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Callus induction and phytochemical profiling of *Yucca carnerosana* (Trel.) McKelvey obtained from *in vitro* cultures

Inducción de callos y perfil fitoquímico de *Yucca carnerosana* (Trel.) McKelvey obtenida de cultivos *in vitro*

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Abstract

It has been demonstrated that some species of the *Yucca* genus are a source of metabolites with functional properties, as is *Yucca carnerosana* (Trel.) McKelvey with antifungal activity. This research aimed to induce the formation of callus tissue in *Y. carnerosana*, to know the growth kinetic, and to analyze the metabolite profile of the formed tissue and plants propagated under *in vitro* and *ex vitro* conditions. Callus induction was achieved using Murashige & Skoog (MS) medium supplemented with 4.4 μM benzyladenine and 4.1 μM 4-aminotrichloropicolinic acid (Picloram). The growth kinetics of callus tissue was characterized by a latency phase achieved at the second week of culture, followed by an exponential growth until the fourth week. The culture showed a specific growth rate of 0.0258 d^{-1}; the doubling time was 26.866 days, and the growth index was 5.9091. The metabolite profile was analyzed using Ultra-High-Performance Liquid Chromatography coupled to Mass Spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS). The chromatographic and mass spectral analysis allowed the separation and identification of 22 compounds in callus tissue, 26 in *in vitro* plants, and 27 in *ex vitro* plants. Our results indicate that the callus tissue and the *in vitro* and *ex vitro* plants of *Y. carnerosana* may be a source of metabolites of interest.

Keywords: UHPLC-MS, polyphenols, *in vitro*, growth regulators, Asparagaceae.

Resumen

Algunas especies del género *Yucca* han demostrado ser una fuente de metabolitos con propiedades funcionales, tal es el caso de *Yucca carnerosana* (Trel.) McKelvey con actividad antifúngica. El objetivo de esta investigación fue inducir la formación de tejido calloso en *Y. carnerosana*, conocer el comportamiento cinético y analizar el perfil de metabolitos del tejido obtenido, así como en plantas propagadas *in vitro* y *ex vitro*. La inducción de callos se logró utilizando medio Murashige & Skoog (MS) suplementado con benciladenina 4.4 μM y ácido 4-aminotricloropicolínico (Picloram) 4.1 μM. La cinética de crecimiento se caracterizó por una fase de latencia alcanzada en la segunda semana de cultivo, seguida de un crecimiento exponencial hasta la cuarta semana. La tasa específica de crecimiento fue de 0.0258 d^{-1}; el tiempo de duplicación fue de 26.866 días y el índice de crecimiento fue de 5.9091. El perfil de metabolitos se analizó mediante cromatografía líquida de ultra alta resolución acoplada a espectrometría de masas (UHPLC-PDA-HESI-Orbitrap-MS/MS). El análisis cromatográfico y espectrométrico permitió la separación e identificación de 22 compuestos en callos, 26 en plantas *in vitro* y 27 en plantas *ex vitro*. Nuestros resultados indican que el tejido calloso de *Y. carnerosana* así como las plantas *in vitro* y *ex vitro* puede ser fuente de metabolitos de interés.

Palabras clave: UHPLC-MS, polifenoles, *in vitro*, reguladores de crecimiento, Asparagaceae.

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1 Introduction

Some of the most representative succulent plants in Mexican flora are those belonging to the *Yucca* genus (Asparagaceae) (Matuda and Piña, 1980), with around 49 species across the Mexican territory (Rocha *et al*., 2006). Some species of the *Yucca* genus represents a source of building material (Soltani *et al*., 2020), food additives (Suzuki *et al*., 2020; Thomas-Popo *et al*., 2019; Tsibranska *et al*., 2020), and cosmetics (Lee *et al*., 2019), as well as compounds with therapeutic potential (Patel, 2012). In this regard, it has been shown that *Yucca shidigera* contains yuccaol A, B and C, resveratrol, and other phenolic compounds with reported biological activities (Oleszek *et al*., 2001) such as anti-inflammatory (Marzocco *et al*., 2004). Similarly, *Yucca filamentosa* and *Y. schidigera* contain steroidal saponins with biological applications such as anti-tumoral, antimicrobial and anti-arthritic; and resveratrol which exhibits antioxidant and anti-inflammatory activities (Patel, 2012). The methanolic extract from the bark of *Yucca periculosa* contains 4,4'-dihydroxystilbene, resveratrol, and 3,3',5,5'-tetrahydroxy-4-methoxystilbene, and has shown growth-regulating activity against the larvae of *Spodoptera frugiperda*, a maize pest (Torres *et al*., 2003). *Yucca aloifolia* has a high linoleic, oleic, and palmitic acid content, so the biodiesel obtained from this species' oil could be used as fuel (Nakashima *et al*., 2016). On the other hand, the antifungal activity of *Y. carnerosana* against the development of postharvest fruit fungi such as *Rhizopus stolonifer*, *Colletotrichum gloeosporioides*, and *Penicillium digitatum* has been demonstrated (Jasso de Rodríguez *et al*., 2011). Furthermore, it has been found that *Y. carnerosana* residues from the fiber industry have biosorption capacity to remove Pb (II) ions present in aqueous solutions due to their phenolic acids and lignans (Medellín-Castillo *et al*., 2017). Thus, a great interest has arisen in taking advantage of these species. Currently, *Yucca* spp. extracts are marketed as capsules, drinks, or powders (Patel, 2012), without phytochemical characterization. These events have led populations to decline; thus, conservation strategies must be employed to protect the environment and increase the number of individuals in populations.

Plant cell tissue and organ culture offer a useful biotechnological tool to enhance the production of biomass and secondary metabolites (Cisneros-Tórres *et al*., 2019; Cortes-Morales *et al*., 2018; Khan *et al*., 2019). This technique provides tools such as *in vitro* mass propagation, leading to greenhouse transfer, which has already been reported for *Yucca* spp. such as *Yucca elephantipes* (Pierik and Steegmans, 1983), *Y. aloifolia* (Atta-Alla and Van Staden, 1997), *Yucca valida* (Arce-Montoya *et al*., 2006), *Yucca coahuilense*, *Y. filamentosa*, and *Y. periculosa* (López-Ramírez *et al*., 2018). In this regard, the induction of callus tissue of *Yucca* spp. has been applied for the regeneration of plants, as is the case of *Yucca gloriosa* (Durmishidze *et al*., 1983), and for the study of steroid substances during morphogenesis (Gogoberidze *et al*., 1992). To the best of our knowledge, information regarding the *in vitro* culture of *Y. carnerosana* is limited in the literature and refers to micropropagation of this species (López-Ramírez *et al*., 2018). Similarly, information regarding the phytochemical profile of *Y. carnerosana* is also limited in the literature; nevertheless, it has been proposed that the fruits and seeds contains sarsapogenin (Romo de Vivar, 1985). Thus, *in vitro* culture and callus generation could allow to generate several lines of research on cell metabolism, cellular response mechanisms to different types of stress, generation of somatic embryogenesis, among others, without the need to extract specimens from their natural habitat, as well as the study and production of compounds under controlled conditions (Efferth, 2019).

