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Evaluación de la actividad antiviral de metabolitos producidos por *Pentalinon andrieuxii*

Tesis que presenta

ISMAEL FERNANDO VILLEGAS ACOSTA

En opción al título de

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CENTRO DE INVESTIGACIÓN CIENTÍFICA DE YUCATÁN, A. C.
POSGRADO EN CIENCIAS BIOLÓGICAS

RECONOCIMIENTO

Por medio de la presente, hago constar que el trabajo de tesis de Ismael Fernando Villegas Acosta titulado **Evaluación de la actividad antiviral de metabolitos producidos por *Pentalinon andrieuxii***, fue realizado en la unidad de Biotecnología, en la línea de Biotecnología de Productos Naturales, en el laboratorio de Química de Productos Naturales, del Centro de Investigación Científica de Yucatán, A.C. bajo la dirección del Dr. Luis Manuel Peña Rodríguez, dentro de la opción en Biotecnología, perteneciente al Programa de Posgrado en Ciencias Biológicas de este Centro.

Esta tesis tiene orientación al desarrollo socioeconómico de la región, pues se estudia a una planta nativa de México con posibles propiedades antivirales.

Atentamente

Dr. José Luis Hernández Stefanoni
Director de Docencia

Mérida, Yucatán, México, a 17 de junio de 2025

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Este trabajo se llevó a cabo en la Unidad de Biotecnología del Centro de Investigación Científica de Yucatán, A.C., y forma parte del proyecto titulado Evaluación de la actividad antiviral de metabolitos producidos por *Pentalinon andrieuxii* bajo la dirección del Dr. Luis Manuel Peña Rodríguez

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A mi familia

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Evaluación de la actividad antiviral de metabolitos producidos por *Pentalinon andrieuxii*

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RESUMEN

Pentalinon andrieuxii es una planta medicinal con antecedentes reportados de actividad antiparasitaria e inmunomoduladora y reconocida por su producción de cardenólidos, metabolitos secundarios que en otros sistemas biológicos se han vinculado con propiedades antivirales. Sin embargo, hasta la fecha no se ha documentado actividad antiviral ni el contenido específico de cardenólidos en esta especie

Para la detección de cardenólidos en *P. andrieuxii*, se utilizó *Nerium oleander* como planta control debido a su producción de oleandrina, un cardenólido con reconocida actividad antiviral. El estudio fitoquímico de *N. oleander* permitió obtener oleandrina pura y descubrir un nuevo cardenólido, identificado como 8-hidroxi-digitoxigenin-3-O-β-D-diginósida. La evaluación de la citotoxicidad en la línea celular Madin-Darby de riñón canino (MDCK) mostró que oleandrina y el nuevo cardenólido son citotóxicos, por lo que se descartó su uso como controles en ensayos antivirales contra Influenza A(H1N1). No obstante, el nuevo cardenólido resultó ser 78 veces menos citotóxico que la oleandrina, a pesar de que ambos cardenólidos presentan estructura química y polaridad similares. Estudios de acoplamiento molecular *docking* mostraron que esta diferencia se debe a una menor afinidad del nuevo cardenólido por la proteína Na+/K+ ATPasa.

La evaluación del efecto citopático del extracto metanólico de hojas de *P. andrieuxii* y sus fracciones de polaridad baja (hexano), media-baja (diclorometano), media-alta (acetato de etilo) y alta (acuosa) frente al virus Influenza A(H1N1) reveló que las fracciones de polaridad media-alta y alta reducen significativamente el efecto citopático, tanto en co-tratamiento como en pos-tratamiento. Esto sugiere que los metabolitos bioactivos interfieren en procesos virales para evitar la infección y muerte celular. El análisis por LC-MS de las diferentes fracciones de *P. andrieuxii* permitió la identificación preliminar de componentes con características de cardenólidos, específicamente urechitoxina, en la fracción de polaridad media-baja y de polifenoles con actividad antioxidante en la fracción bioactiva de polaridad media alta; estos resultados sugieren que los cardenólidos no son responsables de la actividad antiviral contra el virus Influenza A(H1N1) y que los metabolitos responsables de la actividad son de polaridad alta.

ABSTRACT

Pentalinon andrieuxii is a medicinal plant with reported antiparasitic and immunomodulatory activities, and it is also associated with the production of cardenolides—secondary metabolites linked to antiviral properties in other biological systems. However, to date, neither antiviral activity nor specific cardenolide content has been documented for this species.

For the detection of cardenolides in *P. andrieuxii*, *Nerium oleander* was used as a control plant due to its production of oleandrin, a cardenolide with recognized antiviral activity. The phytochemical study of *N. oleander* yielded pure oleandrin and led to the discovery of a new cardenolide identified as 8-hydroxy-digitoxigenin-3-O-β-D-diginoside. The evaluation of cytotoxicity in the Madin-Darby canine kidney (MDCK) cell line showed that oleandrin and the new cardenolide are cytotoxic, so their use as controls in antiviral assays against Influenza A(H1N1) was ruled out. However, the new cardenolide was found to be 78 times less cytotoxic than oleandrin, even though both cardenolides have similar chemical structures and polarities. Molecular docking studies showed that this difference is due to a lower affinity of the new cardenolide for the Na+/K+ ATPase protein.

The evaluation of the cytopathic effect of the methanolic extract of *P. andrieuxii* leaves and its low (hexane), medium-low (dichloromethane), medium-high (ethyl acetate) and high (aqueous) polarity fractions against the Influenza A(H1N1) virus revealed that the medium-high and high polarity fractions significantly reduce the cytopathic effect, both in co-treatment and post-treatment. This suggests that bioactive metabolites interfere with viral processes to prevent infection and cell death. LC-MS analysis of the different fractions of *P. andrieuxii* allowed the preliminary identification of components with cardenolide characteristics, specifically urechitoxin, in the medium-low polarity fraction and polyphenols with antioxidant activity in the medium-high polarity bioactive fraction; these results suggest that cardenolides are not responsible for the antiviral activity against the Influenza A(H1N1) virus and that the metabolites responsible for the activity are of high polarity.

INTRODUCCIÓN

Los virus son entidades biológicas, infecciosas, microscópicas, acelulares, que solo pueden replicarse dentro de las células de otros organismos, los cuales pueden infectar a todo tipo de organismos como bacterias, animales, hongos y plantas (Payne, 2017). Su diversidad es muy alta debido a factores genéticos y evolutivos. Ya que pueden tener genomas de ADN o ARN, y su tamaño y complejidad varían ampliamente. Los virus de ARN, en particular, tienen tasas de mutación muy altas, ya que pueden experimentar recombinación genética, un proceso en el que fragmentos de material genético se intercambian entre diferentes virus que infectan la misma célula, generando nuevas cepas con características únicas (Domingo, 2019).

Una de las problemáticas relacionadas con las enfermedades virales radica en su alta capacidad de propagación y aparición constante de nuevas variantes o cepas que dificultan su control. Esto genera un aumento en la carga para los sistemas de salud, con incrementos en casos de enfermedades respiratorias agudas y brotes epidémicos que pueden superar los niveles basales habituales, como ocurre con la influenza y la COVID-19 (AlMalki et al., 2023; Straussy Strauss, 2008). Su alta diversidad genética y capacidad de evolución rápida permite a los virus adaptarse a nuevos huéspedes y ambientes, lo que dificulta el desarrollo de vacunas y antivirales que sean efectivos contra todas las variantes existentes y futuras (Taylor, 2014). Por ejemplo, el virus de la inmunodeficiencia humana (VIH) presenta múltiples subtipos y formas recombinantes circulantes, lo que obliga a diseñar vacunas específicas para los subtipos predominantes en cada región geográfica (Rivera-Morales et al., 2005), así también, influenza es otro virus que cambia constantemente por mutaciones en sus segmentos de ARN, lo que genera nuevas variantes antigénicas que pueden causar epidemias recurrentes y pandemias, haciendo imposible su erradicación y complicando el desarrollo de vacunas universales (García-Garcíay Ramos, 2006).

Otra dificultad importante es la resistencia a tratamientos antivirales y la falta de vacunas universales eficaces para todos los virus o sus variantes, lo que limita las opciones para prevenir y tratar estas enfermedades. A esto se suma la necesidad de vigilancia epidemiológica constante y la preparación de los sistemas de salud para responder

rápidamente ante brotes o cambios en la distribución geográfica de virus (Luong et al., 2025).

El primer antiviral aprobado como terapia para tratar la infección por VIH / SIDA fue la Azidotimidina (AZT) en 1987; desde entonces y hasta el 218, más de ochenta medicamentos antivirales recibieron la aprobación de la Administración de Alimentos y Medicamentos (FDA), aumentado la oferta y la diversidad de estos agentes, siendo las moléculas pequeñas las que destacan en la lista y teniendo en cuenta que el 48.9 % de antivirales desarrollados son para el tratamiento del VIH, 19.3 % para el virus del Hepatitis C (VHC), 9.1% para el virus del Hepatitis B (VHB), 12.5 % Citomegalovirus, virus herpes simplex (VCM, VHS), 4.6 % para Influenza y 5.7 % para otros virus. En conjunto los antivirales aprobados representan un total del 90 % para tratar infecciones crónicas en comparación con las infecciones agudas (Chaudhuri et al., 2018).

Para el estudio de nuevos antivirales se pueden mencionar dos principales enfoques, dirigido a proteínas celulares y otro dirigido a proteínas virales (De Clercqy Herdewijn, 2010). De los antivirales aprobados por la FDA, se puede identificar aquellos que tienen como blanco, proteínas como la polimerasa 37.5 %, proteasas 23. 6 %, integrasas virales 4.2 %, proteína no estructural del virus de hepatitis C (NS5A) 8.3 % y otros blancos entre 12. 5 % y 13.9 % teniendo como objetivo el hospedero (Chaudhuri et al., 2018). Algunas de las diferentes clases de antivirales y sus mecanismos de acción, como los sulfatos de polivinilo (PVS) y los polivinilsulfonatos (PVAS) que actúan como inhibidores absorbidos por virus se muestran en la (Figura 1).

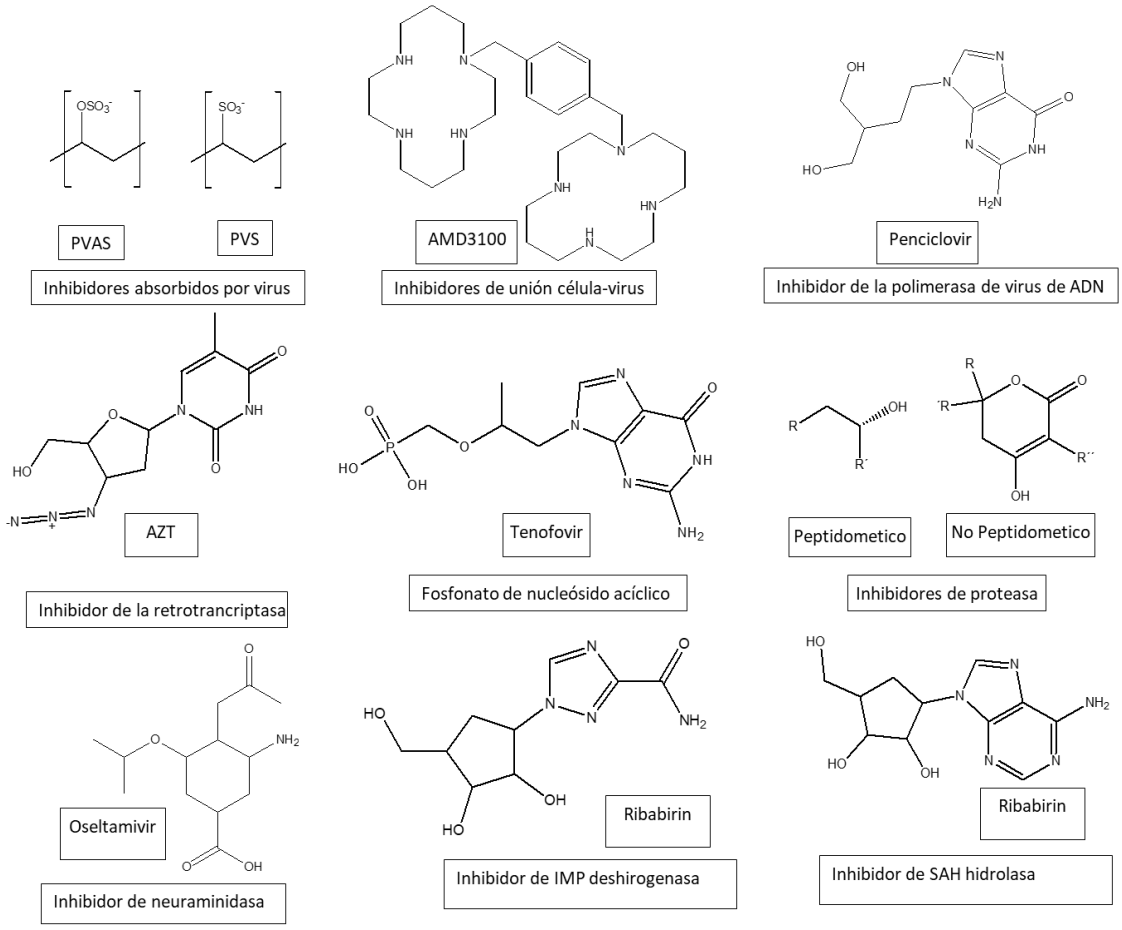

Figura 1 Diferentes clases antivirales disponibles en el mercado (De Clercq & Herdewijn, 2010)

