UNIVERSIDAD AUTONOMA DE AGUASCALIENTES

CENTRO DE CIENCIAS BÁSICAS
DEPARTAMENTO DE QUÍMICA

TESIS

Análisis del perfil de metabolitos presentes en plantas y cultivos *in vitro* de *Coryphantha macromeris* y *C. potosiana*

PRESENTA
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PARA OBTENER EL GRADO DE DOCTOR EN CIENCIAS BIOLÓGICAS

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Aguascalientes, Ags., 20 de mayo del 2019

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DOCTORADO EN CIENCIAS BIOLOGICAS

Estimado alumno:

Por medio de este conducto me permito comunicar a Usted que habiendo recibido los votos aprobatorios de los revisores de su trabajo de tesis y/o caso práctico titulado: ***"ANALISIS DEL PERFIL DE METABOLITOS PRESENTES EN PLANTAS Y CULTIVOS IN VITRO DE CORYPHANTHA MACROMERIS Y C. POTOSIANA"*** hago de su conocimiento que puede imprimir dicho documento y continuar con los trámites para la presentación de su examen de grado.

Sin otro particular me permito saludarle muy afectuosamente.

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Article

Phytochemical Profiling of *Coryphantha macromeris* (Cactaceae) Growing in Greenhouse Conditions Using Ultra-High-Performance Liquid Chromatography–Tandem Mass Spectrometry

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Abstract: Chromatographic separation combined with mass spectrometry is a powerful tool for the characterization of plant metabolites because of its high sensitivity and selectivity. In this work, the phytochemical profile of aerial and radicular parts of *Coryphantha macromeris* (Engelm.) Britton & Rose growing under greenhouse conditions was qualitatively investigated for the first time by means of modern ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS). The UHPLC-PDA-HESI-Orbitrap-MS/MS analysis indicated a high complexity in phenolic metabolites. In our investigation, 69 compounds were detected and 60 of them were identified. Among detected compounds, several phenolic acids, phenolic glycosides, and organic acids were found. Within this diversity, 26 metabolites were exclusively detected in the aerial part, and 19 in the roots. Twenty-four metabolites occurred in both plant parts. According to the relative abundance of peaks in the chromatogram, ferulic and piscidic acids and their derivatives may correspond to one of the main phenolic compounds of *C. macromeris*. Our results contribute to the phytochemical knowledge regarding *C. macromeris* and its potential applications in the pharmaceutical and cosmetic industries. Besides, some metabolites and their fragmentation patterns are reported here for the first time for cacti species.

[rmiq] Editor Decision

JV

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Jaime Vernon-Carter
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Al personal administrativo de la Universidad Autónoma de Aguascalientes.

DEDICATORIAS

A Dios
A mi familia
A mis amigos

En especial dedico este trabajo a mi hijo Sebastian H., quien ha sido el mas paciente en este proceso. Gracias por vivirlo conmigo y comprender mis eventuales ausencias. Dedico también este trabajo a mi esposa Yenny Adriana, quien ha sido parte fundamental de este proyecto de vida.

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RESUMEN

La familia Cactaceae es una de las más amenazadas dentro del reino vegetal y es la más importante en las regiones áridas y semiáridas del Continente Americano. Entre las cactáceas se encuentran especies del género *Coryphantha*, las cuales poseen actividades farmacológicas y usos en la medicina tradicional mexicana, tal es el caso de *Coryphantha macromeris* (Engelm.) Britton & Rose y *C. potosiana* (Jacobi) Glass & Foster. Para estas especies se ha propuesto que las condiciones ambientales de las regiones semiáridas dificultan su germinación, crecimiento y reproducción. En este trabajo se establecieron diferentes sistemas de cultivo *in vitro* (planta *in vitro*, cultivo de callos y células en suspensión) de ambas especies y se analizó el perfil de metabolitos presentes en la sección aérea y radicular de plantas aclimatadas y en los cultivos *in vitro* de *C. macromeris*. Los cultivos de plantas *in vitro* se establecieron a partir de semillas germinadas en tubos de cultivo que contenían medio Murashige y Skoog (MS) (30 g L^{-1} de sacarosa, 10 g L^{-1} de agar, pH 5.7). Los brotes obtenidos *in vitro* se utilizaron como fuente de material vegetal para la propagación y aclimatación de las especies a condiciones de invernadero, así como para la inducción de callos y posterior obtención de las suspensiones celulares. Para el análisis del perfil de metabolitos se utilizó cromatografía de líquidos de ultra alta resolución acoplada a espectrometría de masas en tándem (UHPLC-PDA-HESI-Orbitrap-MS/MS). Bajo las condiciones propuestas, se detectaron 44 metabolitos en las plantas *in vitro* y se identificaron 43 de ellos, mientras que para las plantas de invernadero la diversidad de metabolitos fue mayor, ya que se detectó la presencia de 69 compuestos y 60 fueron identificados. Para ambos casos, los metabolitos que mostraron la mayor abundancia relativa en el cromatograma correspondieron a ácidos orgánicos (ácido cítrico, glucónico y tianshico) y ácidos fenólicos (ácido

piscidico, ferúlico y siríngico y/o sus derivados). En el presente trabajo, también se reporta un método eficiente para la inducción de callos friables obtenidos de *C. macromeris* y *C. potosiana*, su comportamiento cinético, su escalamiento a nivel matraz, así como el perfil de metabolitos presentes en cada sistema de cultivo. Se establecieron cultivos de callos a partir de secciones transversales inoculadas en medio MS suplementado con 6-bencilaminopurina (BAP; 2.20 μM) y Picloram (4.14 μM). Para *C. macromeris*, la mayor producción de biomasa (20,65 g DW L^{-1}) se obtuvo a las 9 semanas de cultivo, similar a lo encontrado para *C. potosiana.* El perfil de metabolitos de los callos de *C. macromeris* se evaluó en la etapa de mayor producción de biomasa. Los análisis realizados indicaron la presencia de 61 compuestos y se identificaron 52 de ellos. Entre los compuestos detectados, se identificaron 11 ácidos orgánicos, 16 ácidos fenólicos, 8 flavonoides y 17 metabolitos de diferentes clases. Para el establecimiento del cultivo de células en suspensión, se tomaron muestras de tejido calloso con características friables y se transfirieron a matraces de 120 mL que contenían 25 mL de medio MS líquido suplementado con los mismos reguladores de crecimiento (BAP 2.20 μM y Picloram 4.14 μM), y se evaluaron tres velocidades de agitación (80, 100 y 120 rpm) para analizar el efecto de la velocidad de agitación sobre la morfología y viabilidad celular. Nuestros resultados indican que una velocidad de agitación de 120 rpm impacta negativamente en la integridad celular ya que las células sufrieron cambios morfológicos y pérdida de viabilidad. Sin embargo, a 80 y 100 rpm, las células sobrevivieron y proliferaron con éxito con porcentajes de viabilidad similares (aproximadamente 97%). El análisis de perfil de metabolitos presentes en cultivos de células en suspensión se realizó utilizando células de dos meses de edad cultivadas a 80 rpm. Los análisis realizados indicaron la presencia de 49 metabolitos y se identificaron 45 de ellos. Entre los compuestos detectados, se identificaron diferentes clases de metabolitos tales como ácidos

fenólicos (derivados del ácido gálico), iridoides (gardósido), estilbenos (tirolobibencil E), lignanos (acantosido B), flavonoides (catequina, lantanosido, sakuranina, afrormosina, kaempferol-7-ramnósido) y feniletanoides, entre otros. Nuestros resultados indican que el perfil de metabolitos de las especies analizadas es afectado por el tipo de cultivo *in vitro* y contribuyen al conocimiento biotecnológico y fitoquímico de *C. macromeris* y *C. potosiana* y sus aplicaciones potenciales en la industria farmacéutica y cosmética, ya que representa una fuente potencial para la obtención de compuestos seleccionados, así como un sistema útil para futuras investigaciones relacionados a la obtención y estudio de metabolitos de alto valor. Además, algunos metabolitos se reportan aquí por primera vez para especies de cactáceas, así como sus patrones de fragmentación.

ABSTRACT

The family Cactaceae is one of the most threatened within the plant kingdom and is the most important in the arid and semiarid regions of America. Among Cacti species, the genus *Coryphantha* is highly endemic and is distributed in the arid and semiarid regions of northern Mexico and southern United States. There are species of the genus *Coryphantha,* which possess pharmacological activities and uses in folk medicine as is the case of *Coryphantha macromeris* (Engelm.) Britton & Rose and *C. potosiana* (Jacobi) Glass & Foster. For this plant species, it has been proposed that the environmental conditions of semiarid regions difficult their germination, growth, and reproduction. In this work, different *in vitro* culture systems (*in vitro* plants, callus, and cell suspension cultures) of *C. macromeris* and *C. potosiana* were established and then the phytochemical profile of aerial and radicular sections of acclimatized plants and *in vitro* cultures of *C. macromeris* was analyzed. *In vitro* cultures were established from seeds germinated in culture vessels containing Murashige and Skoog (MS) medium (30 g L^{-1} sucrose, 10 g L^{-1} agar, pH 5.7). The obtained shoots from the germinating seeds *in vitro* were used as the source of explants for the *in vitro* multiplication cycles and for the acclimatization of plants to greenhouse conditions. The phytochemical profile of plants and *in vitro* cultures was analyzed using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS). Under the proposed chromatographic conditions, 44 metabolites were detected, and 43 of them were identified in *in vitro* plants. Greenhouse plants showed a higher diversity of compounds since 69 metabolites were detected, and 60 of them were identified. Among detected compounds in plants cultivated *in vitro* and under greenhouse conditions, organic acids (citric, gluconic and tianshic acids), and phenolic acids

such as piscidic, ferulic and syringic acid and/or their derivatives were found as the metabolites with the highest relative abundance.

In this work, we also report an efficient method for the induction of friable callus obtained from *C. macromeris and C. potosiana,* their kinetic behavior, as well as the procedure for the obtention of cell suspension cultures, and the phytochemical profile analyzed in each culture system. Callus cultures were established from stem discs inoculated on MS medium supplemented with 6-benzylaminopurine (BAP; 2.2 μM) and Picloram (4.14 μM). For *C. macromeris*, the highest yield in biomass production (20.65 g DW L^{-1}) was achieved at 9 weeks of culture, similar to that found for *C. potosiana.* The chromatographic and mass spectral analysis of the callus tissue obtained from *C. macromeris* after 9 weeks of culture indicated the presence of 61 metabolites, and 52 of them were identified. Among detected compounds, 11 organic acids, 16 phenolic acids, 8 flavonoids, and 17 metabolites of different classes were identified. The friable callus tissue was then transferred to 120 mL flasks containing 25 mL of MS liquid medium supplemented with the same growth regulators (BAP 2.2 μM and Picloram 4.14 μM), and then three agitation velocities (80, 100 and 120 rpm) were evaluated in order to assess the effect of agitation velocity on cell viability and morphology. Our results indicate that an agitation velocity of 120 rpm impacts negatively on cell integrity since cells suffered morphological changes and loss of viability. Nevertheless, at 80 and 100 rpm, cells successfully survived and proliferated with similar viability percentages (ca. 97%). The phytochemical profile of two-month-old cells cultivated at 80 rpm was then analyzed. The chromatographic and mass spectral analysis indicated the presence of 49 metabolites, and 45 were of them were identified. Among detected compounds, different classes of metabolites such as phenolic acids (gallic acid derivatives), iridoids (gardoside), stilbenes (tyrolobibenzyl E), lignans

(acanthoside B), flavonoids (catechin, lantanoside, sakuranin, afrormosin, Kaempferol 7-rhamnoside) and phenylethanoids (phlomisethanoside) were found. Our results suggest that the phytochemical profile of the analyzed species is affected by the type of *in vitro* culture and contribute to the biotechnological and phytochemical knowledge regarding *C. macromeris* and *C. potosiana* and its potential applications in the pharmaceutical and cosmetic industries since it represents a potential source for the obtention of selected compounds, as well a useful system for future investigations regarding the elicitation and study of high valuable metabolites. Besides, some metabolites are reported here for the first time for cacti species as well as their fragmentation patterns.

PRÓLOGO

La presente Tesis Doctoral está integrada por 5 capítulos. Cada capítulo corresponde a manuscritos redactados durante el desarrollo del proyecto. El conjunto de los manuscritos propuestos da respuesta a los objetivos e hipótesis planteadas durante el proyecto titulado "Análisis del perfil de metabolitos presentes en plantas y cultivos *in vitro* de *Coryphantha macromeris* y *C. potosiana*".

El **capítulo I** aborda una revisión sobre la composición y perfil de metabolitos primarios y secundarios presentes en diferentes especies de cactáceas, enfocándose principalmente en el contenido de metabolitos de naturaleza fenólica y alcaloides y la correlación existente con las propiedades funcionales y los usos tradicionales de las diferentes especies.

El **capítulo II** hace referencia al perfil de metabolitos presentes en la sección aérea y radicular de plantas de *Coryphantha macromeris* cultivadas en condiciones de invernadero e identificados mediante cromatografía de líquidos de ultra alta resolución, acoplado a espectrometría de masas en tándem (UHPLC-PDA-HESI-Orbitrap-MS/MS). En este capítulo, los ensayos realizados se encaminan a dar respuesta al Objetivo II del trabajo de tesis, correspondiente analizar el perfil de metabolitos presentes en los tejidos obtenidos bajo diversos sistemas de cultivo y se reporta por primera vez la presencia de 69 compuestos en plantas de *C. macromeris*, haciendo una correlación con las actividades farmacológicas y diversas propiedades funcionales como antioxidante, bactericida, entre otras reportadas previamente en la literatura para los metabolitos identificados.

El **capítulo III** aborda los ensayos realizados para el establecimiento e inducción de brotes en sistema *in vitro* y da continuidad al análisis del perfil de metabolitos presentes en la sección aérea y radicular de plantas de *C. macromeris* cultivadas en condiciones *in vitro* y propone a esta especie como una fuente potencial para la obtención de metabolitos de interés, ya que se detectó la presencia de 44 compuestos y 43 de estos fueron identificados. De la diversidad de compuestos presentes en este sistema, 7 metabolitos fueron identificados por primera vez para *C. macromeris.* Con los ensayos realizados se demostró

que *C. macromeris* cultivada en condiciones controladas posee la capacidad de biosintetizar diferentes grupos de compuestos encontrados en plantas cultivadas en condiciones *ex vitro*, además de otros metabolitos no detectados en plantas cultivadas por métodos convencionales.

En el **capítulo IV** se indican los ensayos realizados para inducir la formación de tejido calloso en *C. macromeris*, así como su comportamiento cinético y el perfil de metabolitos presentes en la etapa de mayor producción de biomasa (9 semanas). Con los resultados presentados en este capítulo, se propone el potencial de esta especie para la producción de biomasa y para la biosíntesis y estudio de metabolitos de interés, ya que se detectó la presencia de 61 compuestos y 52 fueron de estos fueron identificados. Entre los compuestos identificados, 11 metabolitos fueron ácidos orgánicos, 16 ácidos fenólicos, 8 flavonoides y 17 metabolitos pertenecientes a diferentes grupos de compuestos como alcaloides, iridoides, lignanos, cuasinoides, isoprenoides y otros compuestos glucosilados.

El **capítulo V** hace referencia al establecimiento de cultivo de células en suspensión, así como a la evaluación del efecto de la velocidad de agitación sobre la morfología de las células de *C. macromeris* y su perfil de metabolitos evaluado después de dos meses de cultivo bajo las condiciones de agitación consideradas como adecuadas para la producción de biomasa con esta especie. Los resultados presentados en este capítulo sirven como base para realizar futuras investigaciones relacionadas al establecimiento de cultivo de células en suspensión de *Coryphantha* spp. o especies relacionadas y propone su potencial para la biosíntesis de compuesto de interés, ya que se detectaron 49 metabolitos y 45 de estos fueron identificados. Entre los metabolitos identificados, se encontraron ácidos fenólicos, iridoides, flavonoides, entre otros con propiedades funcionales reconocidas.

En la sección de **anexos**, se indican algunos experimentos realizados durante el desarrollo del proyecto de tesis. El anexo 1 indica resultados de los experimentos realizados para inducir la formación de brotes en diferentes especies del género *Coryphantha*. El **anexo 2** indica los experimentos realizados para la transformación genética de especies, el **anexo 3** hace referencia a los alcaloides identificados en diferentes muestras de *C. macromeris* y los

anexos 4 y 5 hacen referencia a los perfiles de separación de alcaloides en diferentes muestras de *C. potosiana.*

Con los resultados presentados en este trabajo de tesis, se concluye que el perfil de metabolitos de *C. macromeris* es afectado por el sistema de cultivo. Se identificaron diferentes grupos de metabolitos en los tejidos obtenidos de *C. macromeris* cultivada en diferentes sistemas. Sin embargo, la planta cultivada en invernadero y el tejido calloso fueron los que presentaron la mayor diversidad de compuestos. Algunos de los metabolitos identificados en los diferentes sistemas poseen propiedades funcionales reconocidas.

INTRODUCCIÓN

1. Importancia de los metabolitos secundarios y su aprovechamiento racional

Los metabolitos secundarios son compuestos biosintetizados por las plantas como un mecanismo natural de defensa química ante las condiciones de estrés [1]. Se ha demostrado que estos compuestos poseen propiedades funcionales sobre los humanos y otros seres vivos [2]. Los metabolitos secundarios se clasifican en función de su naturaleza química y pueden ser divididos en alcaloides, compuestos fenólicos, flavonoides, terpenos, cumarinas y estilbenos [3]. Los diferentes grupos de compuestos pueden encontrarse en la naturaleza formando sistemas conjugados con otras moléculas, como los carbohidratos, proporcionando así mayor estabilidad al compuesto dentro de la célula, incrementando su polaridad y actividades biológicas [4].

Las actividades biológicas relacionadas a la presencia de metabolitos secundarios han sido reconocidas y reportadas para diferentes especies vegetales [3, 5, 6]; sin embargo, también se ha propuesto que los rendimientos en la obtención de los metabolitos pueden ser bajos y con calidad variable en los sistemas de producción convencionales como el cultivo de plantas en campo; por lo que se ha propuesto el uso de herramientas biotecnológicas como el cultivo de células para la producción y extracción de metabolitos de interés [7]. En este sentido, los sistemas de cultivo *in vitro* representan una fuente renovable para la producción de metabolitos difíciles de obtener por síntesis química [8], reduciendo también las labores en campo y la variabilidad estacional. En el capítulo I se aborda una revisión sobre diferentes grupos de compuestos identificados en especies de cactáceas y se hace una breve descripción sobre sus propiedades funcionales.

1.1. Uso de espectrometría de masas para la identificación de metabolitos secundarios

Las plantas están compuestas por diferentes tipos de metabolitos encontrados en una amplia diversidad estructural y en diferentes concentraciones, lo que dificulta su identificación mediante un solo método analítico o bajo un mismo sistema de separación [9]. Para logar la elucidación del metaboloma se han propuesto diversas técnicas que combinan la capacidad de separación de un método, encargado de producir compuestos puros o fracciones enriquecidas con un componente, con la capacidad de identificación de los métodos espectroscópicos, encargados de brindar información estructural que contribuye a identificar los metabolitos mediante el uso de bases de datos o mediante su comparación con estándares de referencia, facilitando así la elucidación de compuestos encontrados en mezclas complejas [10], tal es el caso del acoplamiento entre los sistemas cromatográficos y la espectrometría de masas.

La espectrometría de masas es una técnica que contribuye a detectar e identificar metabolitos presentes en mezclas complejas, basándose en los patrones de fragmentación de cada molécula y a su relación masa/carga (m/z). La fragmentación de las moléculas se logra al bombardearlas con partículas cargadas, provocando así la pérdida de un electrón; los fragmentos generados por el bombardeo de electrones contribuyen a reconstruir la molécula y conocer su identidad. De este modo, se construye el espectro de masas de una muestra en particular, graficando la relación m/z en el eje "x" y la abundancia relativa de cada compuesto, calculada en relación al pico mayoritario en el eje "y".

Se ha realizado la identificación de metabolitos en diferentes sistemas vegetales mediante el acoplamiento de la espectrometría de masas con diferentes técnicas de

separación [11-15], contribuyendo al conocimiento sobre la composición de diferentes especies y su uso potencial. En el capítulo 1 se muestran algunos metabolitos identificados en diferentes especies de cactáceas, utilizando espectrometría de masas y otras técnicas de identificación. Así mismo, en los capítulos II al V, se indica el uso de esta técnica para la identificación de metabolitos presentes en los tejidos de *C. macromeris* obtenidos bajo diversos sistemas de cultivo, como planta de invernadero (Capítulo II), planta *in vitro* (Capítulo III), cultivo de callos (Capítulo IV) y células en suspensión (Capítulo V).

1.2. Especies de cactáceas y su aprovechamiento racional

La familia de las cactáceas comprende alrededor de 1,600 especies distribuidas principalmente en las regiones áridas y semiáridas del continente Americano [16], teniendo a México como el centro de origen y diversificación; posee 4 subfamilias (*Opuntioideae, Pereskioideae, Cactoideae y Maihuenioideae*) y alrededor de 63 géneros; las cactáceas son plantas suculentas que han sufrido cambios anatómicos y fisiológicos para adaptarse al entorno árido [17] y cuya morfología de las semillas varía en función de los géneros y especies; el tamaño promedio de las semillas es de 1-3 mm (Cactoideae), 4 mm (Pereskioideae), 5 mm o mas (Opuntioideae) [18]; Así mismo, son especies caracterizadas por la producción de mucilago, el cual ha sido utilizado por sus diversas propiedades funcionales [19].

En México, los diferentes usos antropogénicos han provocado una sobreexplotación de los ecosistemas, poniendo en riesgo a diferentes especies, por lo que se ha clasificado a la familia de las cactáceas como la más amenazada dentro del reino vegetal [17], estando sujetas a protección por la NOM-059-SEMARNAT-2010 [20].

Se ha demostrado que algunas especies de cactáceas germinan más rápido en espacios con sombra en comparación con los lugares expuestos a radiación solar [21]. En este sentido, se ha propuesto que el porcentaje de germinación puede ser bajo para algunas especies de cactáceas [22, 23], teniendo cada especie requerimientos óptimos de temperatura para lograr una mayor germinación [24], lo que en conjunto con la presencia de depredadores pudieran limitar su crecimiento y posterior dispersión.

En México, diversas especies de cactáceas obtenidas por métodos de cultivo o colectadas de manera silvestre se han utilizado como fuente alimenticia [25] forraje, ornamental y medicinal [26]; esta última debido a sus propiedades como antioxidante [27], bactericida [28], antimutagénica [29], encapsulante [30], entre otras que varían en función de la composición y características bioquímicas de cada especie vegetal.

1.3. Generalidades de *Coryphantha* spp.

Dentro de la familia de las cactáceas se encuentran especies pertenecientes al género *Coryphantha*. El género *Coryphantha* estaba antiguamente unificado con el género *Mammillaria* y fue en 1856 cuando fueron separados [31]. Se han reportado 42 especies pertenecientes al género *Coryphantha* [32], las cuales se encuentran distribuidas en las regiones áridas y semiáridas ubicadas entre la Sierra Madre Oriental y la Sierra Madre Occidental, cubriendo también parte de Arizona, Texas y Nuevo México, EUA (Figura 1). Las condiciones ambientales (baja disponibilidad de nutrientes, agua, alta radiación solar, frio y calor intensos) presentes en estas regiones dificultan su germinación, crecimiento y reproducción [33]. Algunas

especies de este género presentan un alto índice de endemismos [34] y están categorizadas como amenazadas por la NOM-059-SEMARNAT-2010 [20].

Figura 1. Distribución de *Coryphantha* spp. en el territorio mexicano y el sur de Estados Unidos. Tomado de Dicht y Lüthy [35].

1.3.1. *Coryphantha macromeris* (Engelmann) Britton & Rose

Son cactáceas globosas que varían de entre 10 a 15 cm de altura y 5 cm de diámetro; sus areolas miden de 4-5 mm, con espinas radiales que pueden estar dispuestas irregularmente y varían de 9 a 15 unidades de 15 a 28 mm de longitud; sus espinas centrales (3-6) se disponen horizontalmente y miden de 25 a 50 mm de longitud [35]. Sus flores tienen una coloración magenta y varían de 3 a 5 cm de longitud y de 4.5 a 7 cm de diámetro [36]. En la Figura 2a y 2b se muestra a *C. macromeris* formando un aglomerado, así como el aspecto de la flor y espinas.

Coryphantha macromeris es una especie que habita desde los 750 a 1500 msnm y crece en suelos arenosos en zonas abiertas o bajo los arbustos [35], usualmente formando

aglomerados que van desde 15 cm a 1 metro de amplitud [36] y con una altura de alrededor de 20 cm [35]. Está distribuida principalmente en el desierto de Chihuahua y en los Estados de Coahuila, Durango, Zacatecas, y Tamaulipas, así como en el sur de Estados unidos (Nuevo México y Texas) [32, 36, 37]; es reconocida también como *Echinocactus macromeris, Cactus macromeris, Lepidocoryphantha macromeris, Mammillaria heteromorpha, Echinocactus heteromorphus, Cactus heteromorphus y Mammillaria dactylithele* [35]. En México se le conoce comúnmente como "biznaga partida-partida", "doña Ana" y en inglés se conoce como "long mamma cory cactus" y "big niddle cactus" [36, 37].

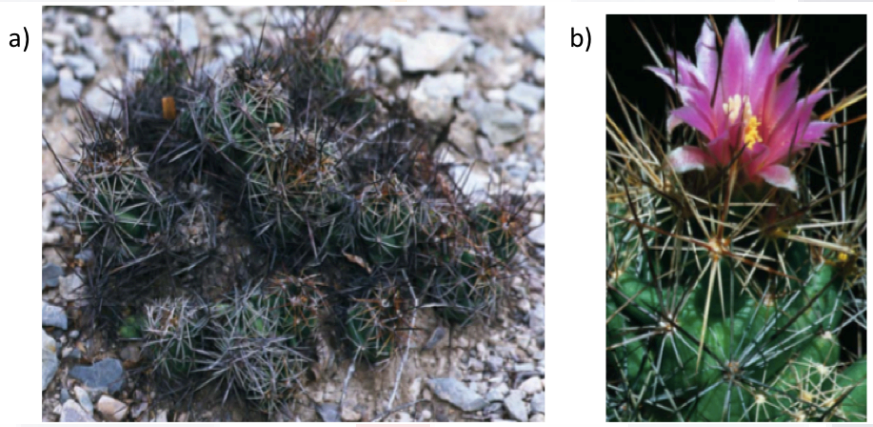

Figura 2. Especie de *Coryphantha macromeris* formando un aglomerado (a) y aspecto de la flor y espinas (b). Imagen tomada de Dicht y Lüthy [35]

1.3.2. *Coryphantha potosiana* (Jacobi) Glass & Foster

Es una especie que puede superar los 25 cm de altura y 8 cm de diámetro; sus areolas frecuentemente no poseen espinas centrales, pero cuando las presentan pueden medir de 8 a 15 mm de longitud; sus espinas radiales pueden variar entre 15 y 18 unidades, de 10-12 mm de longitud. Sus flores son pequeñas (22 mm de longitud) y de color amarillo pálido (Figura 3). Es una especie endémica de San Luis Potosí,

donde crece en planicies y regiones con presencia de agaves y especies del género *Opuntia* [35].

Figura 3. Especie de *Coryphantha potosiana*. Imagen tomada de Dicht y Lüthy [35]

1.4. Composición y características bioquímicas de *Coryphantha* spp

Los trabajos existentes relacionados a la identificación de compuestos en especies del género *Coryphantha* son escasos y hacen alusión a la identificación de alcaloides en *Coryphantha calipensis* [38], *C. macromeris* var. *runyonii* [39] y *C. ramillosa* [40]. Así mismo, en un estudio realizado por Sánchez-Herrera, et al. [41], en el que evaluaron la composición proximal y el perfil fitoquímico de *Coryphantha bumamma, C. clavata* y *C. cornifera,* encontraron que el contenido total de proteínas, grasas, fibra, humedad y cenizas varía de una especie a otra y en función de la sección del tallo (apical, media, basal) o raíz en que se haga la determinación. Así mismo, se detectó la presencia de flavonoides y esteroles en los extractos acuosos y etanólicos, así como de saponinas en el extracto acuoso y hexánico.

1.4.1. Metabolitos secundarios identificados en especies del género *Coryphantha*

Los estudios relacionados a la identificación y/o cuantificación de metabolitos secundarios en especies del género *Coryphantha* son escasos y se han enfocado a la identificación y cuantificación de alcaloides en diferentes especies. Hodgkins, et al. [42] lograron separar diferentes alcaloides en plantas de *C. macromeris* mediante técnicas cromatográficas y espectroscópicas, encontrando a la macromerina como el principal alcaloide e identificando otro alcaloide al que denominaron gigantina (1,2-dimetil-4-hidroxi-6,7-dimetoxi-1,2,3,4-tetrahidroisoquinolina). Por su parte, Hornemann, et al. [43] analizaron mediante cromatografía en capa fina, la presencia de alcaloides en diferentes especies de *Coryphantha (C. cornifera var. echinus, C. pectinata, C. elephantidens, C. duranguensis, C. ottonis, C. poselgeriana* y *C. cornifera*), encontrando que la hordenina, *N*-metiltiramina y la sinefrina estaban presentes en todas las especies analizadas, lo que sugiere que pudieran encontrarse como metabolitos constitutivos en diferentes especies del género *Coryphantha*. Por su parte, Keller, et al. [39] encontraron la presencia de diferentes tipos de alcaloides en muestras de *C. macromeris* var. runyonii, siendo un derivado de la normacromerina el encontrado en mayor concentración. Sánchez-Herrera, et al, [41] detectaron la presencia de alcaloides en extractos polares de *C. bumamma, C. clavata* y *C. cornifera* mediante el uso de pruebas presuntivas.

Todos estos resultados sugieren que diferentes especies de *Coryphantha* poseen alcaloides, por lo que es interesante profundizar en el conocimiento sobre su composición. En el capítulo 1 se aborda una revisión sobre el perfil de alcaloides en diferentes especies de cactáceas. Así mismo, en el anexo 3 indica el perfil de alcaloides presentes en la sección aérea y radicular de plantas de *C. macromeris* cultivadas en condiciones *in vitro* y de invernadero, respectivamente.

1.5. Herramientas biotecnológicas para la multiplicación masiva de brotes

El cultivo de células y tejidos vegetales (CCTV) representan una fuente potencial para la producción de biomasa y de compuestos de interés para el hombre; dichos compuestos pueden ser utilizados en diferentes ramos de la industria [8, 44]. Se ha propuesto que los productos naturales además de tener actividades funcionales, sirven como base para el descubrimiento, diseño o la síntesis de nuevos compuestos con actividades biológicas [45], por lo que el establecimiento de cultivos de células y tejidos vegetales en sistemas *in vitro* se convierte en una tarea fundamental con alcances socioeconómicos, generando sistemas que permitan la producción de metabolitos y al mismo tiempo, la conservación del hábitat y de especies que presentan una capacidad reproductiva limitada, tal es el caso de algunas especies pertenecientes a la familia de las cactáceas [46].

En el CCTV, las especies son propagadas en sistemas con condiciones controladas y bajo procesos continuos con alcance de gran escala. La propagación está basada en la totipotencia celular y en la capacidad de la célula alterar su metabolismo y plasticidad bajo las condiciones de cultivo. En este sentido, cada especie requiere la combinación de diferentes condiciones en el medio para su óptimo desarrollo [47], viéndose afectados también los patrones de crecimiento y enraizamiento [48].

El cultivo de plantas *in vitro* se realiza en un sistema cerrado, en el que la manera de controlar el microambiente del interior del sistema es modificando las condiciones externas, como la temperatura e intensidad lumínica. En este sentido, se ha demostrado que la intensidad lumínica y el tipo de recipiente tienen efecto en la distribución de la temperatura al interior del sistema [49] y que la humedad relativa es mayor al 98 % a lo largo del tiempo de cultivo, mostrándose variaciones en función de la temperatura del aire [50], por lo que el controlar las condiciones se

convierte en una tarea fundamental en orden de disminuir en lo posible la variabilidad entre los individuos.

Se ha propuesto que una de las limitaciones de los cultivos *in vitro* es la poca capacidad que tienen las plantas de regular la pérdida de agua, lo que sugiere que pudiera existir una disfuncionalidad estomática [46], por lo que el proceso de aclimatación de las especies propagadas *in vitro* debe ser gradual en orden de permitir a la planta adaptarse a las nuevas condiciones, recuperando totalmente su funcionalidad.

Para el crecimiento de los diferentes organismos se utilizan reguladores de crecimiento vegetal, mismos que tienen efectos diferenciados entre las especies. Dentro de los reguladores se encuentran las auxinas, citocininas, giberelinas, entre otros [51] que pueden ser afectados por las diferentes condiciones del medio de cultivo, tal es el caso de la presencia de carbón activado y el pH.

El pH del medio afecta el crecimiento y la actividad de los reguladores de crecimiento; el tipo y concentración de reguladores varía en función de cada especie, del tipo de explante utilizado y de la respuesta fisiológica que se desea inducir en el material vegetal.

1.5.1. Cultivo de células y tejidos vegetales con especies de cactáceas

El cultivo *in vitro* de especies de cactáceas ha demostrado ser una alternativa para su propagación [22, 23, 52-54], siendo utilizado para la conservación de especies en peligro [46, 55]. Por su parte, los reportes relacionados a la inducción de callos y cultivos de células en suspensión de cactáceas son escasos. En el Cuadro 1 se indican algunos trabajos realizados con la propagación *in vitro* de diferentes especies de

cactáceas. En los Capítulos II-V se indican los experimentos realizados para obtener diferentes sistemas de cultivo *in vitro* con *C. macromeris,* así como el perfil de metabolitos secundarios evaluado en los diferentes sistemas.

Cuadro 1. Cultivos *in vitro* de diferentes especies de cactáceas.

Especie	Actividad realizada	Referencia
Escobaria minima *Mammillaria pectinifera* *Pelecyphora aselliformis*	Evaluaron el efecto del tipo de explante y de diferentes tipos y concentraciones de reguladores de crecimiento sobre la formación de brotes.	[22]
Cereus peruvianus L.	Evaluaron el efecto de diferentes reguladores de crecimiento sobre la formación de brotes, así como los ciclos de subcultivo y el efecto de diferentes medios sobre la síntesis de pigmentos y la capacidad de enraizamiento.	[56]
Coryphantha elephantidens	Evaluaron el efecto de diferentes reguladores de crecimiento y tipo de explantes sobre la formación de brotes, así como el efecto del tipo y concentración de fuente de carbono sobre la formación de biomasa.	[54]
Obregonia denegrii Fric. *Coryphantha minima* Baird	Demostraron que la propagación *in vitro* de especies de cactáceas es más eficiente que un sistema *ex vitro*.	[46]
Coryphantha elephantidens (Lem.) Lem.	Establecieron un cultivo de callos para la regeneración de plántulas, evaluando también la supervivencia a la aclimatación.	[53]
Notocactus magnificus	Establecieron un protocolo para la propagación *in vitro* y la regeneración de plantas, haciendo uso de diferentes reguladores de crecimiento.	[23]
Opuntia lanigera Salm–Dyck	Establecieron las condiciones de micropropagación, evaluando diferentes orientaciones del explante, tipo y concentración de citocinina utilizada, así como la concentración de fuente de carbono y el efecto de ácido giberélico sobre el crecimiento en condiciones de aclimatación.	[57]
Opuntia ficus-indica (L.) (Mill)	Evaluaron el efecto de diferentes reguladores de crecimiento sobre la formación de callos y desarrollaron un protocolo para la regeneración de plantas.	[58]
Echinocereus cinerascens	Establecieron un protocolo de micropropagación, evaluando el efecto de diferentes reguladores de crecimiento (solos y combinados) sobre la formación de brotes y callos.	[59]
Echinocereus cinerascens	Realizaron la multiplicación de brotes y embriogénesis utilizando medio MS suplementado con diferentes reguladores de crecimiento.	[60]
Mammillaria san-angelensis	Evaluaron el efecto que tienen las auxinas a diferentes concentraciones sobre la respuesta morfogénica en plantas.	[55]

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JUSTIFICACIÓN

México es un país megadiverso; como parte de su riqueza florística existen diferentes especies de cactáceas como lo son *Coryphantha macromeris* y *C. potosiana*, las cuales poseen usos en la medicina tradicional mexicana. Sin embargo, son especies de lento crecimiento y bajo índice de reproducción, lo cual ha puesto en peligro a sus poblaciones. Para atender a esta problemática, se ha propuesto la aplicación de herramientas biotecnológicas que permitan la multiplicación masiva de brotes y la producción de compuestos de interés en medios con condiciones controladas. Ya que se ha demostrado que diferentes especies de cactáceas son una fuente de compuestos de interés para el hombre y que las propiedades funcionales de cada especie varían en función de su composición, es importante realizar estudios para conocer el perfil de metabolitos presentes en plantas y cultivos *in vitro* de *C. macromeris* y *C. potosiana* en orden de contribuir y profundizar en el conocimiento estas especies y sus posibles aplicaciones.

HIPOTESIS

Los diferentes sistemas de cultivo *in vitro* (planta, tejido calloso y células en suspensión), causarán alteraciones en el patrón de acumulación de compuestos en los tejidos *C. macromeris* y *C. potosiana*. Conocer estas alteraciones permitirá seleccionar el mejor sistema de cultivo para la producción *in vitro* de cada tipo de metabolito.

OBJETIVOS

Objetivo general

Analizar el perfil de metabolitos presentes en plantas y cultivos *in vitro* en al menos una de las especies estudiadas.

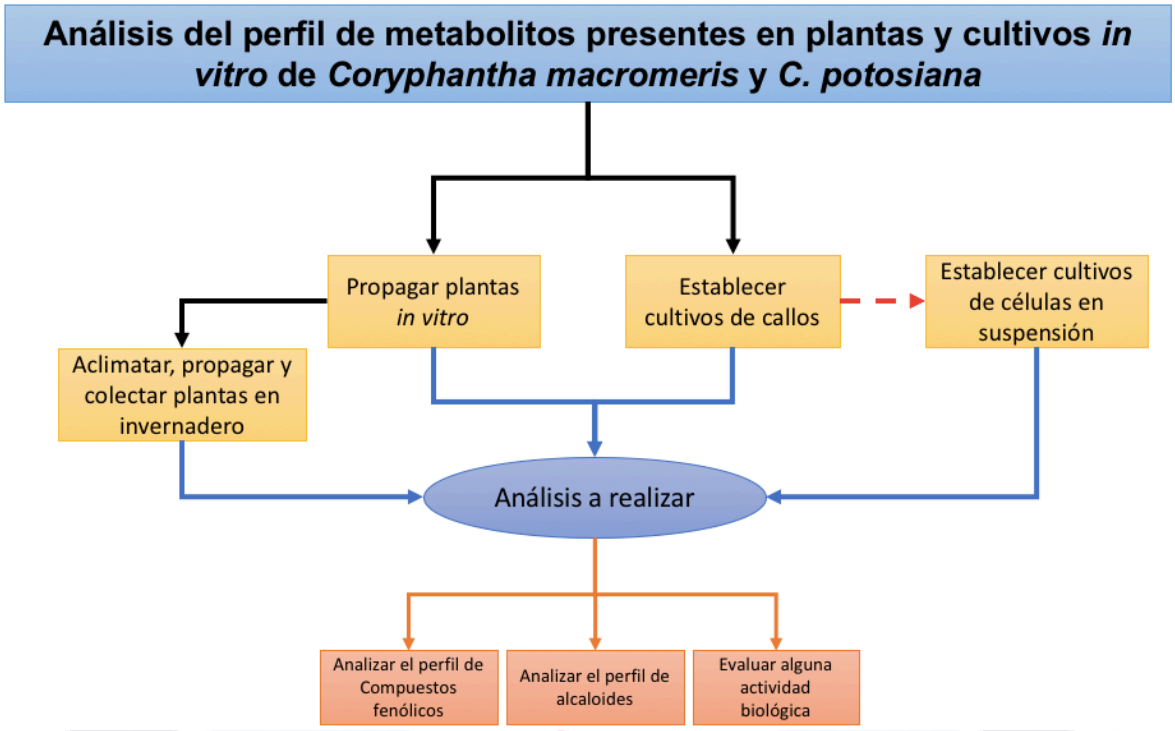
Objetivos específicos

1. Establecer cultivos de *Coryphantha* spp, en diversos sistemas *in vitro*.
2. Analizar el perfil de compuestos fenólicos y compuestos tipo alcaloides en los tejidos obtenidos bajo diversos sistemas *in vitro*.
3. Evaluar alguna actividad biológica de extractos obtenidos a partir de cultivos obtenidos bajo diversos sistemas *in vitro*.

