. potatorum* Zucc. have great potential as a new source of bioactive compounds. That could be associated with biological activities (e.g. antibacterial, antifungal, antioxidant) as previously described by other authors [6].

Before quantifying the phenolic and flavonoids content, the percent of moisture was determined in each section of the leaf, in 2, 4 and 6 years old leaves. However, the percentage of humidity showed a slight variation respect to the leaf position (**M**, **B**, or **A**) and among leaves of different ages, being the average values 88.24 ± 1.63, 85.66 ± 2.12 and $86.19 \pm 1.74\%$ for 2, 4 and 6 -year-old leaves, respectively.

The percentage of humidity in the leaves of *A. potatorum* is within that reported by Jiménez-Muñóz et al. [30], 83.43% humidity in leaf samples of *A. tequilana* Weber, and 91.5% reported by Reyes-Munguía et al. [31] for purple *Agave* leaves. These values are explained because of the role in the internal distribution of minerals and the transport of water to the whole plant, as explained by Gutiérrez [32]. According to the result obtained, in order to carry out its physiological processes, the plant behaves as a single unit, maintaining the same moisture percentage, minerals, and total soluble solids in each position (M, B, or A) of the stalk so that the plant can carry out its secondary metabolism.

The phenolic and flavonoid content were determined as described in the methodology and corrected by the moisture percentage. The average value in each part of the leaf on leaves of different ages is summarized in Table 2.

From Table 2 can be seen that phenolic compounds concentrations are significantly higher in the apical zones (**Ba**, **Ma**, and **Aa**) than in the basal zones (**Bb**, **Mb**, and **Ab**) in 2 years old plant. While in the leaves of 4 and 6 years old only in the basal leaves, there is a significant difference between basal and apical zone. Regardless of the leaf position in the agave, there is not any clear tendency for phenolic compounds distribution. The total phenolic concentration in plants of 2, 4 and 6 years, as the sum of the concentration in each part of the leaves, exhibits that the plants of 4 years showed the lowest values, and the phenolic concentration is almost twice in plants of $6 \times$ years old, respect to that of 2 years old ones. In general, considering the position of the leaf in the plant, as well as the divisions of this, there is a significant difference in the phenolic concentration as a function of the age (see Supplementary Material).

In general, the flavonoids content did not show a statistical difference with respect to the position of the leaf in the plant, neither the part of this (apical or basal), finding only a significant difference in flavonoid concentration as a function of age. The flavonoid content had the lowest values in plants of 4 years which are almost equal in plants of 6 and 2 years old. Additionally, in the leaves of 6 years old, the quantities of both metabolites are more homogenous regardless of the leaf part.

Table 2 Quantitative phytochemical analysis to phenols and flavonoids in *Agave potatorum* leaves at different age and position

Secondary metabolites	Phenols (mg GAE/100 g d.w.)			Flavonoids (mg QE/100 g d.w.)		
Age of plant (years): Sample	2	4	6	2	4	6
Bb	29.81 ± 5.81^d	16.01 ± 9.24^c	131.99 ± 4.81^d	20.98 ± 2.13^b	9.23 ± 1.23^b	28.99 ± 4.55^b
Ba	95.42 ± 6.73^b	72.13 ± 6.58^a	158.11 ± 6.73^b	23.14 ± 5.08^c	14.84 ± 7.33^a	35.58 ± 6.41^a
Mb	28.83 ± 14.87^d	21.70 ± 17.56^c	123.84 ± 11.86^d	26.52 ± 1.65^b	10.38 ± 4.43^a	31.47 ± 6.44^a
Ma	110.33 ± 21.51^b	33.36 ± 4.13^b	125.04 ± 12.30^d	29.67 ± 10.51^a	13.84 ± 1.53^a	14.70 ± 0.46^c
Ab	54.77 ± 6.82^c	8.00 ± 4.60^d	173.08 ± 9.36^a	30.36 ± 7.36^a	5.95 ± 4.97^c	28.66 ± 2.56^b
Aa	142.60 ± 59.84^a	12.94 ± 5.18^c	142.83 ± 6.014^c	36.96 ± 6.32^a	13.73 ± 3.03^a	28.20 ± 8.18^a