En este contexto, la búsqueda de nuevos compuestos antivirales, especialmente aquellos derivados de fuentes naturales se han convertido en una estrategia para la investigación científica, impulsada por la necesidad de abordar patógenos con alta diversidad genética y capacidad de evolución rápida, buscando desarrollar antivirales con acción de amplio espectro, mecanismos de acción innovadores, baja toxicidad y sostenibilidad ambiental (Ianevski et al., 2022). Sin embargo, no existen estudios enfocados en evaluar e identificar metabolitos de *P. andrieuxii* con actividad antiviral, por lo tanto, el presente trabajo tiene como objetivo identificar metabolitos producidos por *P. andrieuxii* que puedan constituir futuros fármacos contra infecciones virales.

CAPÍTULO I

ANTECEDENTES

1.1 Plantas como fuentes potenciales de antivirales

Las plantas tienen la capacidad de producir metabolitos secundarios que pueden usarse como antivirales (Miresmailliy Isman, 2014), mostrando actividad contra virus in vitro e in vivo, con virus de ADN y ARN, con o sin envoltura. Incluso con diferentes cepas de un virus y adicionalmente pueden lograr un efecto de inmunomodulador (Vellingiri et al., 2020). Por mencionar algunas plantas reportadas con la actividad antiviral y sus metabolitos bioactivos: *Aloe vera:* antraquinonas (Huang et al., 2019), *Astragalus membranaceus:* astragalósidos I y IV (Liang et al., 2019), *Camellia sinensis:* TF3, TF2B, teaflavina 3,3′-di-*O*- malato, epigalatocatequina galato (Wu et al., 2020; Xu et al., 2017), *Eucalyptus globulus:* eucaliptol, tereticornaeto A, grandinol, sideroxilina y aceites esenciales (Brezáni et al., 2018; Li et al., 2016; Yang et al., 2010), *Glycyrrhiza glabra:* 2-acetamido-beta-D glucopiranosil amina, las amidas y los conjugados de glicirricina con dos residuos de aminoácidos (Fiore et al., 2008; Yang et al., 2015), *Sambucus nigra*: flavonoides presentes con la actividad antiviral (Roschek et al., 2009). Como se observa, la actividad antiviral de las plantas está asociada a diferentes grupos de metabolitos como compuestos fenólicos, terpenoides, alcaloides, compuestos organosulfurados, poliacetilenos, polisacáridos, por mencionar algunos (Behl et al., 2021).

1.2 Cardenólidos

Dentro de los terpenos y los metabolitos con actividad antiviral se encuentran los cardenólidos, los cuales son un grupo de metabolitos secundarios caracterizados por un núcleo esteroideo (genina o aglicona), una γ-lactona en posición C17, uno o varios glicósidos generalmente en posición C3, además se encuentran sustituidos en diversas posiciones de la genina con grupos metilo, hidroxilo, formilo o acetilo, (Figura 1.1). Su presencia se ha podido identificar en plantas, insectos y humanos jugando un papel muy importante en la defensa de los primeros dos (Agrawal et al., 2024). Los cardenólidos son metabolitos que se conoce su capacidad para interferir en el funcionamiento de la enzima

Na/K ATPasa, resultando en la muerte celular; sin embargo, esas características no han sido impedimento para ser usados como fármacos.

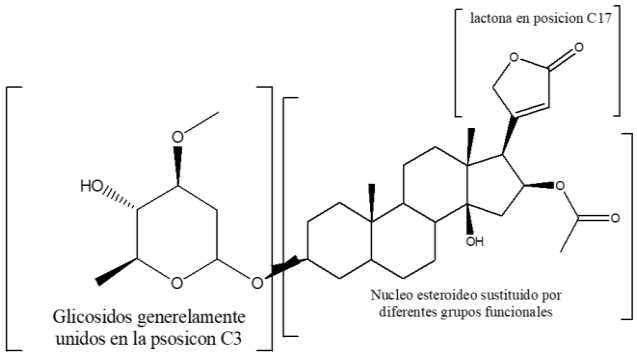

Figura 1.1 Estructura general de un cardenólido (e.g. oleandrina)

1.3 Actividad farmacológica de cardenólidos

El primer reporte del uso de cardenólidos como fármacos, fue 1785 por Willian Withering, describiendo los usos que tenía las infusiones de *Digitalis purpurea,* con actividad intrópica positiva acompañada de una disminución de la frecuencia cardiaca en pacientes con fibrilación muscular, la cual se identificó posteriormente que se debía a los cardenólidos presentes (digoxina). Siendo hoy en día, la digoxina un cardenólido presente en la lista modelo de medicamentos esenciales de la Organización Mundial de la Salud recomendados para los sistemas de salud, convirtiéndose en uno de los medicamentos a base de plantas más usado (WHO, 2017).

Sin embargo, los cardenólidos pueden llegar a ser tóxicos debido a una ingesta accidental elevando la concentración levemente más allá del rango terapéutico (0.8 a 2 ng/m o 1.0 a 2.6 nmol/l), acumulación sistemática a disfunción hepática, renal y acumulación sistemática secundaria a una interacción farmacológica, debido a un modelo de distribución de dos compartimentos donde el fármaco se redistribuye en el compartimento del tejido intracelular después de que se obtienen los niveles plasmáticos máximos (Kanjiy MacLean, 2012). Actualmente, en el mercado se puede encontrar la digitoxina y el Cedilanid (Lanatosido C) para el tratamiento de afecciones cardiacas (Withering, 2014). En Estados Unidos, la digoxina es el único cardenólido comercialmente disponible para recetas. Sin embargo, la presencia de cardenólidos están ampliamente presentes en

múltiples productos botánicos y otras sustancias naturales (Kanjiy MacLean, 2012). Otra aplicación médica estudiada para cardenólidos es la anticancerígena, pues actualmente se encuentra en fase II el fármaco PBI-05204, el cual es un extracto de *N. oleander* presentando actividad contra el adenocarcinoma ductal pancreático metastásico (mPDA) mostrando características prometedoras al combinarse con quimioterapia (Roth et al. 2020). Además de probarse con otras líneas de cáncer de manera *in-vitro*, limitando estos estudios a los cardenólidos más abundantes en cada una de las plantas (Wen et al., 2016).

Por otra parte, además de los usos farmacológicos antes mencionados, también se han evaluado cardenólidos con posibles aplicaciones como antibacteriales (Kamtcha et al., 2018), antiinflamatoria (Ihenetu et al., 2008) y neuro-protectora (Wang et al., 2006) por mencionar algunas.

1.4 Actividad antiviral de cardenólidos

Otra línea de investigación en la que están involucrados los cardenólidos, es la actividad antiviral, donde se ha evaluado su potencial como fármaco con una gran variedad de virus, que de igual forma pueden tener diferentes sitios de acción. Tal es el caso de pruebas contra el virus de gastroenteritis transmisible (TGEV) en las células testiculares porcinas, mostrando que los cardenólidos disminuyen las expresiones de la nucleocapsida y la proteína espiga de TGEV (Yang et al., 2017). Por otra parte, se ha estudiado la actividad antiviral con derivados de cardenólidos como es el caso de la digitoxigenina y la adición de grupos amino contra el virus de la influenza A (AIV) el cual se reportó que estos derivados pueden ser una gran promesa como fármaco ya que afecto la transcripción del ARNm viral y, por lo tanto, alterar la expresión de proteínas virales en las primeras etapas del ciclo de replicación, lo que conduce a una formación deficiente de nuevas partículas virales, estos mismos derivados se evaluaron contra el virus del herpes simple tipo 1 y 2 (HSV-1 y HSV-2) los cuales interfieren con los pasos intermedios y finales de la replicación del HSV (Figura 1.2) (Boff, Schneider, et al., 2020; Boff, Schreiber, et al., 2020).

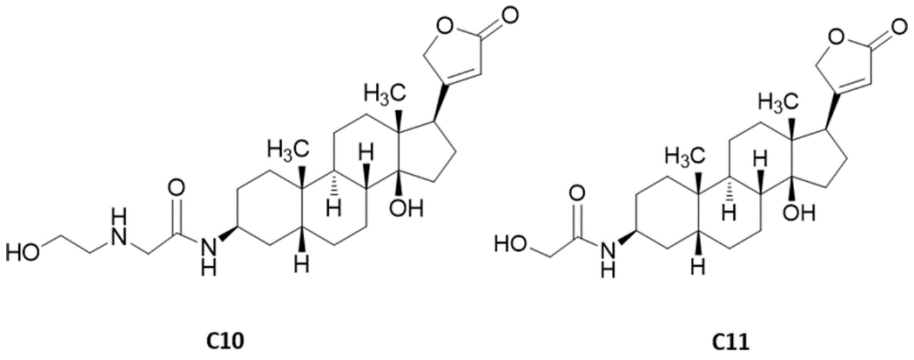

Figura 1.2 Derivados de cardenólidos con actividad biológica 3β - [(N-(2-hidroxietil) aminoacetil] amino-3-desoxidigitoxigenina (C10) y 3β-(hidroxiacetil) amino-3-desoxidigitoxigenina (C11)

Estos ejemplos evidencian su diversa actividad, destacando que la mayoría de los cardenólidos evaluados en los diferentes bioensayos, son los ya usados como fármacos; digoxina, digitoxina, ouabaína, convallatoxina, G-estrofantina y lanatósido C (Tabla 1.1), además de oleandrina por ser el cardenólido más abundante en *N. oleander.*

Tabla 1.1 Principales cardenólidos reportados con actividad antiviral (Amarelley Lecuona, 2018)

Cardenólidos	**virus**
Digoxina, oubaina, digitoxina, convallatoxina	Citomegalovirus
Oubaina, digoxina, digitoxina, G-estranfantina	Virus del herpes simple
Digoxina, digitoxina	Adenovirus
Digoxina	Virus chikungunya
Oubaina, digitoxina	Coronavirus
Oubaina	Virus sincitial respiratorio
Oubaina	Virus del ébola
Oubaina, digitoxina, lanatosido C	Virus de la gripe
Oubaina, digitoxina, lanatosido C, digoxina	Virus de inmunodeficiencia humana (VIH)

1.5 Oleandrina y su actividad antiviral

Oleandrina (Figura 1.3) es conocida por ser el principal cardenólido producido por *Nerium oleander*, planta perteneciente a la familia *Apocynaceae.* Su actividad biológica ha sido ampliamente estudiada, especialmente la actividad antiviral (Tabla 1.2), la cual han posicionado a olenadrina como un posible futuro fármaco.