This work aimed to generate and characterize the growth of callus tissue from *Y. carnerosana* and identify of some of the metabolites present in methanolic extracts prepared with callus and plants cultivated under *in vitro* and *ex vitro* conditions. In this regard, Ultra-High-Performance Liquid Chromatography coupled to Mass Spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS) was employed as an approach for the identification of compounds present in plant extracts (Cornejo *et al*., 2016; Simirgiotis *et al*., 2017). With the results generated, it is possible to contribute to this species' biotechnological management and phytochemical knowledge.

2 Materials and methods

2.1 Plant material

The plants of *Y. carnerosana* were obtained from the *in vitro* germplasm bank of the Universidad Autónoma de Aguascalientes, México, and then micropropagated

according to the method proposed by López-Ramírez *et al*. (2018). For this, axillary buds of *Y. carnerosana* were sliced and cultivated in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (pH 5.7, 30 g $L^{-1}$of sucrose and 10 g L^{-1} of agar as gelling agent). The medium was sterilized in an autoclave at 121 ºC for 20 min and then supplemented with meta-topoline (12.43 μM), filtered through a 0.45 μM nylon filter disc (Acrodisc®). The axillary buds were transferred into 500 mL flasks containing 30 ± 3 mL of the culture medium and incubated at 25 ± 2 °C under a photoperiod of 16 h light (40 μMol m^{-2} s^{-1}) for 4 weeks. The generated *in vitro* shoots (4±1 cm in length) were used for the experiments of callus induction and plant acclimatization. One specimen is available at the Herbarium of the Universidad Autónoma de Aguascalientes (HUAA; Voucher No. 16463).

For *in vitro* rooting, individual shoots were transferred into MS medium (Murashige and Skoog, 1962) without growth regulators. The culture medium was prepared as given before, and the explants were incubated under the mentioned conditions above. After 5 weeks of culture, the seedlings that developed roots were removed carefully from the culture medium and washed with distilled water. The *in vitro* plants were then transferred into pots containing substrate (PROMIX®, United States) and acclimated to greenhouse conditions as reported previously (Pérez-Molphe-Balch *et al*., 2002). Each pot was covered with a transparent polythene bag for 2-3 weeks to prevent desiccation and allow acclimatization. Once the acclimatization process was achieved, each plant was maintained for 64 weeks until harvesting for extract preparation and phytochemical analysis.

2.2 Callus generation

For callus induction, three different explants were used: stem, the base of the leaf, and middle part of the leaf (Figure 1A), as well as different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; 0.0, 4.52, 6.79, and 9.05 μM) combined with different concentrations of benzyladenine (BA; 0.0, 2.22, and 4.44 μM), were evaluated. Another tested combination was 4-aminotrichloropicolinic acid (Picloram; 0.0, 4.14, 6.21, and 8.28 μM) with BA (0.0, 2.22, and 4.44 μM). Thus, a total of 12 different combinations were evaluated (Table 1). The explants were cultivated in MS medium and incubated as given in section 2.1. Three independent experiments composed of three explants were performed in each treatment.

Statistical analyses were performed with GraphPad Prism 8.1.2 (GraphPad Software San Diego, CA, USA). Data were analyzed using one-way ANOVA, and a p-value of 0.05 was considered statistically significant.

2.3 Yucca carnerosana callus growth kinetics

The treatment composed of 4.14 μM of picloram and 4.44 μM BA allowed the generation of callus tissue. Nevertheless, for *Y. carnerosana* callus maintenance and evaluation of its kinetic growth behavior, the concentration of picloram was reduced by half. Thus, 1 g of callus tissue was transferred into flasks containing 60 mL of MS medium, 30 g L^{-1} sucrose, 10 g L^{-1} agar, and 2.05 μM picloram, and 4.4 μM BA. The pH of the culture medium was adjusted to 5.7 before autoclaving at 120 °C for 20 min. The incubation conditions mentioned above were used. Three independent samples were taken each week randomly for 12 weeks. Each sample was dried in an oven at 37 °C ± 2 °C for 1 week in dark conditions and then weighed to obtain the dry weight (DW). The growth kinetics parameters were calculated according to the change in fresh weight (FW) and DW. The growth rate equation was as follows: $\mu = \ln (X_E/X_0)/\Delta t$, where X_0 and X_E are the dry weight of callus at the beginning and the end of the culture period interval (g L^{-1}), respectively; Δt is the culture time interval (days); μ is the specific growth rate (day^{-1}). The doubling time was calculated as follows: $t_d = \ln 2/\mu$ where t_d is the doubling time (days). The growth index was calculated as follows: $GI = (X_E\text{-}X_0)/X_0$ where X_E and X_0 are the end and initial dry weight of callus, respectively (Gómez-Aguirre *et al*., 2012; Maldonado-Magaña *et al*., 2013). A Microsoft Excel (Microsoft 365) spreadsheet was used to perform calculations and graphs.

2.4 Phytochemical analysis using Ultra-High-Performance Liquid Chromatography coupled to Mass Spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS)

2.4.1 Preparation of extracts

For the phytochemical analysis, *in vitro* plants (4-week-old plants), callus tissue (tissue obtained after

12 weeks), and *ex vitro* plants (64-week-old plant leaves) were used. Each sample was collected and then dried in an oven (Ecoshel, Mexico) at 37 °C ± 2 °C for 7 d. The dried material was pulverized in a mortar, and then 50 g of each sample was successively extracted by maceration using 300 mL of hexane, followed by chloroform and methanol (JT Baker, Spain). Each extraction stage was carried out three times in a water bath at 60 ºC for 15 min. Then, each sample was filtered on filter paper (Whatman® grade 41, Argentina) and concentrated in a rotary evaporator (Sev-Prendo, México) to remove the dissolvent. The methanolic extract was used for UHPLC-PDA-HESI-Orbitrap-MS/MS analysis.