En los capítulos II-V se indican los ensayos realizados para dar cumplimiento al objetivo particular número 1. En estos mismos capítulos se indica el perfil de metabolitos de naturaleza fenólica (objetivo 2) y en la sección de anexos se indica el perfil de alcaloides presentes en *C. macromeris*. Así mismo en relación al objetivo número 3, relacionado a la evaluación de actividades biológicas, se encuentra indicado en perspectivas del trabajo, ya que se realizó la evaluación de actividad antioxidante, pero los extractos metanólicos de *C. macromeris* no mostraron actividad bajo los métodos evaluados.

ESQUEMA METODOLÓGICO

A continuación, se indica el esquema general de la estrategia metodológica utilizada en el presente trabajo de tesis. Los detalles específicos de la metodología empleada se encuentran indicados en los capítulos II-V.



CAPÍTULO I
Especies de cactáceas: una revisión sobre el perfil y contenido de metabolitos primarios y secundarios

I.1. Resumen

Las cactáceas corresponden a la familia de especies más amenazada dentro del reino vegetal. Su composición y características varían de una especie a otra y en función de las condiciones del medio en el que se encuentren. Los tejidos y el mucilago de las cactáceas están formados principalmente por carbohidratos y en menor proporción por proteínas, ácidos grasos y minerales. Dentro de los carbohidratos, los principales monosacáridos para algunas especies son la galactosa y la arabinosa. El contenido de proteínas varía en un intervalo de 0.3 a 47.3 g/100 g; los principales minerales son el Na, Mg, Ca, P y K en la mayoría de las especies dentro de esta revisión. Una de las características distintivas de las cactáceas es la presencia de alcaloides y compuestos de naturaleza fenólica. Para el caso de los alcaloides, la hordenina, la tiramina y la anhalinina se encontraron en mayor concentración y para los compuestos fenólicos, el ácido ferúlico y el ácido protocatecuico, en las especies dentro de esta revisión. La información aquí presentada propone a diferentes especies de cactáceas como una fuente de compuestos funcionales y enmarca un vínculo entre sus usos tradicionales y su perfil y contenido de metabolitos secundarios.

I.2. Introducción

Las cactáceas son plantas suculentas que han sufrido cambios anatómicos y fisiológicos para adaptarse al entorno árido y cuya morfología varía en función de cada género y especies; la familia de las cactáceas está conformada por las subfamilias Opuntioideae, Pereskioideae, Cactoideae y Maihuenioideae, dentro de las cuales existen alrededor de 63 géneros [1] y cuyas especies se caracterizan por la producción de mucilago [2], el cual ha sido investigado por sus propiedades reológicas y potencial de uso [3-6].

Se ha propuesto que alrededor del 78% de especies de cactáceas son endémicas [1]; sin embargo, alrededor del 57% de la diversidad cactoflorística es utilizada por el hombre, siendo los fines de horticultura, alimenticio, medicinal, forrajero y para la elaboración de artesanías los usos más comunes [7]. Estos usos antropogénicos en conjunto con el desarrollo de urbanizaciones, han provocado una sobreexplotación y/o deterioro de los ecosistemas, poniendo en riesgo a diferentes especies, por lo que se ha clasificado a la familia de las cactáceas como la quinta más amenazada dentro del reino vegetal, con un 31% de especies amenazadas, siendo México y Brasil los países con mayor número de especies en esta condición [7].

Se ha propuesto que algunas especies de cactáceas son de lento crecimiento y bajo índice de reproducción. Este fenómeno puede deberse al bajo índice de germinación [8, 9], a la naturaleza xenogámica de algunas especies y a que poseen flores efímeras que abren mayoritariamente durante la noche [10] o en horas y días con menor temperatura [11]. En este sentido, Martins, et al. [10] encontraron que para *Cipocereus minensis*, la mayor cantidad de néctar es producido durante la noche, por lo que la presencia de fauna nocturna (murciélagos) es determinante para lograr una mayor polinización. Este fenómeno es similar a lo reportado para *Hylocereus undatus* [12].

Estos eventos en conjunto con la presencia de depredadores pudieran limitar su crecimiento y posterior dispersión.

En México, diversas especies de cactáceas obtenidas por métodos convencionales de cultivo o colectadas de manera silvestre se han utilizado principalmente como alimento y como fuente de compuestos activos en la medicina tradicional [13]. Las propiedades funcionales que poseen las diferentes especies de cactáceas varían de acuerdo a su composición y características bioquímicas, como es la presencia de metabolitos primarios y secundarios. En esta revisión, se abordan algunos aspectos relacionados al perfil de metabolitos primarios y secundarios presentes en diferentes especies de cactáceas, haciendo mayor énfasis en el perfil de alcaloides y compuestos de naturaleza fenólica, en orden de dar una visión general sobre la naturaleza química de estas especies y su potencial de uso.

I.3. Composición y perfil de metabolitos en especies de cactáceas

I.3.1. Perfil y contenido de metabolitos primarios y minerales en especies de cactáceas

Dentro de la familia de las cactáceas, el género *Opuntia* es el más estudiado, debido a su interés alimenticio; una de las características distintivas de las cactáceas es la presencia de mucilago, el cual corresponde a uno de sus mecanismos de defensa que contribuyen a evitar la pérdida de agua y a disminuir los daños por frio [2], comprendiendo alrededor del 16-19% de peso seco de los cladodios en *Opuntia* spp. [14]. Los mucilagos obtenidos de las cactáceas son polímeros hidrofílicos de naturaleza glucoproteica [15], constituidos usualmente por galactosa, arabinosa, ramnosa y ácido glucurónico [2]. El mucilago obtenido de las especies de cactáceas en conjunto con los diferentes tejidos vegetales representan una fuente de metabolitos en la que el perfil fitoquímico y la concentración de compuestos pueden

variar en función de las condiciones ambientales en que la planta se encuentre, de su edad, de la sección de la planta donde se realice la determinación [16] y la etapa de maduración [17, 18].

Guzmán Loayza y Chávez [19] al realizar un análisis comparativo entre cladodios jóvenes (1 mes) y maduros (1 año) de *Opuntia ficus-indica,* encontraron que el contenido total de proteínas, carbohidratos y vitamina "C" fue mayor en los cladodios jóvenes, mientras que la cantidad de humedad, fibra y minerales incrementó con la edad del cultivo y el contenido de grasas permaneció constante (0.17 - 0.11%) a lo largo del desarrollo de la planta. Estos resultados son contrastantes con lo propuesto por Chaparro, et al. [18] para frutos de la misma especie, encontrando variabilidad en el análisis proximal en función de la etapa del desarrollo; lo que sugiere que cada sección de la planta posee un patrón biosintético específico o posibles mecanismos de translocación de compuestos a zonas de consumo/almacenamiento de metabolitos.

Los cladodios y frutos de las cactáceas están formados principalmente por carbohidratos y en menor proporción por proteínas. Dentro de los carbohidratos, la galactosa y la arabinosa son los monosacáridos que se encuentran en mayor concentración. En un estudio realizado por Petera, et al. [20], en el que caracterizaron la composición del mucilago obtenido de los cladodios de *Cereus triangularis*, encontraron que posee un alto contenido de galactosa y arabinosa, similar a lo reportado para el mucilago obtenido del fruto de *Opuntia ficus-indica* [21]; así mismo, para ambas especies se detectó la presencia de ramnosa, glucosa y ácidos galacturónicos (Cuadro I- 1). Por su parte, Matsuhiro, et al. [22], encontraron que el mucilago obtenido del fruto de *Opuntia ficus indica* contiene un 24.3% de ácido galacturónico y que la arabinosa, ramnosa, xilosa y galactosa fueron también

detectados. Estos resultados sugieren que dichos monosacáridos pudieran ser los componentes mayormente encontrados en diferentes especies de cactáceas.

Por su parte, Montoya-Arroyo, et al. [23] encontraron que el pericarpio del fruto de *Hylocereus* spp. (pitahaya) contiene un 81.38% de carbohidratos y un 5.85% de proteínas (Cuadro I- 1), lo que indica que este subproducto pudiera ser una fuente no convencional de dichos compuestos a un bajo costo. De la fracción de carbohidratos, los azucares identificados fueron la glucosa (4.79%), la fructosa (1.10%) y la sacarosa (<1.1%; Cuadro I- 1). Así mismo, al realizar un estudio de la fracción péctica soluble en agua, encontraron que está formada principalmente por ácido anhidrourónico (46.62%), galactosa (6.82%), ramnosa (6.47%), arabinosa (3.76%), xilosa (2.04%), glucosa (1.16%) y manosa (0.73%).

Cuadro I- 1. Contenido total de proteínas, carbohidratos y perfil de monosacáridos en diferentes especies de cactáceas.

	Especie	*Cereus triangularis* [1]	*Hylocereus* sp. [2] **	*Opuntia ficus-indica* [3]
	Parte utilizada	**Cladodios**	**Fruto (pericarpio)**	**Frutos (mucilago)**
	Unidades	**% mol**	**g/100g DW**	**% DW**
	Contenido total de proteínas	3%*	5.85	ND
	Contenido total de carbohidratos	90%*	81.38	ND
Monosacáridos - disacáridos	**Galactosa**	55.4	ND	23.5
	Arabinosa	37	ND	32.7
	Ramnosa	3.81	ND	4.2
	Glucosa	1.69	4.79	1.5
	Ac. Galacturónico	1.63	ND	14.2
	Manosa	0.47	ND	-
	Xilosa	ND	ND	4.5
	Fructosa	ND	1.1	ND
	Sacarosa	ND	<1.1	ND
	Referencia	[20]	[23]	[21]

Monosacáridos identificados por [1] GC/MS-EI; [2] GC-FID; [3] GC; * peso-peso; ** Fracción péctica soluble en agua; ND: No determinado; -: No detectado.

Los estudios relacionados al análisis de la composición proximal y contenido de minerales en diferentes especies de cactáceas son diversos, y hacen referencia mayormente a especies del género *Opuntia*. El Cuadro I- 2 indica la composición proximal de diferentes especies de cactáceas. Allí se puede observar que el contenido total de proteínas varía de una especie a otra, en un intervalo de 0.29 a 47.3 g/100 g; el de lípidos de 0.4 a 12.5 g/100 g y el de minerales de 1.08 a 39.10 g/100 g, siendo el Na, Mg, P, Ca y K los mayoritarios, mientras que el Zn, Cu, Fe, N, Mn y Cr son encontrados en menor proporción. Todos estos resultados sugieren que el contenido y perfil de metabolitos presentes en especies de cactáceas pueden variar en función de la fuente vegetal, de su edad, de las características del suelo donde la planta fue cultivada y en función de las diferentes secciones de la planta.

Cuadro I- 2. Composición proximal y contenido de minerales en diferentes especies de cactáceas.

		*Opuntia humifusa*****	*Opuntia humifusa*	*Opuntia spp*	*Opuntia dillenii****	*Opuntia ficus indica****	*Epiphylum hookeri*	*Opuntia ficus indica****
	Parte utilizada	**Fruto**	**Cladodios**	**Cladodios (mucilago)**	**Cladodios**	**Cladodios**	**Cladodios**	**Fruto**
	Unidades	**%**	**g/100 g DW**	**g/100 g FW**	**g/100 g FW**	**g/100 g FW**	**g/100 DW**	**g/100 g DW**
	Proteínas	4	47.3	6.1-7.9	0.29	0.3	7.86	1.03
	Humedad	6.1	5.5	4.5-5.9	92	94	85.63	87.07
	FDT	22.8	50.3	ND	3.73	2.7	35.54*	ND
	Lípidos	4.5	12.5	ND	ND	ND	2.95**	0.4
	Cenizas	7.6	20.12	34.9-39.1	1.23	1.08	7.23	4.03
Minerales	**Na**	158.4	0.2828	ND	0.0091	0.00171	ND	0.0187
	Mg	382.6	1.4412	ND	0.0845	0.0941	ND	0.0634
	Zn	7.5	0.0204	ND	0.000251	0.000368	ND	0.0126
	Cu	2.8	0.0022	ND	0.000093	0.000063	ND	0.00001
	Fe	10.7	0.0168	ND	0.00013	0.00013	0.037	0.0259
	Ca	601.6	1.9678	10.53-12.67	0.157	0.177	1.13	0.3165
	P	795.8	1.11	ND	0.01632	0.01638	0.19	0.00005
	N	ND	ND	0.98-1.28	ND	ND	ND	ND
	K	ND	ND	1.61-2.01	0.234	0.224	0.94	0.1088
	Mn	ND	ND	ND	0.000426	0.00078	ND	0.0378
	Cr	ND	ND	ND	0.000019	0.000027	ND	ND
	Referencia	[24]	[25]	[14]	[26]		[27]	[28]

FDT: Fibra Dietética Total; DW: peso seco; FW: Peso fresco; * Reportado como fibra cruda; ** Reportado como extracto etéreo. ***Datos de minerales uniformizados para hacer coincidir unidades. **** Datos de minerales reportados como miligramos (%).

I.3.2. Metabolitos secundarios en cactáceas

Los metabolitos secundarios son compuestos sintetizados por las plantas como mecanismo pasivo de defensa química que es inducido por las interacciones ecológicas entre la planta y su entorno (Figura I-1). Entre los metabolitos secundarios destacan los alcaloides, compuestos fenólicos, terpenos, flavonoides, cumarinas, entre otros que contribuyen a facilitar la supervivencia de las plantas [29].

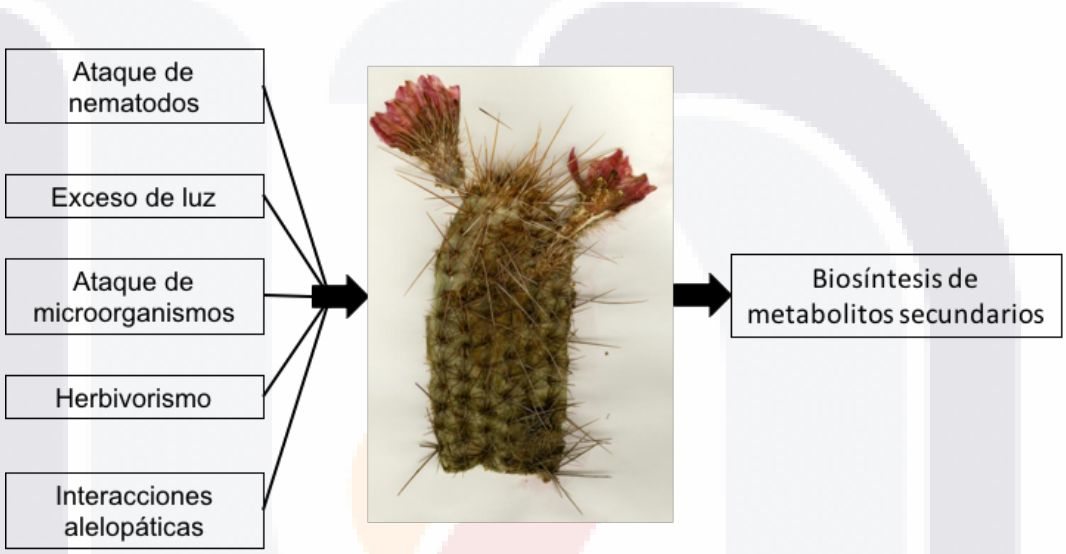


Figura I- 1. Eventos por los que se induce la biosíntesis de metabolitos secundarios. Tomado y modificado de Sepúlveda-Jiménez, et al. [30].

Dentro de la ruta de biosíntesis de algunos metabolitos secundarios intervienen enzimas cuya actividad es determinante para desencadenar los procesos biosintesintéticos, tal es el caso de la fenilalanina amonio liasa (PAL). La PAL es una enzima que cataliza la desaminación de la L-fenilalanina para formar iones de amonio y ácido cinámico, siendo esta conversión el paso intermedio entre el metabolismo primario y el metabolismo secundario de las plantas [31], dando lugar a la ruta de los fenilpropanoides, ruta mediante la cual son sintetizados diferentes metabolitos secundarios.

La actividad la PAL se ha asociado a la respuesta de las plantas ante las condiciones de estrés [32], pudiendo expresarse diferenciadamente en función del tipo de tejido analizado [32]. Se ha propuesto que la enzima PAL tiene algunas isoformas, mostrando cada una condiciones óptimas para su actividad [33]. En este sentido, la actividad de la PAL varía en función del tiempo de que dure la condición de estrés en la planta o es activada mediante el uso de elicitores. En un estudio realizado por Kundu, et al. [34], encontraron que la actividad de la PAL varía en función del tiempo de elicitación y que la producción de metabolitos de interés está directamente relacionada con la actividad de la enzima, por lo que estudiar su actividad y funcionalidad se convierte en una tarea fundamental en orden de dilucidar sus mecanismos de acción para la inducción/producción de metabolitos de con propiedades funcionales.

I.3.2.1. Alcaloides identificados en especies de cactáceas

Una de las características distintiva de diferentes especies de cactáceas es la presencia de alcaloides [35]. Los alcaloides son moléculas nitrogenadas que poseen propiedades alucinógenas y farmacológicas sobre los humanos y otros animales. Las especies de cactáceas que los contienen son comúnmente utilizadas como agentes terapéuticos y medios de diagnóstico en las practicas chamánicas y la medicina tradicional, tal es el caso de *Lophophora diffusa* (Peyote) [36] y de *Echinopsis* spp. (cactus de San Pedro) [37].

En este sentido, ha propuesto que la mescalina es el principio activo en especies del género *Echinopsis* y que la mayor concentración se encuentra en el clorenquima de la planta [37, 38]. En un estudio realizado por Ogunbodede, et al. [37] en el que evaluaron el contenido de mescalina en diferentes especies y cultivares del género

Echinopsis, encontraron que su concentración varia en un intervalo de 0.053 a 4.7 % de peso seco (Cuadro I- 3), dando una correlación directa entre las plantas más utilizadas en la medicina tradicional y su alto contenido de alcaloides; esto también sugiere que cada especie posee diferente capacidad genética para biosintetizar dicho compuesto y que la región (altitud, latitud, precipitaciones, etc.) pudiera ejercer efectos sobre su patrón de acumulación.

Cuadro I- 3. Contenido total de mescalina en especies del género *Echinopsis* (Cactaceae). Cuadro tomado y modificado de Ogunbodede, et al. [37].

Especie	Contenido total de mescalina (% en peso seco) *	Sitio de colecta u origen de las muestras
E. pachanoi	4.7	Matucama, región de lima, Perú.
E. pachanoi cv. Juul´s Giant	1.4	Planta cultivada
E. pachanoi (Long spined)	1.2	Huancabamba, Región de Piura, Perú.
E. scopulicola	0.85	Provincia de Burdet O'Connor, Bolivia.
E. pachanoi	0.82	Región del Rio Marañon, Perú.
E. pachanoi (Short spined)	0.54	Huancabamba, Piura Region, Peru.
E. lageniformis (Monstrose)	0.48	Planta cultivada
E. pachanoi complex cf. *T. pallarensis* Ritter	0.47	Departamento de Áncash, Perú.
E. pachanoi complex cf. *T. Riomizquesis* Ritter	0.40	Región Rio Mizque, Provincia de Campero, Bolivia.
E. santanensis	0.32	Provincia del Santa, Departamento de Ancash, Perú.
E. peruviana	0.24	Región de Matucana, Lima, Perú.
E. lageniformis	0.18	Planta cultivada
E. puquiensis	0.13	Carretera Nazca-Puquio, costado del cañón de Pachán, Departamento de Ayacucho, Perú.
E. uyupampensis	0.053	Planta cultivada

*Datos obtenidos por cromatografía de líquidos de alta resolución (HPLC).

Por su parte, El-Seedi, et al. [39], detectaron la presencia de mescalina (~2%) en muestras arqueológicas de *Lophophora williamsii* (peyote) localizadas en Rio Grande, Texas; encontrando mediante estudios de datación radiocarbónica que las muestras corresponden a los años 3780–3660 antes de Cristo, lo que sugiere que los habitantes nativos pudieron haberlas utilizado, reconociendo sus propiedades psicotrópicas.

En los estudios realizados por Starha [40], Starha, et al. [41], Starha, et al. [42] en el que evaluaron el perfil y contenido total de alcaloides presente en especies de los géneros *Gymnocalycium y Turbinicarpus*, encontraron que el perfil y concentración de alcaloides varía de una especie a otra y que la hordenina es el alcaloide que se encuentra en mayor concentración en la mayoría de las especies analizadas, seguido de la tiramina (Cuadro I- 4). Esto es similar a lo reportado por Follas, et al. [43], quienes al realizar un estudio con 6 especies del género *Lobivia,* (*L. allegriana, L. aurea, L. backebergii, L. binghamiana, L. huashua* y *L. pentlandii*), el cual actualmente clasificado como *Echinopsis,* y una especie del género *Pseudolobivia* (*P. Kermesina*) obtenidas de diferentes fuentes en California y Arizona, EUA., encontraron que la hordenina pudiera estar en mayor concentración para las especies del género *Lobivia* y la 3,4-dimetoxi-β-feniletilamina y tiramina para *P. kermesina*.

Por su parte, Baldizán, et al. [44], al realizar pruebas fitoquímicas preliminares, detectaron la presencia de alcaloides en *Pereskia guamacho*, especie utilizada como forraje para ganado en Venezuela. Así mismo, Baldera-Aguayo y Reyna-Pinedo [38] encontraron que la zona parenquimatosa clorofilica de *Echinopsis peruviana* posee un mayor contenido de alcaloides, comparado con el contenido de otros metabolitos como compuestos fenólicos, triterpenos, esteroles y aminas primarias y secundarias, lo que está relacionado con sus propiedades alucinógenas. Para esta especie, el alcaloide reportado en mayor concentración fue la mescalina (0.19% de peso seco),

entrando dentro del intervalo de concentración reportado por Ogunbodede, et al. [37] en especies del mismo género.

Brown, et al. [35], al evaluar la presencia de alcaloides en 16 especies de cactáceas correspondientes a diferentes géneros y colectadas en el sur de Estados Unidos (Dallas y Texas) en diferentes épocas del año, encontraron que las especies pertenecientes a los géneros *Carnegiea, Lemaireocereus, Trichocereus, Selenicereus, Echinocereus, Echinomastus, Echinocactus, Ferrocactus, Neomammiliaria* y *Coryphantha* contenían alcaloides, mientras que en *Opuntia schotii* no se detectó su presencia, pudiendo haber afectado la época y la zona de colecta, ya que se ha propuesto que diferentes especies del género *Opuntia* poseen alcaloides [45].

Estos resultados sugieren que los alcaloides están presentes en diferentes especies de cactáceas, mostrando un perfil y concentración variable en función de cada especie y de la región donde fue colectada; dando también una correlación directa con sus usos tradicionales. Esto al mismo tiempo abre un campo para realizar investigaciones encaminadas al conocimiento sobre la composición de las cactáceas y su posible aplicación como marcadores quimiotaxonómicos [46].

Cuadro I- 4. Alcaloides identificados en especies del género *Gymnocalycium* y *Turbinicarpus* (Cactaceae). Tomado y modificado de Starha [40], Starha, et al. [41] y Starha, et al. [42].

Especie/Alcaloide identificado	*G. anisitsi* Br. & R.	*G. baldianum* Speg.	*G. bayrianum* Till.	*G. boszingianum* Schutz	*G. calochlorum* lto	*G. cardenansianum* R.	*G. curvinispinum* Fric	*G. delaetii* Backbg.	*G. friedrichii* Paz.	*G. horridispinum* Frank	*G. manzanense* Backbc	*G. megalotheles* Br. & R.	*G. mihanovichii* Br. & R.	*G. fpanzii* Werd	*G. pungens* Fleischer	*G. schickendantzii* Br. & R.	*G. vatteri* Buin
Tiramina	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-metilltiramina	−	−	+	+	+	+	−	+	−	+	+	+	−	+	+	+	+
Hordenina	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mescalina	−	+	−	−	+	−	−	−	−	+	−	−	−	−	−	−	+
N-metilmescalina	+	−	+	+	+	−	+	+	−	+	−	−	−	+	−	−	+
Anhalinina	+	+	+	+	+	+	+	+	−	+	−	+	−	+	−	+	+
Analidina	+	+	−	−	+	−	−	−	−	−	−	−	−	+	−	+	+
Analamina	−	+	−	−	+	−	−	−	−	−	−	−	−	+	−	+	−
Analonidina	+	+	+	+	+	+	+	+	−	−	−	+	−	+	−	−	+
Pellotina	−	+	+	+	+	+	+	+	−	+	−	−	−	+	−	+	+
Analonina	−	+	+	+	−	−	−	−	−	−	−	−	−	+	−	+	+
Lofoforina	−	+	+	+	−	−	−	−	−	−	−	−	−	+	−	+	+
N-N dimetilmescalina	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
O-metilanalidina	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
O-metilanalonidina	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
β-fenetilamina	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Referencia	[40]																

Cuadro I-4 (Continuación). Alcaloides identificados en especies del género *Gymnocalycium* y *Turbinicarpus* (Cactaceae).

Especie/Alcaloide identificado	*Gymnocalycium albispinum* Backbg.	*G. chubutense* Speg.	*G. gibbosum* (Haw.) Pfeiff	*G. marsoneri* (Fric) lto	*G. monvillei* (Lem.) Br. & R.	*G. oenanthemum* Backbg	*G. quehlianum* (Haage) Berg.	*G. stellatum* Speng	*G. uebelmannianum* Rausch	*Turbinicarpus schmiedeckianus* (Bod) Buxb. Et. Backbg.	*T. schmiedeckianus* var. Schwarzii (Shurly) Gil. Et F.	*T. lophophoroides* (Werd.) Buxb. Et. Backbg.	*T. schmiedeckianus* var. Flaviflorus (Frank et Lau) Gi. Et F.	*T. pseudomacrochele* var. Krainzianus (Frank) Gi. Et F.	*T. pseudopectinatus* (Backbg.)	*T. schmiedeckianus* var. Dickisoniae Gi. Et F.
Tiramina	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-metilltiramina	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hordenina	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mescalina	−	−	−	−	+	+	+	+	+	−	+	+	+	+	−	−
N-metilmescalina	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Anhalinina	+	−	+	−	+	−	+	+	+	+	+	+	+	+	+	+
Analidina	−	−	+	−	+	+	−	−	+	ND	ND	ND	ND	ND	ND	ND
Analamina	−	−	+	−	+	+	−	+	+	ND	ND	ND	ND	ND	ND	ND
Analonidina	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	+
Pellotina	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	+
Analonina	+	+	+	−	+	+	+	+	+	ND	ND	ND	ND	ND	ND	ND
Lofoforina	+	+	+	−	+	+	+	+	+	ND	ND	ND	ND	ND	ND	ND
N-N dimetilmescalina	−	−	+	−	+	+	+	+	+	+	+	+	−	+	+	−
O-metilanalidina	−	+	+	−	+	+	−	−	+	+	+	+	+	+	+	+
O-metilanalonidina	+	+	+	−	+	+	+	+	+	ND	ND	ND	ND	ND	ND	ND
β-fenetilamina	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+	+	+	+	+
Referencia	[41]									[42]						

(✚) Identificado en mayor concentración; (+) Identificado; (-) No identificado; ND: no determinado.

I.3.2.2. Compuestos de naturaleza fenólica identificados en especies de cactáceas

Otra de las características de las especies de cactáceas es la presencia de compuestos fenólicos, a los cuales se les ha atribuido propiedades antioxidantes, y se han propuesto como fuente de ingredientes funcionales [47]. Dichas propiedades se ven afectadas por los procesos y solventes de extracción, siendo los solventes más polares los que presentan mejor capacidad de extractiva [48, 49]. Yahia y Mondragon-Jacobo [50] al evaluar la capacidad antioxidante de 10 variedades de *Opuntia* spp., realizando extracciones hidrofílicas y lipofílicas, encontraron que los extractos polares presentaron la mayor actividad, mientras que en los apolares la capacidad antioxidante fue baja.

Se ha demostrado que los compuestos de naturaleza fenólica ocurren comúnmente en forma glucosilada. En este sentido, De Leo, et al. [51] al analizar el perfil y contenido de flavonoides en las flores de *Opuntia ficus-indica* encontraron que la isoramnetina 3-O-robinobiosido fue el flavonoide que se encontró en mayor concentración (42.69 mg/g), seguido de la isoramnetina 3-O-galactosido (9.79 mg/g) y la quercetina 3-O-rutinosido (7.09 mg/g). Estos resultados son similares a lo propuesto por Mata, et al. [52] para jugos preparados con frutos *Opuntia ficus-indica*, quienes detectaron la presencia de diferentes grupos de compuestos fenólicos en su forma glucosilada y para los subproductos obtenidos del xoconostle (*Opuntia joconostle*), en donde los compuestos mayoritarios corresponden a derivados glucosilados de la isoramnetina [53]. Estos resultados coinciden con lo reportado para semillas de *O. Ficus-indica*, en donde los derivados glucosilados del ácido ferúlico fueron los que se encontraron en mayor concentración [54].

Jiménez-Aspee, et al. [13] al evaluar la presencia de compuestos de naturaleza fenólica en frutos obtenidos de *Eulychinia acida,* encontraron la presencia de 6

compuestos principales, siendo la isoramnetina en forma glucosilada la que se detectó en mayor concentración. La isoramnetina también fue detectada en extractos de *Opuntia monacantha,* además del kaempferol [55]. Por su parte, Guevara-Figueroa, et al. [56] al analizar diferentes variedades silvestres y comerciales de *Opuntia* spp., así como 2 productos procesados (tabletas), encontraron a los ácidos ferúlico y salicílico en todas las muestras analizadas, así como la presencia de los ácidos gálico, coumarico, 3,4 dihidroxibenzoico y 4 hidroxibenzoico, en algunas muestras. Para el caso de los flavonoides, se encontró la presencia de nicotiflorina, rutina y narcisina, así como la isoquercetina, isoramnetina en sus formas glucosiladas.

Todos estos resultados sugieren que el perfil y concentración de compuestos varían en función de la especie analizada [57] y pueden verse afectados por los procesos maduración [58], por efecto de las condiciones y tiempo de almacenamiento [59] o en función a las diferentes secciones de la planta, tal es el caso de *Opuntia humifusa* [24, 25]. Así mismo, sugieren que los derivados glucosilados de la isoramnetina se encuentran en diferentes especies de cactáceas, así como diferentes ácidos fenólicos. La forma glucosilada de cada metabolito confiere mayor polaridad, facilitando su movilidad dentro de la célula y pudiendo estar relacionado a sus propiedades funcionales [60]. Por lo anterior, es interesante realizar más investigaciones en orden de profundizar en el conocimiento de la composición de las diferentes especies de cactáceas. En el Cuadro I- 5 se indican algunos compuestos de naturaleza fenólica identificados para diferentes especies de cactáceas.

Cuadro I- 5. Compuestos de naturaleza fenólica identificados en algunas especies de cactáceas.

Especie	*Opuntia humifusa*	*Opuntia humifusa*	*Opuntia Dillenii**	*Opuntia ficus indica**	*Hylocereus polyrhizus*	*Hylocereus undatus*	*Opuntia spp.***	*Opuntia spp.****
Parte utilizada	Fruto	Cladodios	Cladodios	Cladodios	Semillas	Semillas	Semillas	Semillas
Técnica de separación-cuantificación	HPLC-DAD	HPLC-DAD	Folin-Ciocalteu	Folin-Ciocalteu	HPLC	HPLC	RP-HPLC	RP-HPLC
Unidades	mg/g				mg/100g	mg/100g	%	%
Ácido gálico	0.14	0.15	ND	ND	0.25	0.20	4.36	0.85
Ácido protocatecuico	1.79	2.6	ND	ND	0.93	0.93	ND	ND
Ácido clorogénico	0.38	0.37	ND	ND	ND	ND	Nd	1.2
Ácido *p*-hidroxibenzoico	0.24	4.38	ND	ND	0.66	0.72	ND	ND
Ácido siríngico	0.32	0.66	ND	ND	0.08	0.21	6	8.4
Ácido isovanilico	0.4	0.71	ND	ND	ND	ND	ND	ND
Ácido *p*-coumarico	1.53	0.61	ND	ND	0.78	0.79	Nd	2.15
Ácido ferúlico	3.55	2.22	ND	ND	ND	ND	ND	ND
Ácido trans-m-coumarico	0.2	0.2	ND	ND	ND	ND	ND	ND
Ácido salicílico	0.17	0.22	ND	ND	ND	ND	ND	ND
Ácido cinámico	ND	ND	ND	ND	ND	ND	27.7	0.6
Ácido trans-*m*-cinamico	0.04	0.01	ND	ND	ND	ND	ND	ND
Ácido vanilico	ND	ND	ND	ND	0.64	0.70	Nd	6.3
Ácido cafeico	ND	ND	ND	ND	0.08	0.71	2.1	0.9
Total (mg/g)	8.78	12.13	0.16	1.28	ND	ND	ND	ND
Referencia	[24]	[25]	*[26]*	*[26]*	[61]	[61]	[58]	[58]

HPLC-DAD: Cromatografía liquida de alta resolución, acoplado a un arreglo de diodos. RP-HPLC: Cromatografía liquida de alta resolución en fase reversa. * Datos transformados para hacer coincidir unidades; Nd: No detectado; ND: No determinado; ** obtenido por hidrólisis en semillas de fruto maduro; *** obtenido por maceración en semillas de fruto maduro.

I.4. Referencias

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CAPÍTULO II

Phytochemical profiling of *Coryphantha macromeris* (Cactaceae) growing in greenhouse conditions using ultra-high-performance liquid chromatography–tandem mass spectrometry [1]

II.1. Abstract

Chromatographic separation combined with mass spectrometry is a powerful tool for the characterization of plant metabolites because of its high sensitivity and selectivity. In this work, the phytochemical profile of aerial and radicular parts of *Coryphantha macromeris* (Engelm.) Britton & Rose growing under greenhouse conditions was qualitatively investigated for the first time by means of modern ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS). The UHPLC-PDA-HESI-Orbitrap-MS/MS analysis indicated a high complexity in phenolic metabolites. In our investigation, 69 compounds were detected and 60 of them were identified. Among detected compounds, several phenolic acids, phenolic glycosides, and organic acids were found. Within this diversity, 26 metabolites were exclusively detected in the aerial part, and 19 in the roots. Twenty-four metabolites occurred in both plant parts. According to the relative abundance of peaks in the chromatogram, ferulic and piscidic acids and their derivatives may correspond to one of the main phenolic compounds of *C. macromeris*. Our results contribute to the phytochemical knowledge regarding *C. macromeris* and its potential applications in the pharmaceutical and cosmetic industries. Besides, some metabolites and their fragmentation patterns are reported here for the first time for cacti species.

[1]Artículo publicado. Cabañas-García, E.; Areche, C.; Jáuregui-Rincón, J.; Cruz-Sosa, F.; Pérez-Molphe Balch, E., Phytochemical profiling of *Coryphantha macromeris* (Cactaceae) growing in greenhouse conditions using ultra-high-performance liquid chromatography–tandem mass spectrometry. *Molecules* **2019,** 24, (4), 705.

II.2. Introduction

The family Cactaceae is one of the most threatened within the plant kingdom [1], and is the most important plant family of the arid and semiarid regions of America [2], comprising around 1600 species [3]. Cacti are succulent plants which have been used for their functional properties and industrial utility [4-6]. Different cacti species obtained by cultivated or wild-collected methods have been used as food sources and fodder, for ornamental purposes, and for medicinal purposes due to their antioxidant [7], antimutagenic [8], and bactericidal [9] properties, and have been cataloged as a source of bioactive compounds including alkaloids [10, 11] and phenolics [12, 13].

Plants contain different classes of metabolites found in different concentrations and of a wide structural diversity. Their phytochemical profiles and functional properties may vary according to: a) the solvent and extraction processes [14, 15], b) the stage of growth [16], c) storage conditions [17], and d) the specific section of the plant [18, 19]. This complexity makes it difficult to identify plant metabolites through a single analytical method or under a defined separation system [20]. Several techniques have been proposed to achieve the elucidation of the plant metabolome, including the coupling of mass spectrometry with different separation techniques [21, 22]. The knowledge of natural product chemistry is important since identified metabolites may serve as the basis for the discovery or design of novel compounds with biological activities [23] and as chemotaxonomic markers for plant classification [24].

The genus *Coryphantha* is highly endemic, distributed in the arid and semiarid regions located between the Sierra Madre Oriental and the Sierra Madre Occidental in northern Mexico, expanding to the southern regions of Arizona, Texas, and New Mexico, USA [25]. Currently, natural populations of *Coryphantha* species have been reduced due to overexploitation and destruction of habitats.

Coryphantha spp. are slow growing plants and the environmental conditions of semiarid regions difficult their germination, growth, and reproduction; for this reason, biotechnological approaches have been proposed for the *in vitro* propagation of *Coryphantha* spp. and other cacti species [26, 27]. *Coryphantha macromeris* (Engelm.) Britton & Rose is a globose cactus traditionally known as "Dona Ana", "long mamma cory-cactus", "big needle cactus", and "biznaga partida-partida"; it is mainly distributed in northern Mexico and southern United States. In Mexico, *C. macromeris* is used in folk medicine for healing stomach disorders; nevertheless, hallucinogenic properties have been proposed, due to the presence of alkaloids such as macromerine [28] and other β-phenethylamine derivatives [29]. More recently, Kikuchi, et al. [30] evaluated the chemical constituents of a psychotropic herbal product and, using DNA sequence analysis, suggested that *C. macromeris* could be one of its components, as the *rpl16* intron sequence of the herbal product showed 99% of similarity with that of *C. macromeris.* Information regarding the phytochemical composition and bioactive compounds in *C. macromeris* is scarce. Thus, the aim of this study was to identify, using UHPLC coupled with tandem mass spectrometry, the main secondary metabolites present in shoots and roots of *Coryphantha macromeris* growing under greenhouse conditions in order to contribute to the phytochemical knowledge of this species and its potential applications.