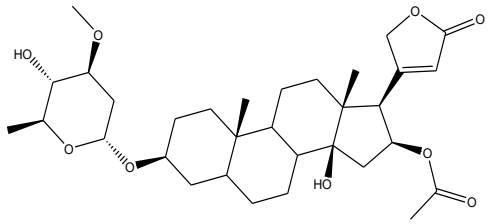

Figura 1.3 Estructura de oleandrina

Tabla 1.2 Actividad antiviral reportada para oleandrina (Newman et al., 2020)

Familia	Virus	Tipo de virus
Retroviridae	HIV-1	Dos copias de RNA (+)
Retroviridae	HTLV-1	Dos copias de RNA (+)
Filoviridae	Ebolavirus	RNA (-)
Filoviridae	Marburgvirus	RNA (-)
Orthomyxoviridae	Influenza	RNA (-)
Togaviridae	Venezuelan equine encephalitis	RNA (+)
Togaviridae	Chikungunya	RNA (+)
Coronaviridae	SARS-CoV-2	RNA (+)
Herpesviridae	HSV-1, HSV-2	DNA
Herpesviridae	Human CMV	DNA

1.6 *Pentalinon andrieuxii*

Por otra parte, *Pentalinon andireuxii* (Müll. Arg.) B.F. Hansen & Wunderlin (*Apocynaceae*) tribu Echites, es una enredadera conocida como bejuco guaco, cantibteac o contrayerba (Figura 1.4), se distribuye en la península de Yucatán, del sur de México hasta Nicaragua; se caracteriza por ser una planta trepadora con látex, flores amarillas, fruto de dos folículos, angostamente terete, de 18–28 cm de largo y 4–7 mm de ancho, con semillas apicalmente comosas. Es conocida por su uso en la medicina tradicional maya, para curar las lesiones de leishmaniosis (úlcera del chiclero), como antiinflamatorio y antidepresivo. Como resultado de la búsqueda de metabolitos bioactivos producidos por esta especie, se identificaron trinorsesquiterpenos (Urechitol A y B) triterpenos, derivados esteroides y esteroles (Pan et al., 2012). A pesar de que, hasta el momento, no se han aislado cardenólidos de esta planta, existe evidencia de la producción de los cardenólidos urechitoxina y oleandrina en *Pentalinon luteum (Hassall, 1951)*. Además, recientemente se estableció que existe una relación entre *N. oleander* y *P. andrieuxii* al identificar que la polilla *Syntomeida epilais* las usa como fuente de alimento asociado al secuestro de cardenólidos como mecanismo de defensa. Los resultados preliminares de este trabajo mostraron que *P. andrieuxii* produce cardenólidos relacionados con la aglicona oleandrigenina con los cardenólidos de *N. oleander*, permitiendo identificar agliconas correspondientes a oleandrigenina y gitoxigenina (Villegas Acosta, 2019).

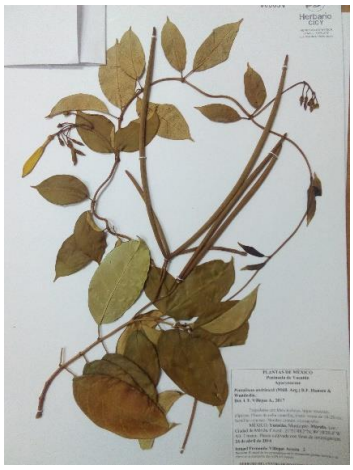

Figura 1.4. Ejemplar de *Pentalinon andrieuxii* montado para la colección del herbario CICY

1.6 Virus de Influenza A(H1N1)

El virus de la Influenza pertenece a la familia Orthomyxoviridae, se puede clasificar en subtipos según la combinación de hemaglutinina (HA) 18 subtipos y la neuraminidasa (NA) 11 subtipos. Actualmente, circulan en los seres humanos los virus de la influenza de subtipo A (H1N1) y A (H3N2). El A (H1N1) también se escribe como A (H1N1) pdm09, ya que causó la pandemia en 2009 y posteriormente reemplazó al virus de la influenza estacional A (H1N1) que había circulado antes de 2009 (Controly Prevention, 2014).

Su trasmisión es por vía respiratoria y los principales síntomas de Influenza están relacionados con enfermedades respiratorias, las cuales incluyen dolor de cabeza, fiebre, dolor muscular, congestión nasal, tos, y diarrea. En los hemisferios norte y sur la presencia de influenza está asociada a los meses más fríos; por otra parte, en los trópicos no hay una asociación con la época climática del año (Lowen et al., 2014).

Para el tratamiento y prevención de las infecciones por el virus de influenza existen vacunas y antivirales, sin embardo, por su alta mutagenicidad o intercambio génico de las partículas virales no siempre son efectivas (Erbelding et al., 2018; Shiny Seong, 2019). Esto impulsa la búsqueda continua de terapias alternativas, donde los enfoques metodológicos priorizan la identificación de compuestos que inhiban etapas críticas del ciclo viral, como la entrada, replicación o liberación de partículas, adaptándose a la dinámica evolutiva del patógeno (Omoto et al., 2018). La evaluación de la reducción del efecto citopático (EC) —manifestado como lisis celular, pérdida de adhesión y disfunción metabólica— constituye un parámetro clave en estudios *in vitro*.(Hechtfischer et al., 1997), en el cual se puede demostrar su utilidad al preservar la viabilidad celular. Estos modelos, junto a otras técnicas de microscopía y cuantificación de citocinas, permiten validar la eficacia antiviral mientras se monitorean mecanismos de resistencia, fundamentales para el desarrollo de terapias contra cepas pandémicas emergentes (Galán-Sánchez et al., 2014).

Por todo lo expuesto anteriormente, en este trabajo se tienen como objetivo evaluar el extracto metanolico, fracciones semipurificadas y productos puros de *P. andrieuxii*, como potenciales antivirales contra el virus de Influenza A(H1N1) con el fin de coadyuvar en la búsqueda de nuevos fármacos que ofrezcan un tratamiento para este tipo de infección.

JUSTIFICACIÓN

En los últimos años, el aumento de las infecciones virales ha provocado graves pérdidas en la salud y el bienestar global. Esta situación se agrava debido al limitado acceso a tratamientos efectivos, lo que dificulta el control de las enfermedades y amplifica sus consecuencias tanto humanas como económicas. Los antivirales tienen un papel muy importante para el tratamiento de estas infecciones y se han usado en conjunto con las vacunas con el fin de erradicarlas. La búsqueda de nuevos antivirales ha incrementado a lo largo del tiempo, sin embargo, los aprobados por la FDA en el mercado son principalmente para infecciones crónicas en humanos.

En este sentido, los cardenólidos por la bibliografía antes citada pueden ser usados como medicamentos antivirales por las características estructurales que presenta, lo que permite tener una mayor capacidad de blancos o sitios de acción que pueden tener con las partículas virales o la célula huésped, y con esto evitar que pueda proliferar la infección de Influenza A(H1N1).

Hoy en día existe múltiples publicaciones científicas relacionando a los cardenólidos con actividad antiviral, como es el caso de los cardenólidos de *Nerium oleander,* el cual cuenta con reportes asociándolos a la actividad antiviral, como la oleandrina. Y aunque aún no se han identificado cuáles son los cardenólidos que está produciendo *Pentalinon andrieuxii,* esta planta presenta una relación quimiotaxinomica con la aglicona oleandrigenina y en la producción de cardenólidos con *N. oleander.* Por lo que el aislamiento e identificación de los cardenólidos presentes en *P. andireuxii* podría servir como una posible fuente de antivirales que aún no se han explorado, con grandes capacidades de resultar con actividad por los antecedentes que presenta la familia *Apocynaceae* en esta área.

HIPÓTESIS

El extracto metanólico, así como las fracciones de distinta polaridad obtenidas del extracto metanólico de las hojas de *Pentanilon andrieuxii* tendrán actividad antiviral, la cual podrá ser comparada con la que presenta *Nerium oleander* y oleandrina, evaluándolos contra los virus de Influenza A(H1N1).

Esta actividad biológica estará relacionada con la producción de metabolitos secundarios, los cuales podrán funcionar como antivirales de amplio espectro.

OBJETIVO GENERAL

Evaluar el potencial antiviral del extracto crudo, fracciones semipurificadas y productos puros de *Pentalinon andrieuxii* contra virus patógenos de humanos, plantas y bacterias.

OBJETIVOS ESPECÍFICOS

1. Obtener el control vegetal de la actividad antiviral: El extracto metanólico, fracción de CH_2Cl_2 y oleandrina a partir de *N. oleander*

2. Identificar por medio de ensayos *in-vitro* el potencial como antiviral del extracto metanólico, fracciones semipurificadas y metabolitos puros de *P. andrieuxii* contra Influenza A(H1N1), usando como referencia el extracto metanolico, la fracción de CH_2Cl_2 y oleandrina de *N. oleander*.

3. Identificar y aislar metabolitos bioactivos de *P. andrieuxii* contra el virus de Influenza A(H1N1)

ESTRATEGIA GENERAL DE INVESTIGACIÓN

A partir del extracto metanólico de hojas de *N. oleander* y *P. andrieuxii* se realizará una partición líquido-líquido con diferentes disolventes con el fin de poder aislar los cardenólidos presentes en esas plantas.

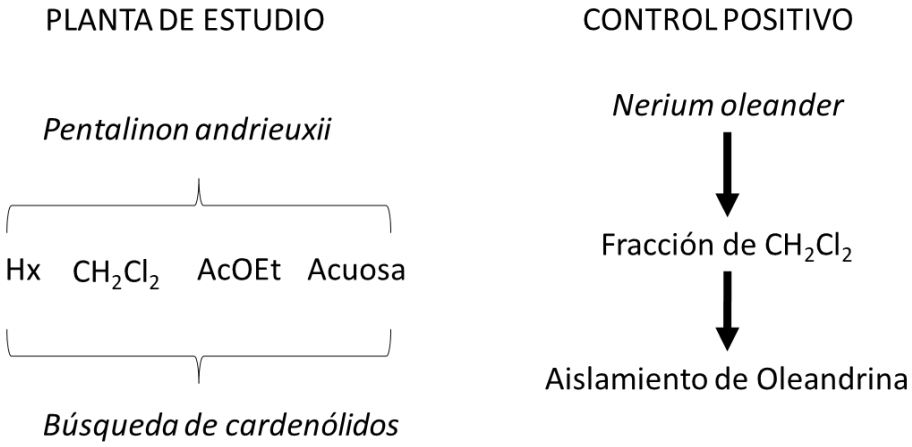

Figura 1.5 Estrategia experimental para el estudio fitoquímico de *P. andrieuxii*

Para la evaluación de la actividad antiviral: se evaluarán los extractos totales, fracciones de *P. andrieuxii* y usando como referencia el extracto, la fracción de CH_2Cl_2 y oleandrina de *N. oleander*.