2.4.2 *Sample preparation and UHPLC-PDA-HESI-Orbitrap-MS/MS conditions*

The sample was resuspended (2.5 mg mL^{-1}) in HPLC-MS-grade methanol and sonicated over 10 min. All samples were filtered (0.22 μM) and injected into a UHPLC system coupled to a mass spectrometer. The phytochemical analysis was performed as previously reported (Cornejo *et al*., 2016; Torres *et al*., 2003), using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Bremen, Germany) with a C18 column (ID: 150 × 4.6 mm, 5 μM; Restek Corporation, Bellefonte, PA, USA), equipped with a Quaternary Series RS pump and a Dionex Ultimate 3000 Series TCC-3000RS column compartment, an Ultimate 3000 Series WPS-3000RS autosampler (Thermo Fisher Scientific) and a rapid separations PDA detector. The detection wavelengths were 254, 280, 320, and 440 nm; PDA was recorded from 200 to 800 nm for peak characterization. The separation was performed in a gradient elution mode composed of a 1% formic aqueous solution (A) and acetonitrile (B). The flow rate was 1.0 mL min^{-1}, and the injection volume 10 μL. The gradient program [time (min), %B] was: (0.00, 5), (5.00, 5), (10.00, 30), (15.00, 30), (20.00, 70), (25.00, 70), (35.00, 5), and 12 min for column equilibration before each injection. The system was controlled by the Chromeleon 7.2 Software (Thermo Fisher Scientific, Waltham, MA, USA, and Dionex Softron GmbH division of Thermo Fisher Scientific) and coupled to a Thermo high-resolution Q Exactive focus mass spectrometer (Thermo Fisher Scientific). The chromatographic system was coupled to the mass spectrometer with a heated electrospray ionization source II (HESI II). Nitrogen (purity>99.999%) was employed as both the collision and damping gas. Nitrogen was obtained from a Genius NM32LA nitrogen generator (Peak Scientific, Billerica, MA, USA). Mass calibration for Orbitrap was performed once a week, in both negative and positive modes. Caffeine and N-butylamine (Sigma-Aldrich, Saint Louis, MO, USA) were the calibration standards for positive ions. Buspirone hydrochloride, sodium dodecyl sulfate, and taurocholic acid sodium salt were used to calibrate the mass spectrometer. These compounds were dissolved in a mixture of acetic acid, acetonitrile, water, and methanol (Merck Darmstadt, Hesse, Germany) and infused using a Chemyx Fusion 100 syringe pump. The XCalibur 2.3 and Trace Finder 3.2 (Thermo Fisher Scientific, San Jose, CA, USA) programs were used for UHPLC control and data processing, respectively. Q Exactive 2.0 SP 2 (Thermo Fisher Scientific, Waltham, MA, USA) was used to control the mass spectrometer (Cabañas-García *et al*., 2020; Cabañas-García *et al*., 2019).

2.4.3 *MS parameters*

The HESI parameters were optimized as follows: sheath gas flow rate 75 units; auxiliary gas flow rate 20 units; capillary temperature 400 °C; auxiliary gas heater temperature 500 °C; spray voltage 2500 V (for ESI-); and S lens RF level 30. Full scan data in negative mode was acquired at a resolving power of 70,000 full widths half maximum (FWHM) at *m/z:* 200. For the compounds of interest, a scan range of *m/z:* 100-1000 was chosen; the automatic gain control (AGC) was set at 3×10^6, and the injection time was set to 200 ms. Scan rate was set at 2 scans s^{-1}. External calibration was performed using a calibration solution in positive and negative modes before each sample series. In addition to the full scan acquisition method, for confirmation purposes, a targeted MS/MS analysis was performed using the mass inclusion list and expected retention times of the target analytes, with a 30 s time window, with the Orbitrap spectrometer operating both in positive and negative mode at 17,500 FWHM (*m/z:* 200). The AGC target was set to 2×10^5, with a maximum injection time of 20 ms. The precursor ions were filtered by the quadrupole operating at an isolation window of *m/z:* 2. The fore vacuum, high vacuum, and ultra-high vacuum were maintained at approximately 2 mbar, from 10^5 to below 10^{10} mbar, respectively. Collision energy (HCD cell) was operated at 30 eV. Detection was based on calculated exact mass and the retention time of target compounds. The mass tolerance window was set to 5 ppm (Cabañas-García *et al*., 2019).

3 Results and discussion

3.1 Callus tissue culture establishment

Callus generation was only observed from stem explants (Figure 1). the base and the middle part of the leaf not generated callous tissue and showed necrotic characteristics after 19-21 days. This event may be due to the higher concentration of auxins in active growth regions of plant such as stems (Jiang *et al*., 2017). Regarding the callus induction experiments, although the treatments composed by 2.22 μM BA+4.52 μM 2,4-D; 4.44 μM BA+4.52 μM 2,4-D, and 4.44 μM BA+9.05 μM 2,4-D showed the highest induction frequency (66%, see Table 1), the formed tissue showed compact, solid, and necrotic characteristics (see Figures 1b, 1e, and 1g). On the other hand, the treatment composed by 4.4 μM BA+4.1 μM picloram showed an induction frequency of 44% (see Table 1), and the formed tissue showed friable and white to yellowish-green characteristics (Figure 1 K) after 4-5 weeks of growth. The induction frequency and the physical characteristics of the formed tissue may be influenced by mutual interactions among plant growth regulators, which exert differentiated responses in different plant tissues (Wang and Irving, 2011). Once the callous tissue was obtained, it was subcultured with the same regulators, reducing the auxin concentration, since it has been proposed that the callous tissues become necrotic if the same concentrations are maintained (Garay-Arroyo *et al*., 2014). In this regard, it has been proposed that auxins are differentially distributed within tissues, which gives rise to various morphogenetic processes with potential herbicide effects at high doses (Quareshy *et al*., 2017). The treatment composed by 4.4 μM BA+4.1 μM picloram was used for growth kinetic evaluation of the formed callus tissue of *Y. carnerosana*. Based on the fresh and dry weight measurements (Figure 2), A lag phase was observed until week 2, then an exponential growth phase from week 2 to 12, without observing the stationary or death phases. The culture showed a growth rate of 0.025 d^{-1}, a doubling time of 26.866 days, and a growth index of 5.9091. To the best of our knowledge, this is the first report dealing with the callus tissue's kinetic behavior evaluation of *Y. carnerosana* and for another *Yucca* spp. With the obtained information, it was possible to quantify the grams of biomass that each gram of callus could generate in the unit of time.