II.3. Materials and Methods

II.3.1. Plant Material

The plants of *Coryphantha macromeris* were obtained from the *in vitro* germplasm bank of the Autonomous University of Aguascalientes, México. *Coryphantha macromeris* was maintained on semisolid Murashige and Skoog medium [31] (3% sucrose, 8 g L^{-1} agar, and pH 5.7) and incubated at 25 °C with fluorescent light (40 μmol m^2 s^{-1}) and 16/8 (light/dark) photoperiod. Each plant was subcultured

for three months during one year with the described conditions, and then *in vitro* plants were acclimated to greenhouse conditions as reported previously [26] and kept for one year until harvesting for phytochemical analysis. The plant material was botanically identified by Professor Miguel Alvarado Rodríguez. A voucher specimen was deposited at the herbarium of the Autonomous University of Aguascalientes (HUAA; Voucher No. 6386).

II.3.2. Sample Preparation

For analysis, one-year-old plants growing under greenhouse conditions were collected and separated into aerial and radicular parts. Each part of the plant was sliced and dried in an oven (40 °C) during 1 week in dark conditions. Dried material was finally pulverized in a mortar and then extracted three times with methanol in an ultrasonic bath (30 min each time). The resultant extract was filtered and evaporated under reduced pressure at 40 °C and freeze-dried (Labconco Freeze Dryer; Labconco Corporation, Kansas City, MO, USAEach freeze-dried 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 in an UHPLC system hyphenated with a mass spectrometer, as given below.

II.3.3. UHPLC-PDA-HESI-Orbitrap-MS/MS Conditions

Phytochemical analysis was performed as reported previously [32-34], 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) and equipped with a Quaternary Series RS pump and a Dionex Ultimate 3000 Series TCC-3000RS column compartments with 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, and PDA was recorded from 200 to 800 nm for peak characterization. The separation was performed in a gradient elution mode composed by 1% formic aqueous solution (A) and acetonitrile (B). The flow rate was 1.0 $mL \cdot min^{-1}$ 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 Chromeleon 7.2 Software (Thermo Fisher Scientific, Waltham, MA, USA and Dionex Softron GmbH division of Thermo Fisher Scientific) and hyphenated with 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 and 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. XCalibur 2.3 software and Trace Finder 3.2 (Thermo Fisher Scientific, San Jose, CA, USA) 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.

II.3.3.1. 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 width 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 30s 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 the 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 105 to below 1010 mbar, respectively. Collision energy (HCD cell) was operated at 30 eV. Detection was based on calculated exact mass and on retention time of target compounds presented in Table II- 1. The mass tolerance window was set to 5 ppm.

II.4. Results and Discussion

Methanolic extracts prepared with aerial and radicular parts of *C. macromeris* cultivated under greenhouse conditions were analyzed by ultra-high-performance liquid chromatography–tandem mass spectrometry and a photodiode array detector (UHPLC-PDA-HESI-Orbitrap-MS/MS). This

technique has been used for the characterization of metabolites in complex mixtures [35], and the chromatographic conditions used in this work have shown efficiency for the separation and identification of different secondary metabolites [32-34]. For *C. macromeris* extracts, UHPLC conditions allowed a good separation of many sample components.

The phytochemical characterization of *C. macromeris* was achieved by comparing the obtained information by UHPLC-PDA-HESI-Orbitrap-MS/MS with spectroscopic evidences existing in the literature or by structure searching and studying the fragmentation pattern of molecules. Additionally, retention time and UV (λmax) spectra were used for peak characterization. As far as we know, metabolite profiling of *C. macromeris* is reported for the first time in this work. Elution profile (Figure II- 1) and mass spectra obtained in negative ion mode indicated a highly complex phenolic composition. Peak characteristics (i.e., retention time, theoretical and measured mass, UV (λmax), and fragmentation pattern) and tentative identification of each compound are summarized in Table II- 1. All detected compounds exhibited an accuracy smaller than 5 ppm. Under the proposed UHPLC-PDA-HESI-Orbitrap-MS/MS method, 69 compounds were separated, and 60 of them were tentatively identified in *C. macromeris* greenhouse plants (Table II- 1). Within this complexity, 26 compounds occurred in the aerial part, 19 in occurred in the roots, and 24 occurred in both plant parts, suggesting a translocation mechanism from actively photosynthetic tissues to metabolite consuming/storage areas.

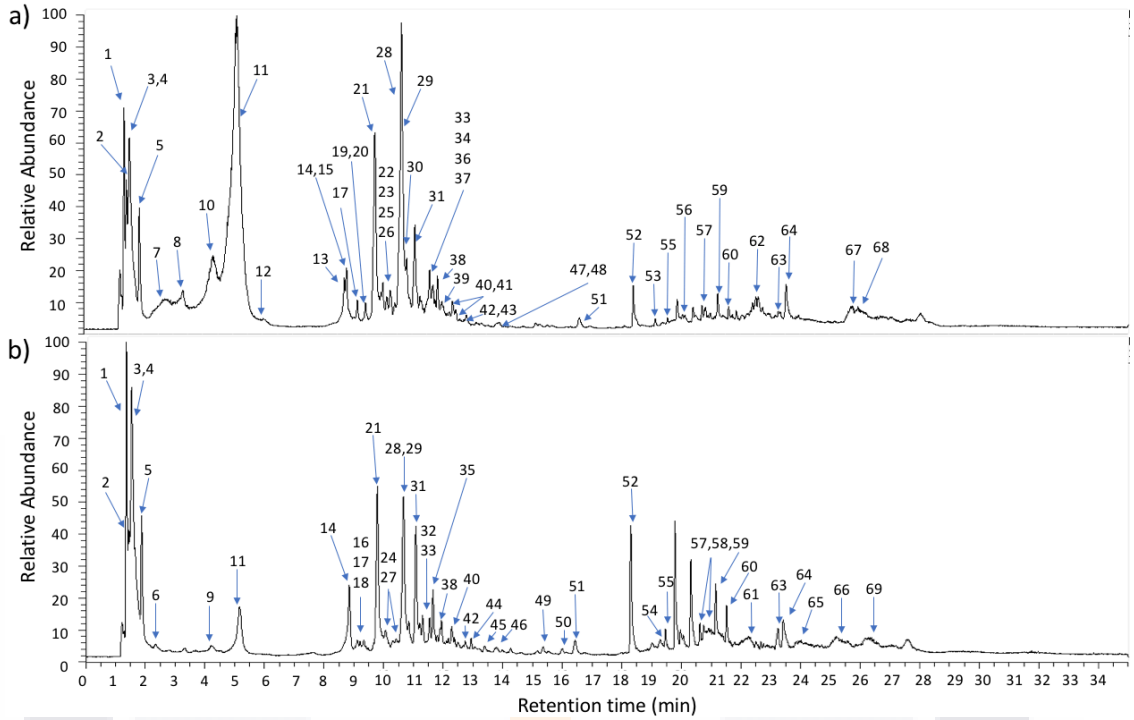

Figure II- 1. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of *Coryphantha macromeris* methanolic extracts prepared with aerial (a) and radicular (b) parts. Peak numbers refer to the metabolites indicated in Table II- 1; repeated numbers in (a) or (b) indicate that the metabolite is found in both plant parts.

Table II- 1. Metabolites identified in aerial and radicular parts of *Coryphantha macromeris* by ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS) data.

Peak	Retention time (min)	UV max (λ_{max})	Tentative identification	Elemental composition [M–H]⁻	Theoretical mass (*m*/*z*)	Measured mass (*m*/*z*)	Accuracy (ppm)	MSn ions	Plant part
1	1.37	220, 272	Vaccihein A	$C_{18}H_{17}O_9^-$	377.08781	377.08600	4.80	361.09076 ([M-H-OH]⁻) 347.07724 ([M-H-OCH_3]⁻) 319.07990 ([M-H-$C_2H_3O_2$]⁻) 289.06958 ([M-H-$C_2H_3O_2$-OCH_3]⁻) 125.02375 ([M-H-$C_8H_9O_4$-$C_3H_5O_2$]⁻)	Root/shoot
2	1.45	220, 272	Dihydroxy methoxy butanoic acid	$C_5H_9O_5^-$	149.04555	149.04498	3.82	135.02925 ([M-H-CH_3]⁻) 131.03433 ([M-H-OH]⁻) 119.03425 ([M-H-CH_3-OH]⁻) 103.03932 ([M-H-OCH_3-OH]⁻)	Root/shoot
3	1.52	220, 274	2-Hydroxy-succinic acid (malic acid)	$C_4H_5O_5^-$	133.01390	133.01363	2.03	115.00297 ([M-H-OH]⁻)	Root/shoot
4	1.55	224, 277	Iso citric acid	$C_6H_7O_7^-$	191.01973	191.01938	1.83	111.00790 ([M-H-CO_2-2OH]⁻)	Root/shoot
5	1.87	277	Iso citric acid isomer	$C_6H_7O_7^-$	191.01973	191.01938	1.83	111.00790 ([M-H-CO_2-2OH]⁻)	Root/shoot
6	2.35	220, 277	3,5-Dihydroxy-4-methyloxolan-2-yl methoxy-6-hydroxymethyl oxane-3,4,5-triol	$C_{12}H_{21}O_9^-$	309.11911	309.11935	0.78	293.12424 ([M-H-OH]⁻) 279.10870 ([M-H-OH-CH_3]⁻) 147.06578 ([M-H-$C_6H_{11}O_5$]⁻) 131.07106 ([M-H-$C_6H_{11}O_5$-OH]⁻)	Root
7	2.74	224, 277	Piscidic acid isomer	$C_{11}H_{11}O_7^-$	255.05103	255.05098	0.20	193.05013 ([M-H-CHO_2-OH]⁻) 165.05521 ([M-H-$C_2H_2O_3$-OH]⁻) 135.04442 ([M-H-$C_2H_2O_3$-CHO_2]⁻) 119.04952 ([M-H-$C_2H_2O_3$-CH_2-OH]⁻) 107.04927 ([M-H-$C_4H_4O_3$]⁻)	Shoot
8	3.34	228, 277	Piscidic acid isomer	$C_{11}H_{11}O_7^-$	255.05103	255.05098	0.20	193.05013 ([M-H-CHO_2-OH]⁻) 165.05521 ([M-H-$C_2H_2O_3$-OH]⁻) 135.04442 ([M-H-$C_2H_2O_3$-CHO_2]⁻) 119.04952 ([M-H-$C_2H_2O_3$-CH_2-OH]⁻) 107.04927 ([M-H-$C_4H_4O_3$]⁻)	Shoot
9	4.22	197, 223, 278	Protocatechuic acid hesoxide	$C_{13}H_{15}O_9^-$	315.07230	315.07239	0.29	255.05104 ([M-H-$C_2H_5O_2$]⁻) 211.06094 ([M-H-$C_2H_5O_2$-CHO_2]⁻) 153.01878 ([M-H-$C_6H_{11}O_5$]⁻) 137.02385 ([M-H-$C_6H_{11}O_5$-OH]⁻) 121.02895 ([M-H-$C_6H_{11}O_5$-2OH]⁻) 109.02863 ([M-H-$C_6H_{11}O_5$-CHO_2]⁻)	Root
10	4.34	223, 276	Piscidic acid isomer	$C_{11}H_{11}O_7^-$	255.05103	255.05098	0.20	193.05013 ([M-H-CHO_2-OH]⁻)	Shoot

								165.05521([M-H-$C_2H_2O_3$-OH]$^-$) 135.04442 ([M-H-$C_2H_2O_3$-CHO_2]$^-$) 119.04952 ([M-H-$C_2H_2O_3$-CH_2-OH]$^-$) 107.04927 ([M-H-$C_4H_4O_3$]$^-$)	
11	5.13	222, 275	Piscidic acid isomer	$C_{11}H_{11}O_7^-$	255.05103	255.05095	0.31	193.05013 ([M-H-CHO_2-OH]$^-$) 165.05521 ([M-H-$C_2H_2O_3$-OH]$^-$) 135.04442 ([M-H-$C_2H_2O_3$-CHO_2]$^-$) 119.04952 ([M-H-$C_2H_2O_3$-CH_2-OH]$^-$) 107.04927 ([M-H-$C_4H_4O_3$]$^-$)	Root/shoot
12	6.02	222, 275	Piscidic acid isomer	$C_{11}H_{11}O_7^-$	255.05103	255.05101	0.08	193.05013 ([M-H-CHO_2-OH]$^-$) 165.05521([M-H-$C_2H_2O_3$-OH]$^-$) 135.04442 ([M-H-$C_2H_2O_3$-CHO_2]$^-$) 119.04952 ([M-H-$C_2H_2O_3$-CH_2-OH]$^-$) 107.04927 ([M-H-$C_4H_4O_3$]$^-$)	Shoot
13	8.74	222, 277	Lucuminic acid	$C_{19}H_{25}O_{12}^-$	445.13515	445.13531	0.36	163.03947 ([M-H-$C_{10}H_{19}O_8$-OH]$^-$) 119.04939 ([M-H-$C_{11}H_{19}O_{10}$-OH]$^-$) 107.04942 ([M-H-$C_{11}H_{19}O_9$-CHO_2]$^-$)	Shoot
14	8.85	223, 276	Hyrtioerectine C	$C_{11}H_{12}NO_4^-$	222.07718	222.07703	0.68	206.08206 ([M-H-OH]$^-$) 198.07718 ([M-H-C_2H_3]$^-$) 180.06580 ([M-H-C_2H_3-OH]$^-$) 178.08685 ([M-H-CHO_2]$^-$)	Root/shoot
15	8.93	227, 283	Piscidic acid isomer	$C_{11}H_{11}O_7^-$	255.05103	255.05104	0.04	193.05013 ([M-H-CHO_2-OH]$^-$) 165.05521([M-H-$C_2H_2O_3$-OH]$^-$) 135.04442 ([M-H-$C_2H_2O_3$-CHO_2]$^-$) 119.04952 ([M-H-$C_2H_2O_3$-CH_2-OH]$^-$) 107.04927 ([M-H-$C_4H_4O_3$]$^-$)	Shoot
16	9.12	255, 207	Protocatechuic aldehyde	$C_7H_5O_3^-$	137.02442	137.02386	4.09	121.02882 ([M-H-OH]$^-$) 109.02884 ([M-H-COH]$^-$)	Root
17	9.19	230, 286	Piscidic acid derivative	$C_{21}H_{27}O_{13}^-$	-	487.14600	-	255.05110 ([piscidic acid]$^-$) 193.05078 ([piscidic acid-CHO_2-OH]$^-$) 165.05516 ([piscidic acid-$C_2H_2O_3$-OH]$^-$ 135.04453 ([piscidic acid-$C_2H_2O_3$-CHO_2]$^-$) 107.04935 ([piscidic acid-$C_4H_4O_3$]$^-$)	Root/shoot
18	9.41	223, 278	Piscidic acid derivative	$C_{20}H_{27}O_{13}^-$	-	475.14606	-	255.05112 ([piscidic acid]$^-$) 193.05037 ([piscidic acid-CHO_2-OH]$^-$) 165.05513([piscidic acid-$C_2H_2O_3$-OH]$^-$) 135.04453 ([piscidic acid-$C_2H_2O_3$-CHO_2]$^-$) 107.04923 ([piscidic acid-$C_4H_4O_3$]$^-$)	Root
19	9.45	231, 295	Sinapic acid derivative	$C_{22}H_{29}O_{14}^-$	-	517.15649	-	223.06104 ([sinapic acid]$^-$) 208.03767 ([sinapic acid-CH_3]$^-$) 179.07083 ([sinapic acid- CHO_2]$^-$)	Shoot

								164.04738 ([sinapic acid-CHO_2-$OH]^-$)	
20	9.50	231, 295	Sinapic acid	$C_{11}H_{11}O_5^-$	223.06070	223.06102	1.43	208.03757 ([M-2H-$CH_3]^-$) 179.07094 ([M-H-$CHO_2]^-$) 164.04730 ([M-2H-CHO_2-$OH]^-$)	Shoot
21	9.77	224, 276	Syringic acid acetate	$C_{11}H_{11}O_6^-$	239.05611	239.05594	0.71	197.04517 ([syringic acid]$^-$) 195.06580 ([M-H-$CHO_2]^-$) 179.03439 ([M-H-2$CH_3O]^-$) 149.06023 [M-H-2CH_3O-OH]$^-$) 135.04456 ([M-H-CHO_2-2$CH_3O]^-$) 107.04944 ([M-H-CHO_2-CH_3O-$C_2H_3O_2]^-$)	Root/shoot
22	9.97	231, 286	Piscidic acid derivative	$C_{21}H_{27}O_{13}^-$	-	487.14603	-	255.05101 ([piscidic acid]$^-$) 193.05025 ([piscidic acid-CHO_2-$OH]^-$) 165.05528 ([piscidic acid-$C_2H_2O_3$-$OH]^-$) 135.04456 ([piscidic acid-$C_2H_2O_3$-$CHO_2]^-$) 107.04945 ([piscidic acid-$C_4H_4O_3]^-$)	Shoot
23	10.04	233, 283	Syringic acid acetate derivative	$C_{19}H_{31}O_8^-$	-	387.20276	-	239.05594 ([syringic acid acetate]$^-$) 197.04517 ([syringic acid]$^-$) 195.06580 ([syringic acid acetate-$CHO_2]^-$) 179.03439 ([syringic acid acetate-2$CH_3O]^-$) 149.06023 ([syringic acid acetate-2CH_3O-OH]$^-$) 135.04456 ([syringic acid acetate-CHO_2-2$CH_3O]^-$) 107.04944 ([syringic acid acetate-CHO_2-CH_3O-$C_2H_3O_2]^-$)	Shoot
24	10.08	231, 283	Caffeic acid	$C_9H_7O_4^-$	179.03498	179.03477	1.17	163.03950 ([M-H-OH]$^-$) 135.04510 ([M-H-$CHO_2]^-$) 109.02870 ([M-H-$C_3H_3O_2]^-$)	Root
25	10.17	231, 286	Sinapic acid hexoside	$C_{17}H_{21}O_{10}^-$	385.11402	385.11438	0.93	223.06099 ([M-H-$C_6H_{11}O_5]^-$) 208.03757 ([sinapic acid-$CH_3]^-$) 179.07095 ([sinapic acid-$CHO_2]^-$) 164.04745 ([sinapic acid-CHO_2-$OH]^-$)	Shoot
26	10.27	234, 283	Propanedioic acid, [5-[[2-[(6-deoxy- α-L-galactopyranosyl) oxy] cyclohexyl] oxy]-Pentyl]	$C_{20}H_{33}O_{10}^-$	433.20792	433.20825	0.76	417.21347 ([M-H-OH]$^-$) 387.20309 ([M-H-$CHO_2]^-$) 287.15030 ([M-H-$C_6H_{11}O_4]^-$) 245.13950 ([M-H-$C_8H_{13}O_5]^-$) 131.07069 ([M-H-$C_{14}H_{23}O_6]^-$)	Shoot
27	10.31	237, 283	Cyclohexanecarboxylic acid, 3-[(6-deoxy-3-*O*-methyl-D-galactopyranosyl)oxy]-1,4,5-trihydroxy	$C_{14}H_{23}O_{10}^-$	351.12967	351.13010	1.22	303.14487 ([M-H-3OH]$^-$) 287.14999 ([M-H-4OH]$^-$) 273.13449 ([M-H-3OH-$CH_3O]^-$)	Root
28	10.63	235, 326	Ferulic acid	$C_{10}H_9O_4^-$	193.05063	193.05032	1.61	179.03455 ([M-H-$CH_3]^-$) 149.06050 ([M-H-$CHO_2]^-$) 163.03963 ([M-H-CH_3-$OH]^-$)	Root/shoot

								147.04456 ([M-H-CH_3-2OH]$^-$)	
29	10.69	235, 327	Ferulic acid derivative (fertaric acid)	$C_{14}H_{13}O_9^-$	325.05651	325.05664	0.40	193.05032 ([ferulic acid]$^-$) 179.03453 ([ferulic acid-CH_3]$^-$) 163.03954 ([ferulic acid-CH_3-OH]$^-$)	Root/shoot
30	10.84	237, 291	7,8,11-Trihydroxyguai-4-en- 3-one 8-*O*-β-D-glucopyranoside	$C_{21}H_{33}O_9^-$	429.21250	429.21335	1.98	267.16003 ([M-H-$C_6H_{11}O_5$]$^-$) 249.14989 ([M-H-$C_6H_{11}O_5$-OH]$^-$)	Shoot
31	11.09	235, 286	Cinnamic acid derivative	$C_8H_{14}O_6^-$	-	206.08205	-	147.04449 ([cinnamic acid]$^-$) 103.05447 ([cinnamic acid-CHO_2]$^-$)	Root/shoot
32	11.22	227, 283	2-Propenoic acid, 2-methyl-, 4-[2-(2,4-dioxo-1,5-dioxaspiro [5.5]undec-3-yl)ethenyl]-6-(2,4-dioxo-1,5-dioxaspiro[5.5]undec-3-ylidene)-4-hexenyl ester	$C_{30}H_{34}O_{10}^-$	554.21629	554.21448	3.27	193.05049 ([M-H-$C_{20}H_{25}O_6$]$^-$)	Root
33	11.54	239, 289, 323	Ferulic acid isomer	$C_{10}H_9O_4^-$	193.05063	193.05048	0.78	179.03458 ([M-H-CH_3]$^-$) 149.06030 ([M-H-CHO_2]$^-$) 163.03954 ([M-H-CH_3-OH]$^-$)	Root/shoot
34	11.61	238, 292, 323	Ferulic acid isomer	$C_{10}H_9O_4^-$	193.05063	193.05038	1.29	179.03441 ([M-H-CH_3]$^-$) 149.06026 ([M-H-CHO_2]$^-$) 163.03937 ([M-H-CH_3-OH]$^-$) 147.04457 ([M-H-CH_3-2OH]$^-$)	Shoot
35	11.66	226, 282	Ferulic acid derivative I	$C_{24}H_{24}O_5^-$	-	392.16193	-	193.05038 ([ferulic acid]$^-$) 149.06030 ([ferulic acid-CHO_2]$^-$) 163.03954 ([ferulic acid-CH_3-OH]$^-$)	Root
36	11.71	236, 286	Ferulic acid isomer	$C_{10}H_9O_4^-$	193.05063	193.05038	1.29	179.03452 ([M-H-CH_3]$^-$) 149.06024 ([M-H-CHO_2]$^-$) 163.03937 ([M-H-CH_3-OH]$^-$) 147.04440 ([M-H-CH_3-2OH]$^-$)	Shoot
37	11.79	235, 286, 380	2-Isoferulic piscidic acid-1-metyl ester	$C_{22}H_{21}O_{10}^-$	445.11400	445.11438	0.85	255.05092 ([piscidic acid]$^-$) 193.050380 ([ferulic acid]$^-$) 165.05516 ([piscidic acid-$C_2H_2O_3$-OH]$^-$) 135.04440 ([piscidic acid-$C_2H_2O_3$-CHO_2]$^-$) 107.04936 ([piscidic acid-$C_4H_4O_3$]$^-$)	Shoot
38	11.86	240, 296, 381	Ferulic acid derivative II	$C_{20}H_{29}O_{10}^-$	-	429.17707	-	193.05029 ([ferulic acid]$^-$) 179.03450 ([ferulic acid-CH_3]$^-$) 163.03937 ([ferulic acid-CH_3-OH]$^-$) 147.04440 ([ferulic acid-CH_3-2OH]$^-$)	Root/shoot
39	12.18	283, 368	Ferulic acid derivative III	$C_{21}H_{31}O_{13}^-$	-	491.17731	-	193.05026 ([ferulic acid]$^-$) 179.03456 ([ferulic acid-CH_3]$^-$) 163.03929 ([ferulic acid-CH_3-OH]$^-$) 147.04446 ([ferulic acid-CH_3-2OH]$^-$)	Shoot
40	12.38	283	Azelaic acid	$C_9H_{15}O_4^-$	187.09758	187.09740	0.96	169.08130 ([M-H-OH]$^-$)	Root/shoot

								125.09650 ([M-H-CHO_2-OH]$^-$)	
41	12.47	284, 368	Ferulic acid derivative IV	$C_{20}H_{29}O_{10}^-$	-	429.17706	-	193.05023 ([ferulic acid]$^-$) 179.03427 ([ferulic acid-CH_3]$^-$) 163.03958 ([ferulic acid-CH_3-OH]$^-$) 147.04450 ([ferulic acid-CH_3-2OH]$^-$)	Shoot
42	12.70	259	2-Phenylethyl β-D-glucopyranoside	$C_{14}H_{19}O_6^-$	283.11871	283.11893	0.78	267.12402 ([M-H-OH]$^-$) 251.12881([M-H-2OH]$^-$) 235.13390 ([M-H-3OH]$^-$) 121.06506([M-H-$C_6H_{11}O_5$]$^-$)	Root/shoot
43	12.75	272, 368	Dalbergioidin	$C_{15}H_{11}O_6^-$	287.05611	287.05618	0.24	271.06094 ([M-H-OH]$^-$) 179.03467 ([M-H-$C_6H_5O_2$]$^-$) 165.05252 ([M-H-$C_6H_5O_3$]$^-$) 163.03951 ([M-H-$C_6H_5O_2$-OH]$^-$) 147.04404 ([M-H-$C_6H_5O_2$-2OH]$^-$) 125.02380 ([M-H-$C_9H_9O_3$]$^-$) 109.02868 ([M-H-$C_9H_7O_4$]$^-$)	Shoot
44	12.95	260	4,8,12-trihydroxy-2,4-dodecadienoic acid,	$C_{12}H_{19}O_5^-$	243.1238	243.12383	0.12	199.1337 ([M-H-CHO_2]$^-$) 139.11221 ([M-H-CHO_2-C_2H_3-2OH]$^-$)	Root
45	13.40	283	Caffeic acid isomer	$C_9H_7O_4^-$	179.03498	179.03481	0.95	163.03954 ([M-H-OH]$^-$) 135.04454 ([M-H-CHO_2]$^-$) 109.02881([M-H-$C_3H_3O_2$]$^-$)	Root
46	13.80	222, 284	β-D-Glucopyranoside, 1,1-dimethyl-5-methylenenonyl	$C_{10}H_{17}O_4^-$	345.22826	345.22849	0.67	327.21780 ([M-H-OH]$^-$) 315.21780 ([M-H-CH_3O]$^-$)	Root
47	13.90	224, 284	Sebacic acid	$C_{10}H_{17}O_4^-$	201.11323	201.11293	1.49	185.11778 ([M-H-OH]$^-$) 157.12276 ([M-H-CHO_2]$^-$)	Shoot
48	13.93	223, 284	alpha-Ionol O-[arabinosyl-(1->6)-glucoside]	$C_{24}H_{39}O_{10}^-$	487.25487	487.25504	0.35	473.24008 ([M-H-CH_3]$^-$) 459.22311 ([M-H-2CH_3]$^-$) 355.21292 ([M-H-$C_5H_9O_4$]$^-$) 341.19687 ([M-H-$C_5H_9O_4$-CH_3]$^-$)	Shoot
49	15.35	283, 368,	Buteine	$C_{15}H_{11}O_5^-$	271.0612	271.06131	0.41	163.03952 ([M-H-$C_6H_5O_2$]$^-$) 137.02380 ([M-H-$C_8H_7O_2$]$^-$ 135.04443 ([M-H-$C_7H_5O_3$]$^-$) 121.02880 ([M-H-$C_8H_7O_2$-OH]$^-$) 108.02104 ([M-H-$C_9H_7O_3$]$^-$)	Root
50	15.98	283	D-xylofuranose tetradecyl glycoside	$C_{22}H_{41}O_9^-$	449.27561	449.27576	0.33	403.27036 ([M-H-CH_3O-OH]$^-$) 316.22061 ([M-H-$C_5H_9O_4$]$^-$) 329.23349 ([M-H-C_4H_9-4OH]$^-$) 117.05499 ([M-H-$C_{17}H_{32}O_5$-OH]$^-$)	Root
51	16.42	283	Corchorifatty acid F isomer	$C_{18}H_{31}O_5^-$	327.21770	327.21799	0.89	309.20665 ([M-H-OH]$^-$) 291.19684 ([M-H-2OH]$^-$) 173.11787 ([M-H-$C_9H_{15}O_2$]$^-$)	Root/shoot

								157.12346 ([M-H-$C_9H_{15}O_2$-OH]$^-$) 125.09643([M-H-$C_3H_5O_2$-$C_7H_{13}O_2$]$^-$)	
52	18.30	283, 368	Tianshic acid	$C_{18}H_{33}O_5^-$	329.23335	329.23361	0.79	165.12788 ([M-H-C_7H_{15}O-3OH]$^-$) 127.11205 ([M-H-$C_{10}H_{19}O_2$-2OH]$^-$)	Root/shoot
53	19.15	283	Dimethyl sebacate (sebacic acid derivative)	$C_{12}H_{21}O_4^-$	229.14453	229.144	0.39	201.11287 ([sebacic acid]$^-$) 215.12865 ([M-H-CH_3]$^-$) 211.13374 ([M-H-O]$^-$) 199.13379 ([M-H-CH_3O]$^-$) 185.11778 ([M-H-CH_3O-CH_3]$^-$) 157.12303 ([M-H-$C_2H_3O_2$-CH_3]$^-$)	Shoot
54	19.28	282, 368	Unknown	$C_{13}H_{27}O_8^-$	-	311.16888	-	-	Root
55	19.47	283, 368	Corchorifatty acid F isomer	$C_{18}H_{31}O_5^-$	327.21770	327.21802	0.98	309.20731 ([M-H-OH]$^-$) 291.19672 ([M-H-2OH]$^-$) 173.11792 ([M-H-$C_9H_{15}O_2$]$^-$) 125.09679 ([M-H-$C_3H_5O_2$-$C_7H_{13}O_2$]$^-$)	Root/shoot
56	20.06	283	Unknown	$C_{13}H_{27}O_8^-$	-	311.16888		-	Shoot
57	20.63	283	Nordihydrocapsiate	$C_{17}H_{25}O_4^-$	293.17583	293.17612	0.99	277.18088 ([M-H-OH]$^-$) 263.16534 ([M-H-CH_3O]$^-$) 247.16968 ([M-H-CH_3O-OH]$^-$) 157.12309 ([M-H-$C_8H_9O_2$]$^-$) 153.05524 ([M-H-C_9H_{17}O]$^-$) 141.12810 ([M-H-$C_8H_9O_3$]$^-$)	Root/shoot
58	20.92	274	Plastoquinone 3	$C_{23}H_{31}O_2^-$	339.23296	339.23322	0.77	203.10753 ([M-H-C_9H_{15}]$^-$) 163.11229 ([M-H-C_6H_{11}-C_5H_7O]$^-$) 149.06009 ([M-H-$C_{13}H_{21}$]$^-$) 135.04454 ([M-H-$C_{14}H_{23}$]$^-$)	Root
59	21.25	283	Decyl gallate (gallic acid derivative)	$C_{17}H_{25}O_5^-$	309.17075	309.17093	0.58	293.17935 ([M-H-OH]$^-$) 169.01381 ([gallic acid]$^-$) 153.01903([M-H-$C_{10}H_{21}$O]$^-$) 125.02367 ([M-H-$C_{11}H_{21}O_2$]$^-$)	Root/shoot
60	21.61	283	Nordihydrocapsiate isomer	$C_{17}H_{25}O_4^-$	293.17583	293.17612	0.99	277.18080 ([M-H-OH]$^-$) 263.16535 ([M-H-CH_3O]$^-$) 247.16990 ([M-H-CH_3O-OH]$^-$) 157.12309 ([M-H-$C_8H_9O_2$]$^-$) 153.05524 ([M-H-C_9H_{17}O]$^-$) 141.12810 ([M-H-$C_8H_9O_3$]$^-$)	Root/shoot
61	22.30	283	Unknown	$C_{13}H_{27}O_8^-$	-	311.16904	-	-	Root
62	22.53	283	Unknown	$C_{24}H_{45}O_{11}^-$	-	509.29691	-	-	Shoot
63	23.36	283, 337	13-Hydroxyoctadecadienoic acid	$C_{18}H_{31}O_3^-$	295.22787	295.22797	0.34	281.21204 ([M-H-CH_3]$^-$) 279.23334 ([M-H-OH]$^-$) 169.12331 ([M-H-C_8H_{15}O]$^-$)	Root/shoot

								153.12767 ([M-H-$C_8H_{15}O$-OH]$^-$)	
64	23.54	283, 337	p-Hydroxynonanophenone	$C_{15}H_{21}O_2^-$	233.1547	233.15462	0.34	219.17544 ([M-H-O]$^-$) 167.14342 ([M-H-C_4H_5O]$^-$) 135.04446 ([M-H-C_7H_{15}]$^-$) 121.02875 ([M-H-C_7H_{15}-CH_3]$^-$)	Root/shoot
65	24.03	283	Unknown	$C_{15}H_{31}O_8^-$	-	339.20029	-	-	Root
66	25.20	283	Unknown	$C_{14}H_{29}O_8^-$	-	325.18463	-	-	Root
67	25.72	283, 337	Unknown	$C_{14}H_{29}O_8^-$	-	325.18457	-	-	Shoot
68	25.98	283, 337	Unknown	$C_{14}H_{29}O_8^-$	-	325.18454	-	-	Shoot
69	26.24	283	Unknown	$C_{14}H_{29}O_8^-$	-	325.18463	-	-	Root

Compounds 1–5 were detected in both parts of *C. macromeris*. Compound 1 was assigned as vaccihein A (Figure II- 2a). This antioxidant metabolite was isolated for the first time in *Vaccinium ashei* (Ericaceae) by Ono, et al. [36] and this is the first time that is reported for cacti species. Compounds 2–5 were assigned as carboxylic acids: compounds 2 and 3 were proposed as dihydroxy methoxy butanoic acid and malic acid [37], respectively, and compounds 4 and 5 as isocitric acid isomers [13]. Citric acid occurs as one of the main hydrophilic constituents in *Opuntia* species (Cactaceae) [13, 38], and its presence may be also related to the crassulacean acid metabolism of cacti species [12].

Among the detected compounds, 31 metabolites were phenolic acids (compounds 6–13, 15–25, 31, 32, 34, 36–42, 44, 48 and 62) and 16 of them occurred in the aerial part (see Table II- 1). In our investigation, compound 6 was assigned as 3,5-dihydroxy-4-methyloxolan-2-yl methoxy-6-hydroxymethyl oxane-3,4,5-triol, due to the presence of fragments ions at *m*/*z*: 293.12424, 279.10870, 147.06578, and 131.07106 (see Table II- 1); Vankudothu and Anwar [39] proposed, through molecular docking, that this metabolite showed high affinity for the ACC2 protein, suggesting an effect against type 2 diabetes. Thus, the elicitation and isolation of this compound may be a potential use for *C. macromeris*. Also, two protocatechuic acid derivatives (compounds 9 and 16) were detected: compound 9 was proposed as protocatechuic acid hexoside (see Figure II- 2b) and compound 16 as protocatechuic aldehyde since pseudomolecular ion at *m*/*z*: 137.02386 yielded fragments at *m*/*z*: 121.02882 and 109.02884 (see Table II- 1). It has been proposed that protocatechuic aldehyde influences the pharmacokinetic activity of medicinal herbal extracts prepared with *Salvia miltiorrhiza* (Lamiaceae) [40], suggesting that the presence of this compound in *C. macromeris* extract may enhance its functional properties. Protocatechuic acid has been found as an aglycone in other cacti species such as *Opuntia ficus*-indica [41]

and *Myrtillocactus geometrizans* [17], and as one of the main constituents in *Opuntia humifusa* [18].

Additionally, compound 13 was only detected in the aerial part and was assigned as lucuminic acid, since the pseudomolecular ion yielded fragments at *m*/*z*: 163.03947, 119.04939 and 107.04942 (see Table II- 1). Lucuminic acid has been reported for *Calocarpum sapota* (Sapotaceae) by Takeda, et al. [42] and this is the first time that is reported for cacti species. In our investigation, sinapic acid (compound 20) and two of its derivatives (compounds 19 and 25) were also detected and characterized as reported previously [43]. Sinapic acid is synthesized from ferulic acid through enzymatic mechanisms [44] and it has been proposed to have anti-inflammatory activities [45].

In the same way, other phenolic metabolites such as two syringic acid derivatives (compounds 21 and 23), two caffeic acid isomers [46] (compounds 24 and 45), and cinnamic and gallic acid derivatives (compound 31 and 59, respectively) were also found (see Table II- 1). In addition, piscidic and ferulic acid and/or their derivatives were detected as the most recurrent phenolic acids in *C. macromeris* extracts: six isomers of piscidic acid (compounds 7, 8, 10, 11, 12, and 15) and four if its derivatives (compounds 17, 18, 22, and 37) were detected and assigned as reported previously [47, 48]. Piscidic acid has been previously identified in juices prepared with Opuntia ficus-indica fruits [13] and according to the number of peaks detected and the relative abundance shown in the chromatogram, piscidic acid may be one of the main constituents of the *C. macromeris* aerial part (see Figure II- 1a, Table II- 1); further studies are required to confirm the structure of this metabolite and its total concentration in each part of the plant. On the other hand, compounds 28, 33, 34, and 36 were assigned as ferulic acid isomers [49], and compounds 29, 35, 38, 39, and

41 as ferulic acid derivatives, since they showed characteristic fragment ions of ferulic acid (see Table II- 1). Our results indicate that ferulic acid is present in both parts of the plant but with a higher relative abundance in the shoots (Figure II-1a). Ferulic acid has been detected in different cacti species [18, 38, 50, 51] and its antiproliferative effect in human colon carcinoma HT29 cell line [52] as well its therapeutic potential against cardiovascular and neurodegenerative disorders [53] has been proposed. Our results suggest that *C. macromeris* could represent a potential source for the isolation of this compound or its derivatives; further studies are required to isolate and characterize the structure of these compounds (isomers) and to know their concentration, elicitation, and production in biotechnological systems.

In addition to compounds 2–5, compounds 26, 27, 32, 40, 44, 47, 51–53, 55, and 63 were also assigned as carboxylic acids. The fragmentation pattern of these metabolites was mainly characterized by the loss of water (see Table II- 1). Among these metabolites, compounds 51 and 55 were detected in both parts of the plant and assigned as corchorifatty acid F isomers. This metabolite has been previously identified in leaves of *Corchorus olitorius* (Tiliaceae) by Yoshikawa, et al. [54] and in aerial parts of *Chaenomeles sinensis* (Rosaceae) [55]. It also confers activity against pathogenic fungus *Pyricularia oryzae* in resistant rice cultivars [56]. To our knowledge, this is the first time that is reported for cacti species. On the other hand, compound 40 was identified as azelaic acid, according to Liu, et al. [57]. This dicarboxylic acid has been used for the treatment of acne and skin disorders [58, 59] and skin hyperpigmentation [60]. Compound 52 was assigned as tianshic acid due to the presence of fragments at *m/z*: 165.12788 and 127.11205 (see Table II- 1). The occurrence of this metabolite has been previously reported for *Sambucus williamsii* (Adoxaceae) by Yang, et al. [61] and no information exists for cacti species.