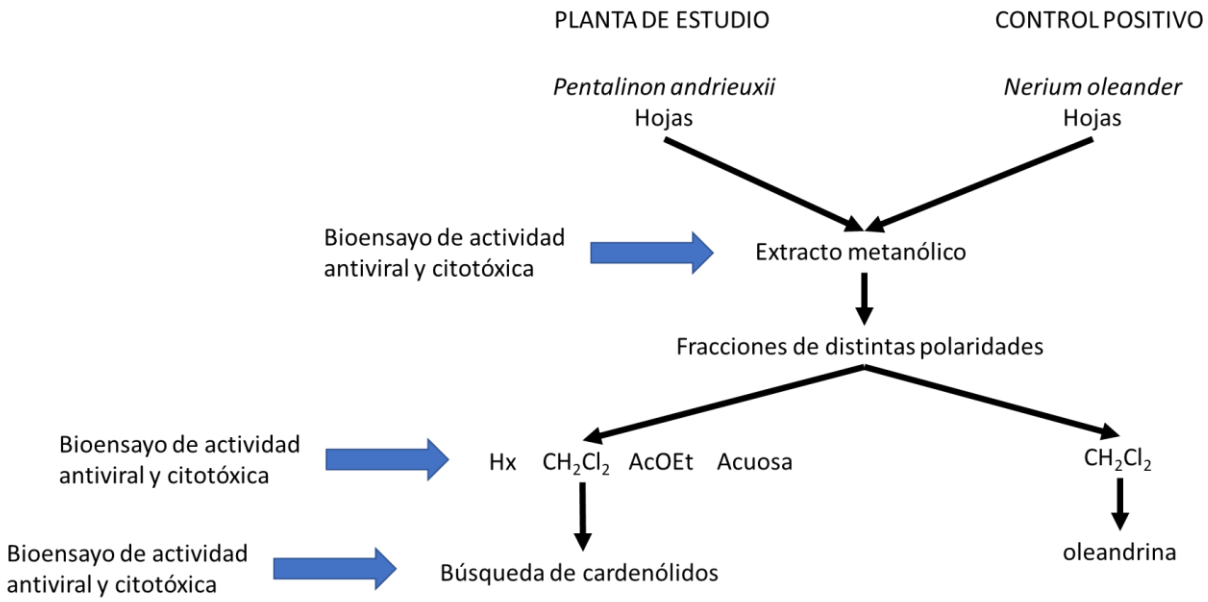

Figura 1.6 Estrategia experimental para el estudio antiviral del extracto de *P. andrieuxii* y de sus fracciones de distinta polaridad en Influenza A(H1N1).

CAPÍTULO II

2 Un nuevo cardenólido bioactivo de *N. oleander*

2.1 Resumen

Las plantas y sus múltiples metabolitos secundarios representan una de las principales fuentes de fármacos potencialmente nuevos. La digoxina, la digitoxina y la ouabaína son un grupo de cardenólidos producidos por plantas de los géneros Digitalis, Strophanthus y Acokenthera que se utilizan en el tratamiento y la gestión de la insuficiencia cardiaca congestiva, las arritmias y la insuficiencia cardiaca. Por otra parte, se ha informado de que varios cardenólidos como la oleandrina, aislada de *Nerium oleander* L., Apocynaceae, muestran actividad antitumoral y antiviral in vitro, en particular contra el COVID-19. Como parte de la búsqueda de nuevos cardenólidos con actividad antiviral a partir de especies vegetales de la familia Apocynaceae en la flora yucateca, el examen del extracto de hoja de *N. oleander* resultó en el aislamiento e identificación del 8-hidroxi-digitoxigenin-3-O-β-D-diginosido, un nuevo cardenólido bioactivo que mostró una actividad citotóxica menor que la de la oleandrina cuando se probó utilizando la línea celular de riñón canino Madin-Darby. El análisis de acoplamiento molecular mostró que el nuevo cardenólido tiene una menor afinidad molecular con la proteína Na+/K+ ATPasa que la oleandrina, lo que sugiere que esta diferencia de afinidad entre los dos cardenólidos podría explicar sus diferencias de actividad citotóxica, a pesar de tener estructuras químicas y polaridades similares.

Villegas-Acosta, I. F., Ayora-Talavera, G., Garcia-Sosa, K., Hernández-Núñez, E., Aguilar-Hernández, V. M., & Peña-Rodriguez, L. M. (2025). A New Bioactive Cardenolide from *Nerium oleander*. *Revista Brasileira de Farmacognosia*, 1-9, https://doi.org/10.1007/s43450-025-00637-9.

Adicionalmente, con el objetivo de identificar los cardenólidos presentes en *P. andrieuxii*, la fracción de media baja polaridad, se procesó usando HPLC-MS bajo las mismas condiciones usadas con los cardenólidos de *N. oleander*, logrando identificar la presencia de un compuesto con características de urechitoxina.

A new bioactive cardenolide from *Nerium oleander*

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2.1 Abstract

Plants and their multiple secondary metabolites represent one of the main sources of potentially new pharmaceuticals. Digoxin, digitoxin and ouabain are a group of cardenolides produced by plants of the genera *Digitalis*, *Strophanthus* and *Acokenthera* that are used in the treatment and management of congestive cardiac insufficiency, arrhythmias, and heart failure. Alternatively, several cardenolides such as oleandrin, isolated from *Nerium oleander* L., Apocynaceae, have been reported to show antitumor and antiviral activity in vitro, particularly against COVID-19. As part of our search for new cardenolides with antiviral activity from plant species of the Apocynaceae family in the Yucatecan flora, our examination of the leaf extract of *N. oleander* resulted in the isolation and identification of 8-hydroxi-digitoxigenin-3-*O*-β-D-diginoside, a new bioactive cardenolide showing a lower cytotoxic activity than that of oleandrin when tested using the Madin-Darby canine kidney cell line. Molecular docking analysis showed that the new cardenolide has a lower molecular affinity to the Na^+/K^+ ATPase protein than oleandrin, suggesting that this difference in affinity between the two cardenolides could explain their differences in cytotoxic activity, despite their having similar chemical structures and polarities.

Keywords: Oleandrin; Apocynaceae; cytotoxic activity; molecular docking; digitoxigenin.

2.2 Introduction

Secondary metabolites are manufactured by plants to make them competitive in their own environment (Ozyigit et al., 2023). These small molecules, which exert a wide range of biological effects on the plant itself and on other living organisms, are of interest to the pharmaceutical industry because of their potential for developing new products or new models for the treatment of various diseases. As of 2015, more than 50,000 secondary metabolites had been discovered in the plant kingdom, many with different types of biological activities and some being used as pharmaceuticals (Newmany Cragg, 2020; Teoh, 2016; Yeshi et al., 2022).

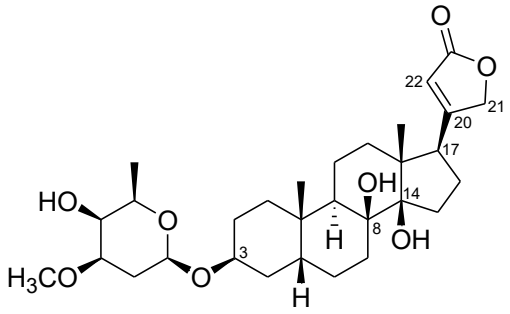
The cardenolides are a group of over 500 terpenoids reportedly found in 60 genera and 12 families of Angiosperms, but occurring mainly in species of the Apocynaceae family (Agrawal et al., 2012). These secondary metabolites are characterized by the cyclopentaneperhydrophenanthrene ring system having an α,β-unsaturated-γ-lactone at C-17 and one or more carbohydrate units at C-3. Cardenolides have been reported to show a wide range of biological activities, including acaricidal, antiviral, anti-inflammatory, and neuroprotective, among others (El-Seedi et al., 2019). To date, three cardenolides, digoxin, digitoxin, and ouabain (Fig S1), are commonly used in the treatment and management of congestive cardiac insufficiency, arrhythmias, and heart failure; both digoxin and digitoxin are obtained from *Digitalis purpurea* L. and *D. lanata* Ehrh, Plantaginaceae, respectively (Formigay Ariza, 2018; Garcia Metz, 2022), while ouabain is isolated from the bark of *Acokanthera ouabaio* [syn. of *Acokanthera schimperi* (A.DC.) Benth. & Hook.f. ex Schweinf.], Apocynaceae, and the seeds of *Strophanthus gratus* Baill., Apocynaceae (Baecher et al., 2014). The three cardenolides have a similar mechanism of action, inhibiting the Na^+/K^+ ATPase pump in cardiac muscle cells that results in an increase in the intracellular Na^+ and Ca^+ stored in the sarcoplasmic reticulum (El-Seedi et al., 2019).

Cardenolides can be highly toxic and can lead to serious health issues if ingested in large amounts; while the mechanism of action for some of the biological activities of cardenolides is still being investigated, they are known for their disruption of the Na^+/K^+ ATPase in host cells interfering with specific enzymatic processes such as the activation of the PI3K/PDK1 pathway (Souza e Souza et al., 2021). The use of computational tools such as molecular docking simulations has been particularly useful to better understand the interactions between cardenolide substrates and the ligand (Wang et al., 2022); these

simulations can offer detailed insights into the molecular interactions and binding mechanisms of the substrate with key amino acid residues in the Na^+/K^+ ATPase target. This knowledge, which can be of great value for the future of drug discovery, demonstrates the importance of computational tools in advancing our understanding of complex biological processes (Rui et al., 2016).

Nerium oleander L. (Fig S2), a plant belonging to the Apocynaceae family, is recognized for its production of cardenolides (Cao et al., 2018). It is a shrub native to Asia and the Mediterranean region, where it has been used in folk medicine to treat leprosy and malaria and as an analgesic and anti-inflammatory (Cao et al., 2018); it has also been reported that various of its bioactive metabolites possess antitumor, anti-HIV, neuroprotective, antimicrobial, and antioxidant activities (Kanwal et al., 2020). Phytochemical investigations have also shown that all parts of *N. oleander* are rich in a mixture of potent cardenolides (Cao et al., 2018; Mishra et al., 2021), with oleandrin (**2**) being considered the main one, together with neridiginoside, nerizoside, and odoroside H (Begum et al., 1999), and the genins oleandrigenin, digitoxigenin, neriin, folinerin, and rosagenin (Cao et al., 2018).

As part of our search for new cardenolides with antiviral activity from plant species of the Apocynaceae family in the Yucatecan flora, we wish to report herein on the isolation and identification of 8-hydroxi-digitoxigenin-3-*O*-β-D-diginoside (**1**) as a new bioactive cardenolide from the leaf extract of *N. oleander*, and the use of molecular docking to explain its comparatively low cytotoxic activity.



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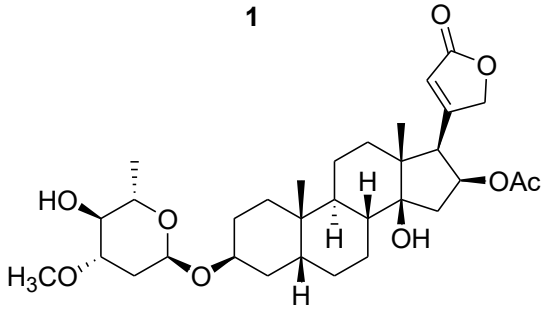


2

2.3 Material and methods

Plant material.

Fresh leaves of *Nerium oleander* L., Apocynaceae, were collected in Merida, Yucatan, Mexico in June 2018 in a cultivated population. A voucher specimen was deposited at the Herbarium of CICY with the number 68854.

Extraction, isolation and identification.

The plant material (3.1 kg) was dried at room temperature for five days, ground and extracted three times by maceration with MeOH (20 l) at room temperature. The solvent was filtered, first through cheesecloth, then cotton, and evaporated under reduced pressure to yield 952 g (29.03%) of crude methanolic extract. A portion (752 g, 79%) of the crude methanolic extract was suspended in a mixture of H_2O/MeOH (3:2, v/v) and the resulting suspension was successively partitioned with hexane (2:1, v/v, three times) and dichloromethane (2:1, v/v, three times) to produce the corresponding low (59.8 g, 8 %) and medium (54.7 g, 7%) polarity semipurified fractions. A portion (25 g, 46%) of the medium polarity fraction was subjected to vacuum liquid chromatography purification (9 cm d, 6 cm h, 250 mL fractions) using a gradient elution with hex:DCM:EtOAc mixtures of increasing polarity; fraction 2 (4 g, 16%), showing oleandrin (**2**) as the main component when analyzed by TLC, was purified by open column (5 cm d, 35 cm h) chromatography using a

gradient elution with mixtures of hex:EtOAc:MeOH to produce fraction 3 (1 g, 25%), which was subjected to gel permeation filtration using Sephadex LH20 (Sigma-Aldrich; column 2.5 cm d, 35 cm h, 10 mL fractions) and a gradient elution with mixtures of hex:EtOAc:MeOH to yield fraction 4 (0.5 g, 50%), which was then purified by flash column chromatography (4 cm d, 38 cm h; 15 mL fractions) using an isocratic elution with a mixture of CH_2Cl_2:acetone 9:1, to produce fractions 5 (65.5 mg, 13%) and 6 (329.1 mg, 66%) identified as 8-hydroxi-digitoxigenin-3-*O*-β-D-diginoside (**1**) and oleandrin (**2**), respectively.