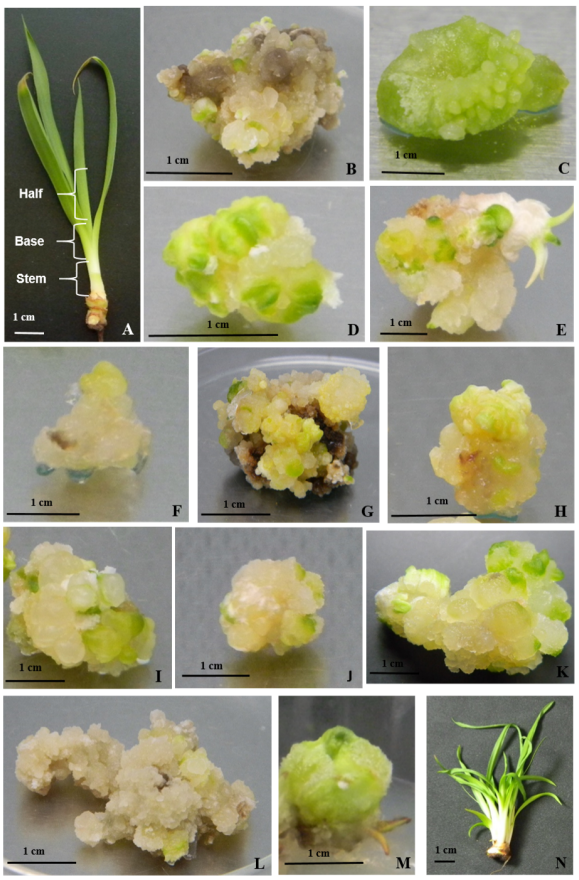

Fig. 1. Effect of growth regulators on callus induction in the stem of *Yucca carnerosana* (Trel.) McKelvey at 21 days. A) Types of *Y. carnerosana* explants used for callus generation. B) 2.2 μM BA + 4.5 μM 2,4-D, C) 2.2 μM BA + 6.8 μM 2,4-D, D) 2.2 μM BA + 9.5 μM 2,4-D, E) 4.4 μM BA + 4.5 μM 2,4-D, F) 4.4 μM BA + 6.8 μM 2,4-D, G) 4.4 μM BA + 9.0 μM 2,4-D, H) 2.2 μM BA + 4.1 μM picloram, I) 2.2 μM BA + 6.2 μM picloram, J) 2.2 μM BA + 8.3 μM picloram, K) 4.4 μM BA + 4.1 μM picloram, L) 4.4 μM BA + 6.2 μM picloram, M) 4.4 μM BA + 8.3 μM picloram, N) Control (free of growth regulators) at 12 weeks of incubation.

3.2 Phytochemical analysis of *Y. carnerosana* methanolic extracts

The phytochemical characterization of extracts was achieved by comparing the information obtained by UHPLC-PDA-HESI-Orbitrap-MS/MS with the spectrometric evidences existing in the literature or by studying the fragmentation pattern of the molecules. For *Y. carnerosana* extracts prepared with different tissues, the chromatographic conditions

Table 1. Effect of BA, 2,4-D and picloram in stem explants on callus induction of *Y. carnerosana* (Trel.) McKelvey.

Growth regulator (μM)			Induction frequency (%)
BA	**2,4-D**	**Picloram**	
0	0	0	0
2.22	4.52		66
2.22	6.79		44
2.22	9.05		0
4.44	4.52		66
4.44	6.79		44
4.44	9.05		66
2.22		4.14	33
2.22		6.21	33
2.22		8.28	11
4.44		4.14	44
4.44		6.21	44
4.44		8.28	0

BA= benzyladenine; 2,4-D= 2,4-dichlorophenoxyacetic acid; Picloram= 4-aminotrichloropicolinic acid. The values represent the average (n=3).

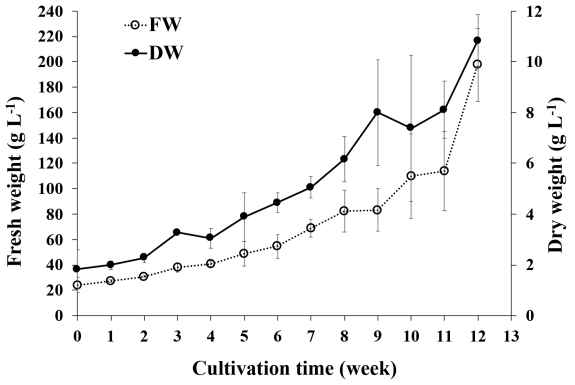

Fig. 2. Callus growth kinetics of *Yucca carnerosana* (Trel.) McKelvey. Each value represents the mean of three replicates ± standard error.

allowed the separation and tentative identification of 64 metabolites (see Figure 3, Table 2). Among detected compounds, 26 occurred *in vitro*, 27 in *ex vitro* condition, and 22 in callus cultures. The characteristics of each peak, such as retention time, theoretical and measured mass, fragmentation pattern, and the tentative identification of each compound, are summarized in the Table 2.

Compounds 1, 2, 4, 5, 7, 8, 14, and 16 were identified as organic acids. Among these compounds, two gluconic acid isomers (compounds 1 and 5), two malic acid isomers (compounds 4 and 7) as well as quinic and citric acids (compounds 2 and 8, respectively) were assigned as proposed by Taamalli *et al*. (2015). These compounds were present *in vitro* and *ex vitro* conditions. Similarly, compound 14 was assigned as shikimic acid (Chen *et al*., 2014) and compound 16 as a malonic acid derivative since pseudomolecular ion at *m/z*: 134.8940 yielded one fragment at *m/z*: 103.0035 (malonic acid). On the other hand, compound 6 was present in callus tissue, and it was identified as resveratrol 3-β-mono-glucoside. For this compound, pseudomolecular ion at *m/z:* 389.1220 yielded fragments at *m/z*: 179.0561 and 211.0717, generated due to the separation of the hexose moiety from the basic polyphenolic structure. The presence of resveratrol has been reported in other *Yucca* species, such as *Y. schidigera* (Cheeke *et al*., 2006) and *Y. periculosa*, showing photoprotective activities against UV rays (García-Bores *et al*., 2010).

On the other hand, compound 10 was only detected in callus tissue, and it was identified as diethyl oximinomalonate since one fragment at *m/z:* 128.0347 was generated due to the loss of ethanol and water. Similarly, compounds 11-13 were detected only in plants cultivated *ex vitro*. In this regard, compound 11 was identified as succinic acid due to the presence of one main fragment at *m/z*: 101.0236 generated due to the loss of water, and compound 12 was identified as dimethyl malate since pseudomolecular ion at *m/z*: 161.0451 yielded one fragment at *m/z:* 129.0187, generated due to the loss of one methyl group and the subsequent elimination of water. Additionally, three piscidic acid isomers (compounds 13, 21, and 22) were detected in plants growing under *in vitro* and *ex vitro* conditions and assigned as reported previously (Cabañas-García *et al*., 2019), see Table 2.