In the same way, other polar compounds were characterized in *C. macromeris* methanolic extracts. Compound 14 was assigned as hyrtioerectine C (alkaloid). Youssef [62] isolated this alkaloid and proposed its cytotoxic activity against HeLa cells. In our research group, studies are being carried out to elucidate the presence of other alkaloids in, and the potential applications of, *C. macromeris* extracts. Signals revealed for compound 30 indicated the presence of a glucopyranoside derivative [63] in the aerial part of *C. macromeris.* Similarly, compound 42 was tentatively identified as 2-phenylethyl β-D-glucopyranoside. This phenolic glycoside has been previously reported in *Pachysandra terminalis* (Buxaceae) [64] and in *Lactuca indica* (Asteraceae) [65]. To our knowledge, this is the first time that it is reported for cacti species. Compound 43 was tentatively identified as dalbergioidin (see Figure II- 2d); this antifungal isoflavone was reported in *Vigna angularis* (Fabaceae) as one of the mechanisms against *Phytophthora vignae* zoospores germination [66], suggesting that its presence in the *C. macromeris* aerial part may correspond to a constitutive adaptation mechanism since no pathogenic conditions were given to the plants; further studies are required to assess this hypothesis. Compounds 46, 48, and 50 were also assigned as metabolites of glycosidic nature (see Table II- 1). On the other hand, the pseudomolecular ion and fragmentation pattern of compound 49 indicated the presence of buteine (chalcone; see Figure II- 2e) in the radicular part of *C. macromeris.* Buteine exists in *Rhus verniciflua* (Anacardiaceae), a traditional herb used for cancer treatment [67], and its antioxidant activity [68] and potential as a chemotherapeutic agent [69] has been proposed. Compounds 57 and 60 were tentatively identified as nordihidrocapsiate isomers (see Figure II- 2f) and compound 58 as plastoquinone 3.

Finally, compounds 54, 56, 61, 62, and 65–69 were not identified since molecular information obtained did not match with theoretical information existing in the

literature. Absorption spectrum of these compounds suggest that they may contain benzoic acid within its structure (UV λmax: 280 nm). Further studies are required for structural elucidation of these metabolites.

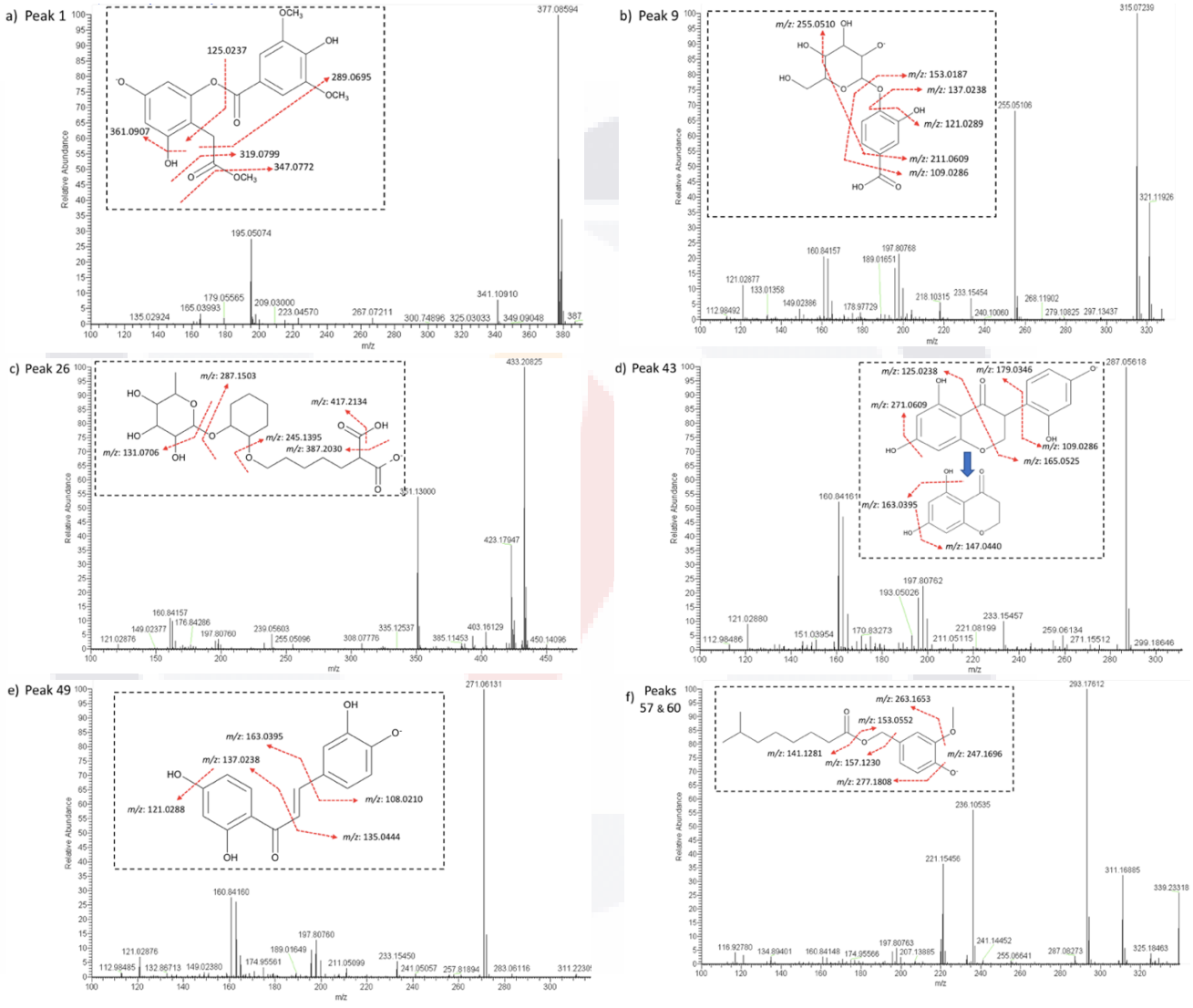

Figure II- 2. Full scan of some metabolites identified for the first time in *Coryphantha macromeris*. Dotted inset represents the molecule and red dotted lines in each inset represent the proposed fragmentation pattern. Peak numbers in each figure, refer to the compounds indicated in Table II- 1.

II.5. Conclusions

The utilized procedures allowed the separation of 69 metabolites present in methanolic extracts of aerial and radicular parts of *C. macromeris,* and the identification of most of them. The aerial part showed higher diversity of metabolites compared with roots, and 24 metabolites occurred in both plant parts. Among detected compounds, ferulic and piscidic acid and their derivatives were the most recurrent phenolic metabolites in *C. macromeris*. In addition, different classes of compounds with reported functional properties were detected. To our knowledge, the phytochemical profile of aerial and radicular parts of *C. macromeris* is reported here for the first time by means of modern ultra-high-performance liquid chromatography–tandem mass spectrometry. Based on the detected compounds, *C. macromeris* may find potential applications in the pharmaceutical and cosmetic industries. The obtained information could be also useful as chemotaxonomic markers for *Coryphantha* species.

II.6. References

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CAPÍTULO III

Phytochemical profile of *Coryphantha macromeris* (Engelm.) Britton & Rose (Cactaceae) obtained from *in vitro* culture [2]

III.1. Abstract

In the last years, the demand for natural products has increased. Different cacti species has demonstrated to be a source for the biosynthesis of secondary metabolites, as is the case of *Coryphantha macromeris* (Engelm.) Britton & Rose. The aim of this work was to evaluate the capacity of *C. macromeris* growing under controlled *in vitro* conditions for the obtention of secondary metabolites. *In vitro* cultures of *C. macromeris* plants were established from seeds using Murashige and Skoog culture medium and then the phytochemical profile of aerial and radicular sections was analyzed by means of ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS). Under the proposed conditions, 43 metabolites were identified; seven of them are reported here for the first time for this cacti species. Organic acids (citric, gluconic and tianshic acids), and phenolic acids such as piscidic, ferulic and syringic acid and/or their derivatives were found as the metabolites with the highest relative abundance. Our results suggest that the *in vitro* culture of *C. macromeris* represents a potential source for the obtention of selected compounds and serve as the basis for future investigations regarding the culture of *C. macromeris* under different biotechnological systems.

[2] Artículo aceptado para su publicación en la Revista Mexicana de Ingeniería Química (RMIQ). Cabañas-García, E.; Areche, C.; Gómez-Aguirre Y.A.; Jáuregui-Rincón, J.; Cruz-Sosa, F.; Pérez-Molphe Balch, E., Phytochemical profile of *Coryphantha macromeris* (Engelm.) Britton & Rose (cactaceae) obtained from *in vitro* cultures.

III.2. Introduction

Biotechnology techniques applied to the *in vitro* growth of cacti species has allowed the production of these plants for commercial and conservation purposes [1]. Some cacti species are slow growing plants in their natural habitat or under traditional vegetative propagation; thus *in vitro* culture has shown to be a suitable alternative for their propagation [2-4]. It has been proposed that *in vitro* culture of cacti species enhances growth rate and biomass production by altering the normal Crassulacean Acid Metabolism (CAM) pathway as is the case of *Coryphantha minima* [5] and also affects the profile and accumulation pattern of secondary metabolites in plants and their functional properties [6, 7].

Secondary metabolites are essential for plant surviving, helping to overcome the environmental stressing conditions [8]. Plant cell tissue and organ culture has become a powerful strategy for plant micropropagation and for the production and study of natural products [9-11] leading to the discovery of novel compounds with biological activities and commercial applications [12]. The potential use of *in vitro* culture is immense since the production of specific metabolites may be enhanced through elicitors or precursor treatments [13, 14] and the biosynthesis of compounds under a defined production system avoids variations in crop quality related with geographical and seasonal constrains, allows a continuous supply of phytocompounds with uniform quality and yield, and comprises a step of a multi-stage strategy for the biosynthesis of secondary metabolites on a larger scale such as callus or cell suspension cultures [15]. Thus, the subsequent dedifferentiation of *in vitro* plant cultures, is a powerful tool to obtain plant active metabolites in greater yield and quality.

The growing demand of natural products with functional properties has increased in the last years [16] and new sources ought to be investigated and research conducted by protecting wild populations of endangered and non-endangered species. Different cacti species has demonstrated to be a source of secondary metabolites with health promoting effects [17-19]. These metabolites may serve as the basis for the discovery, design or synthesis of new compounds with biological activities [20]. For this reason, the establishment of *in vitro* plant cultures becomes a fundamental task with socio-economic scope, generating systems that allow the conservation of the habitat and species with limited reproductive capacity [3] and, at the same time, the production of secondary metabolites in a greater range than in the wild. Few studies have focused on the phytochemical composition of traditionally used cacti species as is the case of *C. macromeris*. *C. macromeris* is traditionally known as "Dona Ana" and in the regions where it is distributed (northern Mexico and southern United States), it is traditionally used for healing stomach disorders. In previous reports we have shown the phytochemical profiling of aerial and radicular sections of *C. macromeris* growing under greenhouse conditions [21], finding the presence of different classes of secondary metabolites with reported functional and potential applications. The aim of this work was to evaluate the potential of *C. macromeris* growing under controlled *in vitro* conditions for the obtention of secondary metabolites in order to increase and deepen in the knowledge of cacti species and their potential applications.

III.3. Materials and methods

III.3.1. Plant material, *in vitro* culture and plant acclimatization

Mature fruits with seeds of *Coryphantha macromeris* (Engelm.) Britton & Rose were collected at the municipality of Concepción del Oro, Zacatecas, México. The plant

material was botanically identified by Professor M. Alvarado Rodríguez. A voucher specimen was deposited at the herbarium of the Autonomous University of Aguascalientes (HUAA; Voucher No. 6386). In the laboratory, the seeds were extracted and disinfected with treatments of 70% (v/v) ethanol (1 min), and 1.8% (v/v) sodium hypochlorite (25 min) and rinsed four times with sterile distilled water under aseptic conditions [22]. Seeds were germinated in culture vessels containing MS medium [23] and 30 g L^{-1} sucrose, solidified with 10 g L^{-1} agar (Sigma-Aldrich, St. Louis, MO, USA). All culture media were adjusted to pH 5.7 with 1N NaOH and then sterilized by autoclaving at 121 °C for 15 min. Cultures were incubated at 25 ºC under fluorescent light (40 μmol m^2 s^{-1}) and 16/8 (light/dark) photoperiod. Seedlings of 4-6 mm in length obtained from the germinating seeds were used as explant source for *in vitro* multiplication. For this, roots were separated, and the aerial sections were inoculated vertically onto: 1) MS basal medium; 2) MS basal medium supplemented with 6-benzylaminopurine (BA, 4.44 μM) and 1-naphthalenacetic acid (NAA, 0.054 μM); and 3) MS basal medium with activated charcoal (AC, 1.0 g L^{-1}) with the purpose of producing new shoots from the areoles. These culture media were selected based in our experience in *in vitro* culture of *Coryphantha* and other closely related species [24]. After 45 days of culture, the average number of shoots and roots formed per explant were evaluated, and a culture media was selected based on the physical characteristics and on the number of shoots generated *in vitro.* Experiments was performed in triplicate. The generated shoots were collected and transferred to the medium which produced higher number of shoots and then subcultured each three month for 12 months and used for phytochemical analysis.

For the acclimatization of micropropagated plants to *ex vitro* conditions, each plant was transferred into pots containing a mix of sand and soil (1:1) and covered with

plastic bags for 3 weeks, as we reported previously [25] in order to prevent desiccation.

III.3.2. Statistical analysis

Statistical analysis was performed using SigmaPlot software (Systat software, version 11.0). Sample differences were determined using one-way ANOVA. Statistical significance of means was considered at $p \leq 5\%$.

III.3.3. Sample preparation and phytochemical analysis by UHPLC-PDA-HESI-Orbitrap-MS/MS

C. macromeris extracts were prepared as reported previously [20]. Phytochemical analysis was performed using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific) with a C18 column (ID: 150 x 4.6 mm, 5 μm; Restek Corporation) and equipped with a quaternary Series RS pump and a Dionex Ultimate 3000 Series TCC-3000RS column compartments with 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, and Photodiode Array Detector (PDA) was recorded from 200 to 800 nm for peak characterization. The separation was performed (flow rate: 1.0 mL min^{-1}, injection volume: 10 μL) in a gradient elution mode composed by 1% formic aqueous solution (A) and acetonitrile (B). 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 Chromeleon 7.2 Software (Thermo Fisher Scientific, Waltham, MA, USA and Dionex Softron GmbH division of Thermo Fisher Scientific) and hyphenated with 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 and 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. XCalibur 2.3 software and Trace Finder 3.2 (Thermo Fisher Scientific, San Jose, CA, USA) were used for UHPLC control and data processing, respectively. Q Exactive 2.0 SP 2 (Thermo Fisher Scientific) was used to control the mass spectrometer.

III.3.3.1 MS parameters

The HESI parameters were optimized as follows: sheath gas flow rate 75 units; auxiliary gas unit flow rate 20; capillary temperature 400 ^{o}C; auxiliary gas heater temperature 500 ^{o}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 width 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 $3x10^{6}$ 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 30s-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 $2x10^5$, with the maximum injection time of 20 milliseconds. The precursor ions were filtered by the quadrupole operating at an isolation window of *m*/*z* 2. The fore vacuum, high vacuum and ultrahigh vacuum were maintained at approximately 2 mbar, from 105 to below 1010 mbar, respectively. Collision energy (HCD cell) was operated at 30 eV. Detection was based on calculated exact mass and on retention time of target compounds presented in Table III- 1. Metabolite identification was achieved by comparing spectrometric data with evidences existing in literature or analyzing the fragmentation pattern of each molecule. The mass tolerance window was set to 5 ppm.

III.4. Results and discussion

Seeds of *C. macromeris* were germinated *in vitro* and then based on our experience in plant cell tissue and organ culture of cacti species, and using the effective growth regulator combinations proposed by our research group for *Coryphantha spp.* and other closely related species [24], three different treatments were evaluated in order to find possible improvements on shoot and root formation. The generated explants into the different treatments showed similar morphological characteristics. Nevertheless, treatment composed by MS + BA (4.44 μM) + NAA (0.054 μM) or AC did not contribute to shoot or root formation since a maximum of 2 new shoots or roots were generated (Figure III- 1d). Statistically ($p \leq 5\%$), MS basal medium without plant growth regulators (PGR) or AC is considered as the best of the evaluated treatments, generating an average of 3.5 new and vigorous shoots per explant after 45 days of incubation (Figure III- 1d). The number of shoots produced is similar to

that reported previously by our research group for *C. duranguensis, C. clavata and C. radians* cultivated under similar conditions [24]; nevertheless, it is slightly lower when is compared with that reported by Smith, Burdick and Reilley [4] for *C. macromeris* plantlets regenerated from 4 years old callus cultures, yielding as many as 20 shoots per culture tube and formed sporadically after 42 to 56 days of culture. Thus, considering that in our investigation each explant produced an average of 3.5 new shoots *in vitro*, an average of 17.5 new shoots per flask containing 5 explants may be achieved after 45 days of culture. These shoots generated *in vitro* can be used as a source of explants for new multiplication cycles, due to this, shoots can be generated permanently without the need to collect and establish new plant material *in vitro*. The *in vitro* generated shoots with this medium successfully rooted (Figure III- 1d) and showed an acclimatization efficiency to *ex vitro* conditions of 72% (data no shown). The lower number of shoots and roots formed by the treatment composed by the combination of MS basal medium with BA and NAA may be related with the formation of callus tissue in the basal area of the explants [26] (Figure III- 1a) or a possible antagonistic interaction between PGR [27]. These results are similar to that reported by Giusti, et al. [28] for shoot proliferation in other closely related species such as *Escobaria minima, Mamillaria pectinifera* and *Pelecyphora aselliformis*, finding that a combination between the NAA and BA at different concentrations, inhibited the formation of shoots, suggesting the antagonistic effects between both PGR for shoot formation in some cacti species. Thus, based in our results, for the *in vitro* culture of *C. macromeris*, we recommend using MS medium without the evaluated PGR or AC.

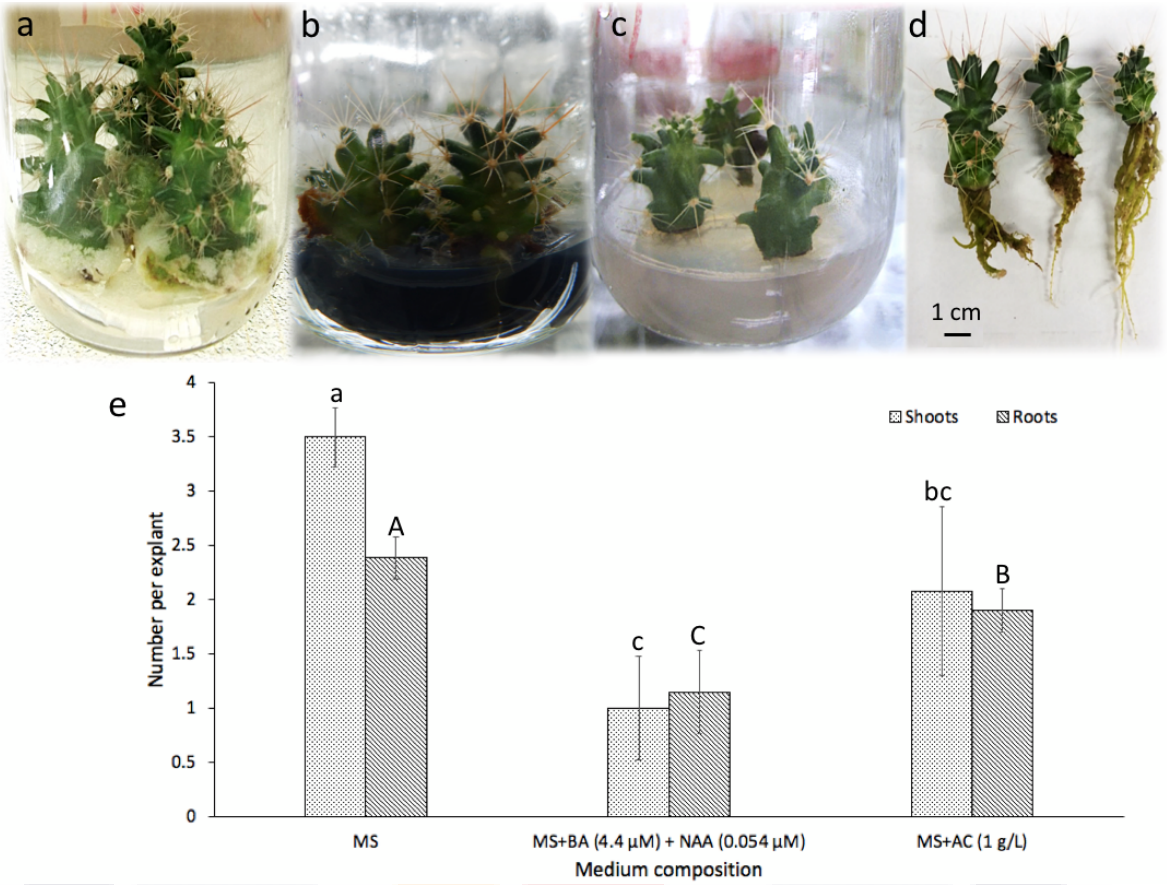

Figure III- 1. *In vitro* culture and propagation of *Coryphantha macromeris.* (a) Shoots produced in medium with BA (4.44 µM) + NAA (0.054 µM); (b) shoots in medium with AC; (c) plants transferred to MS basal medium; (d) Complete plants generated *in vitro* and used for phytochemical analysis; e) number of shoots and roots produced per explant of *C. macromeris* after 45 days of culture using different treatments. Each value represents the average (n=12) and error bars the standard deviation. Histobars with same letters are not significantly different (p≤5%). Lowercase letters refer to differences among shoots and uppercase among roots.

In this work, we also analyzed the phytochemical profile of *C. macromeris* growing under controlled *in vitro* conditions (Figure III- 1d) in order evaluate its biosynthetic capacity and to increase and deepen in the phytochemical knowledge of cacti species and their potential applications for the obtention of secondary metabolites. Under

the proposed chromatographic conditions, 44 metabolites were detected and 43 of them were tentatively identified by tandem mass spectrometry. Within this diversity, 18 metabolites were exclusively detected in shoots, 20 in roots and 6 in both plant sections namely citric, piscidic and tianshic acids, nordihydrocapsiate isomers and p-hydroxynonanophenone (compounds 4, 7, 34, 36, 40 and 43; Table III-1). Among detected compounds, 37 metabolites were assigned as we reported previously for *C. macromeris* plants growing under greenhouse conditions (peaks 2 to 25, 27-30, 32-34, 36, 37, 40, 42-44) [21], and 7 metabolites were exclusively biosynthesized by the *in vitro* plants, including gluconic acid (compound 1), one ferulic acid derivative (compound 26), two tianshic acid derivatives (compounds 38 and 41) and other polar compounds such as D-myo-Inositol, 2-butanoate 4,5-dihexanoate (compound 31), 6,8-dihydroxy-3-(penta-1,3-dien-1-yl) isochroman-1-one (compound 35) and 4-methyl-5-phenylpentanoate (compound 39). The occurrence of the above-mentioned metabolites is reported here for the first time for cacti species growing under controlled *in vitro* conditions. The elution profile of *C. macromeris* aerial and radicular sections is shown in Figure III- 2a and 2b, respectively; peak characteristics as is retention time, theoretical and measured mass, UV (λ_{max}) and tentative identification of each compound are summarized in Table III- 1. All the proposed metabolites showed an accuracy smaller than 5 ppm.

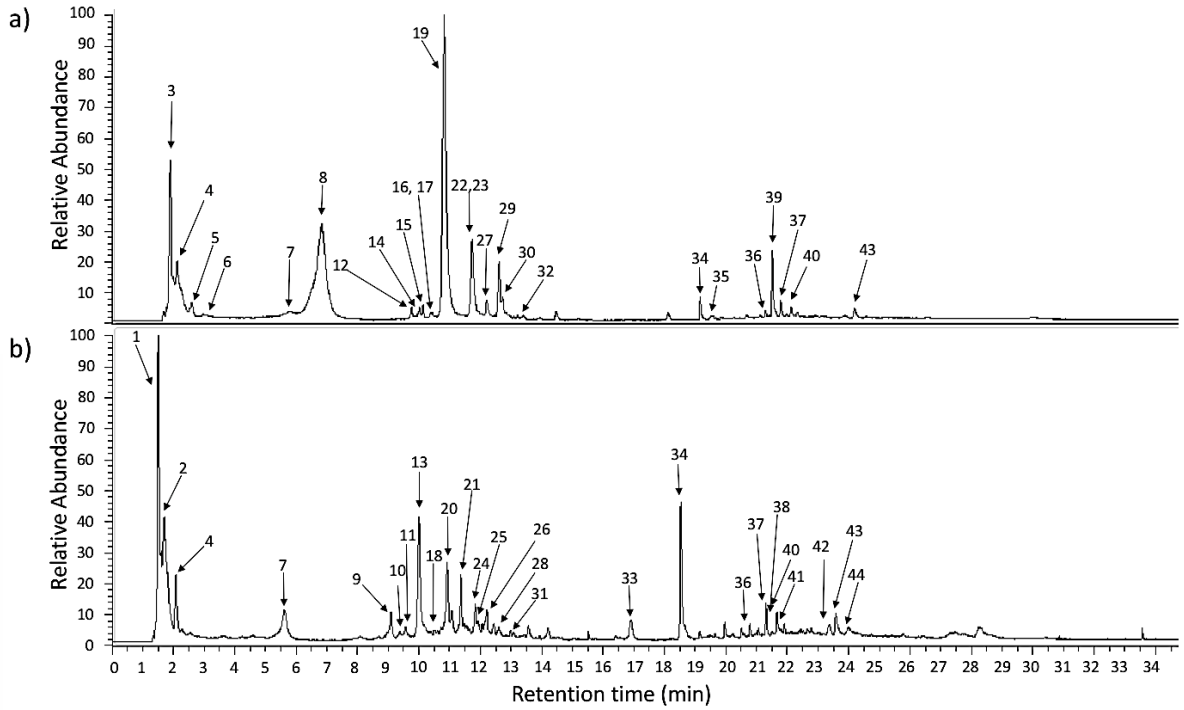

Figure III- 2. UHPLC chromatogram of *C. macromeris* methanolic extracts prepared whit aerial (a) and root section (b) of plants growing *in vitro*. Peak numbers refer to that metabolites indicated in Table III- 1; repeated numbers in each (a) or (b) refers to that metabolites found in both sections.

Compound 1 was assigned as gluconic acid as proposed by Taamalli, et al. [29]. Gluconic acid showed the highest relative abundance in the chromatogram (Figure III- 2b) and was only detected in radicular section. For other systems it has been proposed that this metabolite may be involved in the mechanisms of phosphorus solubilization in the medium [30], which may explain its exclusive presence in roots of *in vitro* plants, rather than in radicular sections of plants growing under greenhouse conditions [21]. Signals revealed for compounds 2, 4 and 5 indicated the presence of isocitric acid isomers and signals for compound 3, the presence of vaccihein A; compounds 6, 7 and 8 were assigned as piscidic acid isomers and compounds 10 and 15 as two of their derivatives (Table III- 1). According to the number of peaks detected for piscidic acid and the relative abundance shown in the

chromatogram (Figure III- 2a and 2b), our results suggest that piscidic acid or its derivatives may correspond to one of the main constituents of *C. macromeris* growing under controlled conditions, similar to that for greenhouse plants [21]. This is interesting since *in vitro* plants of *C. macromeris* shows the capacity for the biosynthesis of several metabolites found in plants cultivated *ex vitro* and offers a controlled system for the obtention and elicitation of selected compounds.

On the other hand, compounds 9 and 14 were assigned as the alkaloid hyrtioerectine C. Our results suggest that this metabolite may exist as constitutive, rather than adaptative since no stressing conditions were given to *in vitro* plants; further studies are required to asses this hypothesis. Compounds 11 and 17 were assigned as sinapic acid isomers [31] and compound 16 as their derivative since characteristic fragment ions of sinapic acid were detected (Table III- 1). On the other hand, compound 12 was assigned as lucuminic acid and compounds 13 and 19 as syringic acid acetate (Table III- 1). According to the relative abundance shown in the chromatogram (Figure III- 2a and 2b), syringic acid acetate may also correspond to one of the main constituents of *C. macromeris* aerial and root section, similar to that found for piscidic acid isomers and their derivatives (Table III- 1 and Figure III- 2a and 2b).

Compound 18 was assigned as a propanedioic acid derivative and compounds 20, 22, 24, 29 and 30 as ferulic acid isomers [32]. Compounds 23, 25 and 26 were proposed as ferulic acid derivatives, since characteristic fragment ions of ferulic acid were detected (Table III- 1). Among these metabolites, the presence of compound 26 is reported for the first time in *in vitro* cultures of *C. macromeris*; further studies are required to characterize the structure of compound 26. Compounds 21 and 27 were assigned as cinnamic acid derivatives and compounds 28 and 32 as azelaic acid isomers. Azelaic acid was detected in both sections of *in vitro* plants, similar to that

previously reported for plants growing under greenhouse conditions [21]. It has been proposed that the presence of organic acids in radicular sections may be related to mechanisms of phosphorus uptake and heavy metals tolerance [33] thus we hypothesized that this metabolite may occur as constitutive and a translocation or radicular secretion mechanisms may exist in *C. macromeris.*

In addition to compound 1 and 26, other 5 compounds were detected and reported here for the first time for cacti species growing under controlled *in vitro* conditions (compounds 31, 35, 38, 39 and 41), as well their fragmentation pattern. Among these metabolites, compound 31 was assigned as D-myo-Inositol, 2-butanoate 4,5-dihexanoate due to the presence of fragments at *m/z*: 375.20310, 343.21277, 245.13945, 269.13974, 229.14453 and 131.07074 (Figure III- 3a). It has been proposed that inositol and its derivatives may act as growth factor in plant cells since several functional roles on plant metabolism and cell wall polysaccharides biogenesis has been proposed [34]. Thus, this inositol derivative may form part as cell wall constituents of *C. macromeris.* Similarly, compound 35 was assigned as 6,8-dihydroxy-3-(penta-1,3-dien-1-yl) isochroman-1-one since pseudomolecular ion yielded fragments at *m/z*: 151.03943, 137.02382, 121.02871, 111.08681, 109.02865 (Figure III- 3b). As far as we know, this is the first time that these metabolites are reported for cacti species. On the other hand, signals revealed for compounds 34, 38 and 41 indicated the presence of tianshic acid (compound 34) and two of its derivatives (compounds 38 and 41; Table III- 1). Compound 39 was assigned as 4-methyl-5-phenylpentanoate due to the presence of one main fragment at *m/z:* 175.07587 generated due to the loss of one methyl group. On the other hand, compound 33 was assigned as corchorifatti acid F and compounds 36 and 40 as nordihydrocapsiate isomers. Compound 37 was proposed as gallic acid derivative (decyl gallate) and compounds 42 and 43 as 13-hydroxyoctadecadienoic acid and p- hydroxynonanophenone, respectively (Table

III- 1). Peak 44 was not identified since molecular information obtained did not match with theoretical information existing in literature.

Our results suggests that the phytochemical profile of *C. macromeris* is slightly affected by the *in vitro* culture since the diversity of metabolites was lower compared with that reported for greenhouse plants [21], in which, 69 metabolites were detected. Nevertheless, several compounds with functional properties are present in *C. macromeris* plants growing under controlled conditions *in vitro*. It has been shown that the *in vitro* culture of plants enhances the biomass production in shorter periods of time [3], especially for slow growing cacti species, suggesting the feasibility of the system for the obtention of *C. macromeris* metabolites. Compounds detected under *in vitro* conditions may occur as constitutive rather than adaptative, since no stressing conditions were given to plants in the culture media and compounds which were exclusively detected under controlled *in vitro* conditions (compounds 1, 26, 31, 35, 38, 39 and 41) may be present due to the highest nutrimental availability in the medium when compared with plants growing under greenhouse conditions [21].

In conclusion, our results contribute to the phytochemical knowledge of cacti species and suggest the potential of *C. macromeris* growing *in vitro* as a valuable source for the biosynthesis and study of selected metabolites. The quantification of target compounds is required to assess the suitability of the system for the *in vitro* obtention of different metabolites and its commercial feasibility. Our investigation also offers a useful system for future investigations related with plant, cell tissue and organ culture of cacti species.

Table III- 1. Metabolites identified in aerial and radicular parts of *Coryphantha macromeris* growing *in vitro* by UHPLC-PDA-HESI-Orbitrap-MS/MS data using HESI in negative ion mode.

Peak	Retention time (min.)	UV Max (λ_{max})	Tentative identification	Elemental composition [M-H]$^-$	Theoretical mass (*m*/*z*)	Measured mass (*m*/*z*)	Accuracy (ppm)	MSn ions	Plant part
1	1.51	270	Gluconic acid	$C_6H_{11}O_7^-$	195.05103	195.05101	0.10	129.018801	Root
2	1.71	222, 274	Citric acid*	$C_6H_7O_7^-$	191. 01973	191.01945	1.46	111.00801	Root
3	1.92	200	Vaccihein A*	$C_{18}H_{17}O_9^-$	377.08778	377.08572	4.72	361.09021 347.07446 319.07958 289.06955 125.02381	shoot
4	2.08	218, 273	Iso citric acid isomer*	$C_6H_7O_7^-$	191. 01973	191.01945	1.46	111.00790	Root/shoot
5	2.63	220, 275	Iso citric acid isomer*	$C_6H_7O_7^-$	191. 01973	191.01935	1.98	111.00790	shoot
6	3.02	220, 275	Piscidic acid isomer*	$C_{11}H_{11}O_7^-$	255.05103	255.05092	0.43	193.05013 165.05515 135.04445 119.04955 107.04945	Shoot
7	5.87	222, 275	Piscidic acid isomer*	$C_{11}H_{11}O_7^-$	255.05103	255.05095	0.31	193.05009 165.05511 135.04472 119.04937 107.04933	Root/shoot
8	6.88	223, 275	Piscidic acid isomer*	$C_{11}H_{11}O_7^-$	255.05103	255.05084	0.74	193.05009 165.05515 135.04449 119.04932 107.04932	Shoot
9	9.09	225, 276	Hyrtioerectine C*	$C_{11}H_{12}NO_4^-$	222.07718	222.07692	1.17	206.08186 198.07663 178.08684	Root
10	9.38	233, 273	Piscidic acid derivative*	$C_{21}H_{27}O_{13}^-$	487.14571	487.14587	0.32	255.05106 193.05022 165.05505 135.04446 107.04950	Root

11	9.66	231	Sinapic acid*	$C_{11}H_{11}O_5^-$	223.05997	223.06097	4.48	208.03757 179.07094 164.04730	Root
12	9.81	222, 272	Lucuminic acid*	$C_{19}H_{25}O_{12}^-$	445.13515	445.13525	0.22	163.03951 119.04940 107.04953	Shoot
13	10.01	225, 276	Syringic acid acetate*	$C_{11}H_{11}O_6^-$	239.05611	239.05594	0.71	197.04456 195.06580 179.03436 149.06012 135.04456 107.04944	Root
14	10.08	223, 275	Hyrtioerectine C*	$C_{11}H_{12}NO_4^-$	222.07718	222.07692	1.17	206.08183 198.07677 180.06609 178.08684,	Shoot
15	10.18	228	Piscidic acid derivative*	$C_{21}H_{27}O_{13}^-$	487.14571	487.14572	0.02	255.05087 193.05019 165.05513 135.04459 107.04922	Shoot
16	10.44	230	Sinapic acid derivative*	$C_{22}H_{29}O_{14}^-$	-	517.15637	-	223.06093 208.03735 179.07088 164.04736,	Shoot
17	10.49	230	Sinapic acid*	$C_{11}H_{11}O_5^-$	223.05997	223.06096	4.43	208.03757 179.07094 164.04730	Shoot
18	10.5	236, 283	Propanedioic acid, [5-[[2-[(6-deoxy-α-L-galactopyranosyl) oxy] cyclohexyl] oxy]-Pentyl]*	$C_{20}H_{33}O_{10}^-$	433.20792	433.20819	0.62	417.21247 387.20291 287.14960 245.13959 131.07077	Root
19	10.88	225, 275	Syringic acid acetate*	$C_{11}H_{11}O_6^-$	239.05611	239.05589	0.92	197.04605 195.06590 179.03441 149.06023 135.04465 107.04942	Shoot

20	10.92	236, 326	Ferulic acid*	$C_{10}H_9O_4^-$	193.05063	193.05028	1.8	179.03455 149.06018 163.03961 147.04457 135.03998	Root
21	11.37	237, 286	Cinnamic acid derivative*	$C_8H_{14}O_6^-$	-	206.08197	-	147.04456 103.05459 135.04437	Root
22	11.78	236, 327	Ferulic acid*	$C_{10}H_9O_4^-$	193.05063	193.05022	2.12	163.03963 149.06049 135.03993	Shoot
23	11.79	236, 327	Ferulic acid derivative (Fertaric acid) *	$C_{14}H_{13}O_9^-$	325.05651	325.05649	0.06	193.05032 163.03928 149.06001 135.03989	Shoot
24	11.83	237, 321	Ferulic acid isomer*	$C_{10}H_9O_4^-$	193.05063	193.0504	1.19	163.03963 149.06050 135.04468	Root
25	12.06	241	Ferulic acid derivative*	$C_{20}H_{29}O_{10}^-$	429.17662	429.17649	0.3	193.05081 163.03934 149.06033 135.04446,	Root
26	12.19	237, 282	Ferulic acid derivarive	$C_{18}H_{33}O_6^-$	-	345.22845	-	193.04984 149.06029 135.04456	Root
27	12.27	236, 290	Cinnamic acid derivative*	$C_8H_{14}O_6^-$	-	206.08195	-	147.04454 103.05433 135.04437	Shoot
28	12.59	282, 328	Azelaic acid*	$C_9H_{15}O_4^-$	187.0985	187.09732	0.96	125.09640	Root
29	12.69	236, 326	Ferulic acid isomer*	$C_{10}H_9O_4^-$	193.05063	193.05023	2.07	163.03963 149.05992 135.03996	Shoot
30	12.8	237, 313	Ferulic acid isomer*	$C_{10}H_9O_4^-$	193.05063	193.05028	1.8	163.03963 149.06020 135.03969	Shoot
31	12.98	282	D-myo-Inositol, 2-butanoate 4,5-dihexanoate	$C_{22}H_{37}O_9^-$	445.2421	445.24460	0.33	375.20310 343.21277 245.13945 269.13974 229.14453 131.07074	Root

32	13.45	282	Azelaic acid*	$C_9H_{15}O_4^-$	187.0985	187.09729	1.12	125.09640	Shoot
33	16.92	284	Corchorifatty acid F*	$C_{18}H_{31}O_5^-$	327.2177	327.21786	0.48	309.20665 291.19690 173.11787 157.12346 125.09642	Root
34	18.53	283, 368	Tianshic acid*	$C_{18}H_{33}O_5^-$	329.23335	329.23349	0.42	165.12788 127.11205	Root/Shoot
35	19.66	283, 403	6,8-dihydroxy-3-(penta-1,3-dien-1-yl) isochroman-1-one	$C_{14}H_{13}O_4^-$	245.0819	245.08174	0.65	151.03943 137.02382 121.02871 111.08681 109.02865	Shoot
36	20.79	282	Nordihydrocapsiate*	$C_{17}H_{25}O_4^-$	293.17583	293.17606	0.78	263.16559 247.16969 157.12259 141.12785	Root/Shoot
37	21.32	283	Decyl gallate (Gallic acid derivative)*	$C_{17}H_{25}O_5^-$	309.17075	309.17102	0.87	293.17578 169.01399 153.01965 125.02386	Root
38	21.52	282	Tianshic acid derivative	$C_{18}H_{33}O_4^-$	-	313.23859	-	165.12788 127.11205 125.09650	Root
39	21.61	283	4-methyl-5-phenylpentanoate	$C_{12}H_{15}O_2^-$	191.10775	191.10742	1.72	175.07587	Shoot
40	21.67	282	Nordihydrocapsiate*	$C_{17}H_{25}O_4^-$	293.17583	293.17609	0.88	277.18082 263.16519 247.17030 153.05557	Root/shoot
41	21.91	283	Tianshic acid derivative	$C_{18}H_{31}O_4^-$	-	311.2229	-	165.12788 127.11205 125.09650	Root
42	23.37	283, 336	13-hydroxyoctadecadienoic acid*	$C_{18}H_{31}O_3^-$	295.22787	295.22815	2.87	281.21198 279.23285	Root
43	23.58	283, 333	p-Hydroxynonanophenone*	$C_{15}H_{21}O_2^-$	233.1547	233.15462	0.34	219.17459 135.04431 121.02875	Root/shoot
44	24	283, 335	Unknown*	$C_{13}H_{27}O_8^-$	-	311.16888	-	-	Root

*Previously detected and identified in *C. macromeris* greenhouse plants [20].