HPLC-Orbitrap

Mass spectrometry analysis was performed using an Ultimate 3000 UHPLC (Thermo Scientific, Waltham, MA, USA) coupled to an UV/Vis detector (Ultimate 3000 UV/VIS detector, Dionex), and an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a Heated Electrospray Ionization interface (HESI-II, Thermo Fisher Scientific, Waltham, MA, USA). A sample volume equal to 10 µl was introduced into the system, then moved to a stainless-steel loop, and ultimately moved to a Hypersil Gold C18 column (100 × 2.1 mm, 1.9 µm particle size) with a flow rate of 300 µl/min. Using a binary gradient consisting of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in methanol (solvent B); the sample was eluted from the column with a flow rate of 300 µl/min. The gradient settings were as follows: 2 min 5% B, 2-12 min from 5% to 100% B, isocratic for 3 min 100% B, then returning to 5% B and isocratic for 7 min for column reconditioning. UV data collected at 220 nm. MS spectra data were acquired in positive mode in data-dependent acquisition for the top ten ions: MS1 resolution 60,000, scan range 100-1000 *m/z*, and MS2 resolution 60,000. The fragmentation method employed was higher-energy collisional dissociation (HCD). MS data were analyzed with Xcalibur 4.1 software (Thermo Scientific, USA) and Mass Frontier (Thermo Scientific, USA). Infrared spectra were obtained using an FT-IR spectrometer MOBILE-IR II diamond analyzer. Optical rotations were measured at 25 °C with an Anton Paar MW-325 polarimeter.

^{1}H NMR (600 MHz), ^{13}C NMR (150 MHz), $^{1}H^{1}H$ COSY, HMQC, HSQC, DEPT 135 spectra were conducted at 25 °C in a Varian-Agilent AR Premium Compact (14.1T) spectrometer. Approximately 20 mg of the pure product was dissolved in 800 µl of $CDCl_3$ and transferred to a 5 mm NMR tube.

8-Hydroxi-digitoxigenin-3-O-β-D-diginoside (**1**): Obtained as colorless oil, TLC R_f 0.25 (CH_2Cl_2:acetone 9:1), optical rotation: $[\alpha]_D^{25°}$+14.20° (*c*.1.0, $CHCl_3$), IR (neat): 3458, 2957, 2924, 2860, 1749, 1631 cm^{-1}. LC-MS: t_R 13.04 min, *m/z* 534.3312 $[M]^+$ (error 22.3 ppm) for $C_{30}H_{46}O_8$ and *m/z* 373.2295 for $C_{23}H_{33}O_4$. ^1H NMR and ^{13}C NMR data (See Table 2.1). COSY, HMQC, HSQC, and DEPT 135 experiments see (Fig S3 to S11).

Oleandrin (**2**): ^1H NMR and, ^{13}C NMR see (Table S1 and Fig S14 and S15). LC- MS: t_R 12.79 min, *m/z* 577.3246 $[M+H]^+$ (error 21.6 ppm) for $C_{32}H_{48}O_9$ and *m/z* 433.2493 for fragment ion peak $[M-C_7H_{14}O_3+H]^+$ (Fig S12 and S13).

Cytotoxicity evaluation

The cytotoxicity assays of oleandrin (**2**) and 8-hydroxi-digitoxigenin-3-*O*-β-D-diginoside (**1**) were carried out following the methodology previously reported (Ortiz-López et al., 2022).

Statistical Analyses

Data are presented as the mean (±) standard deviation of three independent experiments; statistical significance was calculated by a one-way ANOVA analysis and Dunnett´s test, with *p*-values < 0.05 considered as significant using the GraphPad Prism 7 software.

Docking Molecular Analyses

The crystallographic structure of Na^+/K^+ ATPase (target protein) complexed with ouabain was obtained from the Protein Data Bank (PDB ID: 4HYT with a 3.40 Å resolution, using the A subunit of Na^+/K^+ ATPase). The magnesium ion and three interstitial water molecules were kept in the binding site. Next, a rigid re-dock of ouabain was carried out to validate the system (RMSD 0.22 A). The docking analyses were conducted using AutoDock Vina 1.0.2. The applied search algorithm was Iterated Local Search Global Optimizer for global optimization. In this process, a succession of steps with mutation and local optimization. In this study, a grid box was defined as a cube with the geometric center in ouabain, with dimensions of 20x20x24 A, spaced points of 1.0 Å and X, Y and Z coordinates of -27.065, 20.469 and -69.469, respectively. All molecular modeling figures were constructed using the Discovery Studio Visualizer (https://www.3ds.com/products/biovia/discovery-studio) version free and pymol free academic version (https://www.pymol.org, Fig S16, Table S2).

The best conformation was chosen based on the lowest binding energy after the docking search was completed. 1000 modes and exhaustiveness 1000 (exhaustiveness of the global search, roughly proportional to time) with Autodock Vina were performed in all cases of each ligand structure and for each run, with the best pose being saved. The average binding energy for best poses was taken as the final binding energy value (Cob-Calan et al., 2019). This process was repeated ten times (Rocha et al., 2014).

Tabla 2.2 Spectroscopic 13C (150 MHz, CDCl3) and 1H RMN (600 mHz, CDCl3) data of 8-hydroxi-digitoxigenin-3-O-β-D-diginoside (1)

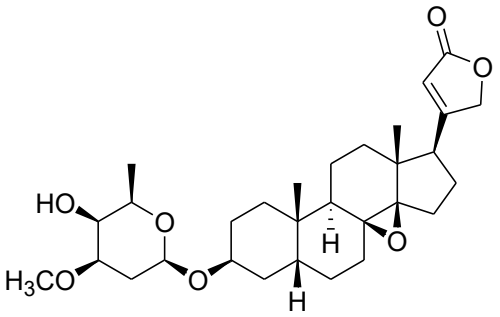
C/H	δ_C	δ_H (J Hz)	COSY	HMBC
1	30.35			
2	27.02			
3	72.31	4.08 (bs)		C-1 (^3J), C-5 (^3J), C-1' (^3J)
4	26.81			
5	36.39			
6	16.08			
7	26.59			
8	65.38			
9	36.70			
10	36.89			
11	24.65			
12	29.84			
13	41.92			
14	70.55			
15	36.97			
16	25.69			
17	51.46	2,57 (bdd, 11.6, 6.2)	H- 22	C- C-13, C-14, C- 15, C-16, C-20, C-21, C-22
18	16.18	0.84 s		C-13, C-14, C- 15, C-17
19	24.63	0.99 s		C- 1, C-9
20	169.62			
21	73.02	a) 4.80 (d, 17.2); b) 4.70 (d, 17.5)	H-22, H-17	C-20, C-22, C-23
22	116.89	5.87 (s)	H-17, H21a/b	C- 17, C-20, C-21, C-23
23	173.69			
1´	99.77	4.47 (bd,9.8)		C-3, C- 2´, C-3´, C-5´
2´	32.03			
3´	78.00	3.34, (ddd 12.32, 5.14, 3.02)		C-2 ´, C-4´, C-7´
4´	67.19	3.68 (bs)	H-3´	C-2´, C-3´, C-6´
5´	70.38	3.43 (bq 6.6)	H-6´	C-1´, C-4´, C- 6´
6´	16.81	1.33 (d, 6.4)	H-5´	C- 4´, C-5´
7´	55.72	3.40 (bs)		C- 3´

2.4 Results and discussion

Purification of the methanolic extract of *N. oleander* using different fractionation and chromatographic procedures resulted in the isolation and identification of oleandrin (**2**) and the new cardenolide 8-hydroxi-digitoxigenin-3-*O*-β-D-diginoside (**1**). While the identity of **2** was confirmed by comparing its spectroscopic data (^{1}H/^{13}C NMR) with those reported in the literature (Carney et al., 2023), the ^{1}H NMR spectrum of the new cardenolide did not show the signals corresponding to the C-16 acetyl group present in oleandrin (**2**) (Table 2.1). However, the ^{1}H NMR spectrum did display an olefinic proton signal at δ 5.87 and the signals for an AB quartet at δ 4.80 and δ 4.70 (*J* = 17.5 and 17.2 Hz), characteristic of the H-22 and H-21a/H-21b proton signals, respectively, in the α,β-unsaturated lactone ring of a cardenolide structure. This was confirmed by the ^{1}H-^{1}H correlation observed between the H-17 (δ 2.57) and the olefininc proton (δ 5.87) signals in the COSY experiment, together with the long-range heteronuclear correlations observed between the H-17 proton and the C-21 (^{3}J) and C22 (^{3}J) carbons in the HMBC experiment (Table 2.1).

The presence of the monosaccharide unit in the structure of **1** was indicated by the signals at δ_C 99.7 and δ_H 4.47 (*J* = 9.8 Hz) in its ^{13}C- and ^{1}H-NMR spectra, respectively, corresponding to the protonated anomeric carbon, where the large coupling constant of the anomeric proton signal confirms its axial orientation (Plazinski et al., 2021). Additional correlations observed between the H-3'/H-4' and H-5'/H-6' in the COSY experiment of **1**, together with the coupling patterns and coupling constant values observed for the different protons (Table 2.1), indicated the proposed stereochemistry of the monosaccharide. Similarly, a detailed analysis of the HMBC experiment showed a ^{3}J correlation between H-1´ and C-3, which confirmed the glycosylation position, together with ^{2}J and ^{3}J correlations between H-1' and C-2' (^{2}J), C-3' (^{3}J), and C-5' (^{3}J); additional correlations observed between H-3' and C-2' (^{2}J), C-4' (^{2}J), and C-7'(^{2}J), together with those of protons H4'/H5'/H6'/H7' with the corresponding neighboring carbons (Table 2.1), also confirmed the structure of the monosaccharide. A search of the literature confirmed that the chemical shift values of the carbons and protons of the monosaccharide corresponded to β-D-digitalose (Bai et al., 2010), while the ^{13}C- NMR data of **1** (Table 2.1) proved to be similar to those in adynerin (**3**) (Bai et al., 2010), a structurally similar cardenolide also isolated from *N. oleander*. While the ^{13}C NMR of the new cardenolide showed the presence of two oxygenated carbons corresponding to C-8 (δ 65.38) and C-14 (δ 70.55), the HPLC-MS analysis of **1** showed a molecular ion peak $[M+H]^{+\bullet}$ with *m/z* 534.3312 (error 22.3 ppm),

corresponding to a molecular formula $C_{30}H_{46}O_8$, together with a fragment ion peak $[M-C_7H_{13}O_4+H]^+$ at *m/z* 373.2295, corresponding to the aglycone of **1**, indicating that the new cardenolide had one unsaturation site less and an extra oxygen atom when compared to (**3**), which contains an epoxide ring connecting C-8 and C-14. Taking into account that the chemical shift values of C-8 and C-14 in the ^{13}C-NMR of **1** coincide with those reported for **3** (Bai et al., 2010), and that the chemical shift values reported for C-8 and C-14 in cerdollagenin (**4**), a cardenolide having a diol with the *syn*-configuration at the same positions, cannot be compared with those of **1** since the reported spectroscopic data for **4** was obtained in a different deuterated solvent ($CDCl_3$ vs CD_3OD and C_5D_5N) (Abey Yamauchi, 1992; Pádua et al., 2006), the *syn* orientation is proposed for the diol in carbons 8 and 14 of the new structure.