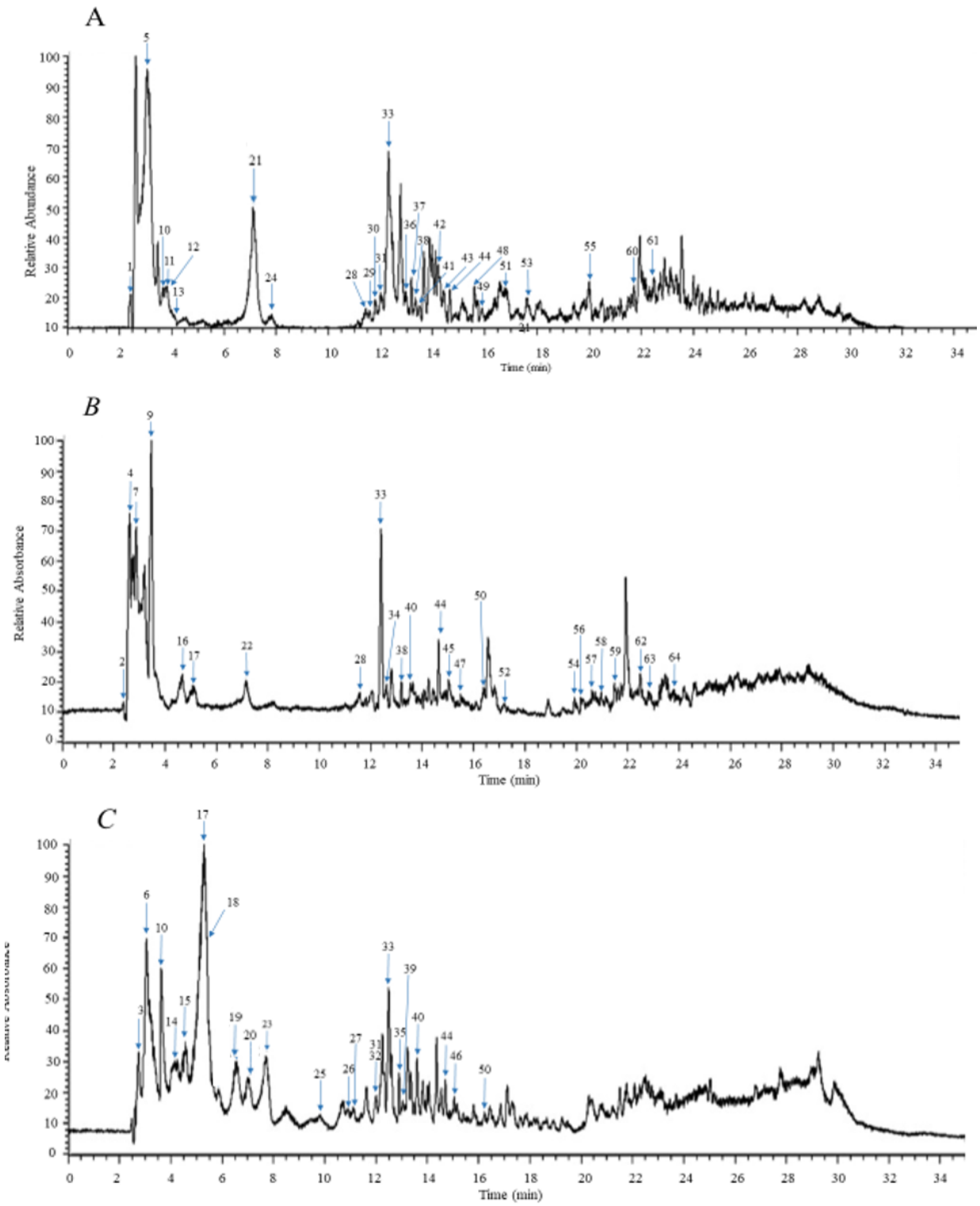

Fig. 3. UHPLC-PDA-HESI- Orbitrap-MS/MS chromatograms of methanolic extracts prepared with *Yucca carnerosana* (Trel.) McKelvey plants. A) *ex vitro* plant, B) *in vitro* plant, and C) callus tissue.

Table 2. Identification of metabolites in callus tissues, *ex-vitro* and *in vitro* plants of *Y. carnerosana* by UHPLC-PDA-HESI-Orbitrap-MS/MS.