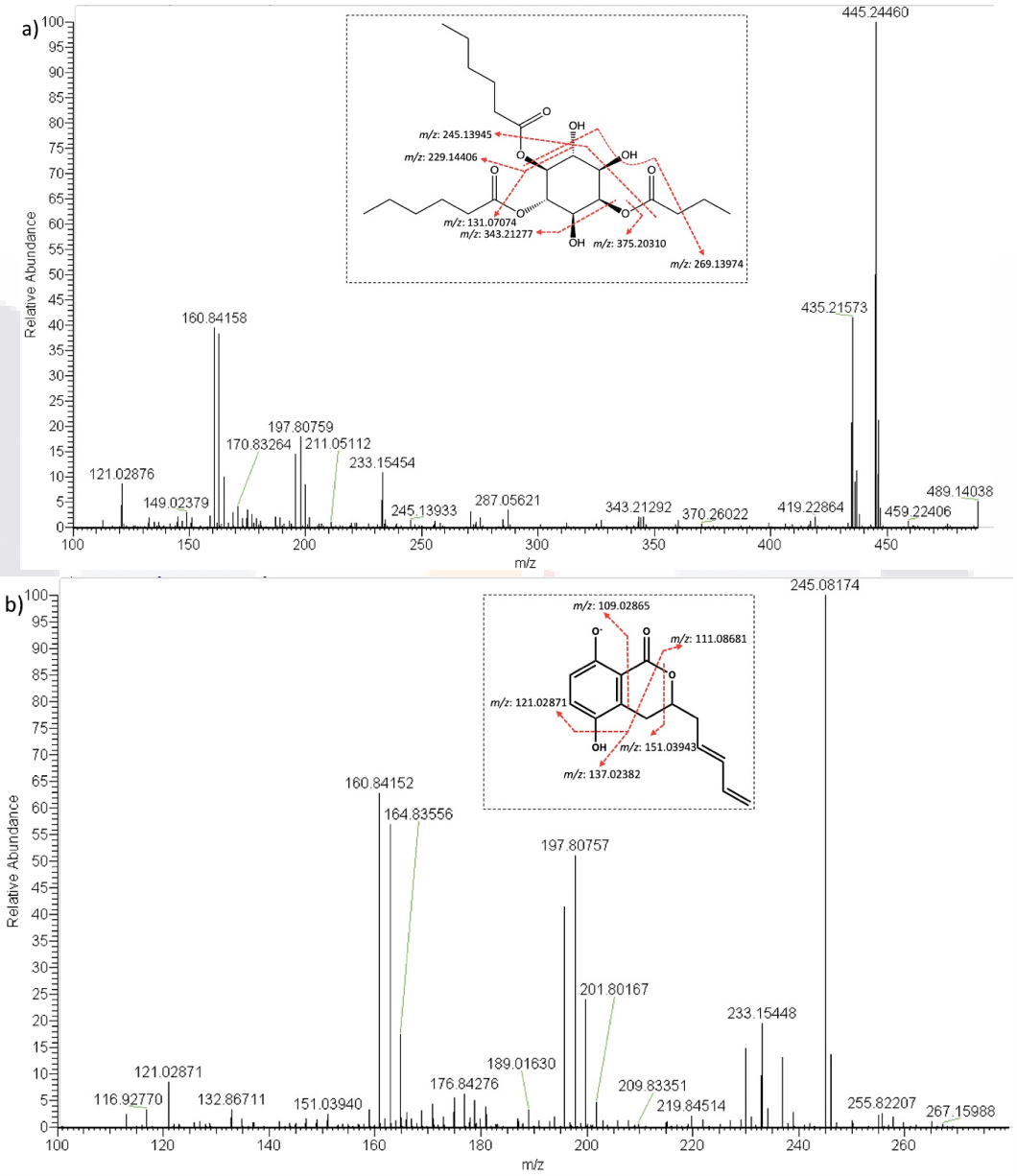

Figure III- 3. Full scan spectra of peaks 31 (a) and 35 (b), two metabolites identified for the first time for cacti species. Dotted inset represents the molecule and red dotted lines in each inset, the proposed fragmentation pattern.

III.5. References

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CAPÍTULO IV

Biomass production and secondary metabolites identification in callus cultures of *Coryphantha macromeris* (Cactaceae), a traditional medicinal plant [3]

IV.1. Abstract

Plant cell, tissue, and organ culture have become a powerful tool for the production of biomass and for the investigation and biosynthesis of a variety of plant secondary metabolites. In this work, we report an efficient method for friable callus induction applied to the medicinal cactus *Coryphantha macromeris* (Engelm.) Britton & Rose, its kinetic behavior and the phytochemical profile analyzed at the maximum biomass production phase. Callus cultures were established from stem discs inoculated on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP; 2.2 μM) and Picloram (4.14 μM). The highest biomass production (20.65 g DW L^{-1}) was achieved at 9 weeks of culture and then the phytochemical profile was analyzed by means of Ultra-High-Performance Liquid Chromatography-tandem Mass Spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS). Chromatographic and mass spectral analysis indicated the presence of 61 metabolites and 52 of them were identified. Among detected compounds, 11 organic acids, 16 phenolic acids, 8 flavonoids and 17 metabolites of different classes were identified. Our results contribute to the knowledge of tissue culture of cacti species and the potential applications of *in vitro* callus culture of *C. macromeris*. Also, we report for the first time the presence of some metabolites in cell culture of cacti species and their fragmentation pattern.

[3]Artículo preparado para someter a la revista *Industrial Crops and Products*. Autores: Cabañas-García, E.; Areche, C.; Gomez Aguirre, Y.A.; Borquez-Ramirez, J.; Muñoz, R.; Jáuregui-Rincón, J.; Cruz-Sosa, F.; Pérez-Molphe Balch, E., Titulo del trabajo: Biomass production and secondary metabolites identification in callus cultures of *Coryphantha macromeris* (Cactaceae), a traditional medicinal plant.

IV.2. Introduction

Cactaceae is one of the most threatened plant family which has changed its anatomy and physiology as an adaptation mechanism to harsh environments [1, 2]. It has been proposed that 57% of the cacti diversity is used by humans for horticultural, nutritional, medicinal, fodder and for artisanal handicraft elaboration [3]. Plant cell, tissue and organ culture technology has demonstrated to be a powerful tool for the micropropagation of cacti species [4-7] and for the production/elicitation of plant active metabolites [8-10]. A number of research works focused on the generation of callus and cell suspension cultures for the production of high value secondary metabolites from medicinal plants have been reported in the last years [11, 12]; nevertheless, despite medicinal properties, few studies have focused on callus induction for the study and production of metabolites from traditionally used cacti species [13-15]. Studies devoted to callus induction on cacti species have been carried out in order to achieve systems for plant regeneration, including *Coryphantha* species [4-7, 16-18]. Secondary metabolites obtained from tissue cultures can be generated on a continuous production system and without environmental constraints since physical conditions such as temperature, light regime, nutritional availability, pH and son on, may be controlled under *in vitro* conditions [8, 19] and the production of phytocompounds may be enhanced throw elicitor or by feeding precursors treatments [20] as is the case of resveratrol obtained from cell cultures of *Vitis vinifera* [21], taxol from *Taxus chinensis* cell culture [22], phenylethanoid glycosides from *Cistanche deserticola* cell cultures [23], berberine from *Coptis japonica* cell cultures [24] and alkaloids from *Cereus peruvianus* [13, 25]. Thus, the knowledge about the kinetic growth behavior of undifferentiated cells is important since optimal subculture stages and harvesting periods at maximum biomass or metabolite accumulation can be achieved. Information regarding the kinetic growth

behavior of undifferentiated cell cultures of cacti species is limited in the literature and refers to that of *Nopalea cochenillifera* [26] and cell suspension cultures of *Opuntia ficus* indica [27].

In our research group, studies are being carried out, using biotechnological approaches for the propagation, conservation and rational use of cacti species, and for the investigation of natural products derived from succulent plants as a source of bioactive metabolites with potential applications in pharmaceutical and cosmetic industries [28, 29]. *Coryphantha macromeris* (Engelm.) Britton & Rose is a medicinal cactus distributed in northern Mexico and southern United States; there is evidence that *C. macromeris* has been traditionally used for its hallucinogenic or sympathomimetic effect due to the presence of alkaloids [30, 31]; nevertheless, traditional uses also suggest properties for healing stomach disorders. *In vitro* culture of cacti species has not been extensively studied, and the potential of *C. macromeris* callus culture for the production of biomass and for the biosynthesis of secondary metabolites has not been analyzed. Thus, the aim of this work was to induce the formation of friable callus of *C. macromeris* and to analyze its phytochemical profile at maximum biomass production phase using UHPLC-PDA-HESI-Orbitrap-MS/MS in order to increase and deepen in the phytochemical knowledge of this species and their potential applications for the isolation and study and of bioactive compounds.

IV.3. Materials and methods

IV.3.1. Plant material, callus induction and kinetic growth evaluation

The plants of *Coryphantha macromeris* were obtained as reported previously [28]. For callus induction, stem discs (5-10 mm) of *in vitro* plants were inoculated on

Murashige and Skoog (MS) medium [32] with 30 g L^{-1} sucrose, 6-benzylamino purine (BAP, 2.2 μM) and Picloram (4.14 μM), that was solidified with 8 g L^{-1} agar; pH was adjusted to 5.7 before autoclaving at 121 °C for 15 min. The cultures were kept at 25 ºC with cool white fluorescent light tubes (40 μmol m^{-2} s^{-1}) and 16/8 (light/dark) photoperiod in C1775 culture vessels (PhytoTechnology Laboratories). Information regarding callus induction percentage, texture, and color were recorded after 9 weeks of culture. Callus biomass accumulation was assessed based on the fresh (FW) and dry (DW) weight measurements as reported previously [4]. For this, 1.0 g of friable fresh biomass was inoculated in 100 mL flasks, containing 25 mL of culture medium prepared as described. To determine callus growth, 3 flasks were collected at intervals of 7 days for 91 days. For dry biomass weight, each sample was dried in an oven at 40 ºC in dark conditions. The kinetic growth parameters were calculated as follows: $\mu = \ln (X_E / X_0)/\Delta t$; where X_0 and X_E are the amounts of callus biomass 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 growth index was calculated as follows: $GI = (X_F - X_0)/X_0$ where X_F and X_0 are the final and initial callus biomass, respectively.

IV.3.2. Sample preparation for phytochemical analysis

Callus were collected at maximum biomass production phase (9 weeks of culture; Figure IV- 2). Collected callus were dried in an oven (40 ºC) during 1 week in dark conditions. Dried material was finally pulverized in a mortar and then extracted three times with methanol in an ultrasonic bath (30 min each time). The resultant extract was filtered and evaporated under reduced pressure at 40 ºC and freeze-dried (Labconco Freeze Dryer). Each freeze-dried sample was resuspended (2.5 mg

mL^{-1}) in HPLC-Mass Spectrometry grade methanol and sonicated during 10 min for phytochemical analysis.

IV. 3.2.1. Phytochemical analysis using UHPLC-PDA-HESI-Orbitrap-MS/MS.

Phytochemical analysis was performed as reported previously [33-35], using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific) with a C18 column (ID: 150 x 4.6 mm, 5 μm; Restek Corporation) and equipped with a quaternary Series RS pump and a Dionex Ultimate 3000 Series TCC-3000RS column compartments with an Ultimate 3000 Series WPS-3000RS autosampler (Thermo Fisher Scientific) and a rapid separations Photodiode Array Detector (PDA). The detection wavelengths were 254, 280, 320 and 440 nm, and PDA was recorded from 200 to 800 nm for peak characterization. The separation was performed in a gradient elution mode composed by 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 Chromeleon 7.2 Software (Thermo Fisher Scientific, Waltham, MA, USA and Dionex Softron GmbH division of Thermo Fisher Scientific) and hyphenated with 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 and 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. XCalibur 2.3 software and Trace Finder 3.2 (Thermo Fisher Scientific, San Jose, CA, USA) were used for UHPLC control and data processing, respectively. Q Exactive 2.0 SP 2 (Thermo Fisher Scientific) was used to control the mass spectrometer.

IV.3.2.1.1. MS parameters

The HESI parameters were optimized as follows: sheath gas flow rate 75 units; auxiliary gas unit flow rate 20; capillary temperature 400 ^{o}C; auxiliary gas heater temperature 500 ^{o}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 width 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 $3x10^{6}$ and the injection time was set to 200 milliseconds (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 30s-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 $2x10^{5}$, with the 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 ultrahigh vacuum were maintained at approximately 2 mbar, from 105 to below 1010 mbar, respectively. Collision energy (HCD cell) was operated at 30

eV. Detection was based on calculated exact mass and on retention time of target compounds. The mass tolerance window was set to 5 ppm.

IV.4. Results and discussion

IV.4.1. Callus induction and kinetic behavior

Friable callus culture of *C. macromeris* was stablished. The best treatment for callus induction was MS medium supplemented with BAP (2.2 μM) and Picloram (4.14 μM), achieving the maximum callus induction frequency (100%), while control (growth regulator free) treatments induced the lowest value (10%, data no shown). The obtained tissue grew up mainly in the basal area of the explant, showed friable characteristics and was white to yellowish green in color (Figure IV- 1). Our results are similar to that found by Wakhlu and Bhau [4], who achieved an induction frequency of 96% in transversal sections of *Coryphantha elephantidens* treated with 2,4-dichlorophenoxyacetic acid (2,4-D) and Kinetin. In previous reports, Smith, et al. [17] proposed that *C. macromeris* was susceptible to callus induction by using medium rich in sucrose (20 g L^{-1}) and BAP combined with 2,4-D; nevertheless, information regarding the induction percentages and the characteristics of the formed tissue and its kinetic behavior was not given. On the other hand, Bhau [18] found that the maximum percentage of callus induction in root segments of *C. elephantidens* was achieved with 2,4-D at low concentrations (4.5 and 9.0 μM), while no induction was found for the control treatment after 4 weeks of culture. These results are in contrast to that found for *C. macromeris*, in which, MS basal medium produced an induction frequency of 10% (data no shown). These events may be related to the genotypic differences between species and the utilized explant [36] and suggest the presence of endogenous growth regulators in *C. macromeris* that exerts this effect on cell signaling [37]. For other cacti species such as *Opuntia ficus*

indica, it has been proposed that the presence of Picloram and other compounds such as casein hydrolysate in the culture medium, is a determinant condition for friable callus tissue formation [27], thus our results agree with the use of Picloram for callus induction in some cacti species; it is therefore recommended to use MS supplemented with BAP (2.2 μM) and Picloram (4.14 μM) as induction and maintenance media in *C. macromeris* callus cultures.

On the other hand, our results indicate that the kinetic growth behavior of *C. macromeris* callus culture is slow when is compared with other plant species such as *Eysenhardtia polystachya* (Leguminosae), which achieved a maximum biomass accumulation (14 g DW L^{-1}) at 12 days of culture [38] and *Armeria maritima* (Plumbaginaceae) cell cultures, with a maximum biomass accumulation of 13 g DW L^{-1} at 14 days [39]. In our investigation, the highest yield of callus biomass was achieved at 9 weeks of culture, with an average of 20.65 g DW L^{-1} (Figure IV- 2). The specific growth rate was 0.92 day^{-1}. The growth curve determined by measurements of the dry and fresh weight presented two distinct phases: exponential and deceleration phase (Figure IV- 2). The exponential phase began at the first week and finished after 3 weeks of culture. During this period, the cellular proliferation was observed resulting in a fast growth of callus. The deceleration phase occurred after 11 weeks and do not finished in the evaluated culture time (13 weeks). The culture showed a growth index of 14. At the end of culture period, no oxidation was observed, and callus tissue maintained its healthy and friable characteristics. These results are in contrast to that findings for cell cultures of *Nopalea cochenillifera* [26], in which, cells were cultured for 91 days and, after 40 to 50 days, cultures lost vigorous characteristics and turned brown. This is interesting since our results suggest that *C. macromeris* callus is a long living culture and subcultures may be performed after 9 to 13 weeks of incubation. Thus, cells can overcome to the deficit of water and

nutriments, similar to whole plants of cacti species [40]. In addition, despite advantages of *in vitro* propagation of plants over conventional propagation techniques [41], our experience with plant cell tissue and organ culture of cacti species indicates the feasibility of the system for the production of biomass, in a shorter period of time when compared with the *in vitro* culture of whole plants [2]. The culture system reported here may be used for further investigations related with the isolation, analysis, and elicitation of plant active metabolites, plant regeneration, and for the study of the biochemical and molecular mechanisms of adaptation and survival to harsh environments of cacti species; thus, the study for the isolation and characterization of genes associated with the control of abiotic stress tolerance may be achieved under a controlled system.

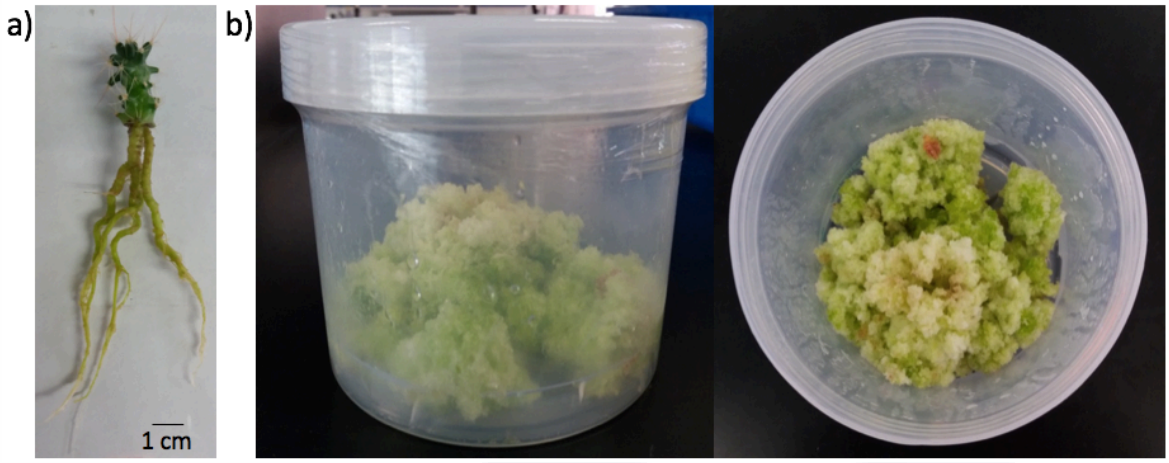


Figure IV- 1. *In vitro* culture of *Coryphantha macromeris.* (a) Plant growing *in vitro* and collected after 3 months of growth; (b) callus growth after 9 weeks of culture in C1775 culture vessels (PhytoTechnology Laboratories®) with 100 mL of medium.

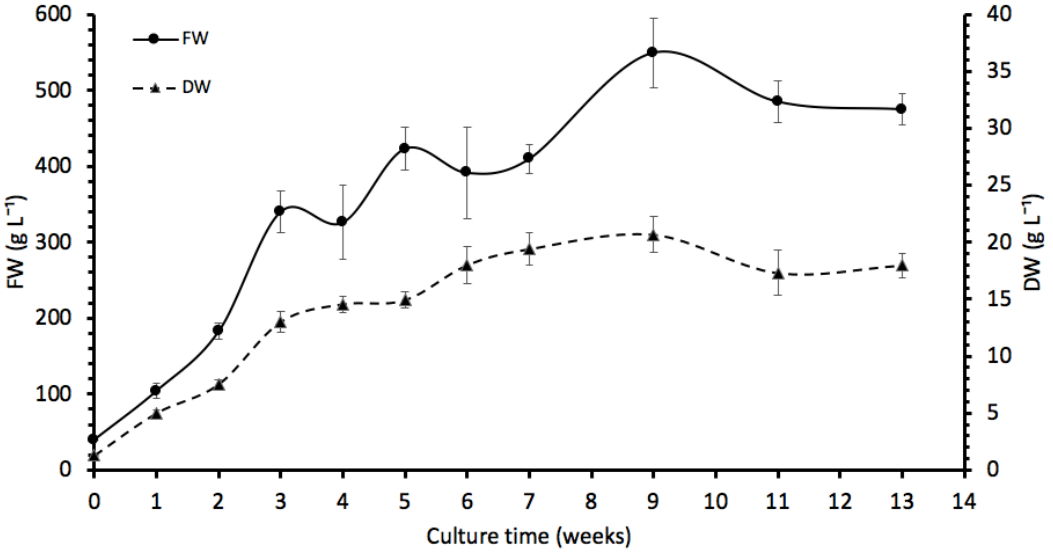


Figure IV- 2. Growth kinetics of callus cultures of *Coryphantha macromeris* originated from stems discs and growing in MS basal medium supplemented with BAP (2.2 μM) and Picloram (4.14 μM). FW: fresh weight; DW: dry weight. Each point represents the average (n=3) and error bars the standard error.

IV.4.2. Phytochemical analysis of *C. macromeris* callus culture

The methanolic extracts of *C. macromeris* were analyzed by UHPLC-PDA-HESI-Orbitrap-MS/MS; since this technique has been used for characterization of metabolites in complex mixtures [42]. Chromatographic conditions used in this work have shown efficiency for the separation and identification of different secondary metabolites [33-35]. Under the proposed chromatographic and spectrometric analysis conditions, 61 metabolites were separated (Figure IV- 3) and 52 of them were identified at the highest biomass production phase (week 9) of *C. macromeris* callus culture (Figure IV- 2). Most of the detected metabolites showed a mass accuracy under 5 ppm, except for that of compound 59. Among detected compounds, 11 organic acids (Table IV- 1), 16 phenolic acids (Table IV- 2), 8 flavonoids (Table IV- 3) and 17 metabolites of different classes (Table IV- 4), were characterized. Also, 9 compounds (compounds 1, 41, 48, 50, 52, 53, 54, 58 and 61)

were not identified since spectroscopic evidences did not match with theoretical information existing in the literature; nevertheless, their absorption pattern (UVmax: ca. 283 nm) suggest that may contain benzoic acid within the structure (data no shown). This is interesting since these compounds may correspond to new unreported plant metabolites. Further studies are required for its separation and structural elucidation of these compounds. On the other hand, compounds 2-4, 9, 10, 13, 17, 25-27, 36, 37, 39, 42, 55, and 60 were assigned as we reported previously for greenhouse plants [28].

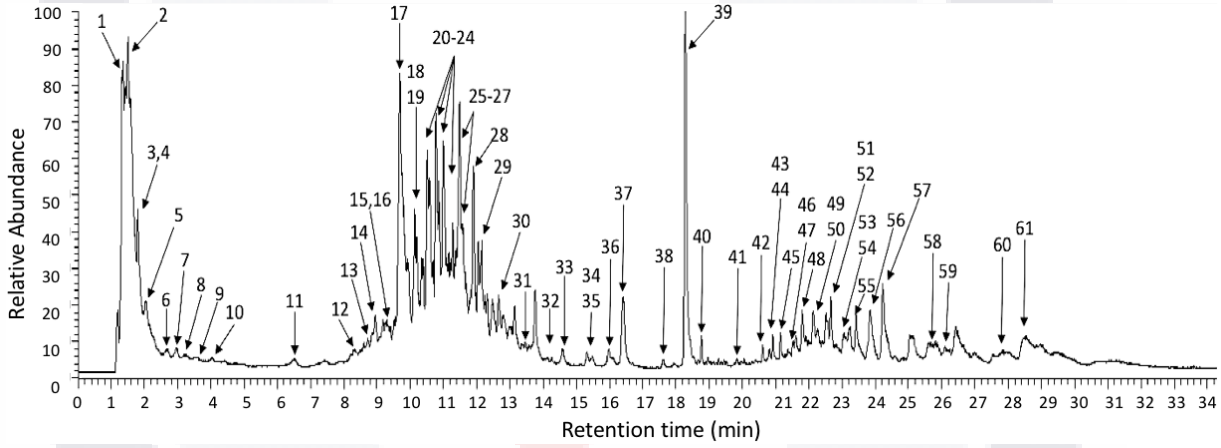


Figure IV- 3. UHPLC chromatogram of *Coryphantha macromeris* methanolic extracts prepared whit callus cultured for 9 weeks. Peak numbers refer to that metabolites indicated in Tables IV- 1-4.

IV.4.2.1. Identification of organic acids

Organic acids are molecules bearing a carbonyl group attached to a hydroxyl group in the same carbon atom. Organic acids occurs at different stages of cell growth cycles [43] and act as key metabolites for metal tolerance, pathogenic resistance and nutrimental uptake mechanisms [44]. The presence of organic acids in cacti species has been proposed due to the crassulacean acid metabolism pathway [45]. In our

research, 11 metabolites were assigned as organic acids (compounds 2-6, 8, 14, 37, 39, 46 and 47). The characteristic fragments were mainly generated due to the loss of carboxylic or hydroxyl groups (Δ*m*/*z*: 44.9976 and Δ*m*/*z:* 17.0027, respectively*)* as proposed by Ledesma-Escobar, et al. [46]. The identification parameters for organic acids are shown in Table IV- 1.

Table IV- 1. Organic acids and related compounds identified in *Coryphantha macromeris* methanolic extracts using UHPLC-PDA-HESI-Orbitrap-MS/MS.* previously reported for greenhouse plants [28].

Peak	Retention time (min.)	UV Max (λ_{max})	Tentative identification	Elemental composition [M-H]$^-$	Theoretical mass (m/z)	Measured mass (m/z)	Accuracy (ppm)	MSn ions
2	1.51	210, 269	2-Hydroxy-Succinic Acid (malic acid) *	$C_4H_5O_5^-$	133.01390	133.01347	3.23	115.00297 ([$C_4H_3O_4$]$^-$)
3	1.81	225, 274	Citric acid isomer *	$C_6H_7O_7^-$	191.01973	191.01924	2.57	111.00790 ([$C_5H_3O_3$]$^-$)
4	1.87	220, 274	2-Hydroxy-Succinic Acid (malic acid) isomer*	$C_4H_5O_5^-$	133.01390	133.01347	3.23	115.00297 ([$C_4H_3O_4$]$^-$)
5	2.06	225, 263	2-hydroxy-4-methoxy-4-oxobutanoic acid (malic acid derivative)	$C_5H_7O_5^-$	147.02990	147.02917	4.96	133.01349 ([$C_4H_5O_5$]$^-$) 115.00297 ([$C_4H_3O_4$]$^-$) 101.02349 ([$C_4H_5O_3$]$^-$)
6	2.68	228, 278	Pentanedioic acid, 2-hydroxy-, 5-methyl ester isomer	$C_6H_9O_5^-$	161.04555	161.04489	4.10	145.04999 ([$C_6H_{11}O_4$]$^-$) 117.05496 ([$C_5H_9O_3$]$^-$) 101.02353 ([$C_4H_5O_3$]$^-$) 113.02351 ([$C_5H_5O_3$]$^-$)
8	3.19	210, 258	Pentanedioic acid, 2-hydroxy-, 5-methyl ester isomer	$C_6H_9O_5^-$	161.04555	161.04492	3.91	145.04999 ([$C_6H_{11}O_4$]$^-$) 117.05496 ([$C_5H_9O_3$]$^-$) 101.02353 ([$C_4H_5O_3$]$^-$) 113.02351 ([$C_5H_5O_3$]$^-$)
14	8.96	225, 279	Isopropylmalic acid	$C_7H_{11}O_5^-$	175.06120	175.06076	2.51	131.07072 ([$C_6H_{11}O_3$]$^-$) 145.05013 ([$C_6H_9O_4$]$^-$) 115.03941 ([$C_5H_7O_3$]$^-$) 101.02353 ([$C_4H_5O_3$]$^-$)
37	16.39	283	Corchorifatty acid F *	$C_{18}H_{31}O_5^-$	327.21770	327.21762	0.24	309.20679 ([$C_{18}H_{29}O_4$]$^-$) 291.19672 ([$C_{18}H_{27}O_3$]$^-$) 125.09654 ([$C_8H_{13}O$]$^-$)
39	18.28	283,368	Tianshic acid *	$C_{18}H_{33}O_5^-$	329.23335	329.23322	0.39	165.12788 ([$C_{11}H_{17}O$]$^-$) 127.11205 ([$C_8H_{15}O$]$^-$)
46	21.36	283,335	Tianshic acid derivative	$C_{18}H_{33}O_4^-$	-	313.23834	-	125.09639 ([$C_8H_{13}O$]$^-$)
47	21.51	283,335	Tianshic acid derivative	$C_{18}H_{31}O_4^-$	-	311.22275		-

IV.4.2.2. Identification of phenolic acids

Phenolic acids are molecules which contain at least one phenol group bearing at least one carboxylic substituent; according to the carbon units in the lateral chain, phenolic acids may occur as cinnamic or benzoic acids and as conjugated systems with other organic molecules such as carbohydrates [47]. Phenolic metabolites have gained strong attention since their functional properties such as antioxidant, anticarcinogenic, anti-inflammatory and other pharmacological activities, has been demonstrated [48]. In our investigation, 16 phenolic acids (compounds 9, 10, 12, 16, 17, 18, 20, 21, 23, 25, 26, 27, 33, 34, 43 and 49) were separated and tentatively identified. The first fragmentation step for glycosylated phenolic acids was the cleavage of the glycosylic bound as proposed by Ledesma-Escobar, et al. [46]; thus, characteristic fragment ions were mainly generated due to the loss of sugars, methyl ($\Delta m/z$: 15.0234), hydroxyl ($\Delta m/z$: 17.0027) and carboxylic ($\Delta m/z$: 44.9976) groups. As an example, see supplementary material (Figure IV- S1a). Table IV- 2 shows the identification parameters of phenolic acids.

IV.4.2.3. Identification of flavonoids

Flavonoids are plant polyphenolic metabolites which has gained attention due to their health promoting effects in diseases such as cancer, Alzheimer disease and others, and due to their functional applications in cosmetic, pharmaceutical and medicinal industries [49]. Conformational characteristics of molecules strongly affects the functional properties of plant extracts [50]. In our investigation, eight metabolites were assigned as flavonoids or related compounds (compounds 15, 29, 30, 31, 32, 35, 44 and 45). One of the main differences in flavonoid conformation is the glycosidic substitution or the degree of hydroxylation and/or methylation of the

polyphenolic basic structure. The first fragmentation step for glycosylated flavonoids was the cleavage of glycosidic bond. As an example, please refer to supplementary material (Figure IV- S1b); for flavonoids found as aglycones, the cleavage of C-C bond in the C ring was observed [51]. The cleavage of methyl ($\Delta m/z$: 15.0234) and hydroxyl ($\Delta m/z$: 17.0027) substituents were also observed as diagnostic fragment ions. Table IV- 3 shows the identification parameters of flavonoids and related compounds.

Table IV- 2. Phenolic acids and their derivatives identified in *Coryphantha macromeris* methanolic extracts using UHPLC-PDA-HESI-Orbitrap-MS/MS. * previously reported for greenhouse plants [28].

Peak	Retention time (min.)	UV Max (λ_{max})	Tentative identification	Elemental composition [M-H]$^-$	Theoretical mass (*m*/*z*)	Measured mass (*m*/*z*)	Accuracy (ppm)	MSn ions
9	3.55	223, 278	Protocatechuic acid hexoside *	$C_{13}H_{15}O_9^-$	315.07230	315.07211	0.60	255.05084 ($[C_{11}H_{11}O_7]^-$) 211.06100 ($[C_{10}H_{11}O_5]^-$) 153.01865 ($[C_7H_5O_4]^-$) 137.02362 ($[C_7H_5O_3]^-$) 135.04411 ($[C_8H_7O_2]^-$) 121.02869 ($[C_7H_5O_2]^-$) 109.02883 ($[C_6H_5O_2]^-$)
10	4.04	215, 283	Protocatechuic acid hexoside isomer *	$C_{13}H_{15}O_9^-$	315.07230	315.07211	0.60	255.05083 ($[C_{11}H_{11}O_7]^-$) 211.06064 ($[C_{10}H_{11}O_5]^-$) 153.01854 ($[C_7H_5O_4]^-$) 137.02364 ($[C_7H_5O_3]^-$) 135.04411 ($[C_8H_7O_2]^-$) 121.02862 ($[C_7H_5O_2]^-$) 109.02872 ($[C_6H_5O_2]^-$)
12	8.32	208, 283	Caffeic acid 3-glucoside	$C_{15}H_{17}O_9^-$	341.08781	341.08780	0.03	179.03412 ($[C_9H_7O_4]^-$) 135.04428 ($[C_8H_7O_2]^-$)
16	9.33	223, 274	Caffeic acid 3-glucoside isomer	$C_{15}H_{17}O_9^-$	341.08781	341.08795	0.41	179.03438 ($[C_9H_7O_4]^-$) 135.04434 ($[C_8H_7O_2]^-$)
17	9.68	224, 277	Syringic acid acetate *	$C_{11}H_{11}O_6^-$	239.05560	239.05573	0.54	195.06573 ($[C_{10}H_{11}O_4]^-$) 179.03423 ($[C_9H_7O_4]^-$) 149.06006 ($[C_9H_9O_2]^-$) 135.04436 ($[C_8H_7O_2]^-$) 107.04939 ($[C_7H_7O]^-$)
18	9.91	224, 283	Feruloyl glucoside	$C_{16}H_{19}O_9^-$	355.103046	355.10342	1.05	193.05000 ([ferulic acid]$^-$) 157.04996 ($[C_7H_9O_4]^-$) 149.05980 ($[C_9H_9O_2]^-$) 135.04434 ($[C_8H_7O_2]^-$) 119.04925 ($[C_8H_7O]^-$) 109.02853 ($[C_6H_5O_2]^-$)

20	10.59	228, 281	Dihydroferulic acid 4-glucuronide	$C_{16}H_{19}O_{10}^-$	371.09837	371.09836	0.03	193.05014 ([ferulic acid]$^-$) 179.03445 ([$C_9H_7O_4$]$^-$) 149.06004 ([$C_9H_9O_2$]$^-$) 135.04437 ([$C_8H_7O_2$]$^-$) 123.04433 ([$C_7H_7O_2$]$^-$)
21	10.7	220, 281	Piscidic acid derivative	$C_{28}H_{37}O_{13}^-$	-	581.22339	-	255.05070 ([piscidic acid]$^-$) 193.05014 ([$C_{10}H_9O_4$]$^-$) 165.05504 ([$C_9H_9O_3$]$^-$) 135.04437 ([$C_8H_7O_2$]$^-$) 119.04933 ([C_8H_7O]$^-$) 107.04900 ([C_7H_7O]$^-$)
23	11.17	227, 279	Piscidic acid derivative isomer	$C_{28}H_{37}O_{13}^-$	-	581.22345	-	255.05046 ([piscidic acid]$^-$) 193.05005 ([$C_{10}H_9O_4$]$^-$) 165.05521 ([$C_9H_9O_3$]$^-$) 135.04445 ([$C_8H_7O_2$]$^-$) 119.04959 ([C_8H_7O]$^-$) 107.04916 ([C_7H_7O]$^-$)
25	11.49	284,321	Ferulic acid isomer *	$C_{10}H_9O_4^-$	193.05063	193.05023	2.07	179.03418 ([$C_9H_7O_4$]$^-$) 149.06026 ([$C_9H_9O_2$]$^-$) 163.03923 ([$C_9H_7O_3$]$^-$) 147.04459 ([$C_9H_7O_2$]$^-$)
26	11.69	230, 281	2-Isoferulic piscidic acid-1-metyl ester *	$C_{22}H_{21}O_{10}^-$	445.11410	445.11414	0.09	255.05046 ([piscidic acid]$^-$) 193.05016 ([$C_{10}H_9O_4$]$^-$) 165.05505 ([$C_9H_9O_3$]$^-$) 135.04422 ([$C_8H_7O_2$]$^-$) 107.04953 ([C_7H_7O]$^-$)
27	11.77	229, 283	Ferulic acid derivative *	$C_{20}H_{29}O_{10}^-$	-	429.17688	-	193.05016 ([ferulic acid]$^-$) 179.03430 ([$C_9H_7O_4$]$^-$) 163.03940 ([$C_9H_7O_3$]$^-$) 147.04443 ([$C_9H_7O_2$]$^-$) 135.04437 ([$C_8H_7O_2$]$^-$)
33	14.59	221, 284	Benzoic acid, 3-(β-D-glucopyranosyloxy)-4,5-dihydroxy-, decyl ester	$C_{23}H_{35}O_{10}^-$	471.22357	471.22357	0.00	309.17163 ([$C_{17}H_{25}O_5$]$^-$) 221.15479 ([$C_{14}H_{21}O_2$]$^-$) 193.05006 ([$C_{10}H_9O_4$]$^-$) 169.01370 ([$C_7H_7O_5$]$^-$) 135.04440 ([$C_8H_7O_2$]$^-$)

34	15.32	283	4-[(2-Methylbutanoyl)oxy]phenyl 4-(nonyloxy)benzoate	$C_{27}H_{35}O_5^-$	439.24902	439.24683	4.94	247.16962 ($[C_{16}H_{23}O_2]^-$) 219.17502 ($[C_{15}H_{23}O]^-$) 137.02362 ($[C_7H_5O_3]^-$) 135.04468 ($[C_8H_7O_2]^-$) 121.02873 ($[C_7H_5O_2]^-$)
43	20.81	283	4-methyl-5-phenylpentanoate	$C_{12}H_{15}O_2^-$	191.10736	191.10739	0.16	175.07591 ($[C_{11}H_{11}O_2]^-$)
49	22.13	283	16-sinapoyloxypalmitate	$C_{27}H_{40}O_7^-$	476.27850	476.27826	0.50	255.23267 ($[C_{16}H_{31}O_2]^-$) 193.05011 ($[C_{10}H_9O_4]^-$) 151.03928 ($[C_8H_7O_3]^-$) 119.04930 ($[C_8H_7O]^-$)

Table IV- 3. Flavonoids and related metabolites identified in *Coryphantha macromeris* methanolic extracts using UHPLC-PDA-HESI-Orbitrap-MS/MS.