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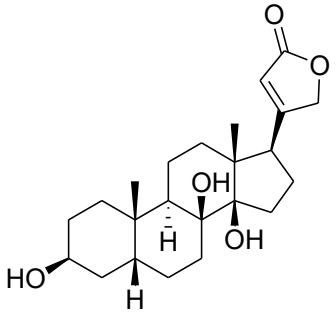

4

Once the structure of the new cardenolide was confirmed, and as the first step to explore their antiviral activity, both **1** and **2**, together with the crude extract from *N. oleander* and the CH_2Cl_2 fraction, were evaluated for their cytotoxic activity using the MDCK cell line. The results showed that all four samples were cytotoxic to the MDCK cell line, indicating that none could be further evaluated for their antiviral activity. However, the high cytotoxicity shown by **2** (CC_{50} of 0.57± 0.72 µM) (Fig 2.1), and the fact that this metabolite is known to be the main cardenolide in the leaf extract of *N. oleander* (Hutchison et al., 2019), can explain the significant levels of apoptosis caused by both the crude extract and

the CH_2CH_2 fraction. These findings coincide with previous reports about the potent cytotoxic activity and the potential antitumor and antiviral activity of **2** (Pan et al., 2017; Zhai et al., 2022) and other cardenolides such as digitoxigenin . Alternatively, the low cytotoxic activity of **1** (CC_{50} 44.64± 0.08 µM) (Fig 2.1) coincides with that reported for digoxin when tested using the same cell line (Akimova et al., 2005; Boff, Schreiber, et al., 2020).

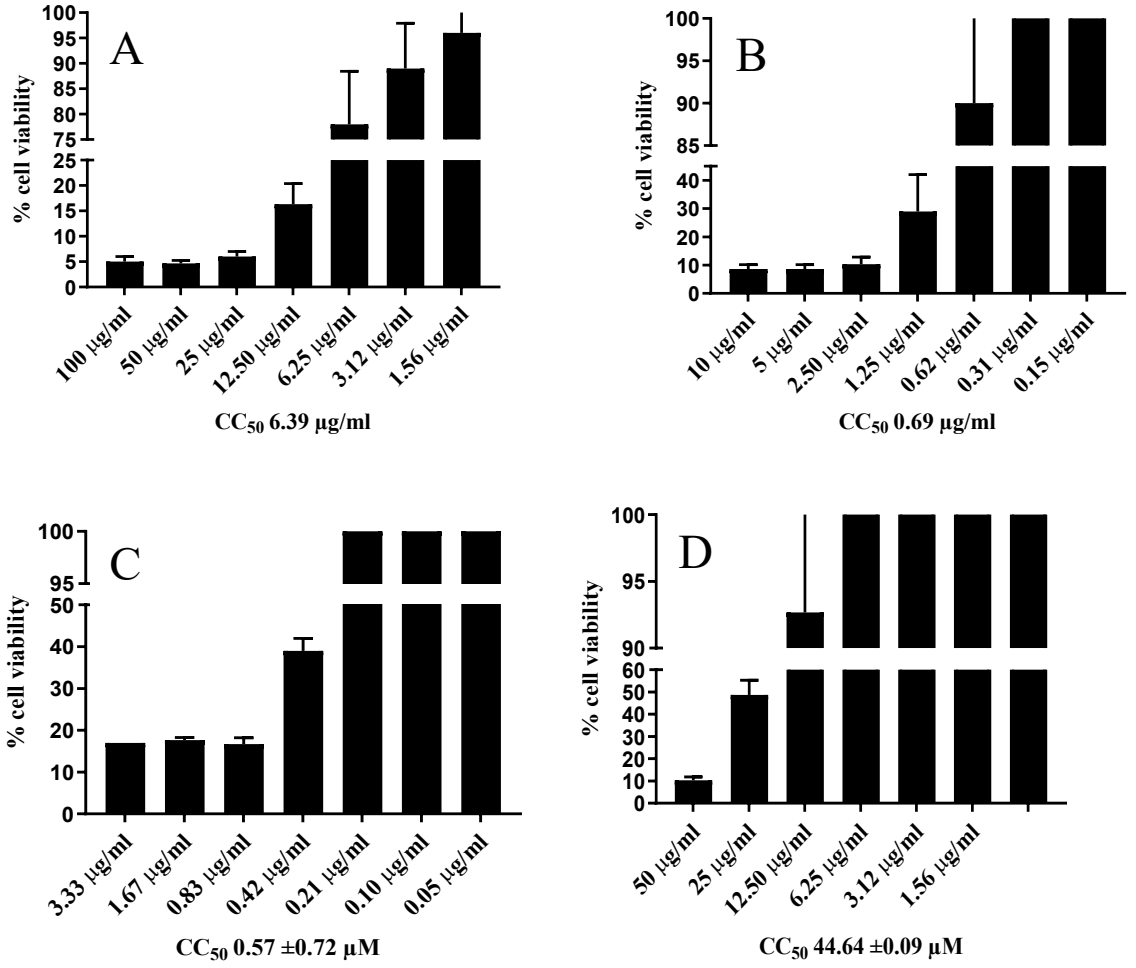

Figura 2.1 Cytotoxic activitiy of the crude extract of Nerium oleander (A), dichloromethane fraction of N. oleander (B), oleandrin (2) (C), and 8-hydroxi-digitoxigenin-3-O-β-D-diginoside (1) (D) in MDCK celular lines; CC50: 50% cytotoxic concentration.

The significant differences in the cytotoxic activity of **2** and the new cardenolide **1** could be explained by the small differences in their chemical structures, *i.e*. an extra hydroxyl and a missing acetyl group in the structure of **1** when compared to that of **2**. It is well known that

small changes in the chemical structure of secondary metabolites can have an effect on their biological activity, *e.g.* the case for digoxin and digitoxin (Fig S1), where the differences in cytotoxic activity could be explained by the presence/absence of a hydroxyl group at C-12 in the structures of the two cardenolides (Drugbank, 2024). Similarly, it has been reported that, on the basis of SAR/QSAR analyses, the antiproliferative capacity of cardenolides in the human lung cancer cell line A549 is related to the substituent groups in C-3, the lipophilicity of the molecule, and its capacity to act as hydrogen bond acceptors (Meneses-Sagrero et al., 2021). Since it has been reported that the main mechanism of action for the cytotoxic activity of **2** involves the inhibition of Na^+/K^+ ATPase (Ruta et al., 2020), and the fact that an interesting feature of Na^+/K^+ ATPase is the highly conserved nature of the ouabain-binding site, suggesting its playing a significant physiological role (Lingrel, 2010), it was decided to use molecular docking to evaluate the interactions of four cardenolides, oleandrin (**2**), the new cardenolide **1**, ouabain, and adynerin (**3**) with the Na^+/K^+ ATPase protein (Fig 2.2) to explain or predict the effect of the variations in their chemical structures, with their levels of cytotoxic activity.

Molecular docking is a computational simulation tool used to predict the preferred orientation of a ligand (key) that binds to a protein (lock); binding affinity and interactions between a ligand and a protein are determined using scoring functions based on force fields, empirical functions, knowledge-based methods, and consensus approaches. While it is widely used in different areas, the application of molecular docking to drug discovery has allowed the identification of potentially therapeutic metabolites (Agu et al., 2023). Currently, the available crystallographic data describe high affinity complexes of Na^+/K^+ ATPase with cardenolides, independently of the variations in their steroidal core structure and the degree of glycosylation (Ladefoged et al., 2021); accordingly, the molecular docking study carried out in this investigation showed all four cardenolides having a good affinity with the enzyme complex. However, while oleandrin (**2)** and ouabain showed the highest affinity levels, the new cardenolide **1** showed the lowest binding values to the protein; furthermore, **1** showed the most hydrogen bonds, while oleandrin (**2**) showed more lipophyllic interactions. These significant differences in binding affinity between **1** and **2** could explain the differences in cytotoxic activity between the two cardenolides when tested against the MDCK cell line (Table S2). These findings are also in agreement with reports that cardenolides having high docking and stability scores in molecular dynamics simulations showed higher levels of cytotoxic activity (Weigand et al., 2014). Alternatively,

and even though previous docking studies with cardenolides emphasize the importance of their interaction with Phe783 and Phe786 of αM5, presumably for stabilizing the steroid ring structure through stacking interactions, and with Gln111 and Asn122 of αM1 and αM2, which appear to be within direct, hydrogen bonding distances of the ouabain molecule are crucial for the high-affinity binding of ouabain (Yatime et al., 2011); in this investigation, these interactions were only observed with ouabain but not with **1** or **2**, strongly suggesting that these interactions may not be decisive in indicating a good affinity for the enzyme (Table S2).

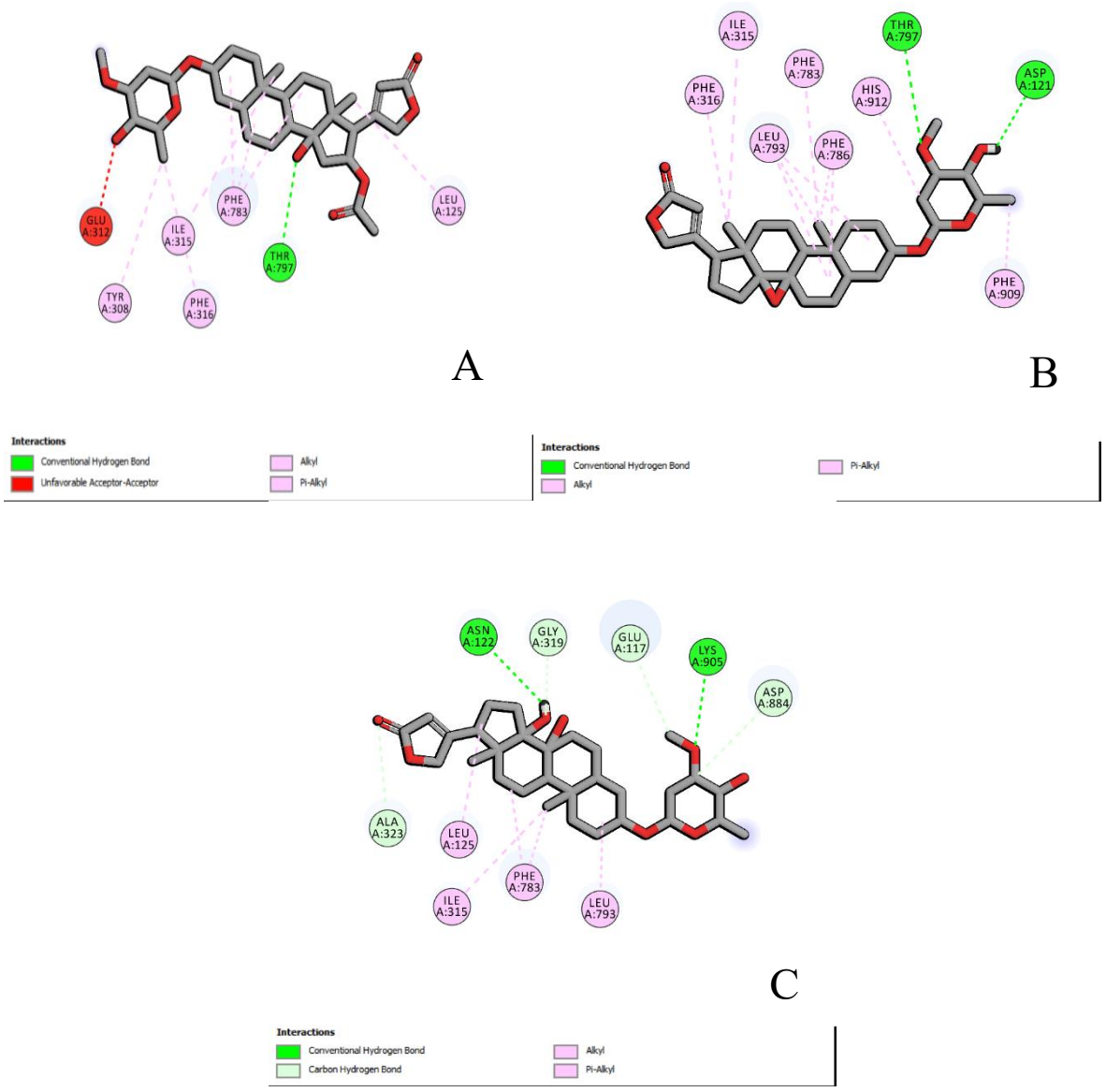

Figura 2.2 Detailed molecular interactions for Discovery Studio software between the binding pocket of Na+/K+ ATPase and oleandrin (2) (A), adynerin (3) (B) and 8-hydroxi-digitoxigenin-3-O-β-D-diginoside (1) (C). Interactions between Na+/K+ ATPase and ligand compo

2.5 Conclusions

This study has identified a new cardenolide that is the least cytotoxic among those evaluated in the MDCK cell line to date. This finding suggests that the new cardenolide may have potential as a less toxic alternative to other cardenolides in certain medical applications, mainly due to the signaling cascade generated by the cardenolides upon binding to the Na^+/K^+ ATPase protein. The discovery of a less cytotoxic cardenolide is a promising development in the field of cardenolide research and highlights the importance of ongoing efforts to identify and evaluate new compounds with potential therapeutic

2.6 Authors' contributions

IFVA carried out all experiments, identification the new cardenolide, and participated in preparing the manuscript. GAT supervised cytotoxic analysis with MDCK cell lines and revised the manuscript. KGS technical assistance in the purification of the new cardenolide. EHN analysis of docking molecular and revised the manuscript. VAH injection and analysis for HPLC-MS. LMPR designed the study, supervised the laboratory work, and contributed to the preparation and critical reading of the manuscript. All the authors have read the final manuscript and approved the submission. Data Availability Data is available upon request to the corresponding author.