The results obtained are expressed as mean ± standard deviation of measurements taken in samples of 2, 4, and 6 years old plants. The different superscript letters in the same column indicate a significant difference ($p < 0.05$, Tukey). *QE* quercetin equivalents, *d.w.* dry sample, *GAE* gallic acid equivalents. Leaves localization: apical (A), basal (B), medium (M); leaf region: apical (a), basal (b), medium (m)

Fig. 1 Thin layer chromatography of the carbohydrates extracted from the leaves of 4 and 6 years old agave plants at field conditions. *RSE* raftilose, *RNE* raftiline, *Bb*, *Mb* and *Ab* part of plant, and 4 or 6 correspond to their age, C anion (*Allium cepa*) fructans, *FOS* fructan standards
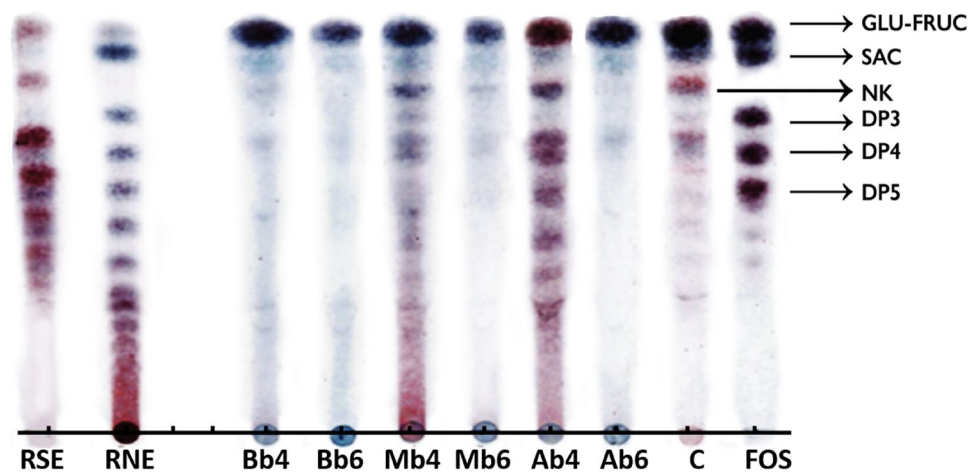
Also, independently of the age, the phenolic content in *A. potatorum* is lower than that reported by Ahumada-Santos et al. [15] for six agave species (*A. tequilana, A. ornithobroma, A. impressa, A. rzedowskiana, A. schidigera*, and *A. angustifolia*), in which that content ranged from 2.06 to 12.37 mg gallic acid equivalents/g d.w. Equally, flavonoid content is lower compared to that in *A. americana* leaves, which ranged from 0.96 to 4.90 mg quercetin equivalents/g of d.w., according to Hamissa et al. [33].

This agrees with the result obtained in this work in which a greater quantity of flavonoids was obtained from the 2 years old leaves (apical part). It is explained because the flavonoids participate in the growth of the plant, increasing again their availability at 6 years old, when the reproductive age of the plant begins its floral scape and its coloration and aroma to obtain gain in pollination.

Based on the concentration of phenolic and flavonoids compound in the leaves, it was expected that the extracts showed antioxidant capacity.

Water Soluble Carbohydrates

To obtain information about water-soluble carbohydrates of *A. potatorum* leaves of 2, 4, and 6 years old, these values were determined. Table 3 shows the content of total carbohydrates, fructose, and fructans. Considering only the basal zone of the leaves, they show a gradual increment from the apical leaves to the basal ones is detected measured in the 4 and 6 years old plants, with a highly significant difference ($p \leq 0.001$), while the plants of 2 years of age showed no significant difference.

All agave leaves presented a direct reducing sugar content (glucose and fructose free) of 4.0 ± 0.24 at 15.23 ± 2.54 mg/g d.w., from the apical to the basal leaves, regardless of the age of the plant (data not shown). Fructose was the most abundant carbohydrate from the total water-soluble carbohydrates, representing an average of 81%. These carbohydrates are related to the fructans metabolism.