Peak	Retention time (min.)	UV Max (λmax)	Tentative identification	Elemental composition [M-H]$^-$	Theoretical mass (*m*/*z*)	Measured mass (*m*/*z*)	Accuracy (ppm)	MSn ions
15	9.17	200, 225, 278	Aspalathin	$C_{21}H_{23}O_{11}^-$	451.12459	451.12466	0.16	289.07153 ([$C_{15}H_{13}O_6$]$^-$) 203.07053 ([$C_{12}H_{11}O_3$]$^-$) 137.02365 ([$C_7H_5O_3$]$^-$) 135.04436 ([$C_8H_7O_2$]$^-$) 125.02345 ([$C_6H_5O_3$]$^-$) 121.02866 ([$C_7H_5O_2$]$^-$) 109.02861 ([$C_6H_5O_2$]$^-$)
29	12.15	228, 281	6-O-Acetylglycitin	$C_{24}H_{23}O_{11}^-$	487.12459	487.12436	0.47	283.06094 ([$C_{16}H_{11}O_5$]$^-$) 175.03908 ([$C_{10}H_7O_3$]$^-$) 135.04428 ([$C_8H_7O_2$]$^-$) 121.02871 ([$C_7H_5O_2$]$^-$)
30	12.67	258	4',5,6-Trimethylscutellarein 7-glucoside	$C_{24}H_{25}O_{11}^-$	489.14024	489.14021	0.06	327.08700 ([$C_{18}H_{15}O_6$]$^-$) 297.07635 ([$C_{17}H_{13}O_5$]$^-$) 149.06026 ([$C_9H_9O_2$]$^-$) 137.02371 ([$C_7H_5O_3$]$^-$) 135.04424 ([$C_8H_7O_2$]$^-$)
31	13.47	226, 283	Pneumatopterin A	$C_{29}H_{36}O_{15}^-$	624.20652	624.20770	1.89	301.10776([$C_{17}H_{17}O_5$]$^-$) 175.03879 ([$C_{10}H_7O_3$]$^-$) 149.05997 ([$C_9H_9O_2$]$^-$) 135.04434 ([$C_8H_7O_2$]$^-$)
32	14.26	221, 284	1-Benzopyran-4-one, 7-(dodecyloxy)-5,6-dihydroxy-2-phenyl-	$C_{27}H_{33}O_5^-$	437.23335	437.23117	4.99	233.15442 ([$C_{15}H_{21}O_2$]$^-$) 151.03943 ([$C_8H_7O_3$]$^-$) 125.02364 ([$C_6H_5O_3$]$^-$) 207.17833 ([$C_{14}H_{23}O$]$^-$)
35	15.49	283	Apigenin 8-C-(6''-acetylgalactoside)	$C_{23}H_{21}O_{11}^-$	473.10894	473.10880	0.30	269.04498 ([$C_{15}H_9O_5$]$^-$) 253.05089 ([$C_{15}H_9O_4$]$^-$) 173.08141 ([$C_8H_{13}O_4$]$^-$) 137.02370 ([$C_7H_5O_3$]$^-$)

44	20.93	283	Dimethyl alpinum isoflavone	$C_{22}H_{19}O_5^-$	363.12380	363.12387	0.19	145.02887 ($[C_9H_5O_2]^-$) 117.03355 ($[C_8H_5O]^-$)
45	21.14	283	Dimethyl alpinum isoflavone isomer	$C_{22}H_{19}O_5^-$	363.12381	363.12387	0.17	145.02878 ($[C_9H_5O_2]^-$) 117.03385 ($[C_8H_5O]^-$)

IV.4.2.4. Other identified metabolites

In our investigation, 17 metabolites of different classes were also identified. Among detected compounds, phenylethanoid glycosides such as Magnoloside U isomers (compounds 22 and 24), alkaloids (hyrtioerectine C), lignans (syringaresinol), iridoids (agnucastoside B), quassinoids (picrasinoside G), phenolic ketones (*p*-hydroxynonanophenone), isoprenoids (plastoquinone 3) and other sugar and related compounds were detected and identified. Table IV- 4 shows the identification parameters for different classes of metabolites detected in *C. macromeris* callus cultures.

Table IV- 4. Other metabolites identified in *Coryphantha macromeris* methanolic extracts using UHPLC-PDA-HESI-Orbitrap-MS/MS. * previously reported for greenhouse plants [28].

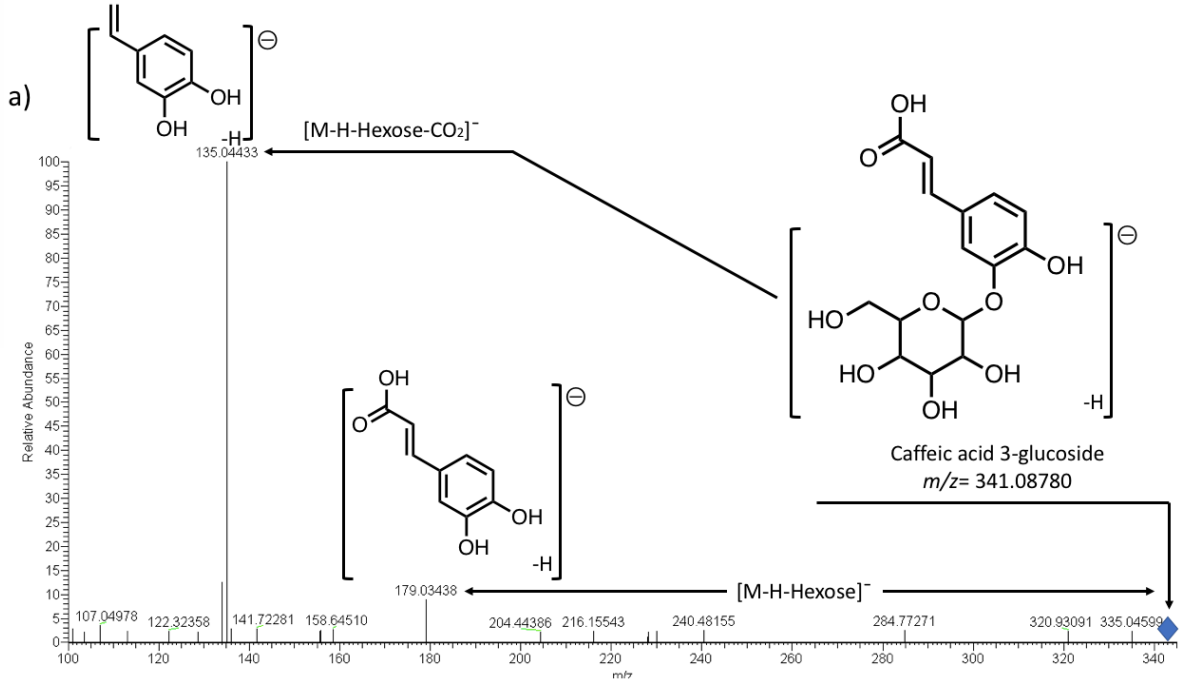
Peak	Retention time (min.)	UV Max (λmax)	Tentative identification	Elemental composition [M-H]$^-$	Theoretical mass (*m*/*z*)	Measured mass (*m*/*z*)	Accuracy (ppm)	MSn ions
7	2.96	220, 283	Rhynchosporoside	$C_{15}H_{27}O_{12}^-$	399.15080	399.15112	0.80	383.15561 ([$C_{15}H_{27}O_{11}$]$^-$) 367.16089 ([$C_{15}H_{27}O_{10}$]$^-$) 353.14526 ([$C_{14}H_{25}O_{10}$]$^-$) 325. 11325 ([$C_{12}H_{21}O_{10}$]$^-$) 309.11902 ([$C_{12}H_{21}O_9$]$^-$) 293.12323 ([$C_{12}H_{21}O_8$]$^-$) 279.10812 ([$C_{11}H_{19}O_8$]$^-$)
11	6.52	220, 277	2-(hydroxymethyl)-6-[-3,4,5-trihydroxy-6-(4-hydroxybutoxy) oxan-2-yl]methoxy]oxane-3,4,5-triol	$C_{16}H_{29}O_{12}^-$	413.16645	413.16632	0.31	367.16083 ([$C_{16}H_{29}O_{12}$]$^-$) 341.10876 ([$C_{12}H_{21}O_{11}$]$^-$) 325.15033 ([$C_{13}H_{25}O_9$]$^-$) 233.15428 ([$C_{15}H_{21}O_2$]$^-$) 281.08157 ([$C_{17}H_{13}O_4$]$^-$) 251.11301 ([$C_{10}H_{19}O_7$]$^-$)
13	8.73	208, 279	Hyrtioerectine C *	$C_{11}H_{12}NO_4^-$	222.07718	222.07677	1.85	206.08206 ([$C_8H_{14}O_6$]$^-$) 198.07718 ([$C_6H_{14}O_7$]$^-$) 180.06580 ([$C_6H_{12}O_6$]$^-$) 178.08685 ([$C_7H_{14}O_5$]$^-$)
19	10.4	228, 282	β-D-Galactopyranoside, 6-hydroxyhexyl 6-O-β-D-galactopyranosyl	$C_{18}H_{33}O_{12}^-$	441.19775	441.19778	0.07	407.19241 ([$C_{18}H_{31}O_{10}$]$^-$) 395.19238 ([$C_{17}H_{31}O_{10}$]$^-$) 217.10789 ([$C_{10}H_{17}O_5$]$^-$) 213.11278 ([$C_{11}H_{17}O_4$]$^-$) 135.04436 ([$C_8H_7O_2$]$^-$) 121.02924 ([$C_7H_5O_2$]$^-$)
22	11.14	227, 279	Magnoloside U	$C_{30}H_{39}O_{14}^-$	623.23453	623.23358	1.52	389.16052 ([$C_{21}H_{25}O_7$]$^-$) 179.05548 ([$C_6H_{11}O_6$]$^-$) 135.04437 ([$C_8H_7O_2$]$^-$)
24	11.39	225, 281	Magnoloside U isomer	$C_{30}H_{39}O_{14}^-$	623.23453	623.2337	1.33	389.16052 ([$C_{21}H_{25}O_7$]$^-$) 179.05550 ([$C_6H_{11}O_6$]$^-$) 135.04437 ([$C_8H_7O_2$]$^-$)

28	11.89	227, 277	Syringaresinol	$C_{22}H_{25}O_8^-$	417.15549	417.15546	0.07	181.04993 ($[C_9H_9O_4]^-$) 166.02646 ($[C_8H_6O_4]^-$) 135.04434 ($[C_8H_7O_2]^-$) 121.02868 ($[C_7H_5O_2]^-$) 109.02860 ($[C_6H_5O_2]^-$)
36	15.97	283	D-xylofuranose tetradecyl glycoside *	$C_{22}H_{41}O_9^-$	449.27561	449.27548	0.29	403.2701 ($[C_{21}H_{39}O_7]^-$) 329.23325 ($[C_{18}H_{33}O_5]^-$) 117.05499 ($[C_5H_9O_3]^-$)
38	17.62	283	Agnucastoside B	$C_{26}H_{39}O_{12}^-$	543.24470	543.24438	0.59	303.18130 ($[C_{15}H_{27}O_6]^-$) 199.09685 ($[C_{10}H_{15}O_4]^-$) 191.05533 ($[C_7H_{11}O_6]^-$)
40	18.76	283	Picrasinoside G	$C_{28}H_{43}O_{12}^-$	571.27599	571.27551	0.84	205.15915 ($[C_{14}H_{21}O]^-$)
42	20.61	283,332	Nordihydrocapsiate *	$C_{17}H_{25}O_4^-$	293.17583	293.17584	0.03	221.15424 ($[C_{14}H_{21}O_2]^-$) 148.05229 ($[C_9H_8O_2]^-$) 108.02074 ($[C_6H_4O_2]^-$)
51	22.68	283	Mezzettiaside 10	$C_{30}H_{51}O_{12}^-$	603.33860	603.33783	1.28	453.28436 ($[C_{25}H_{41}O_7]^-$)
55	23.4	283	p-Hydroxynonanophenone *	$C_{15}H_{21}O_2^-$	233.15470	233.15443	1.16	219.17544 ($[C_{15}H_{23}O]^-$) 135.04451 ($[C_8H_7O_2]^-$) 121.02868 ($[C_7H_5O_2]^-$)
56	23.86	283	Sucrose, 6-palmitate	$C_{28}H_{51}O_{12}^-$	579.33860	579.33795	1.12	255.23233 ($[C_{16}H_{31}O_2]^-$)
57	24.23	274	1-(2,4-dihydroxy-5-methoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl) heptane-3,5-dione	$C_{21}H_{23}O_7^-$	387.14493	387.14499	0.15	177.05505 ($[C_{10}H_9O_3]^-$) 151.07574 ($[C_9H_{11}O_2]^-$)
59	26.09	283	4,7,10,13-Tetraoxahexadecane-1,2,15,16-tetrol	$C_{12}H_{25}O_8^-$	297.15549	297.15302	8.31	161.07907 ($[C_7H_{13}O_4]^-$) 133.04987 ($[C_5H_9O_4]^-$)
60	27.75	283	Plastoquinone 3 *	$C_{23}H_{31}O_2^-$	339.23296	339.23288	0.24	163.11201 ($[C_{11}H_{15}O]^-$)

IV.5. Conclusion

In the present study, we induced the formation of callus tissue in *C. macromeris* and evaluated the phytochemical profile at the highest biomass production phase by means of modern UHPLC-PDA-HESI-Orbitrap-MS/MS. A total of 61 compounds were detected and 52 of them were tentatively identified by analyzing the fragmentation and absorption patterns of molecules. As far as we know, this is the first time that the kinetic behavior and the phytochemical profile of callus tissue obtained from *C. macromeris* is evaluated; several compounds of different classes were identified, and their fragmentation pattern is reported here. The obtained information may be useful for future investigations related to cell tissue culture of *Coryphantha* spp. and other cacti species, as well for the study and elicitation of secondary metabolites, and for performing investigations related to the resistance mechanisms to harsh environments of cacti species.

IV.6. Supplementary material



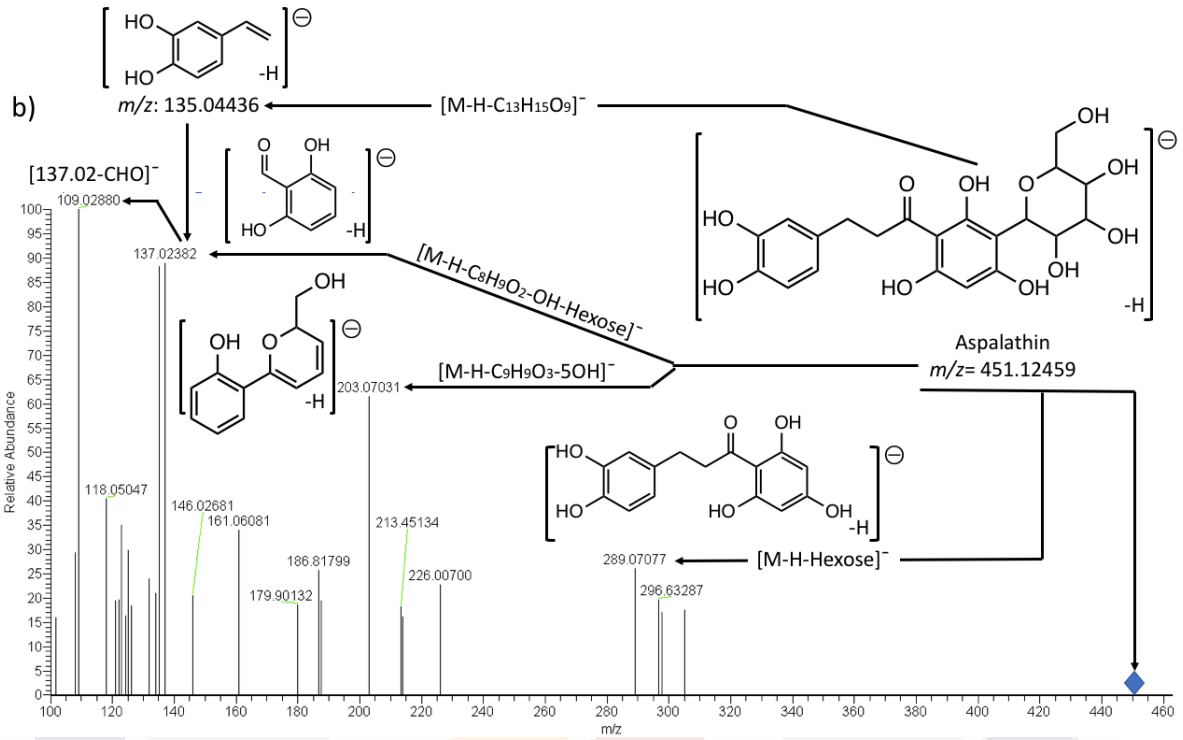


Figure IV-S- 1. Proposed fragmentation pattern of caffeic acid 3-glucoside (a) and aspalathin (b), two phenolic compounds identified for the first time for *Coryphantha species*.

IV.7. References

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CAPÍTULO V

Coryphantha macromeris cell suspension cultures: phytochemical profiling and agitation velocity effect on cell morphology and viability [4]

V.1. Abstract

Cell suspension cultures represents an alternative for the production of plant active metabolites. It has been demonstrated that agitation velocity is one of the main conditions to evaluate in order to achieve high cell viability and high yields in metabolite production. In this work, we analyzed the effect of agitation velocity on cell viability and morphology of *C. macromeris* (Engelm.) Britton & Rose cell suspension cultures; additionally, we analyzed by means of Ultra-High-Performance Liquid Chromatography-tandem Mass Spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS, the phytochemical profile of two-month-old cells cultivated at 80 rpm. Our results indicate that an agitation velocity of 120 rpm impacts negatively on cell integrity. Nevertheless, at 80 and 100 rpm, cells successfully survived and proliferated with similar viability percentages (ca. 97%). The chromatographic and mass spectral analysis indicated the presence of 49 metabolites and 45 of them were identified. Among detected compounds, different classes of metabolites such as phenolic acids (gallic acid derivatives), iridoids (gardoside), stilbenes (tyrolobibenzyl E), lignans (acanthoside B), flavonoids (catechin, lantanoside, sakuranin, afrormosin, Kaempferol 7-rhamnoside) and phenylethanoids (phlomisethanoside) were found. Our results contribute to the phytochemical knowledge of cacti species and offers the basis for future investigations regarding cell suspension cultures of *C. macromeris* and related plant species.

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V.2. Introduction

Coryphantha macromeris (Engelm.) Britton & Rose is a small cactus, which may reach 20 cm in height. This specie habits in sandy soils or under shrubs in regions from 750 to 1500 mamsl [1], forming agglomerates which may vary from 0.15 to 1 m in diameter [2]. It is mainly distributed in the Chihuahuan desert and in Coahuila, Durango, Zacatecas and Tamaulipas, México and in southern United States (New México and Texas) [2-4]. It has been proposed that *C. macromeris* plants may serve as a source for the study and biosynthesis of high valuable secondary metabolites, since several compounds with functional properties has been identified [5]. Despite functional potential, studies regarding cell suspension cultures of *C. macromeris* has not been reported. Cell suspension cultures offers a platform for the analysis of cells at physiological, molecular and biochemical level, and for the biosynthesis and elicitation of high valuable secondary metabolites [6]. Information regarding cell suspension cultures of cacti species is limited in literature and refers to that of *Opuntia ficus- índica* [7] and information regarding the measurement of the effect of hydrodynamic stress on cell viability of cacti species do not exist.

In previous reports we have shown the phytochemical profile of *C. macromeris* cultivated under different conditions such as greenhouse (Chapter II), *in vitro* (Chapter III) and callus cultures (Chapter IV). In this section we analyzed the effect of agitation velocity on cell viability and morphology of *C. macromeris* (Engelm.) Britton & Rose cell suspension cultures. Also, we analyzed phytochemical profile of

two-month-old cells cultivated at 80 rpm in order to know the potential applications of *C. macromeris* cell suspension cultures for the biosynthesis of high valuable metabolites.

V.3. Materials and methods

V.3.1. Plant material

V.3.1.1. Cell suspension cultures establishment and agitation velocity evaluation

Callus cultures of *Coryphantha macromeris* were obtained as we reported previously (Chapter IV). For the establishment of *C. macromeris* cell suspension cultures, 1.0 g of friable callus were transferred (n=8) into 125 mL flasks containing 25 mL of Murashige and Skoog (MS) medium [8] with 30 g L^{-1} sucrose and supplemented with 6-benzylamino purine (BAP, 2.2 μM) and Picloram (4.14 μM). Cells were cultivated for one month under three different agitation velocities (n=8): a) 80 rpm, b) 100 rpm and, c) 120 rpm on a rotatory shaker (Prendo) and under an illumination regime of 35 μmol m^{-2} s^{-1} using cool white fluorescent light tubes.

V.3.1.1.1. Cell viability

Cells were cultured for one-month under the described conditions. Cell viability was measured as proposed by Shigaki and Bhattacharyya [9] with slight modifications. For this, 0.5 mL of culture medium containing cells growing under different conditions were taken and incubated for 5 min. with 0.5 mL of Evans blue aqueous solution (1 mM) and then filtered using filter paper and washed five times with distilled water. Each sample (n=3) was placed on 75x25 mm slides and observed under a defined area of 5 cm^2, using a microscope (Zeizz). Images were taken and processed using Image J version 1.8.0_112.

V.3.1.2. Sample preparation for phytochemical analysis

After an agitation system was selected, cells were subcultured six times for one year (two-month subcultures) and then collected. Thus, for phytochemical analysis, two-month-old cells were used. Methanolic extracts were prepared as we reported previously (see Chapter II) and injected in a UHPLC system hyphenated with a Mass Spectrometer.

V.3.2. UHPLC-PDA-HESI-Orbitrap-MS/MS Conditions

Phytochemical analysis was performed as reported previously [10-12], 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) and equipped with a Quaternary Series RS pump and a Dionex Ultimate 3000 Series TCC-3000RS column compartments with 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, and PDA was recorded from 200 to 800 nm for peak characterization. The separation was performed in a gradient elution mode composed by 1% formic aqueous solution (A) and acetonitrile (B). The flow rate was 1.0 $mL \cdot min^{-1}$ 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 Chromeleon 7.2 Software (Thermo Fisher Scientific, Waltham, MA, USA and Dionex Softron GmbH division of Thermo Fisher Scientific) and hyphenated with 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 and 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. XCalibur 2.3 software and Trace Finder 3.2 (Thermo Fisher Scientific, San Jose, CA, USA) 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.

V.3.2.1. 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 width 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 30s-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 the 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 105 to below 1010 mbar, respectively. Collision energy (HCD cell) was operated at 30 eV. Detection was based on calculated exact mass and on retention time of target compounds presented in Table V- 2. The mass tolerance window was set to 5 ppm.

V.3.3. Statistical analysis

Statistical analysis was performed using SigmaPlot software (Systat software, version 11.0). Sample differences were determined using one-way ANOVA. Statistical significance of means was considered at $p \leq 5\%$.

V.4. Results and Discussion

V.4.1. Morphology and cell viability

In cell suspension cultures, it has been proposed that one of the conditions that affects cell proliferation, morphology and the production of compounds is the amount of agitation in the culture medium [13]. In our investigation, three agitation velocities (80, 100 and 120 rpm) were evaluated in order to find an agitation system which may ensure the proliferation and viability of *C. macromeris* cell suspension cultures. At 120 rpm, cells were damaged by hydrodynamic stress and cell fragments were observed (Figure V- 1d); thus, quantitation of viable cells was difficult to achieve. At lower agitation velocities (80 and 100 rpm), cells successfully survived and proliferated (Figure V- 1b and 1c). Nevertheless, some morphological changes such as cell elongation were observed at 100 rpm (Figure V- 1a and 1c).

Thus, since cell viability of *C. macromeris* cultured at 80 and 100 rpm were similar (ca. 97%; see Table V- 1) and no deformation was observed in cells cultivated at 80 rpm (see Figure V- 1b), an agitation velocity of 80 rpm was selected for the maintenance of *C. macromeris* cells suspension cultures. Cell sensibility of *C. macromeris* may indicate that possess high-sized vacuoles and non-rigid cell wall when compared with cells which can overcome hydrodynamic stress such as *Thevetia peruviana* [14]. Cell resistance to hydrodynamic stress may be associated with the presence of plant growth regulators in the culture medium. It has been proposed that during the signalization process, growth regulators induces an ionic bombardment [15], modifying the inner pH of cells, and activating cellulases, allowing cell expansion in proportion to the pressure exerted by the intracellular content, and increasing cell size and cell sensibility to hydrodynamic stress.

For other plant systems it has been proposed that the agitation velocity is a determinant condition for achieving high cell viability and for secondary metabolites production. Hooker, et al. [16] evaluated the effect of hydrodynamic stress on cell viability and metabolite accumulation in cell suspension cultures of *Nicotiana tabacum* (Solanaceae), finding that the extent of hydrodynamic stress directly affects cell viability and the release of phenolic compounds to the culture medium. On the other hand, Sun and Linden [17] found that the control of agitation velocity is a determinant condition for Taxol production in cell suspension cultures of *Taxus cuspidata* (Taxaceae), suggesting that the regulation of agitation velocity is a condition which directly affects cell growth and bioactive compounds accumulation. These results agree with the findings of Tanaka et al. [18] for *Catharanthus roseus* (Apocynaceae) cell suspension cultures, in which, the production of compounds, as well as the growth rate and the distribution of cell

aggregates varies according to the type of flask and with the extent of hydrodynamic stress generated to the culture system.

One of the characteristics of some cacti species is their ability to biosynthesize pigments, as is the case of betacyanins and other isoforms identified in fruits of *Hylocereus polyrhizus* [19] and in different clones of *Opuntia* spp. [20]. Our results suggest that *C. macromeris* cell suspension cultures may synthesize red-violet pigments (see Figure V- 1d, 1f and 1g). This behavior of cells is not restricted to high agitation velocities, since at 80 and 100 rpm, red-violet pigmentation was also observed.

Table V- 1. Cell viability measurements of *Coryphantha macromeris* cell suspension cultures at different agitation velocities*.

Agitation velocity (rpm)	No. of viable cells	No. of death cells	Cell viability (%)
80	1300	26	97.51[a]
100	2255	44	97.84[a]
120	-	-	-

-: not quantified. Values represent the average (n=3). *information taken from institutional thesis [21].

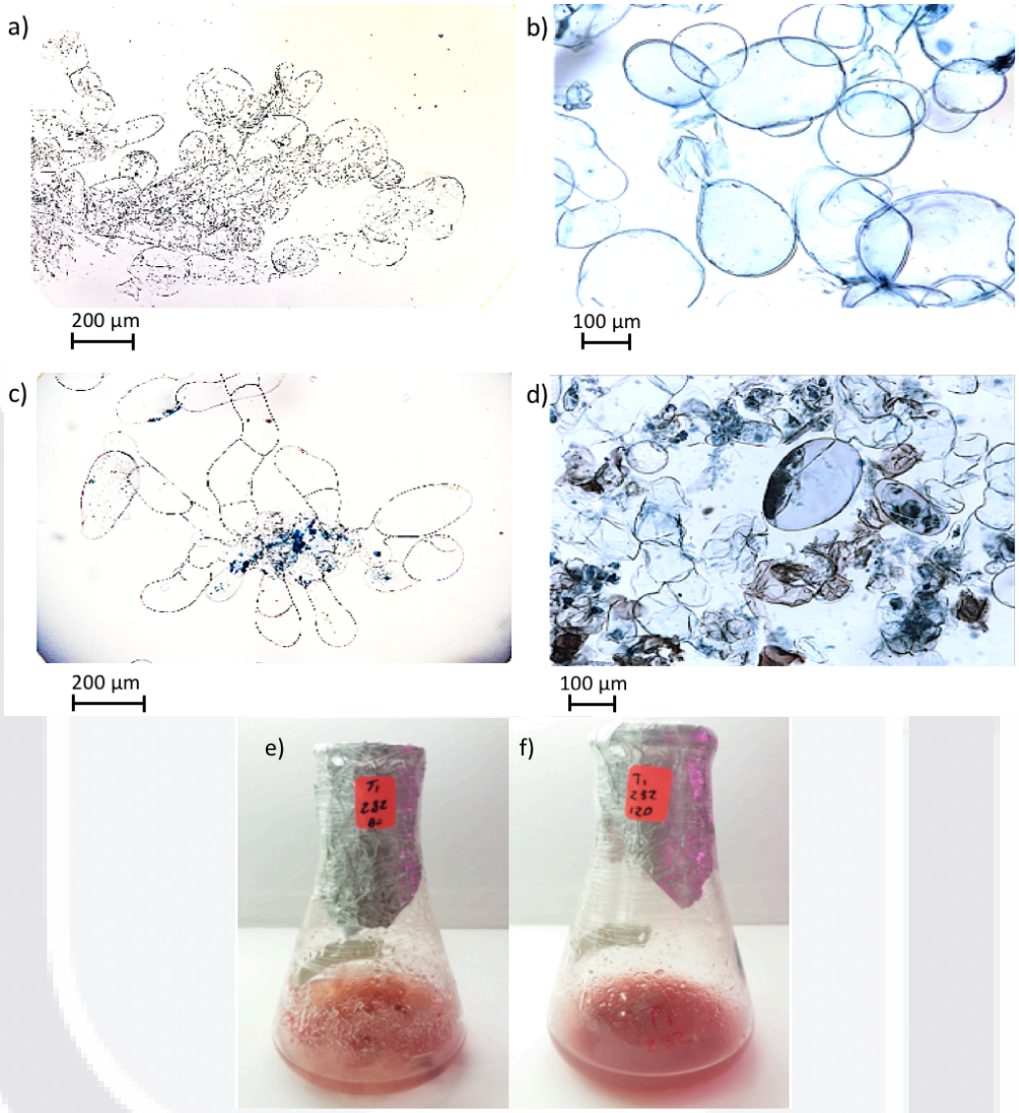


Figure V- 1. Morphological differences in *Coryphantha macromeris* cell suspension cultures growing under different conditions: a) callus culture (control); cell cultures cultivated at: b) 80 rpm; c) 100 rpm; d) 120 rpm.; e-f) general aspect of cells growing at 80 and 120 rpm, respectively. a and c taken from institutional thesis [21].

V.4.2. Phytochemical analysis of *C. macromeris* cell suspension cultures

Under the proposed chromatographic conditions, 49 metabolites were separated and 45 of them were tentatively identified by tandem mass spectrometry operated in high resolution mode. Among detected compounds, 16 metabolites are reported

here for the first time for *Coryphantha macromeris* (peaks 4, 6, 7, 10, 11, 17, 20, 24, 25, 26, 29, 30, 31, 33, 35 and 36; Table V- 2). On the other hand, compounds indicated with an asterisk (*) in Table V- 2 were assigned as we reported previously for *C. macromeris* plants growing under different conditions such as greenhouse (see Chapter II) *in vitro* (see Chapter III), or in callus cultures (see Chapter IV). Thus, this section focuses on the description of that 16 metabolites identified for the first time for *C. macromeris*.

Compound 4 was assigned as succinic acid since pseudomolecular ion at *m/z:* 117.01878 yielded one main fragment at *m/z:* 101.02361 generated due to the loss of water. Compound 6 was proposed as the phenolic glycoside leonuriside A due to the presence of one main fragment at *m/z*: 153.01900 (see figure V- 3a) and compound 7 as gardoside. For this metabolite, pseudomolecular ion yielded fragments at *m/z*: 211.06065, 195.06609 and 179.05550, which were consistent with the separation of the hexose moiety and the subsequent elimination of one and two molecules of water, respectively. On the other hand, compound 10 was assigned as a gallic acid derivative (butyl gallate), since characteristic fragment ions of gallic acid were detected [22] and peak 11 as catechin as reported previously [23]. Peak 17 was assigned as tyrolobibenzyl E (see Figure V- 3b) and peak 20 as acanthoside B since pseudomolecular ion yielded fragments at *m/z*: 417.15588 403.14023 which were consistent with the elimination of one hexose and the subsequent elimination of one methyl group, respectively.

Peaks 24, 31 and 25 were proposed the glycosylated flavonoids sakuranin and lantanoside, respectively. For sakuranin, two isomers were detected (peaks 24 and 31), and the pseudomolecular ion of these compounds generated three fragments at *m/z*: 285.07697, 269.08231 and 193.05005. Fragments at *m/z*: 285.07697 and 269.08231,

were generated due to the cleavage of the hexose moiety and the subsequent elimination of water, respectively, and fragment at *m/z*: 193.05005 due the subsequent separation C-C bond in the B ring of the basic flavonoid structure. For peak 25, fragments at *m/z*: 313.07199, 283.06635 and 267.06635 were detected. These fragments were generated due to the loss of one hexose and the subsequent elimination of water and methoxyl groups. In our investigation, one phenylethanoid glycoside was also detected (compound 26) and assigned as phlomisethanoside since pseudomolecular ion yielded fragments at 383.13522 ([M-H-benzoic acid-CH_3-OH]$^-$) and at *m/z*: 181.05020 ([$C_9H_9O_4$]$^-$), 135.04463 ([$C_8H_7O_2$]$^-$), and 121.02883 ([$C_7H_5O_2$]$^-$).

In addition to compounds 24, 25 and 31, other 5 metabolites were assigned as flavonoids (compounds 29, 30, 33, 35 and 36). Compound 29 was assigned as Aformosin, compound 30 as kaempferol 7-rhamnoside, compound 33 as liquiritigenin, compounds 35 and 36 as coumestrol and Irilone, respectively. The fragmentation pattern of these metabolites was characterized by the cleavage of methyl (Δ*m/z*: 15.0234) and hydroxyl (Δ*m/z:* 17.0027) groups and the cleavage of C-C bond in the C ring [24]. For glycosylated flavonoids, the separation of hexoses was observed. Table V- 2 shows the identification parameters of the detected compounds in *C. macromeris* cell suspension cultures.

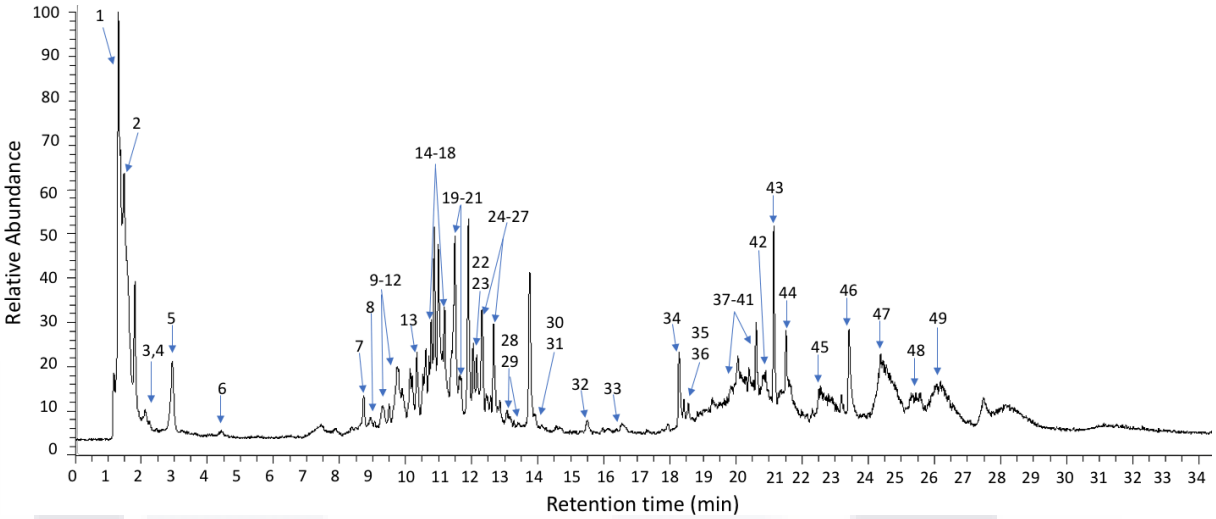
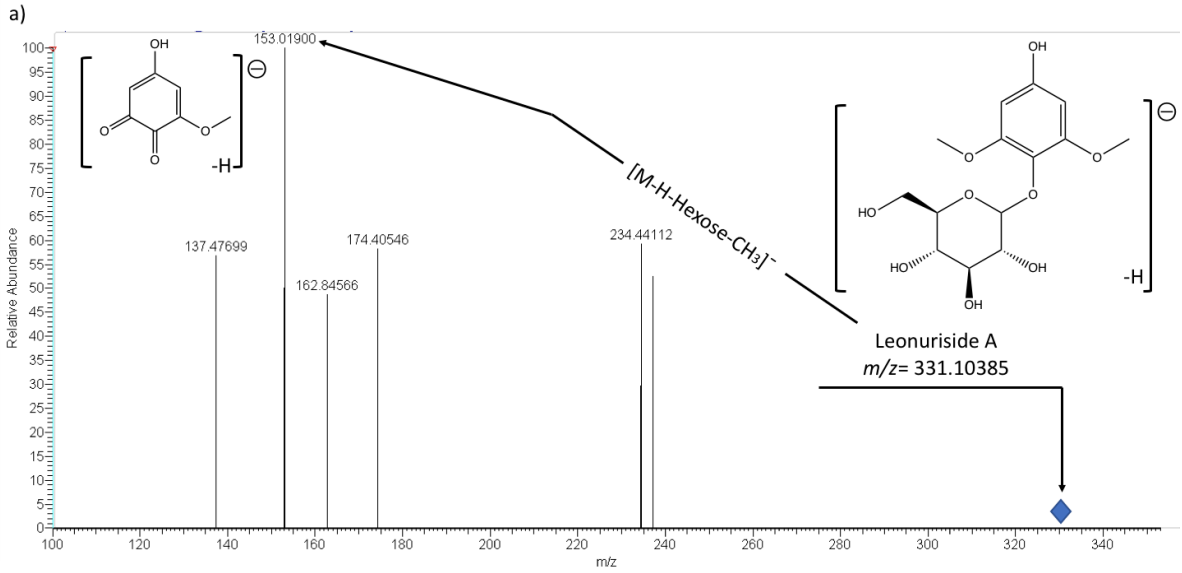


Figure V- 2. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with *Coryphantha macromeris* cell suspension cultures cultivated at 80 rpm in 125 mL flasks. Peak numbers refer to the metabolites indicated in Table V- 2.



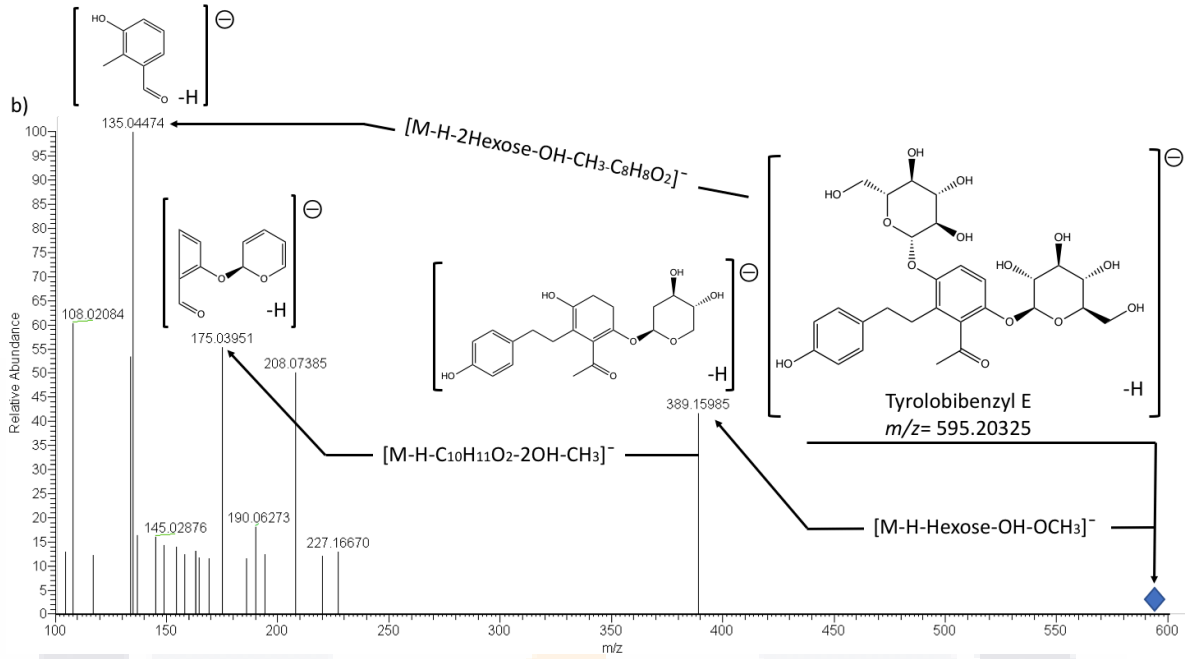


Figure V- 3. Proposed fragmentation pattern of peaks 6 (a) and 17 (b). Two metabolites identified for the first time for *Coryphantha macromeris.* Peak numbers refer to the compounds indicated in Table V- 2.