2.7 Acknowledgements

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2.8 Conflicts of interest

The authors declare no conflict of interest.

2.9 Identificación de cardenólidos en *P. andrieuxii* usando HPLC-MS

En la búsqueda de cardenólidos presentes en *Pentalinon andrieuxii*, se analizó la fracción de media baja polaridad mediante LC-MS, bajo las mismas condiciones empleadas para la planta *Nerium oleander* y sus cardenólidos puros. Se lograron identificar componentes con tiempos de retención a 12.79, 12.87, 13.68, 13.75, 15.20 y 15.93 minutos, todos ellos presentando un fragmento de 354.21 m/z, característico del núcleo esteroidal de los cardenólidos (Ravi et al., 2020), Figura 2.3.

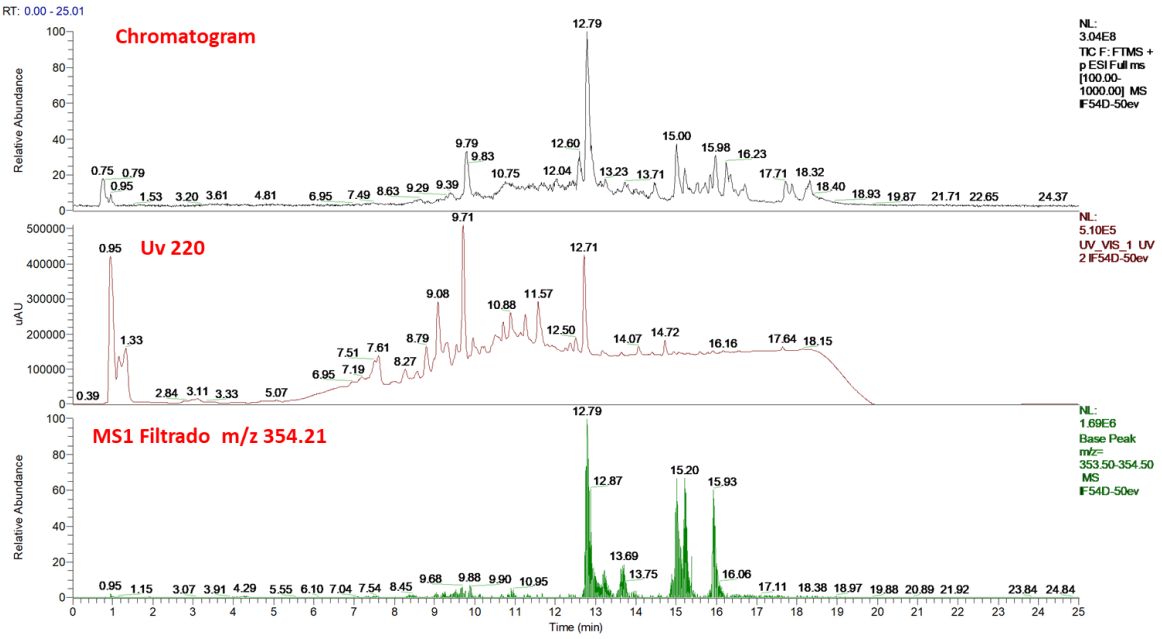

Figura 2.3 Perfil cromatográfico por HPLC-MS de la fracción de CH_2Cl_2 de *P. andrieuxii*. Perfil MS, perfil Uv a 220 y perfil filtrado a un fragmento de 354.21 m/z.

El análisis del patrón de fragmentación del componente más abundante (tR: 12.79 min) permitió su asociación estructural con el cardenólido urechitoxina. Se identificó un fragmento a m/z 331.22, correspondiente a la porción glucídica del compuesto (glucosa y oleandrosa) con adición de sodio (307 + Na). Además, se detectó un ion a m/z 353.2,

vinculado al núcleo esteroidal del cardenólido, y otro a m/z 661.43, que sugiere la pérdida secuencial del grupo acetato y una molécula de alcohol a partir de la estructura completa de urechitoxina (Figuras 2.4 y 2.5). Este perfil de fragmentación coincide con las características estructurales reportadas para urechitoxina (Hassall, 1951), que incluyen un sistema de azúcares unido a la genina del cardenólido mediante enlace glicosídico. Los datos respaldan la identificación preliminar de este metabolito en la muestra analizada.

Finalmente, la información obtenida por LC-MS de la fracción de media baja polaridad confirma la presencia de cardenólidos en dicha fracción y sienta las bases para su futura purificación

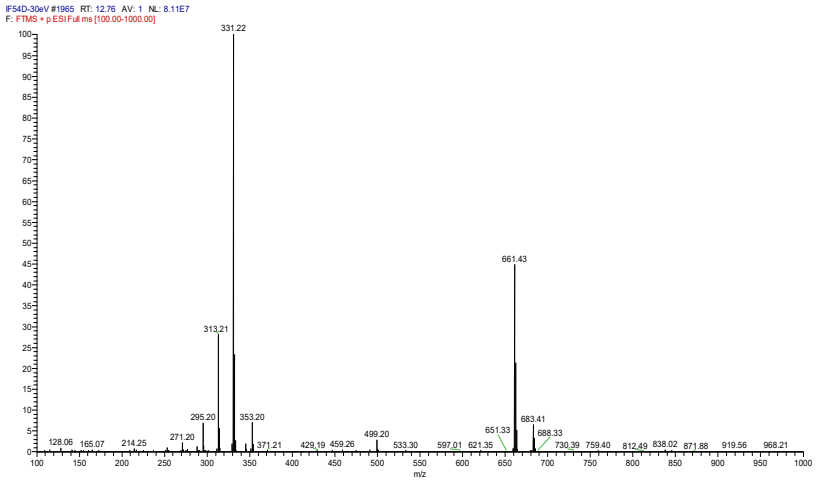

Figura 2.4 Patrón de fragmentacion del componente a 12.79 min en el perfil cromatografico por HPLC-MS de la Fx de CH_2CH_2.

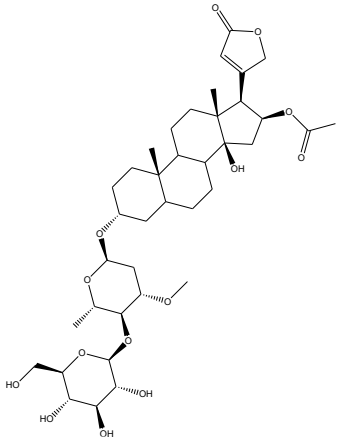

Figura 2.5 Estructura química de la Urechitoxina (738.9 g/mol)

CAPÍTULO III

3 *Pentalinon andrieuxii,* una planta medicinal con actividad antiviral contra el virus de influenza A(H1N1)

3.1 Resumen

Las infecciones respiratorias causadas por virus, entre ellas la gripe, son un importante reto sanitario mundial, cada año provocan epidemias estacionales. El brote de 2009, desencadenado por una nueva cepa del virus de la gripe A(H1N1), marcó la primera pandemia de gripe del siglo XXI y causó más de 200.000 víctimas mortales en más de 214 países. En la actualidad y pese a la disponibilidad de vacunas y medicamentos antivirales, las continuas mutaciones de estos virus obligan a seguir buscando tratamientos antivirales nuevos y más eficaces.

Pentalinon andrieuxii, una enredadera originaria de la Península de Yucatán, se utiliza tradicionalmente en la medicina maya para tratar las mordeduras de serpiente y las lesiones cutáneas causadas por la leishmaniasis cutánea. El conocimiento fitoquímico actual de *P. andrieuxii* incluye informes de trinorsesquiterpenos, triterpenos, derivados de esteroides y esteroles. Sin embargo, hasta la fecha no existen informes sobre la actividad antiviral del extracto o los metabolitos secundarios de esta planta. Como parte de nuestra búsqueda de nuevos metabolitos antivirales de plantas de la familia Apocynaceae, en este trabajo se infroma de los resultados obtenidos de la inhibición del efecto citopático de las fracciones semipurificadas del extracto de hoja de P. adrieuxii evaluados contra la cepa A/Yucatan/2370/09 (H1N1) del virus de Influenza A. El análisis LC-MS de la fracción bioactiva mostró la presencia de metabolitos polifenólicos con propiedades antioxidantes

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Full article

Pentalinon andrieuxii, a medicinal plant with antiviral activity against the Influenza A(H1N1) virus

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3.1 Abstract

Respiratory viral infections, including influenza, continue to pose a significant global health challenge, leading to seasonal epidemics every year. The 2009 outbreak, triggered by a new strain of the influenza A(H1N1) virus, marked the first influenza pandemic of the 21st century that resulted in over 200,000 fatalities across more than 214 countries. Presently, and despite the availability of vaccines and antiviral medications, the ongoing mutations of these viruses necessitates continuing the search for new and more effective antiviral treatments.

Pentalinon andrieuxii, a vine native to the Yucatán Península, is traditionally used in Mayan medicine to treat snake bites and the skin lesions caused by cutaneous leishmaniasis. Current phytochemical knowledge of *P. andrieuxii* includes reports of trinorsesquiterpenes, triterpenes, steroid derivatives, and sterols. However, to date, there are no reports on the antiviral activity of the extract or secondary metabolites from this plant. As part of our search for new antiviral metabolites from plants of the Apocynaceae family, we wish to report herein on the inhibition of the cytopathic effects of the semipurified fractions from the leaf extract of P. adrieuxii, when tested against the A/Yucatan/2370/09 (H1N1) strain of the influenza A virus, and the identification of polyphenolic metabolites in the bioactive fraction.

Keywords cytopathic effect; Apocynaceae; Influenza; polyphenolics

3.2 Introduction

Currently, seasonal influenza infections are responsible for approximately 300,000 deaths annually worldwide (Rajãoy Pérez, 2018). International travel and migration are believed to have facilitated the spread of pathogenic strains of virus and microorganisms, with cities becoming important hubs for the transmission of infectious diseases. Several rural or wild pathogens have adapted to urban environments, while others have emerged or re-emerged in urban areas (Alirol et al., 2011; Kapiririy Ross, 2020).

Influenza A(H1N1) belongs to the Orthomyxoviridae family that includes seven genera and of these, influenza A, B and C can affect humans. These viruses, considered pleomorphic particles (80-120 nm) and characterized by having a lipid coat, have their genomes made up of segmented negative-strand RNA and their own RNA-dependent polymerase complex and are associated with the expression of two important glycoproteins in the process of infection: hemaglutinin (HA) and neuraminidase (NA). Even though the natural reservoir of the influenza virus is wild waterfowl (Eisfeld et al., 2015), it is transmitted via the respiratory system and the main symptoms of influenza are related to respiratory diseases which include headache, fever, muscle pain, nasal congestion, cough, and diarrhea. In the northern and southern hemispheres the presence of influenza is associated with the colder months, while in the tropics there is no association with the climatic time of the year (Lowen et al., 2014).