Peak	Retentiontime (min)	Compound	Elemental composition [M-H]$^-$	Theoretical mass (*m/z*)	Measured mass (*m /z*)	Accuracy (ppm)	MSn ions	Reference	Tissue
1	2.61	Gluconic acid isomer I	$C_6H_{11}O_7^-$	195.0510	195.0506	2.05	129.0186	(Taamalli *et al*., 2015)	E
2	2.62	Quinic acid	$C_7H_{11}O_6^-$	191.0561	191.0557	2.09	127.0392	(Taamalli *et al*., 2015)	I
3	2.73	Sugar	$C_6H_{11}O_6^-$	-	179.0556	-	-		C
4	2.80	Malic acid isomer I	$C_4H_5O_5^-$	133.0142	133.0136	4.51	115.0029		I
5	3.06	Gluconic acid isomer II	$C_6H_{11}O_7^-$	195.0510	195.0506	2.05	129.0186	(Taamalli *et al*., 2015)	E
6	3.07	Resveratrol 3-β-mono-D-glucoside	$C_{20}H_{21}O_8^-$	389.1242	389.1220	5.65	179.0561, 211.0717	(Taamalli *et al*., 2015)	C
7	3.15	Malic acid isomer II	$C_4H_5O_5^-$	133.0142	133.0136	4.51	115.0029		I
8	3.45	Citric acid	$C_6H_7O_7^-$	191.0197	191.0194	1.57	111.0079	(Taamalli *et al*., 2015)	E
9	3.50	Pyroglutamic acid	$C_5H_6NO_3^-$	128.0353	128.0347	4.69	-		I
10	3.62	Diethyl oximinomalonate	$C_7H_{10}NO_5^-$	188.0564	188.0560	2.13	128.0347		C
11	3.68	Succinic acid	$C_4H_5O_4^-$	117.0193	117.0187	5.13	101.0236		E
12	3.82	Dimethyl malate	$C_6H_9O_5^-$	161.0455	161.0451	2.48	129.0187		E
13	4.09	Piscidic acid isomer I	$C_{11}H_{11}O_7^-$	255.0510	255.0510	0.00	165.0551, 135.0442	(Cabañas-García *et al*., 2019)	E
14	4.17	Shikimic acid	$C_7H_9O_5^-$	173.0455	173.0450	2.89	155.0344, 129.0557, 111.0452	(Chen *et al*., 2014)	C
15	4.66	Rhynchosporoside isomer I	$C_{15}H_{27}O_{12}^-$	399.1508	399.1508	0.00	353.1455, 309.1192, 279.1088	(Cabañas-García *et al*., 2021)	I, C
16	5.07	Malonic acid derivative	$C_3H_3O_6^-$	-	134.8940	-	103.0035		I, C
17	5.31	Rhynchosporoside isomer II	$C_{15}H_{27}O_{12}^-$	399.1508	399.1508	0.00	353.1455, 309.1192, 279.1088	(Cabañas-García *et al*., 2021)	C
18	5.85	Rhynchosporoside isomer III	$C_{15}H_{27}O_{12}^-$	399.1508	399.1508	0.00	353.1455, 309.1192, 279.1088	(Cabañas-García *et al*., 2021)	C
19	6.57	2-O-(2-hydroxyethyl)-4-O-[2-O-(2-hydroxypropyl)hexopyranosyl] hexopyranose isomer I	$C_{17}H_{31}O_{13}^-$	443.1770	443.1767	0.68	427.2464, 369.1401		C
20	6.98	Pantothenic acid	$C_9H_{16}NO_5^-$	218.1034	218.1029	2.29	146.0815	(Rodríguez-Pérez *et al*., 2013)	C
21	7.11	Piscidic acid isomer II	$C_{11}H_{11}O_7^-$	255.0510	255.0510	0.39	165.0551, 135.0442		E, I
22	7.16	Piscidic acid isomer III	$C_{11}H_{11}O_7^-$	255.0510	255.0510	0.39	165.0551, 135.0442		I
23	7.71	2-O-(2-hydroxyethyl)-4-O-[2-O-(2-hydroxypropyl) hexopyranosyl] hexopyranose isomer II	$C_{17}H_{31}O_{13}^-$	443.1770	443.1767	0.68	427.2464, 369.1401		C
24	7.83	2-(Benzoyloxy)-3-hydroxysuccinic acid	$C_{11}H_9O_7^-$	253.0353	253.0353	0.00	117.0186, 121.0288		E
25	9.82	2-(hydroxymethyl)-6-[3,4,5-trihydroxy-6-(4-hydroxybutoxy) oxan-2-yl] methoxy oxane-3,4,5-triol	$C_{16}H_{29}O_{12}^-$	413.1664	413.1663	0.24	367.1608, 251.1130		C
26	10.91	Verbasoside isomer I	$C_{20}H_{29}O_{12}^-$	461.1664	461.1664	0.00	123.0444, 153.0550		C
27	11.11	Cyclohexanecarboxylic acid, 3-[(6-deoxy-3-*O* -methyl-d-galactopyranosyl)oxy]-1,4,5-trihydroxy	$C_{14}H_{23}O_{10}^-$	351.1296	351.1296	0.00	303.1448, 287.1499, 273.1344		C
28	11.57	3-oxo-5-amino-hexanoic acid	$C_6H_{10}NO_3^-$	144.0666	144.0660	4.16	128.0347		E, I
29	11.58	2,4,6-Trihydroxyacetophenone-2,4-di-O-β-D- glucopyranoside	$C_{20}H_{27}O_{14}^-$	491.1406	491.1408	0.41	329.0883, 167.0346		E
30	11.81	2-Cyclohexylethyl 4-O-α-D-glucopyranosyl-β-D-glucopyranoside	$C_{20}H_{35}O_{11}^-$	451.2185	451.2188	0.66	189.1660		E
31	12.00	Isopropylmalic acid	$C_7H_{11}O_5^-$	175.0612	175.0609	1.71	143.0713		E, I, C
32	12.06	Cistanoside G	$C_{20}H_{29}O_{11}^-$	445.1715	445.1715	0.00	119.0493		C
33	12.48	Syringic acid acetate isomer I	$C_{11}H_{11}O_6^-$	239.0561	239.0559	0.84	195.0658, 179.0344, 149.0601	(Cabañas-García *et al*., 2019; Chen *et al*., 2014)	E, I, C
34	12.60	Caffeic acid	$C_9H_7O_4^-$	179.0349	179.0348	0.84	135.0447	(Iswaldi *et al*., 2013)	I
35	12.90	Verbasoside isomer II	$C_{20}H_{29}O_{12}^-$	461.1664	461.1664	0.00	123.0444, 153.0550		C
36	13.00	6,7-dihydroxy-2-oxo-1-benzopyran-4-carboxylic acid	$C_{10}H_5O_6^-$	221.0092	221.0090	0.90	177.0190		E
37	13.18	Syringic acid acetate derivative	$C_{22}H_{31}O_{14}^-$	-	519.1718	-	239.0561, 149.0602, 179.0345		E
38	13.21	Syringic acid acetate isomer II	$C_{11}H_{11}O_6^-$	239.0561	239.0559	0.00	195.0658, 179.0344, 149.0601	(Cabañas-García *et al*., 2019) (Chen *et al*., 2014)	E, I
39	13.23	β-D-galactopyranoside, 6-hydroxyhexyl 6-O-β-D-galactopyranosyl	$C_{18}H_{33}O_{12}^-$	441.1977	441.1975	0.45	395.1923, 217.1078		C
40	13.59	Ferulic acid derivative	$C_{21}H_{31}O_{12}^-$	-	475.1821	-	193.0501, 179.0346	(Cabañas-García *et al*., 2019)	I, C
41	13.65	Ethyl 2-methyl-3-(methylamino)-3-oxopropanoate	$C_7H_{12}NO_3^-$	158.0823	158.0817	3.80	-		E
42	14.22	Eriodictyol	$C_{15}H_{11}O_6^-$	287.0554	287.0560	2.09	151.0032	(Taamalli *et al*., 2015)	E
43	14.42	2-[(3-methylbenzoyl)amino]acetic acid	$C_{12}H_{12}NO_3^-$	192.0666	192.0664	1.04	176.0711, 119.0496		E
44	14.70	Sinapic acid	$C_{11}H_{11}O_5^-$	223.0610	223.0609	0.45	169.0865, 179.0708, 164.0428	(Cabañas-García *et al*., 2019; Liu *et al*., 2015)	E, I, C
45	15.02	Magnoloside U	$C_{30}H_{39}O_{14}^-$	623.2345	623.2352	1.12	179.0558, 135.0444	(Cabañas-García *et al*., 2021)	I
46	15.04	3,4,5-triacetyloxy-6-[(4-oxo-2,3-dihydro-cyclopentachromen-7-yl)oxy]oxan-2-yl]methyl acetate	$C_{26}H_{27}O_{12}^-$	531.1508	531.1505	0.56	134.0365		C
47	15.51	Naringenin 7-O-rutinoside	$C_{27}H_{31}O_{14}^-$	579.1719	579.1721	0.35	266.0659		I
48	15.61	3,4,5-triethoxybenzyl alcohol	$C_{13}H_{19}O_4^-$	239.1289	239.1287	0.84	209.1178, 165.0916		E
49	15.88	9,10-Dihydroxy octadecanedioic acid	$C_{18}H_{33}O_6^-$	345.2283	345.2282	0.29	329.2333	(Ledesma-Escobar *et al*., 2015)	E
50	16.22	Rhynchosporoside isomer IV	$C_{15}H_{27}O_{12}^-$	399.1508	399.1508	0.00	353.1455, 309.1192	(Cabañas-García *et al*., 2021)	I, C
51	16.84	Leeaoside	$C_{24}H_{39}O_{11}^-$	503.2498	503.2510	2.38	371.2056	(Zhang *et al*., 2015)	E
52	17.20	1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-5-oxo-3-heptanyl β-D-xylopyranoside	$C_{24}H_{29}O_9^-$	461.1817	461.1816	0.22	-		I
53	17.64	2-Benzoylisoquinolinium	$C_{16}H_{11}NO^-$	233.0834	233.0850	6.86	157.0497		E
54	19.94	9,12,13-trihydroxy-10,15-octadecadienoic acid isomer I	$C_{18}H_{31}O_5^-$	327.2177	327.2178	0.31	309.2075	(Ledesma-Escobar *et al*., 2015)	I
55	20.01	Alpinetin	$C_{16}H_{13}O_4^-$	269.0819	269.0819	0.00	223.0737, 193.0506		E
56	20.23	9,12,13-trihydroxy-10,15-octadecadienoic acid derivative	$C_{18}H_{31}O_6^-$	343.2126	343.2126	0.00	327.2177, 309.2071	(Ledesma-Escobar *et al*., 2015)	I
57	20.64	9,12,13-trihydroxy-10,15-octadecadienoic acid isomer II	$C_{18}H_{31}O_5^-$	327.2177	327.2178	0.31	309.2075	(Ledesma-Escobar *et al*., 2015)	I
58	20.98	9,10-Dihydroxy octadecanedioic acid	$C_{18}H_{33}O_6^-$	345.2283	345.2282	0.29	329.2333	(Ledesma-Escobar *et al*., 2015)	I
59	21.52	5,8,12-Trihydroxy-9-octadecenoic acid	$C_{18}H_{33}O_5^-$	329.2335	329.2335	0.00	315.2175	(Ledesma-Escobar *et al*., 2015)	I
60	21.72	Sakuranetin	$C_{16}H_{13}O_5^-$	285.0768	285.0769	0.35	193.0506, 179.0344, 125.0237		E
61	22.48	9,12,13-trihydroxy-10,15-octadecadienoic acid	$C_{18}H_{31}O_5^-$	327.2177	327.2178	0.31	309.2075	(Ledesma-Escobar *et al*., 2015)	E
62	22.50	Persicogenin	$C_{17}H_{15}O_6^-$	315.0874	315.0876	0.63	297.0763		I
63	22.85	5,8,12-Trihydroxy-9-octadecenoic acid isomer	$C_{18}H_{33}O_5^-$	329.2335	329.2335	0.00	315.2173	(Ledesma-Escobar *et al*., 2015)	I
64	23.80	Nordihydrocapsiate	$C_{17}H_{25}O_4^-$	293.1758	293.1759	0.34	277.1807	(Cabañas-García *et al*., 2019)	I