Table V- 2. Metabolites identified in cell suspension cultures of *Coryphantha macromeris* by ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS) data.

Peak	Retention time (min)	UV max (λ_{max})	Tentative identification	Elemental composition $[M-H]^-$	Theoretical mass (*m*/*z*)	Measured mass (*m*/*z*)	Accuracy (ppm)	MSn ions
1	1.33	214	Vaccihein A *	$C_{18}H_{17}O_9^-$	377.08781	377.086	4.80	347.07681 289.06912 125.02363
2	1.47	220, 274	2-Hydroxy-succinic acid (malic acid) *	$C_4H_5O_5^-$	133.0139	133.0137	1.50	115.00301
3	2.09	220, 265	2-hydroxy-4-methoxy-4-oxobutanoic acid (malic acid derivative) *	$C_5H_7O_5^-$	147.0299	147.02954	2.45	133.01381 115.00230 101.02362
4	2.14	220, 265	Succinic acid	$C_4H_5O_4^-$	117.01933	117.01878	4.70	101.02361
5	2.94	218, 274	Rhynchosporoside *	$C_{15}H_{27}O_{12}^-$	399.1508	399.15128	1.20	353.14581 325. 11478 309.11945 293.12369 279.10941
6	4.45	225, 274	Leonuriside A	$C_{14}H_{19}O_9^-$	331.10346	331.10385	1.18	153.01900
7	8.73	226, 282	Gardoside	$C_{16}H_{21}O_{10}^-$	373.11402	373.11392	0.27	211.06065 195.06609 179.05550
8	8.93	230, 282	Isopropylmalic acid *	$C_7H_{11}O_5^-$	175.0612	175.06097	1.31	145.05013 131.07072 115.03935 101.02354
9	9.3	230, 282	Caffeic acid 3-glucoside *	$C_{15}H_{17}O_9^-$	341.08781	341.08826	1.32	179.03453 135.04454 119.04953
10	9.5	232, 282	Gallic acid derivative (Butyl gallate)	$C_{11}H_{13}O_5^-$	-	225.07674	-	169.01331 125.02370
11	9.53	232, 282	Catechin	$C_{15}H_{13}O_6^-$	289.07176	289.07199	0.80	245.08217 205.05008 179.03450

12	9.9	233, 283	Feruloyl glucoside *	$C_{16}H_{19}O_9^-$	355.10346	355.10382	1.01	193.05035 157.05016 149.06020 135.04436 119.04961 109.02890
13	10.38	234, 285	b-D-Galactopyranoside, 6-hydroxyhexyl 6-O-b-D-galactopyranosyl *	$C_{18}H_{33}O_{12}^-$	441.19775	441.19827	1.18	407.19366 395.19290 217.10799 215.12865 135.04466 121.02882
14	10.61	234, 282	Piscidic acid derivative *	$C_{28}H_{37}O_{13}^-$	-	581.22412	-	193.05032 165.05527 135.04463 119.04950
15	10.71	234, 282	Piscidic acid derivative *	$C_{28}H_{37}O_{13}^-$	-	581.22388	-	193.05034 165.05521 135.04440 119.04974
16	11	231, 281	Piscidic acid derivative *	$C_{28}H_{37}O_{13}^-$	-	581.224	-	193.05026 165.05530 135.04474 119.04965
17	11.04	232, 282	Tyrolobibenzyl E	$C_{28}H_{35}O_{14}^-$	595.20323	595.20325	0.03	389.15985 175.03951 135.04472
18	11.19	235, 281	Piscidic acid derivative *	$C_{28}H_{37}O_{13}^-$	-	581.2244	-	193.05023 165.05508 135.04431 119.04958
19	11.4	235, 282	Magnoloside U *	$C_{30}H_{39}O_{14}^-$	623.23453	623.23431	0.35	389.16101 179.05579 135.04497
20	11.5	233, 278	Acanthoside B	$C_{28}H_{35}O_{13}^-$	579.20831	579.20819	0.21	417.15588 403.14023

21	11.68	241, 282	2-Isoferulic piscidic acid-1-metyl ester *, **	$C_{22}H_{21}O_{10}^-$	445.1141	445.1145	0.90	193.05029 135.04482 119.04971
22	12.16	241, 268	6-O-Acetylglycitin *	$C_{24}H_{23}O_{11}^-$	487.12459	487.12497	0.78	283.06131 135.04459 121.02879
23	12.19	243, 282	Azelaic acid *, **, ***	$C_9H_{15}O_4^-$	187.09758	187.09735	1.23	125.09654
24	12.31	241, 281	Sakuranin isomer	$C_{22}H_{23}O_{10}^-$	447.12967	447.1301	0.96	285.07697 269.08231 193.05005
25	12.34	241, 282	Lantanoside	$C_{25}H_{25}O_{12}^-$	517.13515	517.13544	0.56	313.07199 283.06635 267.06635
26	12.58	273	Phlomisethanoside	$C_{27}H_{33}O_{14}^-$	581.18758	581.18756	0.03	383.13522 181.05020 135.04463 121.02883
27	12.67	260	4',5,6-Trimethylscutellarein 7-glucoside *	$C_{24}H_{25}O_{11}^-$	489.14024	489.14066	0.86	297.07665 137.02371 135.04443
28	13.08	267	4',5,6-Trimethylscutellarein 7-glucoside isomer I *	$C_{24}H_{25}O_{11}^-$	489.14024	489.14056	0.65	137.02388 135.04440
29	13.42	227, 283	Afrormosin	$C_{17}H_{13}O_5^-$	297.07685	297.077	0.50	287.06650 283.06128 109.02867 177.05516
30	14.12	221, 284	Kaempferol 7-rhamnoside	$C_{21}H_{19}O_{10}^-$	431.09837	431.09879	0.97	285.04059 269.04538 135.04449
31	14.26	221, 284	Sakuranin isomer	$C_{22}H_{23}O_{10}^-$	447.12967	447.1301	0.96	285.07697 269.08231 193.05005
32	15.5	283	4,5,6-Trimethylscutellarein 7-glucoside isomer II *	$C_{24}H_{25}O_{11}^-$	489.14024	489.14056	0.65	135.04466
33	16.54	285	Liquiritigenin	$C_{15}H_{11}O_4^-$	255.06628	255.06633	0.20	137.02390 121.02879
34	18.28	283	Tianshic acid *, **, ***	$C_{18}H_{33}O_5^-$	329.23335	329.2337	1.06	165.12794

35	18.43	282	Coumestrol	$C_{15}H_7O_5^-$	267.0299	267.03	0.37	177.01904 161.02126
36	18.55	283	Irilone	$C_{16}H_9O_6^-$	297.04046	297.04062	0.54	269.04553
37	19.68	282	Plastoquinone 3 isomer I *,**	$C_{23}H_{31}O_2^-$	339.23296	339.23325	0.85	163.11252 149.06015 135.04449
38	19.84	282	Plastoquinone 3 isomer II *,**	$C_{23}H_{31}O_2^-$	339.23296	339.23331	1.03	163.11232 149.06003 135.04451
39	20.05	282	Plastoquinone 3 isomer III *,**	$C_{23}H_{31}O_2^-$	339.23296	339.23334	1.12	163.11235 135.04433
40	20.39	282	Plastoquinone 3 isomer IV *,**	$C_{23}H_{31}O_2^-$	339.23296	339.23334	1.12	163.11229 135.04457
41	20.61	282	Nordihydrocapsiate isomer *, **, ***	$C_{17}H_{25}O_4^-$	293.17583	293.17618	1.19	247.17038 141.12770
42	20.82	282	4-methyl-5-phenylpentanoate *,***	$C_{12}H_{15}O_2^-$	191.10775	191.10759	0.84	175.07578
43	21.14	282	Decyl gallate (Gallic acid derivative) *, ***	$C_{17}H_{25}O_5^-$	309.17075	309.17105	0.97	169.01410 153.01871 125.02354,
44	21.51	282	Nordihydrocapsiate isomer *, **, ***	$C_{17}H_{25}O_4^-$	293.17583	293.17624	1.40	247.17038
45	22	282	Unknown	$C_{18}H_{35}O_4^-$	-	315.25443		-
46	23.42	282	p-Hydroxynonanophenone *, **, ***	$C_{15}H_{21}O_2^-$	233.1547	233.15471	0.04	219.17551, 121.02884
47	24.37	282	Unknown*	$C_{14}H_{29}O_8^-$	-	325.18466	-	-
48	25.37	282	Unknown*	$C_{14}H_{29}O_8^-$	-	325.18463	-	-
49	26.06	282	Unknown*	$C_{14}H_{29}O_8^-$	-	325.18463	-	-

Metabolites previously identified in: **greenhouse plants (Chapter II); ****in vitro* plants (Chapter III); *callus culture (Chapter IV).

V.5. Conclusion

In our investigation, the effect of agitation velocity on cell viability and morphology of *C. macromeris* cell suspension cultures were evaluated. Based in our results, an agitation velocity of 80 or 100 rpm may be used for *C. macromeris* cell suspension cultures maintenance and growth. Also, 49 metabolites were separated and 45 of them were identified. Among detected compounds, several flavonoids and phenolic acids were identified. Our results contribute to the phytochemical knowledge of cacti species and offers the basis for future investigations regarding the production of secondary metabolites from cell suspension cultures of *C. macromeris* and related plant species.

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CONCLUSIONES GENERALES

1. Para los fines de este trabajo, se seleccionó el tratamiento con medio MS, como el mejor para la propagación *in vitro* de *C. macromeris*. Se requieren mayores estudios en orden de optimizar la producción de brotes.

2. La planta de *C. macromeris* cultivada *in vitro* produce diferentes tipos de compuestos. Las raíces presentan una mayor diversidad de metabolitos comparada con la sección aérea. Algunos de los compuestos identificados no habian sido reportados previamente para especies de cactáceas.

3. De los sistemas de cultivo analizados, las plantas de *C. macromeris* cultivadas en invernadero, así como los callos obtenidos de esta especie presentaron mayor diversidad de metabolitos. La cantidad de metabolitos presentes en ambos sistemas es similar. Sin embargo, el perfil de compuestos es variable. Algunos de los compuestos identificados en ambos sistemas poseen propiedades funcionales reconocidas.

4. Para el cultivo de células en suspensión de *C. macromeris*, la velocidad de agitación de 120 rpm provoca mayor deformación y lisis celular, por lo que se considera adecuado realizar el cultivo de células de esta especie a velocidades de 80 a 100 rpm en orden de garantizar una mayor viabilidad. Algunos de los metabolitos identificados en este sistema poseen propiedades funcionales reconocidas.

PERSPECTIVAS

- En el presente trabajo, se identificaron diferentes compuestos con potencial de uso para fines farmacéuticos; se recomienda realizar cuantificaciones en orden de evaluar el rendimiento en la producción de metabolitos seleccionados, así como su elicitación y analizar el efecto farmacológico de los extractos de *C. macromeris* sobre modelos biológicos.

- En algunos casos no se logró obtener la identidad de los metabolitos presentes en las diferentes muestras, ya que no existieron coincidencias con las evidencias existentes en la literatura. Esto sugiere que pudiera tratarse de nuevos compuestos. Se recomienda realizar mayores análisis en orden de dilucidar la estructura de esos metabolitos y sus posibles aplicaciones.

- Se demostró que las células de *C. macromeris* cultivadas en medio liquido son sensibles al estrés hidrodinámico, pero al mismo tiempo poseen la capacidad de biosintentizar compuestos con propiedades funcionales reportadas. Se recomienda analizar los rendimientos en la producción de compuestos, realizar análisis biológicos y analizar las condiciones para realizar en escalamiento a nivel biorreactor.

- Se debe analizar el perfil de metabolitos presentes en las muestras de *C. potosiana* (ver anexo 4). Así como sus propiedades funcionales.

MATERIAL ANEXO

Anexo 1. Inducción de brotes en diferentes especies del género *Coryphantha*

Para los tratamientos de inducción de brotes y descripción de los métodos utilizados, favor de referirse al Capítulo III.

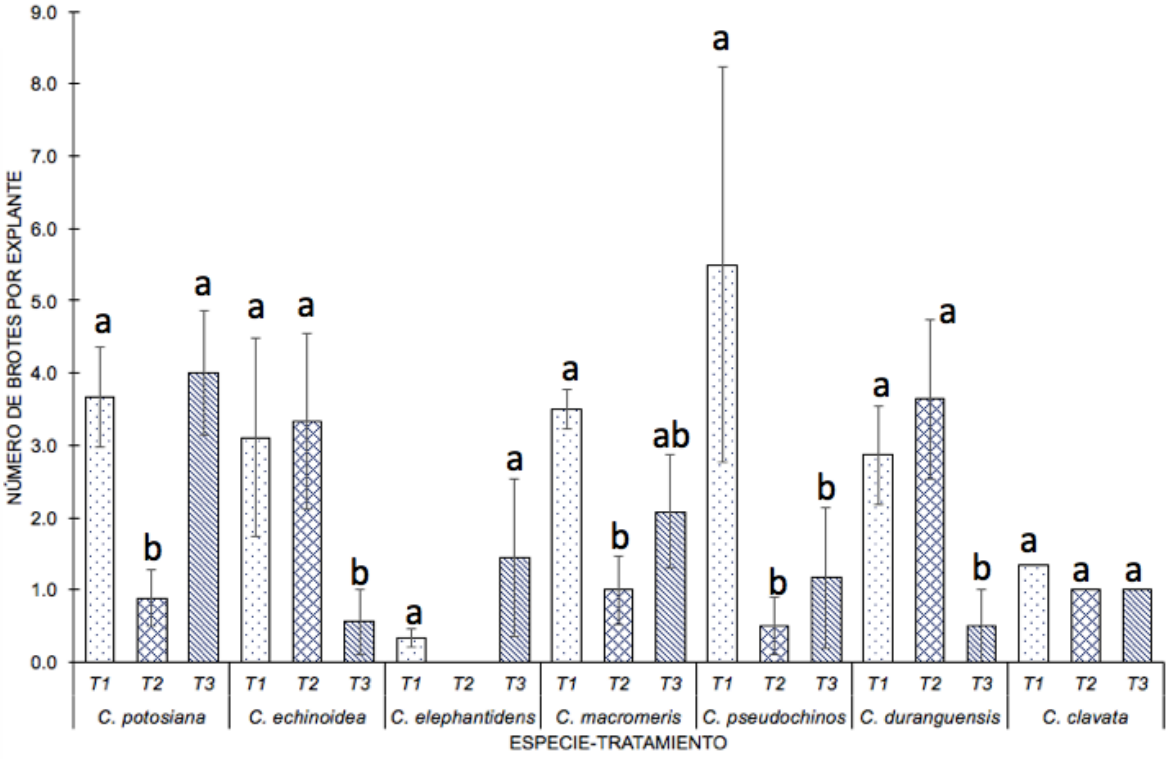

Figura A1-1. Número de brotes producidos por explante en las diferentes especies de *Coryphantha*. Los resultados representan el promedio (n=3 frascos, con 4 explantes cada uno). T1: Medio MS; T2: Medio MS+BA (4.4 µM) + NAA (0.054 µM); T3: Medio MS + carbón activado (1 g L-1). Las barras la desviación estándar. Letras iguales dentro de cada especie indican que no hay diferencias significativas (p≤ 5%).

Anexo 2. Generación de raíces transformadas de *Coryphantha macromeris* y *C. potosiana*

Materiales y métodos

Para la transformación genética se utilizó la cepa bacteriana de *Agrobacterium rhizogenes* (A4), misma que contiene el plasmido pRiA4 y el vector binario pESC4. Para el cultivo de la bacteria se utilizó medio YM enriquecido con rifampicina (50 mg L^{-1}) y la bacteria inoculada se incubó por 72 horas a 25 °C.

Para realizar el cocultivo para la obtención de raíces transformadas, se tomaron 5 mL del cultivo bacteriano y se resuspendieron en 45 mL de medio MS liquido enriquecido con 3% de sacarosa, acetosiringona (1.11 mM) y antioxidantes (100 mg L^{-1} de ácido cítrico y 100 mg L^{-1} de ácido ascórbico). Se realizaron cortes transversales (5-10 mm) de *C. macromeris* y *C. potosiana* y se pusieron en contacto con el medio durante 45 minutos. Una vez transcurrido este tiempo, se retiraron los explantes y el exceso de humedad fue absorbido con una gasa estéril. Cada explante (n=6) fue transferido a cajas Petri (n=5) que contenían medio MS sólido, enriquecido con 3% de sacarosa y 8 g L^{-1} de agar y se incubaron durante 72 horas a 25 °C en condiciones de oscuridad. Posteriormente los explantes fueron retirados y resuspendidos durante 30 minutos en medio MS líquido enriquecido con 30 g L^{-1} de sacarosa y 500 mg L^{-1} de Claforán; una vez transcurrido este tiempo cada explante se transfirió a medio sólido (8 g L^{-1} de agar) enriquecido con 30 g L^{-1} de sacarosa, 50 mg L^{-1} de kanamicina y 250 mg L^{-1} de claforán y se incubó durante 45 días a 25 °C en condiciones de oscuridad y se monitoreó el porcentaje de formación de raíces después de este periodo. Las raíces generadas se transfirieron a medio MS líquido y se incubaron a 25 °C en condiciones de oscuridad, a una velocidad de agitación de 80 rpm.

Resultados

Para el tratamiento de transformación genética se encontró que *C. macromeris* produce una mayor cantidad de raíces en comparación con *C. potosiana* (Cuadro A2-1); las características físicas de las raíces generadas por ambas especies son similares y tienen aspecto piloso (Figura A2-1). Las raíces generadas se transfirieron a medio MS líquido; no se mostró un incremento en la cantidad de biomasa después de 3 meses de cultivo (Figura A2-2).

Cuadro A2-1. Porcentaje de inducción de raíces en *Coryphantha macromeris* y *C. potosiana*.

Especie	Inducción (%)
C. macromeris	46
C. potosiana	8

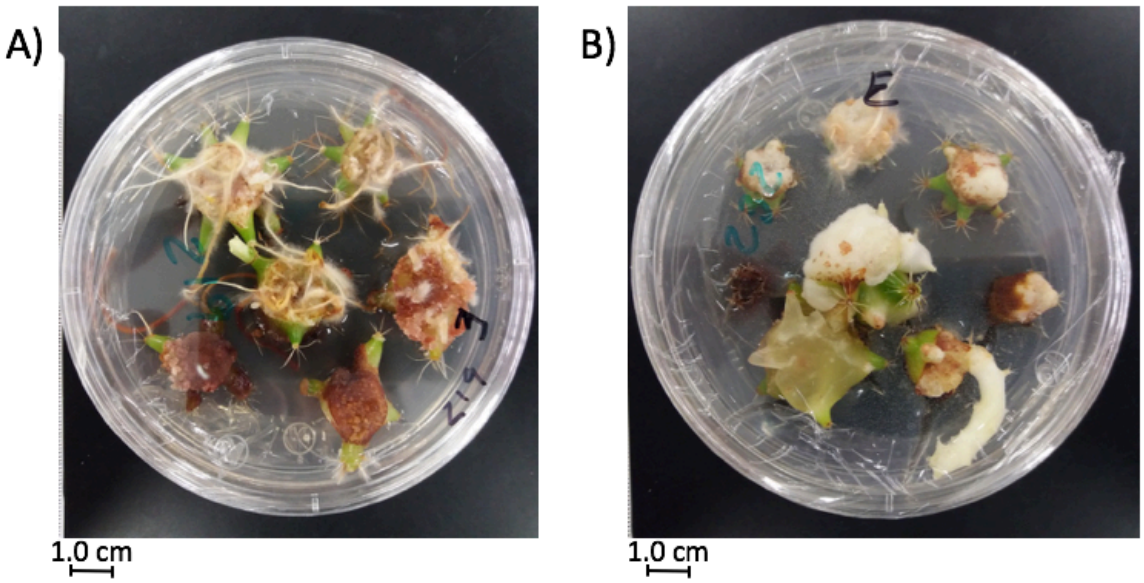

Figura A2-1. Raíces generadas durante el proceso de transformación genética. A) *C. macromeris*; B) *C. potosiana.*

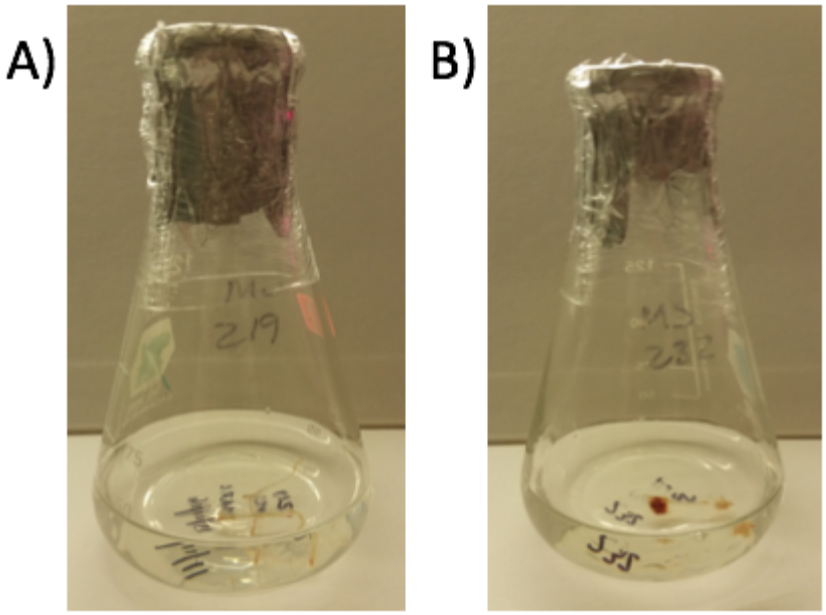

Figura A2-2. Raíces presuntamente transformadas transferidas a medio líquido. A) *C. macromeris*; B) *C. potosiana.*

Anexo 3. Perfil de alcaloides en plantas de *Coryphantha macromeris*.

A.3.1. Perfil de alcaloides presentes en la sección aérea de *Coryphantha macromeris* cultivada en sistema *in vitro*.

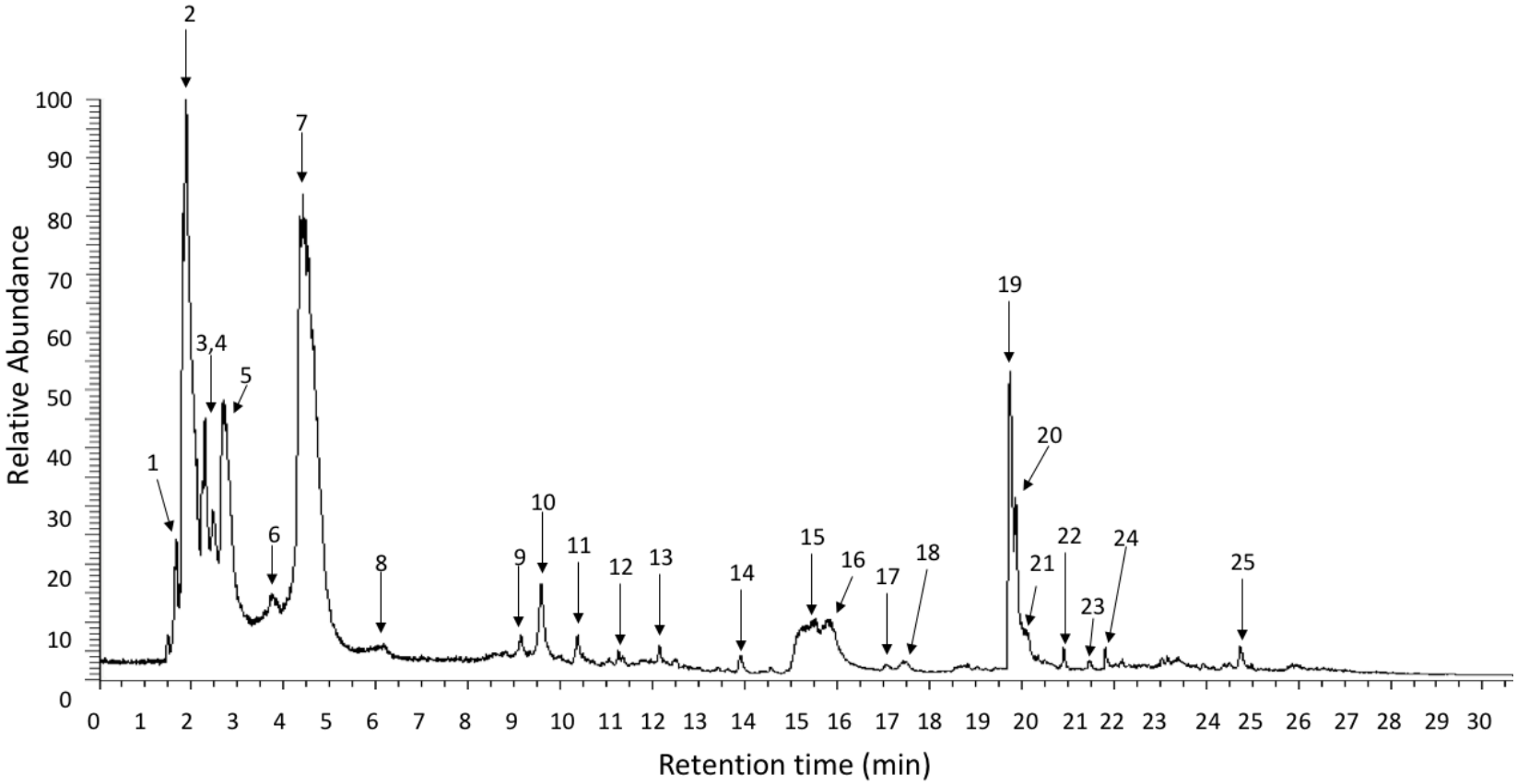

Figura A3-1. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with aerial parts of *Coryphantha macromeris* (*in vitro* plants).

Cuadro A3-1. Alkaloids identified in aerial parts of *Coryphantha macromeris* (*in vitro* plants) by ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS) data using HESI in positive ion mode.

Peak	Retention time (min)	UV max (λ_{max})	Tentative identification	Elemental composition $[M+H]^+$	Theoretical mass (m/z)	Measured mass (m/z)	Accuracy (ppm)	MSn ions
1	1.68	215, 272	Unknown	$C_{20}H_{15}NO_7^+$	-	381.08340	-	-
2	1.87	220, 276	N-Methyltiramine	$C_9H_{14}NO^+$	152.10699	152.10728	-1.91	121.06520 118.06561 103.05464
3	2.28	222, 275	N-Methylphenylalanine	$C_{10}H_{14}NO_2^+$	180.10191	180.10245	3.00	148.07608 120.08121 107.04957
4	2.45	220, 276	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11787	1.60	121.06506 152.10736 118.06570 148.07605
5	2.7	220, 275	N-Methyltiramine isomer	$C_9H_{14}NO^+$	152.10699	152.10736	2.43	121.06514 118.06551 103.05461
6	3.73	205, 226, 276	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11819	3.25	121.06504 152.10736 118.08657 148.07605
7	4.41	205, 228, 278	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11816	3.09	121.06512 152.10744 118.08670 148.07619
8	6.16	205, 226, 278	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11818	3.19	121.06511 152.10742 118.08670 148.07614
9	9.14	222, 275	1,2-Ethanediol, 1-(3-ethenylphenyl)	$C_{10}H_{13}O_2^+$	165.09101	165.09151	3.03	135.08086 121.06520

								107.04955 135.04448
10	9.59	224, 276	Hordenine	$C_{10}H_{15}NO^{+}$	166.12264	166.12312	2.89	152.10699 105.07029 103.05458
11	10.38	223, 275	Maclurin	$C_{13}H_{11}O_{6}^{+}$	263.05501	263.05426	2.85	111.04456 127.03951 123.04430
12	11.26	237, 327	Unknown	-	-	177.05531	-	-
13	12.15	238, 324	6-Phenyl-11-aza-6a-azonia-benzo[a]fluorene-2-ol	$C_{21}H_{15}N_{2}O+$	312.12517	312.12552	1.12	238.10825 222.11336 146.06052
14	13.91	255, 308	Unknown	-	-	314.14124	-	-
15	15.47	221, 284	M-Toluic acid	$C_{8}H_{9}O_{2}^{+}$	137.05971	137.06015	3.21	107.04952 121.06512 109.06516
16	15.82	221, 283	M-Toluic acid	$C_{8}H_{9}O_{2}^{+}$	137.05971	137.06013	3.06	107.04951 121.06517 109.06547
17	17.08	221, 286	M-Toluic acid	$C_{8}H_{9}O_{2}^{+}$	137.05971	137.06015	3.21	107.04962 121.06518 109.06514
18	17.45	285	Pisceín	$C_{14}H_{19}O_{7}^{+}$	299.11253	299.11237	0.53	137.06013 121.06530 107.04910
19	19.75	283	Lauryldiethanolamine	$C_{16}H_{36}NO_{2}^{+}$	274.27406	274.27557	5.51	256.26431 230.24864 102.09165
20	19.86	283	M-Toluic acid derivative			230.24869	-	137.06015
21	20.09	276	4-Amino-2-dodecyltetrahydro-3-furanol	$C_{16}H_{34}NO_{2}^{+}$	272.25841	272.26004	5.99	254.24910 137.06006 240.23325
22	20.91	283	M-Toluic acid derivative	-	-	387.18469	-	137.06001 121.06515

23	21.48	284	Methyl-3-methyl-2-(phenoxycarbonylamino)butanoate	$C_{13}H_{18}NO_4^+$	252.12303	252.12433	5.16	196.06097 178.05022 150.05534
24	21.82	284	Unknown	-	-	415.21704	-	-
25	24.74	285	Unknown	-	-	399.25565	-	-

A.3.1.1. Perfil de alcaloides presentes en la sección radicular de *C. macromeris* cultivada en sistema *in vitro*.

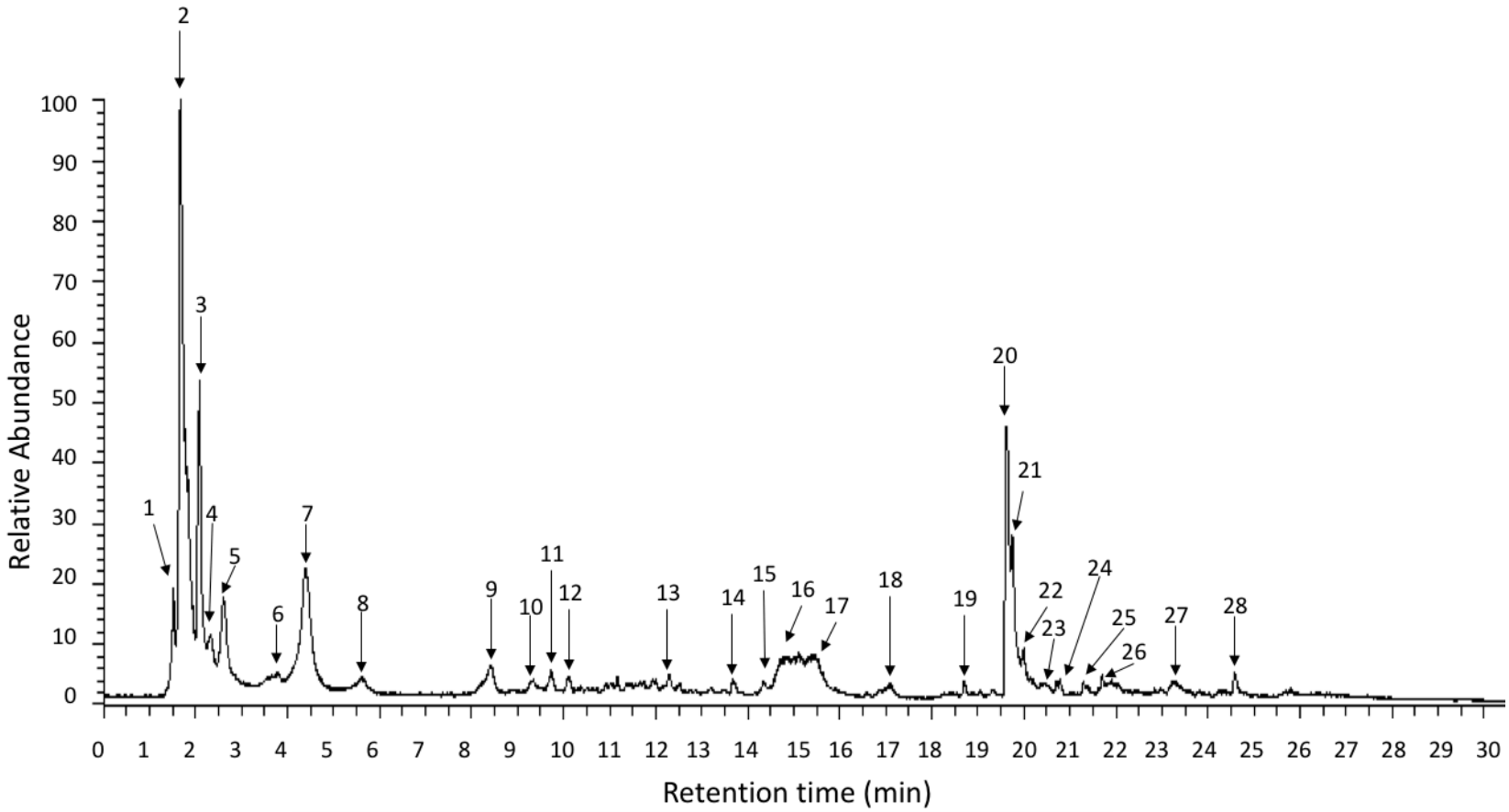

Figura A3-2. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with radicular parts of *Coryphantha macromeris* (*in vitro* plants).

Cuadro A3-2. Alkaloids identified in radicular parts of *Coryphantha macromeris* (*in vitro* plants) by ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS) data using HESI in positive ion mode.

Peak	Retention time (min)	UV max (λ_{max})	Tentative identification	Elemental composition $[M+H]^+$	Theoretical mass (m/z)	Measured mass (m/z)	Accuracy (ppm)	MSn ions
1	1.52		Unknown*	$C_{20}H_{15}NO_7^+$		381.08380	-	-
2	1.68		N-Methyltiramine derivative	$C_9H_{12}NO^+$	-	150.09177	-	121.06516 118.06556 103.05470
3	2.1		N-Methyltiramine derivative isomer	$C_9H_{12}NO^+$	-	150.0918	-	121.06516 118.06556 103.05470
4	2.32		(3-ethenylphenol) N-Methyltiramine derivative	$C_8H_9O^+$	121.06479	121.06529	4.13	118.6554 103.05466
5	2.6		N-Methyltiramine*	$C_9H_{14}NO^+$	152.10699	152.10739	2.63	121.06523 118.06554 103.05465
6	3.78		3,4-Methylenedioxymethamphetamine*	$C_{11}H_{16}NO_2^+$	194.11756	194.11826	3.61	121.06528 152.10744 118.08667 148.07614
7	4.41		3,4-Methylenedioxymethamphetamine*	$C_{11}H_{16}NO_2^+$	194.11756	194.11818	3.19	121.06517 152.10735 118.08655 148.07610
8	5.62		N-Methylphenylalanine derivative	$C_{10}H_{14}NO^+$	-	164.10757	-	148.07593 120.08100 107.04950
9	8.39		N-Allylphthalimide	$C_{11}H_{10}NO_2^+$	188.0706	188.07123	3.35	150.09134 118.06557 107.04945
10	9.35		Unknown	$C_{13}H_{24}NO_3^+$	-	243.18417	-	

11	9.73	N-Allylphthalimide isomer	$C_{11}H_{10}NO_2^+$	188.0706	188.07126	3.51	150.09175 118.06551 107.04957
12	10.1	Naphthalen-1-amine	$C_{10}H_{10}N^+$	144.08078	144.08122	3.05	120.08112 118.06548
13	12.27	1,5-diphenyl-3,7-di(pyridin-2-yl) pyrazolo indazole-4,8-dione	$C_{30}H_{19}O_{2N6}^+$	495.1564	495.15656	0.32	-
14	13.69	Unknown*	-	-	314.14124	-	-
15	14.34	Unknown	-	-	344.15259	-	-
16	14.75	M-Toluic acid*	$C_8H_9O_2^+$	137.05971	137.0601	2.85	107.04958 121.06522 109.06519
17	15.42	M-Toluic acid*	$C_8H_9O_2^+$	137.05971	137.06009	2.77	107.04951 121.06519 109.06519
18	17.09	Pisceín*	$C_{14}H_{19}O_7^+$	299.11253	299.11237	0.53	137.06015 121.06521 107.04968
19	18.7	M-Toluic acid derivative	-	-	353.23343	-	137.06015 121.06541
20	19.62	Lauryldiethanolamine	$C_{16}H_{36}NO_2^+$	274.27406	274.27563	5.72	256.26459 230.24864 102.09173
21	19.74	M-Toluic acid derivative	-	-	230.24869		137.06005 121.06506
22	19.99	4-Amino-2-dodecyltetrahydro-3-furanol	$C_{16}H_{34}NO_2^+$	272.25841	272.26007	6.10	254.24910 137.06010 240.23355
23	20.41	Lauryldiethanolamine derivative	$C_{16}H_{34}NO_3^+$		288.25543	-	274.27582 230.24890 102.09188
24	20.75	Lauryldiethanolamine derivative	$C_6H_{17}N4O_9^+$		289.09921	-	274.27567 230.24881 102.09185

25	21.33	Methyl-3-methyl-2-(phenoxycarbonylamino)butanoate	$C_{13}H_{18}NO_4^+$	252.12303	252.12437	5.31	196.06102 178.05037 150.05536
26	21.7	Unknown	-	-	258.28049	-	-
27	23.23	Unknown	-	-	550.4588		
28	24.57	Unknown	-	-	399.25571	-	-

A.3.2. Perfil de alcaloides presentes en la sección aérea de *Coryphantha macromeris* cultivada en invernadero.