The concentration of fructans distributed throughout the leaves was found in a range of 2–37%. The lowest concentration of fructans was found in **Aa** leaves (2.12%) of 2 years

Table 3 Quantitative phytochemical analysis to water-soluble carbohydrate content in *Agave potatorum* leaves of 2, 4, and 6 years old

Age of plant (years): Sample	Total carbohydrates (mg/g d.w.)			Fructose (mg/g d.w.)			Fructans (%)		
	2	4	6	2	4	6	2	4	6
Bb	90.55 ± 4.20^a	200.44 ± 5.34^c	406.08 ± 8.91^d	80.42 ± 3.24^a	191.20 ± 4.21^c	308.30 ± 3.62^d	8.58 ± 2.03^a	19.35 ± 2.45^b	37.23 ± 4.54^c
Ba	90.89 ± 7.31^a	132.05 ± 2.31^{ab}	123.71 ± 2.00^{ab}	78.56 ± 4.21^a	88.20 ± 4.62^a	110.98 ± 3.53^b	2.25 ± 0.55^a	3.55 ± 0.98^a	4.33 ± 1.00^a
Mb	110.89 ± 3.28^a	170.52 ± 2.62^b	210.59 ± 1.52^c	68.90 ± 4.32^a	128.18 ± 3.43^b	180.18 ± 2.89^c	9.68 ± 1.67^a	14.45 ± 2.01^b	16.55 ± 2.00^b
Ma	78.00 ± 2.33^a	84.07 ± 2.00^a	104.09 ± 2.05^a	66.55 ± 4.20^a	78.00 ± 3.01^b	89.45 ± 7.09^b	2.57 ± 0.87^a	4.35 ± 0.98^a	3.45 ± 0.65^a
Ab	96.04 ± 1.92^a	88.02 ± 1.25^a	123.00 ± 1.81^b	58.02 ± 2.51^a	84.80 ± 3.42^b	110.50 ± 4.27^b	6.28 ± 1.00^b	10.34 ± 0.35^c	3.22 ± 1.23^a
Aa	92.35 ± 2.60^a	82.41 ± 5.56^a	108.2 ± 4.92^a	59.88 ± 3.56^a	77.71 ± 2.33^b	60.55 ± 6.05^b	2.12 ± 0.78^a	3.34 ± 0.89^a	2.32 ± 0.99^a

The results obtained are expressed as mean ± standard deviation of measurements taken in samples of 2-, 4-, and 6 years old plants. The different superscript letters in the same column indicate a highly significant difference ($p < 0.001$, Tukey)

old plants, and the highest value was found in **Bb** leaves (37.23%) of 6 years old plants.

Regardless of the position of the leaf in the plant, the apical areas of the leaves (**Ba**, **Ma**, and **Aa**) have the lowest values of the content of fructans, while a preferential accumulation in the basal parts is evident (**Bb**, **Mb**, and **Ab**). Additionally, it is possible to see a positive gradient of fructans through the leaves in the plant from the apical to the basal ones, regardless of the age. This gradient was the sum of fructan content in **Aa + Ab**, **Ma + Mb**, and **Ba + Bb**.

Fructans extracted from *A. potatorum* Zucc leaves of 2, 4, and 6 years of age were analyzed by TLC. Fructans were identified by the retention factor (Rf) and the color of the stain of each carbohydrate present (Fig. 1). The TLC, developed together with a mixture of standards and revealed with aniline–diphenylamine–phosphoric acid in acetone, allowed the identification of aldose and keto-derivate carbohydrates according to spot color, bluish, and reddish, respectively [27].

Glucose, fructose, and sucrose were present in all samples, regardless of the position of the leaf and the age of the plant. In 4 years old plants, the leaves show an important content of short degree polymerization fructans (SDP) in the apical and middle zones, and in their basal part, they were practically absent, indicating that most of them corresponded to the long degree of polymerization (LDP), similar to those observed for the basal leaves of 6-year-old plants; as for the middle and apical leaves, plants of this age still show an important content of sucrose, the substrate for the formation of fructans. These results agree with those by Saldaña-Oyarzábal et al. [34], which show similar tendencies in fructans in the leaves in *A. tequilana* Weber var. Azul.