C= callus tissue; E= *ex vitro plant*; I= *in vitro* plant.

Compounds 15, 17, 18, and 50 were mainly detected in callus tissue and were identified as rhynchosporoside isomers as reported previously for callus cultures of *Coryphantha macromeris* (Cactaceae) (Cabañas-García *et al*., 2021). Similarly, compounds 19 and 23 were detected in callus tissue, and identified as 2-O-(2-hydroxyethyl)-4-O-[2-O-(2-hydroxypropyl) hexopyranosyl] hexopyranose isomers. For this metabolite, the loss of one methyl group generated one fragment at *m/z*: 427.2464, and the loss of one $C_3H_7O_2^-$ radical generated one fragment at *m/z*: 369.1401. Compound 20 was proposed as pantothenic acid, as reported previously (Rodríguez-Pérez *et al*., 2013) and compound 24 as a succinic acid derivative since pseudomolecular ion generated two fragments at *m/z*: 121.0288 and at *m/z*: 117.0186 (succinic acid), which were consistent with the separation of the benzoic acid moiety and the subsequent elimination of water. On the other hand, compound 25 was assigned as 2-(hydroxymethyl)-6-[3,4,5-trihydroxy-6-(4-hydroxybutoxy) oxan-2-yl] methoxy oxane-3,4,5-triol. For this metabolite, the loss of a methoxy group and the subsequent elimination of water generated one fragment at *m/z*: 367.1608, and the loss of the hexose moiety produced one fragment at *m/z*: 251.1130. Compounds 26 and 35 were identified as verbasoside isomers (see Figure S1). This compound occurred exclusively in callus tissue, which is the first time reported for *Yucca* species.

Compound 27 was only detected in callus tissue, and it was assigned as a cyclohexane carboxylic acid derivative since pseudomolecular ion at *m/z*: 351.1296 yielded fragments at *m/z*: 303.1448, 273.1344, and 287.1499. Fragments at *m/z*: 303.1448 and 273.1344 were generated due to the loss of 3 molecules of water and the subsequent elimination of one methoxy group; fragment at *m/z*: 287.1499 corresponded to the elimination of four molecules of water. On the other hand, compound 28 was only detected in plants, and it was tentatively identified as 3-oxo-5-amino-hexanoic acid since pseudomolecular ion yielded one fragment at *m/z*: 128.0347 generated due to the loss of water. Similarly, compounds 29 and 30 corresponded to glycosidic nature metabolites and were present only in plants cultivated under *ex vitro* conditions. The loss of the hexose moieties characterized the fragmentation pattern of these compounds. Compound 31 was detected in all tissues, and it was assigned as isopropylmalic acid since pseudomolecular ion at *m/z*: 175.0612 yielded one fragment at *m/z*: 143.0713 generated by the loss of two water molecules.