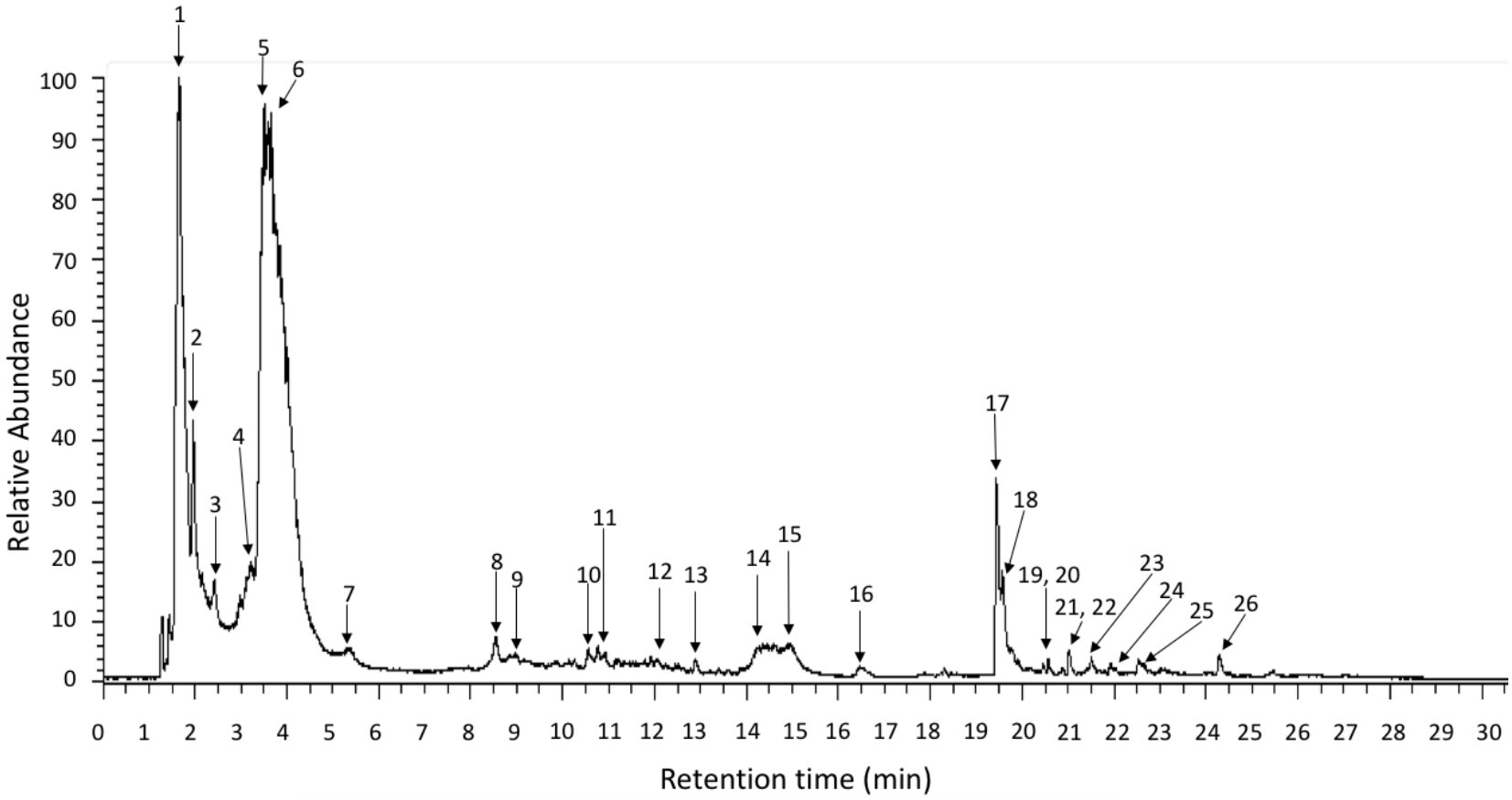

Figura A3-3. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with aerial parts of *Coryphantha macromeris* (greenhouse plants).

Cuadro A3-3. Alkaloids identified in aerial parts of *Coryphantha macromeris* (greenhouse plants) by ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS) data using HESI in positive ion mode.

Peak	Retention time (min)	UV max (λ_{max})	Tentative identification	Elemental composition $[M+H]^+$	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Accuracy (ppm)	MSn ions
1	1.67	225, 277	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11795	2.01	152.10718 148.07596 121.06513 118.06546
2	1.97	220, 277	N-Methylphenylalanine	$C_{10}H_{14}NO_2^+$	180.10191	180.10252	3.39	148.07611 120.08121 107.04962
3	2.42	221, 276	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11827	3.66	152.10748 148.07616 121.06530 118.06545
4	3.2	220, 275	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11818	3.19	152.10745 148.07608 121.06520 118.06560
5	3.51	220, 275	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11819	3.25	148.07607 121.06514 118.06550
6	3.65	220, 276	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11819	3.25	148.07607 121.06526 118.06551
7	5.34	220, 277	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11824	3.50	148.07617 121.06522 118.06552

8	8.55	219, 276	1,2-Ethanediol, 1-(3-ethenylphenyl)	$C_{10}H_{13}O_2^+$	165.09101	165.09148	2.85	135.08086 121.06512 107.04951 135.04440
9	8.98	220, 275	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11819	3.25	148.07611 121.06509 118.06541
10	10.56	232, 284	3,4-Dihydro-8-hydroxy-6,7- dimethoxy-1-methylisoquinoline	$C_{12}H_{15}NO_3^+$	222.11247	222.11336	4.01	148.07616 130.06552
11	10.77	235, 327	Unknown			395.10004	-	-
12	12.07	238, 284	1,5-diphenyl-3,7-di(pyridin-2-yl) pyrazolo indazole-4,8-dione	$C_{30}H_{19}O_{2N6}^+$	495.1564	495.15656	0.32	-
13	12.90	260	Unknown	-	-	253.10837	-	-
14	14.29	221, 284	M-Toluic acid	$C_8H_9O_2^+$	137.05971	137.06009	2.77	107.04950 121.06521 109.06549
15	14.94	221, 284	M-Toluic acid	$C_8H_9O_2^+$	137.05971	137.06012	2.99	107.04951 121.06528 109.06546
16	16.5	284	Pisceín	$C_{14}H_{19}O_7^+$	299.11253	299.1124	0.43	137.06013 121.06514 107.04955
17	19.44	282	Lauryldiethanolamine	$C_{16}H_{36}NO_2^+$	274.27406	274.2757	5.98	256.26462 230.24869 102.09187
18	19.56	282	M-Toluic acid derivative	-	-	230.24879	-	137.06015
19	20.46	284	Lauryldiethanolamine derivative	$C_6H_{17}N4O_9^+$	-	289.09921	-	274.27567 230.24878
20	20.56	283	Lauryldiethanolamine derivative	-	-	387.18494	-	137.06005 121.06512
21	20.86	283	1-Oxo-2-phenyl-phenalen-3-yl benzoate	$C_{26}H_{17}O_3^+$	377.11722	377.1178	1.54	123.04075

22	21.03	266	Alibendol	$C_{13}H_{18}NO_4^+$	252.12303	252.12439	5.39	193.08658 196.06108 178.04987 150.05534
23	21.5	284	Unknown	-	-	415.21753	-	-
24	21.94	284	Unknown	-	-	333.12656	-	-
25	22.54	284	Unknown	-	-	487.29688	-	-
26	24.29	284	Unknown	-	-	399.25586	-	-

A.3.2.1. Perfil de alcaloides presentes en la sección radicular de *Coryphantha macromeris* cultivada en invernadero.

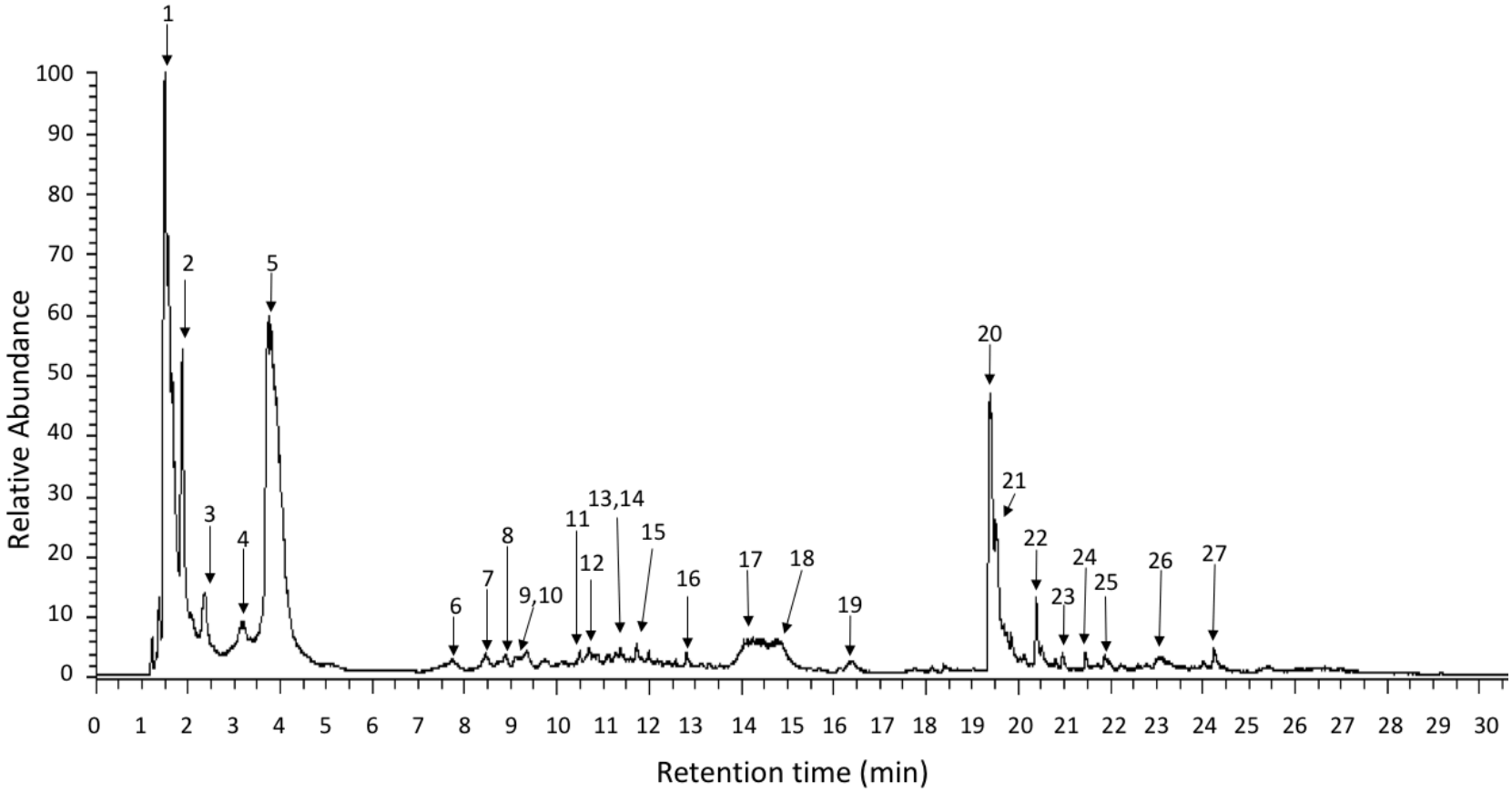

Figura A3-4. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with radicular parts of *Coryphantha macromeris* (greenhouse plants).

Cuadro A3-4. Alkaloids identified in radicular parts of *Coryphantha macromeris* (greenhouse plants) by ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS) data using HESI in positive ion mode.

Peak	Retention time (min)	UV max (λ_{max})	Tentative identification	Elemental composition $[M+H]^+$	Theoretical mass (*m*/*z*)	Measured mass (*m*/*z*)	Accuracy (ppm)	MSn ions
1	1.51	221, 274	N-Methyltiramine derivative	$C_9H_{12}NO^+$	-	150.09175	-	121.06510 118.06573 103.05462
2	1.88	225, 274	N-Methyltiramine derivative	$C_9H_{12}NO^+$	-	150.0918	-	121.06517 118.06550 103.05457
3	2.36	220, 276	N-Methyltiramine	$C_9H_{14}NO^+$	152.10699	152.10742	2.83	121.06519 118.06563 103.05462
4	3.16	226, 276	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11821	3.35	121.06516 152.10741 118.08663 148.07611
5	3.75	228, 277	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11823	3.45	121.06512 152.10734 118.08645 148.07605
6	7.76	225, 279	N-Methyltiramine derivative	$C_{11}H_{10}NO_2^+$	-	188.0713	-	150.09181 121.06519 118.06558 103.05456
7	8.47	230, 279	1,2-Ethanediol, 1-(3-ethenylphenyl)	$C_{10}H_{13}O_2^+$	165.09101	165.09161	3.63	135.08086 121.06522 107.04957 135.04456

8	8.82	224, 279	3,4-Methylenedioxymethamphetamine	$C_{11}H_{16}NO_2^+$	194.11756	194.11839	4.28	121.06503 152.10739 118.08645 148.07612
9	9.13	256	Unknown		-	243.18423	-	-
10	9.35	221, 279	N-Allylphthalimide	$C_{11}H_{10}NO_2^+$	188.0706	188.07132	3.83	150.09183 118.06558 107.04964
11	10.48	234, 277	3,4-Dihydro-8-hydroxy-6,7- dimethoxy-1-methylisoquinoline	$C_{12}H_{15}NO_3^+$	222.11247	222.11337	4.05	148.07611 130.06554
12	10.67	236, 326	Unknown	-	-	213.06676	-	-
13	11.38	228, 283	Unknown	-	-	556.24213	-	-
14	11.72	227, 282	Unknown	-	-	358.15884	-	-
15	11.99	234, 277	1,5-diphenyl-3,7-di(pyridin-2-yl) pyrazolo 3,4-indazole-4,8-dione	$C_{30}H_{19}O_2N_6^+$	495.1564	495.15695	1.11	-
16	12.82	259	Unknown	-	-	253.10838	-	-
17	14.26	221, 284	M-Toluic acid	$C_8H_9O_2^+$	137.05971	137.06013	3.06	107.04949 121.06505 109.06507
18	14.74	221, 283	M-Toluic acid	$C_8H_9O_2^+$	137.05971	137.0601	2.85	107.04951 121.06518 109.06513
19	16.39	283	Pisceín	$C_{14}H_{19}O_7^+$	299.11253	299.11234	0.64	137.06012 121.06523 107.04951
20	19.4	275	Lauryldiethanolamine	$C_{16}H_{36}NO_2^+$	274.27406	274.2757	5.98	256.26443 230.24867 102.09177
21	19.51	282	M-Toluic acid derivative	-	-	230.24876	-	137.06013 121.06499

22	20.39	243, 291	M-Toluic acid derivative	-	-	289.09909	-	137.06009 121.06517
23	20.95	274	methyl-3-methyl-2-(phenoxycarbonylamino)butanoate	$C_{13}H_{18}NO_4^+$	252.12303	252.12439	5.39	196.06100 178.05042, 150.05533
24	21.46	282	Unknown	-	-	415.21716	-	-
25	21.9	285	Unknown	-	-	333.1264	-	-
26	23.07	278	Unknown	-	-	550.45911	-	-
27	24.24	283	Unknown	-	-	399.25577	-	-

Anexo 4. Análisis realizados con *Coryphantha potosiana* para la separación e identificación de metabolitos de naturaleza fenólica

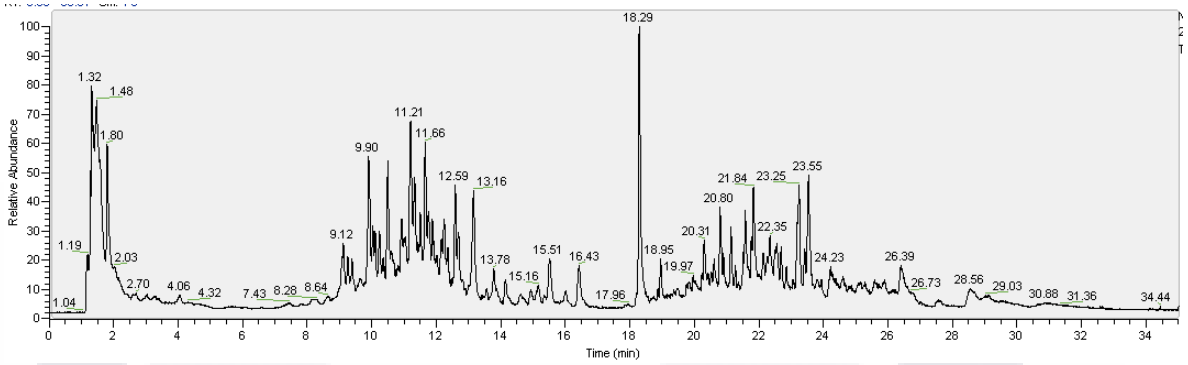

Figura A4-1. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with aerial parts of *Coryphantha potosiana* (*in vitro* plants).

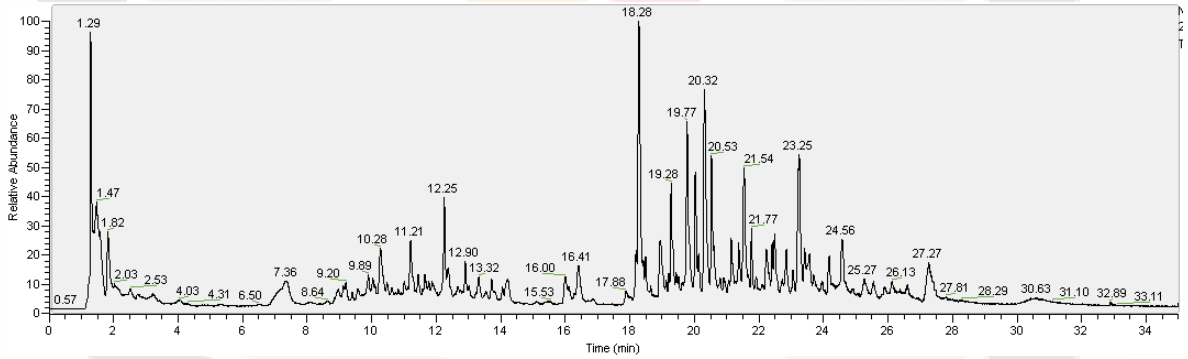

Figura A4-2. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with radicular parts of *Coryphantha potosiana* (*in vitro* plants).

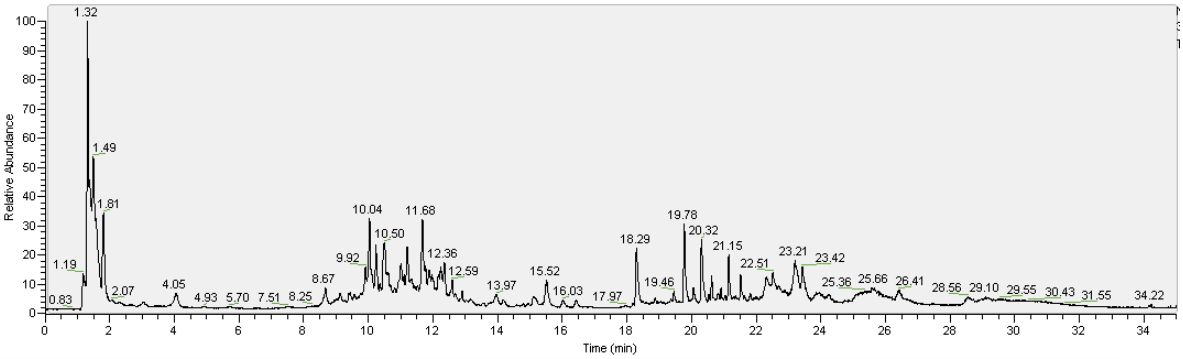

Figura A4-3. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with aerial parts of *Coryphantha potosiana* (*greenhouse* plants).

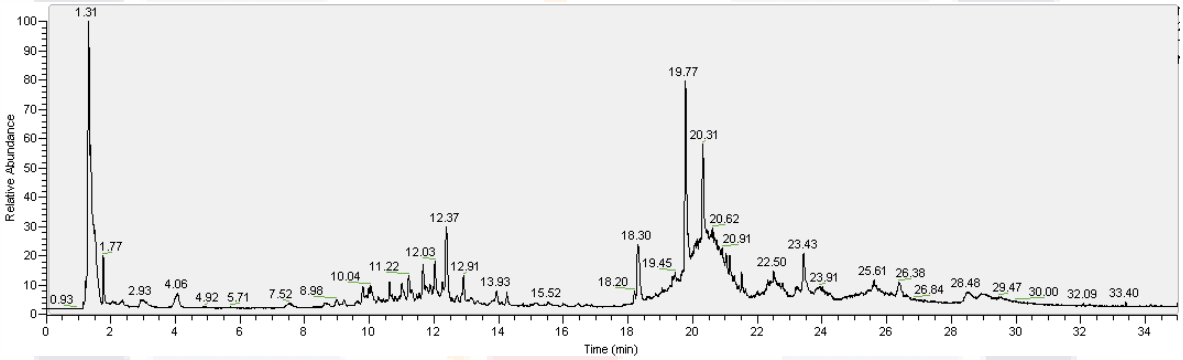

Figura A4-4. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with radicular parts of *Coryphantha potosiana* (*greenhouse* plants).

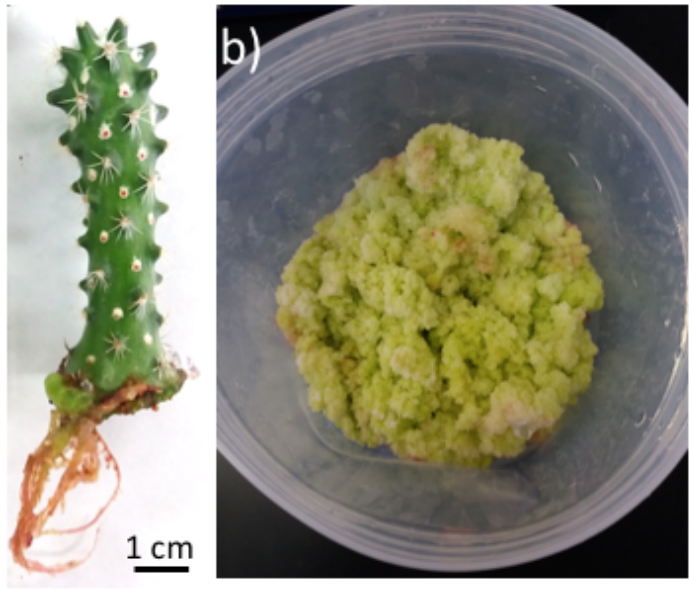

Figura A4-5. *In vitro* culture of *Coryphantha potosiana.* (a) Plant growing *in vitro* and collected after 3 months of growth; (b) callus growth after 9 weeks of culture in C1775 culture vessels (PhytoTechnology Laboratories®) with 100 mL of medium.

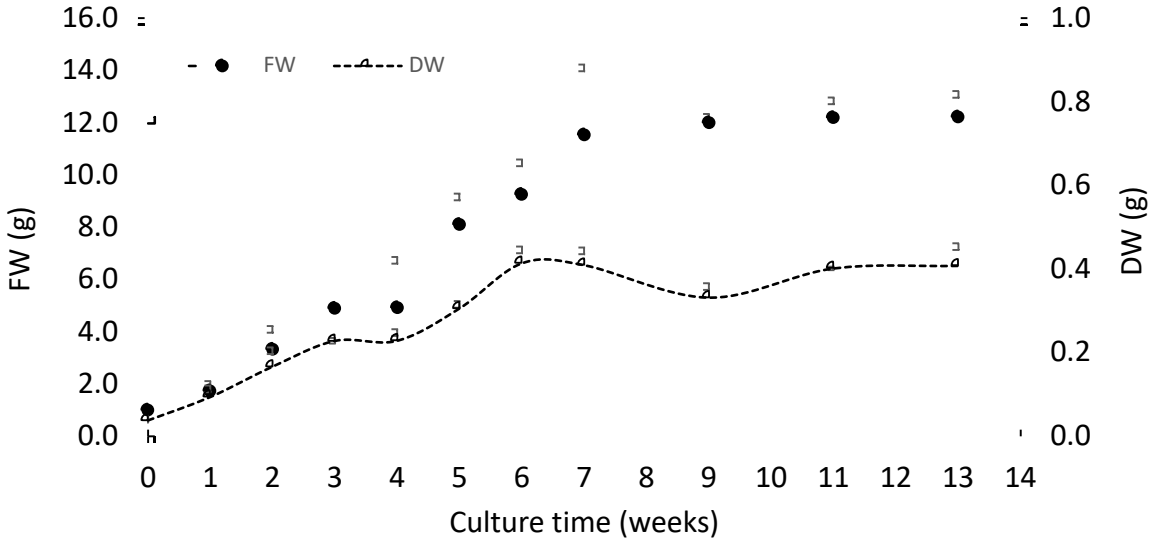

Figura A4-6. Growth kinetics of callus cultures of *Coryphantha potosiana* originated from stems discs and growing in MS basal medium supplemented with BAP (2.2 µM) and Picloram (4.14 µM). FW: fresh weight; DW: dry weight. Each point represents the average (n=3) and error bars the standard error.

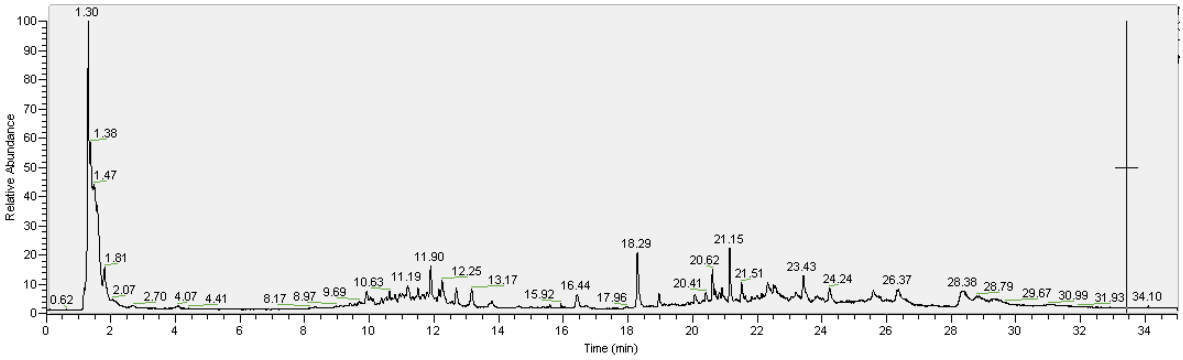

Figura A4-7. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with callus cultures of *Coryphantha potosiana.* Biomass was collected after 7 weeks of culture.

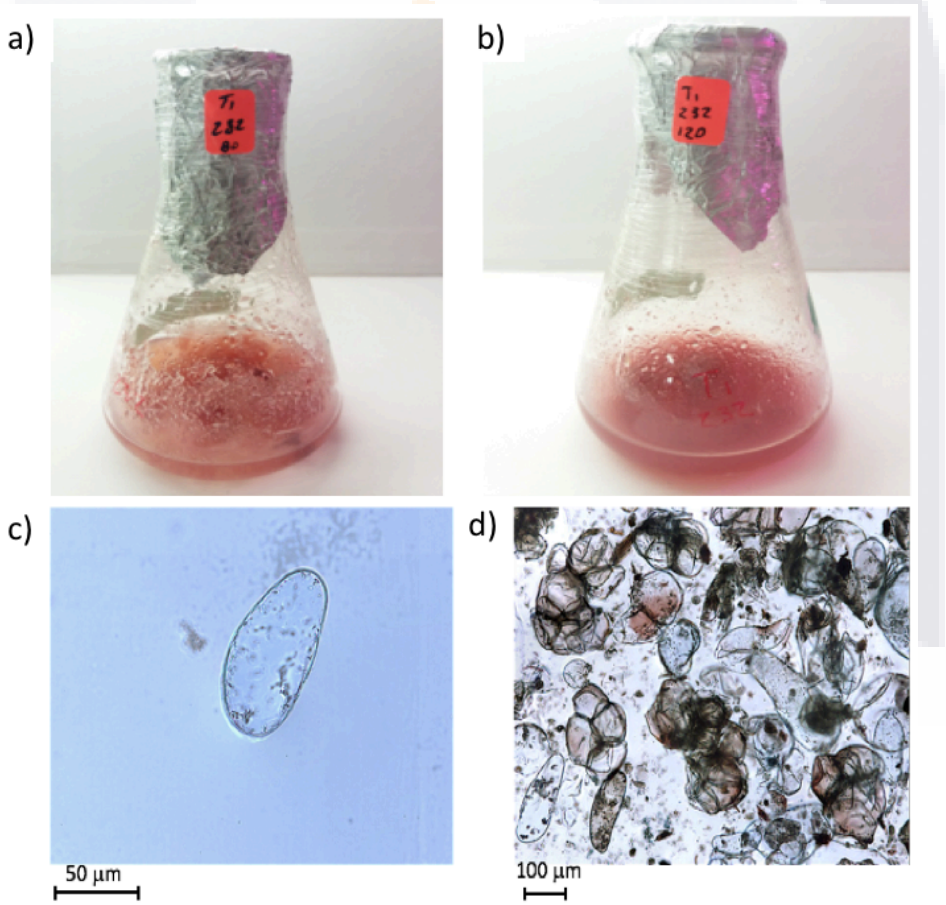

Figura A4-8. Morphological differences in *Coryphantha potosiana* cell suspension cultures growing under different conditions: a and c: 80 rpm; b and d: 120 rpm.

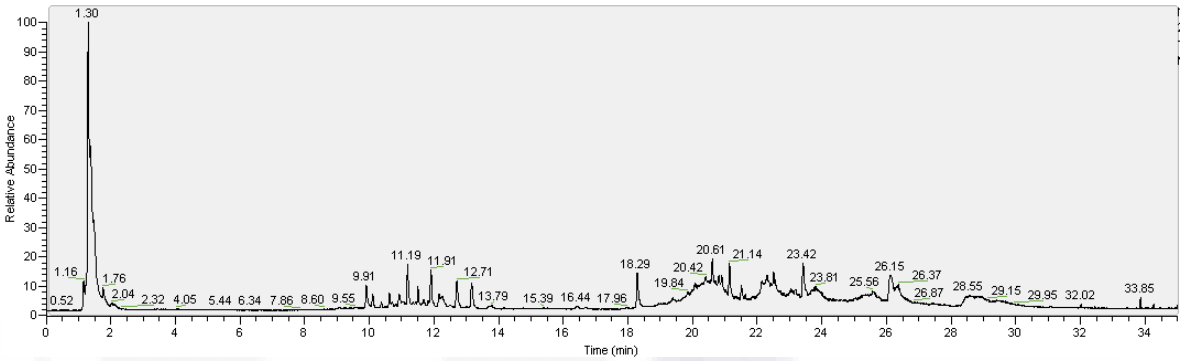

Figura A4-9. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with cell suspension cultures of *Coryphantha potosiana.* Biomass was collected after two months of culture.

Anexo 5. Análisis realizados con *Coryphantha potosiana* para la separación e identificación de alcaloides

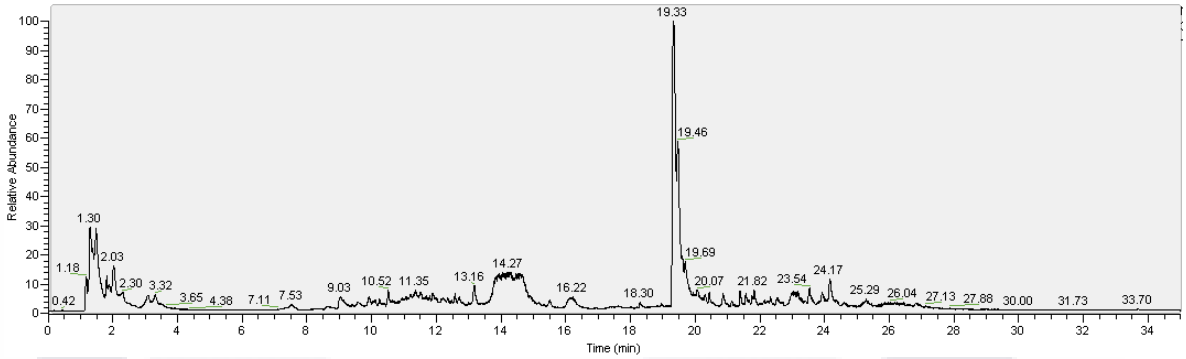

Figura A5-1. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with aerial parts of *Coryphantha potosiana* (*in vitro* plants).

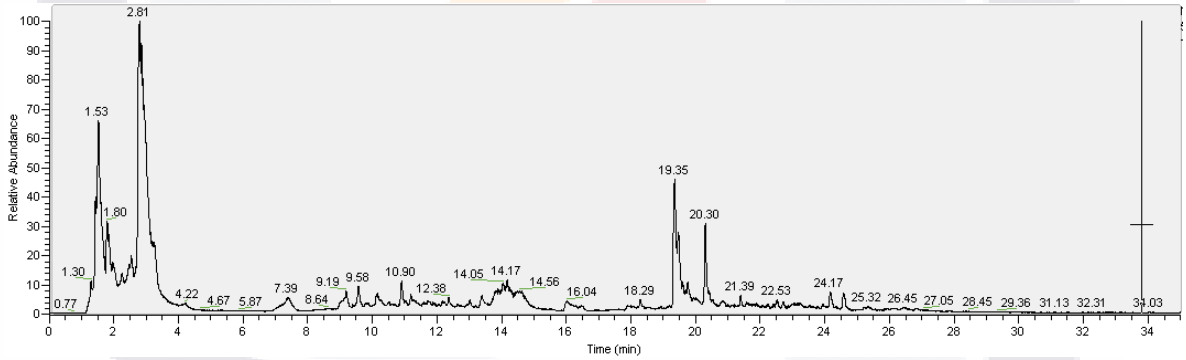

Figura A5-2. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with radicular parts of *Coryphantha potosiana* (*in vitro* plants).

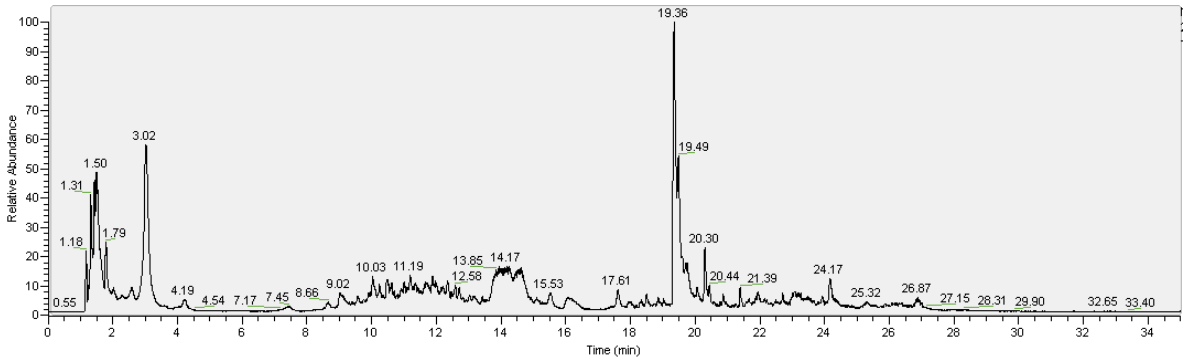

Figura A5-3. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with aerial parts of *Coryphantha potosiana (greenhouse plants).*

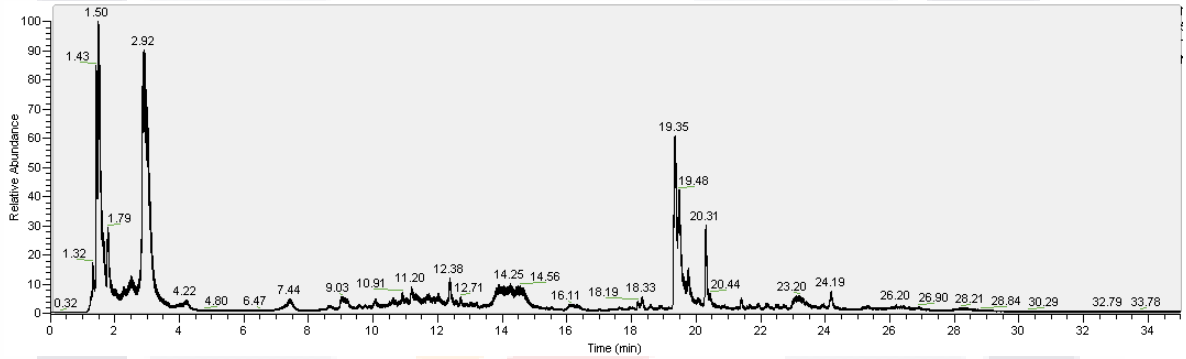

Figura A5-4. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with radicular parts of *Coryphantha potosiana (greenhouse plants).*

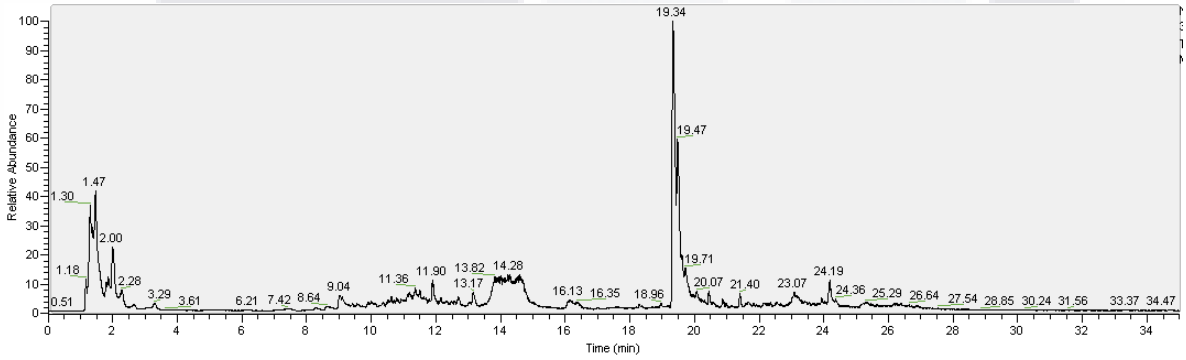

Figura A5-5. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with callus cultures of *Coryphantha potosiana.* Biomass was collected after 7 weeks of culture.

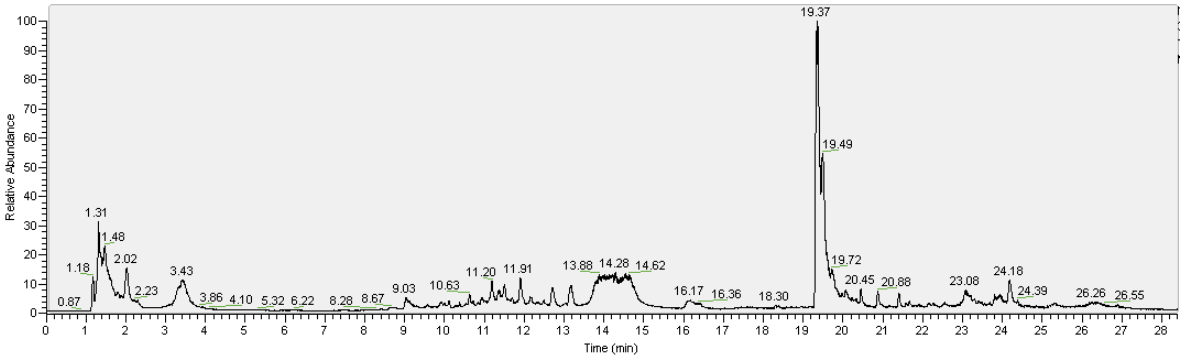

Figura A5-6. Ultra-high-performance liquid chromatography (UHPLC) chromatogram of methanolic extracts prepared with cell suspension cultures of *Coryphantha potosiana.* Biomass was collected after two months of culture.