The presence of SDP fructans, especially in the basal zone of the middle and apical 4-year-old agave leaves (**Bb** and **Mb**), may be involved in protection mechanisms against

stress at low temperatures and/or the maintenance of the hydric balance [35], both conditions present in the region where the agaves were collected or the degradation of high DP fructans present in the basal part of the leave. The presence of SDP fructans in *A. potatorum* leaves indicates that sucrose produced by photosynthesis is metabolized to produce fructans rather than starch [36]. Li et al. [37], previously shown positive correlations between the accumulation of SDP fructans and tolerance to cold stress in plants. Regardless of polymerization degree, the accumulation of fructans in the leaf serves as a short-term carbohydrate reserve, where synthesis and mobilization of these carbohydrates contrast with the fructans in the mesontle, which function as a long-term carbohydrate reserve

Biological Activity

Considering that antifungal activity has been associated with phenolic and flavonoid compounds [6], and since in the leaves of *A. potatorum* these metabolites were quantified (phenolic compounds: 8.00 to 158.12 mg GAE/100 g d.w.; and flavonoids: 5.95 to 36.97 mg QE/100 g d.w.), the effect of ethanolic extracts of fresh *A. potatorum* of 2, 4 and 6 years old leaves were evaluated over *B. cinerea* mycelia growth at 1000 to 30,000 ppm.

The effect of ethanolic extracts of *A. potatorum* leaves from 0 to 30,000 ppm was assessed in the mycelial growth of *B. cinerea*. Figure 2 shows results opposite to those expected because ethanolic extracts of agave improved the mycelia growth of *B. cinerea* at any time and concentration with significant difference (Tukey < 0.05, Supplementary Material) respect to the control at 0 ppm of extract, and independently of the agave age.

According to the statistical analysis (Tukey < 0.05), the control maintains the lowest growth, significantly different
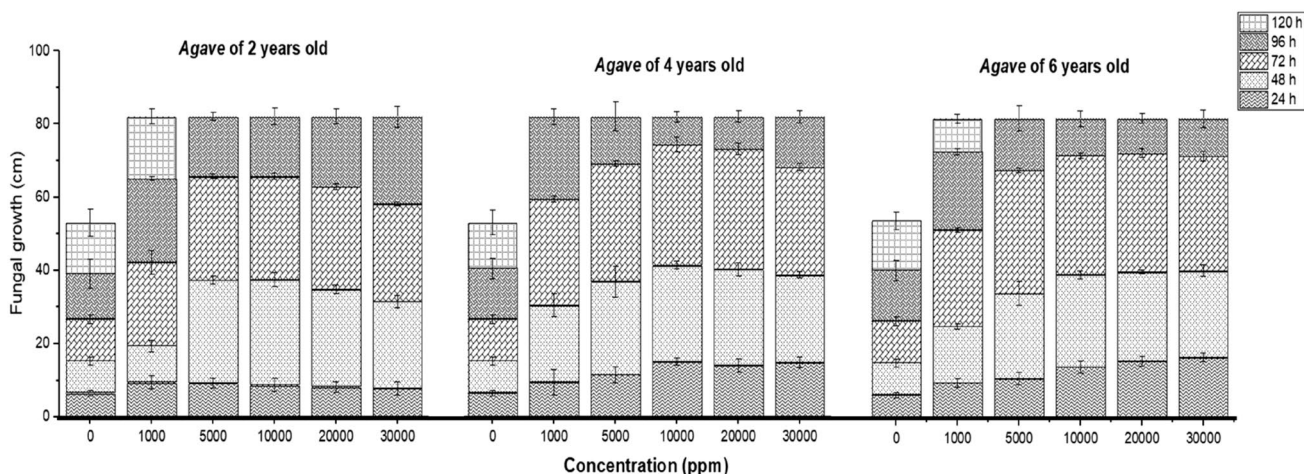
Fig. 2 *Botrytis cinerea* growth (average values in cm), during 120 h of incubation at 25 °C as function of the concentration of ethanolic extract

from the samples with agave leaves extract, regardless of the age of the agave leaves. The greatest radial growth under the extract effect occurs in the first 72 h reaching more than 50% at 1000 ppm of 2 years old leaves, and until 90% to 10,000 ppm of 4 years old leaves. In this sense, the maximum diameter was reached at 96 h, while the control has not reached 50% of growth.