Compound 32 was tentatively identified as cistanoside G since pseudomolecular ion at *m/z*: 445.1715 yielded one main fragment at *m/z*: 119.0493 ($[M\text{-}H\text{-}C_{12}H_{21}O_{10}]^-$). This phenylethanoid glycoside occurred exclusively in callous tissue. This metabolite was reported for the first time in Cistanche plants (Orobanchaceae) (Deyama *et al*., 2006) and this is the first report for *Yucca* genus and Asparagaceae family. To the best of our knowledge, the biological activities of cistanoside G have not been reported in the literature. Nevertheless, phenylethanoid glycosides have been reported in several plant sources employed in traditional Chinese medicine since they possess biological activities such as neuroprotective, antioxidant, anti-inflammatory, antivirus, antibacterial and, antiosteoporotic properties (Xu *et al*., 2017; Xue and Yang, 2016). Our results suggest that *Y. carnerosana* callus culture may be a source of material to perform this group of metabolites' isolation and study.

Additionally, compounds 33, 34, 37, and 38 were assigned as metabolites of phenolic nature (caffeic acid and syringic acid and one of its derivatives) as reported previously (Cabañas-García *et al*., 2019; Chen *et al*., 2014; Iswaldi *et al*., 2013). For these compounds, antioxidant properties have been proposed (Alvarado-Ambriz *et al*., 2020; Cikman et al., 2015) and anti-inflammatory, anticancer, antidiabetic, neuroprotective, cardioprotective, and hepatoprotective effects (Srinivasulu *et al*., 2018). Compound 36 occurred in plants cultivated under *in vitro* conditions, and it was assigned as 6,7-dihydroxy-2-oxo-1-benzopyran-4-carboxylic acid since pseudomolecular ion at *m/z*: 221.0090 yielded one main fragment at *m/z*: 177.0190 ($[M\text{-}H\text{-}CHO_2]^-$). For compound 39, spectrometric evidences suggested the presence of *β*-D-galactopyranoside, 6-hydroxyhexyl 6-O-*β*-D-galactopyranosyl in callus tissue of *Y. carnerosana*. The pseudomolecular ion at *m/z*: 441.1975 yielded two fragments at *m/z*: 395.1923 ($[M\text{-}H\text{-}OH\text{-}CH_3O]^-$) and 217.1078 ($[M\text{-}H\text{-}hexose\text{-}C_2H_5O]^-$). Similarly, compound 40 occurred in callus tissue and *in vitro* plants, and it was assigned as a ferulic acid derivative (Cabañas-García *et al*., 2019). It has been proposed that ferulic acid inhibits the growth of *Phytophthora cinnamomi*, a fungus responsible for root rot in a wide range of hosts, producing significant economic and ecological losses worldwide (Matei *et al*., 2020). Thus, this derivative may be interesting for further studies.

Eriodictyol (Compound 42) was assigned as reported previously (Taamalli *et al*., 2015), and

compound 43 as 2-[(3-methyl benzoyl) amino] acetic acid, since two fragments were detected at *m/z*: 176.0711 ($[M-H-OH]^-$) and 119.0496 ($[M-H-C_2H_3NO_2]^-$). On the other hand, compound 44 was detected in all samples, and it was assigned as sinapic acid as proposed by Cabañas-García *et al*. (2019) and Liu *et al*. (2015). For this metabolite, its therapeutic effect against UVB-induced photo-aging of the skin has been proposed (Jeon *et al*., 2019). Compound 45 was assigned as magnoloside U as reported previously (Cabañas-García *et al*., 2021), and compound 46 was assigned as 3,4,5-triacetyloxy-6-[(4-oxo-2,3-dihydro-cyclopentachromen-7-yl)oxy]oxan-2-yl]methyl acetate since pseudomolecular ion at *m/z*: 531.1505 yielded one fragment at *m/z*: 134.0365, and compound 47 was proposed as the glycosylated flavonoid naringenin 7-O-rutinoside due to the presence of one main fragment at *m/z*: 266.0659, generated by the loss of the glycosidic moiety.

Compound 48 (3,4,5-triethoxybenzyl alcohol) yielded fragments at *m/z*: 209.1178 and 165.0916 generated due to the elimination of one methoxy group and the subsequent elimination of the C_2H_6O radical. For compound 51, spectrometric evidences suggested the presence of leeaoside (Zhang *et al*., 2015) in *ex vitro* plants.

In addition to citric, malic, gluconic, succinic, and pantothenic acids, other organic acids were detected (compounds 49, 54, 56-59, 61, and 63). The fragmentation pattern was mainly characterized by the loss of water and CO_2, as proposed by Ledesma-Escobar *et al*. (2015). Similarly, compounds 55, 60, and 62 were also assigned as flavonoids (alpinetin, sakuranetin, and persicogenin, respectively). The fragmentation steps were mainly characterized by the B-ring loss from the basic flavonoid structure and the loss of water and methyl groups. Finally, compound 64 was assigned as nordihydrocapsiate, as previously reported (Cabañas-García *et al*., 2019).

Several phenolic acids, phenolic glycosides and organic acids were identified in the methanolic extracts of *Y. carnerosana* obtained from *in vitro* cultures. It has been demonstrated that for achieving the highest diversity of bioactive compounds present in plant extracts, the extraction processes and conditions should be investigated to determine the optimal methodology (Sánchez-Rangel *et al*., 2014; Vallejo-Castillo *et al*., 2020). Thus, further studies should be performed to find the optimal conditions that may ensure the highest diversity of metabolites in each sample of *Y. carnerosana*.

Conclusions

It was determined that the best medium for friable callus induction was MS supplemented with 4.4 μM BA + 4.1 μM picloram. The callus growth curve of *Y. carnerosana* showed a lag phase and an exponential growth phase in a 2-12-week period. However, the stationary phase could not be observed within the time considered for the experiment. The methanolic extract prepared with *Y. carnerosana* callous tissue showed the presence of 22 compounds. On the other hand, 26 compounds occurred in the extract prepared with plants growing *in vitro*, and 27 in plants cultivated under *ex vitro* conditions. Our results suggest that the callus tissue culture of *Y. carnerosana* is a promising source for the study and production of biomass and plant metabolites.

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