Respect to the effect of the concentrations in the first 72 h, before the Petri dishes started to be filled up, for leaves of 2 years old, the highest growth was reached at 5000 and 10,000 ppm, and between them, there is no statistical difference, while among the other concentrations, a statistical difference was observed. To the extract of agave leaves of 4 and 6 years old, in general, the concentrations of 10000, 20000 and 30000 ppm induced the highest growth and did not show a significant difference among them, but these are statistically different from 5000 to 1000 ppm, and these are different.

The extract of 4 years old agave leaves showed the highest growth, with significant difference respect to the extract of 2 years old agave leaves at all concentrations evaluated, and with 6 years old agave leaves only at 1000 and 10,000 ppm, because at 5000 and 20,000 ppm are statistically equal (see Supplementary Material). This behavior agrees with the lowest content of phenolic and flavonoid compounds.

The unexpected results in the mycelia growth of *B. cinerea*, despite phenolic compounds, terpenes, and flavonoids present in the extract, which previously have been associated with antifungal activity, could be explained due to the lowest concentration of these metabolites, in comparison with that in other *Agaves* [15]. Furthermore, the absence of saponins that have a fungal activity as well [6], together with the presence of carbohydrates also present in the extract, produced a mycelial growth acceleration.

Although an inhibition in the growth of the *B. cinerea* was expected, the previous results are valuable. The potential that *A. potatorum* leaf extracts can have in the growth of commercial or medicinal mushrooms is attractive, considering that 6 years old agave leaves are truly a residue in the use of *A. potatorum*, which until today has focused mainly on obtaining mezcal.

Conclusions and Future Trends

This work provides the phytochemical characterization of an ethanolic extract of *A. potatorum* Zucc. leaves of 2, 4, and 6 years of age. In these, the presence of coumarins, tannins and phenolic compounds, triterpenoids, and cardiac glycosides (only in 6 years old leaves) were positive, but the presence of saponins was not evidenced. The phenolic and flavonoid content of the leaves of *A. potatorum*, regardless of the age or position of the leaves in the plant, as well as the part of the leaf, were in general lower than in other *Agave* species. The quantifying of soluble carbohydrates evidenced a positive gradient of fructans though the leaves in the plant from apical to basal ones, regardless of the age. Four years old leaves mainly contain SDP fructans, while in 6 years old leaves, LDP fructans are predominant. Ethanolic extract of the fresh leaves of *A. potatorum* showed an increase in the mycelial growth of *B. cinerea*, regardless of the age of *Agave* or concentration of the extract in the medium. The data showed that plant age is the most important factor influencing the content of bioactive compounds.

This study opens the possibility to propose new uses of the waste that *A. potatorum* leaves represent in the mezcal industry. Firstly, the leaves can be a source of cardiac glucosides, specifically in plants of 6 years of age, to ameliorate heart disease. Specially the SDP fructans in 4 years old agave leaves would allow them to be used as substrates for probiotic bacteria, and the fructans in 6 years old agave leaves could be used as an ingredient in the elaboration of functional foods. In addition, the extract exhibited a fungal growth enhancing effect, suggesting a biotechnological advantage that can improve the growth of beneficial fungi in agricultural crops, *Trichoderma* as an example. However, potential biological effects should be assessed.

Finally, this research provides the basis for a knowledge-based integral use of the *A. potatorum* Zucc. waste. Nevertheless, isolation, characterization, and quantification of these metabolites to establish the relationship structure–activity is the next step. Though, the main limitation of this type of study is the high variability that exists when working with wild species, due to the environmental factors that influence their growth.

Compliance with Ethical Standards

Conflict of interest None of the authors has any conflict of interest that could affect the performance of the work or the interpretation of the data.

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