Presently, the overall share of antivirals developed for the treatment of influenza infections is only 4.6%, with 10% of these being antivirals approved for the treatment of acute infections (Chaudhuri et al., 2018). The antivirals used against influenza that are approved by the US Food and Drug Administration (FDA) include neuraminidase (NA) inhibitors (zanamivir, oseltamivir and peramivir). While there are a number of antivirals being developed, including Baloxavir marboxyl (zofluza), VIS-410, MEDI-8852, Pimodivir, JNJ-5806, NT300, Fludase, Laminavivir Octanoate, Radavisen and DAS181 (Chaudhuri et al., 2018), the existence of oseltamivir-resistant mutants emphasizes the urgent need to use known targets to search for new molecules with antiviral activity from different natural sources (Shiny Seong, 2019).

To date, a number of natural products from plants have been shown to posses antiviral activity (Miresmailliy Isman, 2014), particularly against the Influenza A virus (IAV) (Perez,

2003). The Apocynaceae family is known to be one of the most diverse angiosperm families, rich in alkaloids, flavonoids, carbohydrates, and terpenoids such as cardenolides and steroids (Yadav et al., 2024). A number of plants from this family have demonstrated antiviral activity, with cardenolides identified as the key bioactive metabolites contributing to these effects (Yadav et al., 2024). Recently, *Pentalinon andiieuxii* (Müll. Arg.) B.F. Hansen & Wunderlin (Apocynaceae) (Fig 3.1), has been reported to contain cardenolides structurally-related to oleandrin, that are used by lepidopterans as part of their own defense mechanism against predators (Villegas Acosta, 2019). Even though there are limited reports on the biological activity of the phytochemicals identified from *P. andrieuxii*, sterols isolated from the roots have been reported to show immunomodulatory effect when treated with activated macrophages (Pan et al., 2012). However, in view of the recent reports about the cardenolide oleandrin having antiviral activity against COVID-19 , and the fact that cardenolides structurally-related to oleandrin have been detected in the leaf extracts of *P. andrieuxii*, we describe here the evaluation of the antiviral activity of the leaf crude extract and its semipurified fractions.

Fig 3.1 Flowering plant of *Pentalinon andrieuxii.*

3.3 Experimental

Plant material. Fresh leaves of *P. andrieuxii* were collected in Merida, Yucatan, Mexico in June 2018 from a population kept in the nursery of Centro de Investigación Científica de Yucatán. A voucher specimen was deposited at the Herbarium of CICY under collection number 68850.

Extraction, isolation and identification. The plant material (600 g) was dried at room temperature for three days, and then it was ground and extracted three times by maceration with methanol (20 L) at room temperature. The solvent was filtered, first through cheesecloth, then cotton, and evaporated under reduced pressure to yield 59 g (23%) of crude methanolic extract (A1). A portion (15 g) of the crude extract (A1) was suspended in a mixture of H2O/methanol (3:2, v/v) and the resulting suspension was successively partitioned with hexane (three times, 2:1, v/v,), dichloromethane (three times, 2:1, v/v,), and ethyl acetate (three times, 2:1, v/v,) to produce the corresponding low (1A, 8.75 g, 58%), medium-low (1B, 0.42 mg, 3%) and medium (1C, 0.70 g, 5%) polarity semipurified fractions, together with the aqueous residue or polar fraction which was frozen and freeze-dried (1D, 3.80 g, 25%).

Quantification of in vitro antioxidant activity. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was carried out following the methodology previously reported (Williamsy Benkeblia, 2018) Briefly, a 20 µL aliquot of each sample: The medium polarity fraction of *P. andrieuxii* (100 µg/mL), control; methanolic extract of *Camellia sinensis* (1000 µg/mL) or blank (MeOH) was mixed with 180 µL of a 0.1 mM ethanolic solution of DPPH in a 96-well plate. After incubation in the dark for 30 minutes, the decrease in absorbance was measured at 517 nm using six replicates per sample. Antioxidant activity was calculated as milligrams of Trolox equivalents (TE) per gram of sample using a Trolox standard calibration curve (Fig S1).

Determination of total phenol content (TPC). The total phenol content (TPC) was determined following the Folin-Ciocalteu methodology adapted to the 96-well plate assay, as described by (Hubery Rupasinghe, 2009) a stock solution of gallic acid (500 mM) was used to prepared serial dilutions (20 to 200 µg/mL) to build a standard curve. The medium polarity fraction were evaluated at a concentration of 100 µg/mL (six replicates); the solvent and an the methanolic extract of *Camellia sinensis* (1000 µg/mL) were used as blank and positive controls, respectively. For the test, 100 µl of 0.2 N Folin-Ciocalteu´s reagent was combined with 20 µl of sample; after incubating for 5 min at room temperature (27±1°C), 80 µl of a 75% (w/v) solution of sodium carbonate was added to the mixture. The plate assay was incubated in the dark for two more hours under shake (100 rpm) conditions. The absorbance of the samples was recorded twice at 760 nm using a microplate reader (Cytation 3, BioTek Instrument Inc. USA). The results were expressed as mg of gallic acid equivalents (EAG) per gram of sample (Fig S2).

Cells and virus strains. The influenza virus strain was provided by the Virology Laboratory of the Centro Regional de Investigaciones 'Dr. Hideyo Noguchi' (Universidad Autónoma de Yucatán). The virus, identified as A/Yucatan/2370/09 (H1N1) pdm (sensitive to oseltamivir carboxylate), was propagated in MDCK cells in the presence of 1 µg TPCK-trypsin (SIGMA) per mL and stored at -70 °C until use. Viral titre was determined in MDCK cells using a standard plaque assay protocol.

Cytotoxicity Assay

The cytotoxicity assay was performed using the MDCK (Madin-Darby Canine Kidney) cell line (donated by the Instituto de Diagnóstico y Referencia Epidemiologico InDRE/IRR FR-58); the MDCK cells were maintained in Dulbecco's minimal essential media (DMEM) enriched with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin (GIBCO), and 1M HEPES, and incubated at 37 °C in a 5% CO_2 environment. For the assay, MDCK cells were placed in 96-well plates at a density of 1×10^5 cells per well and incubated at 37 °C with 5% CO2 for 24 hours. After washing twice with phosphate-buffered saline (PBS) solution, each well received 100 µL of the extract or semipurified fractions at one of seven concentrations: 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 µg/mL. Each concentration was replicated four times, with a control well containing only DMEM. The plates were incubated for an additional 72 hours under the same conditions. After removing the inoculum, the cells were washed once with PBS, stained with 0.4% crystal violet in methanol for 30 minutes, then washed again with running tap water and allowed to dry. Absorbance was measured at 490 nm using a Multilabel Plate Reader (Victor 3x Perkin-Elmer). Cell viability was calculated as the ratio of the optical density (OD) of treated cells to that of the control cells, expressed as a percentage: Cell viability = (OD treated cells /OD cell control)*100

The concentration causing cell death in 50% of the cells (CC_{50}) was determined by plotting extract concentration against cell viability percentage and performing regression curve analysis using the GraphPad Prism 7 software; for each sample tested, a cell control with only DMEM was included.

Cytopathic effect reduction assay in pre-treatment. MDCK cells were grown in DMEM supplemented with 10% FBS for 24 h before washing with PBS and incubating for another

24 hours with the extract or semipurified fractions in quadruplicate. After removing the inoculum, cells were exposed to virus at MOI: 0.001 A/Yucatan/2370/2009. After washing with PBS again, cells were incubated for 72 hours in DMEM containing 1μg/mL TPCK trypsin.

Cytopathic effect reduction assay in co-treatment. Cells were similarly prepared as described above, but combined with the extract or semipurifid fractions and the virus before incubation at room temperature for one hour. The mixtures were then added to the cells and incubated under standard conditions.

Cytopathic effect reduction assay in post-treatment. Cells were similarly prepared as described above, seeded until confluent and infected with the virus before being treated with various dilutions of the crude extract or semipurified fractions in DMEM supplemented with TPCK trypsin for 72 h.

Chromatographic analysis by UPLC-PDA-ESI-MS/MS

Chromatographic analysis was conducted using a Waters Acquity H-Class UPLC system (Milford, MA, USA) equipped with a quaternary pump, an automatic injector, and a photodiode array (PDA) detector. The separation was carried out using a Waters Acquity UPLC BEH C18 column (1.7 µm, 100 × 2.1 mm ID) and a mobile phase consisting of 0.1% formic acid in ultrapure water (A) and 0.1% formic acid in acetonitrile (B). The PDA detector recorded wavelengths in the 190–400 nm range, with absorbance measured at 290 nm.

For mass spectrometry (MS/MS), a Waters Xevo TQ-S micro instrument was employed under previously reported but modified conditions (Ana et al., 2018). The negative ion mode used a collision energy of 10 eV. Mass spectra were collected in full scan mode over a range of 50–700 m/z. Data acquisition and processing were performed using MassLynx V4.1 software. Tentative identification of components was achieved by comparing the chromatographic and MS experimental data with those in literature references and public databases such as the European Mass Bank (https://massbank.eu/MassBank/index.html) and ReSpect for phytochemicals (http://spectra.psc.riken.jp/menta.cgi/respect/index).

Statistical Analyses

Data are presented as the mean (±) standard deviation of three independent experiments; statistical significance was calculated by a one-way ANOVA analysis and Dunnett´s test, with p-values < 0.05 considered as significant using the GraphPad Prism 7 software.

3.4 Results and discussion

Evaluation of the cytotoxicity of the crude leaf extract of *P. andrieuxii* and its semipurified fractions in MDCK cells showed that neither the extract, nor the semipurified fractions were cytotoxic (Fig 3.2 and Fig S3). These findings coincide with those previously reported about the leaf extract of *P. andruexii* not being toxic when tested in the Brine Shrimp lethality assay, commonly used as an indirect assay to detect cytotoxic metabolites im plant extracts Once it was established that none of the samples were cytotoxic, they were evaluated for their capacity to reduce the cytopathic effect, which refers to the ability of a particular sample or product to prevent the damage caused by viruses in infected cells, which can lead to cell death or alterations in cell function (Fields et al., 2007). The crude extract and the semipurified fractions were tested using three different strategies to reduce the cytopathic effect, pre-treatment, co-treatment and post-treatment. Bioassay results showed the aqueous fraction of the *P. andrieuxii* best preventing the cytopathic effect, with a 100% cell viability at all concentrations tested (50-1.56 µg/mL) using the co-treatment strategy (Fig 3.2). Similarly, co-tretament with the medium polarity fraction also showed an important inhibition of the cytopathic effect, with over 80% cell viability at all concentrations tested, and an SI value of >64 for both fractions. Interestingly, post-treatment with the aqueous fraction only showed moderate antiviral activity (58% cell viability) at 50 µg/mL, with an IC_{50} of 46.27 µg/mL and an SI of 2.1, while post-treatment with the medium polarity fraction resulted in good antiviral activity (87% cell viability) when tested in the post-treatment at 50 µg/mL, having an IC_{50} of 23.7 µg/mL and an SI of 4.2. The lack of antiviral activity when testing the extract and the semipurified fracions using the pre-treatment strategy suggests that the bioactive metabolites do not play a significant role in preventing the viral infection of the MDCK cells. However, the activity observed when testing the fractions using the co-treatment strategy implies that the bioactive metabolites act by either blocking the viral replication pathways, which reduces cell damage associated with infection, or by protecting the cells from virus-induced lysis, maintaining cell integrity and reduction of the cytopathic effect (Kim et al., 2020; Suchmany Blair, 2007). The fact that the leaf crude extract did not show a significant capacity to reduce the cytopathic effect,

while its semipurified fractions did, demonstrate the importance of an early fractionation process to uncover metabolites with antiviral, or any other, activity.

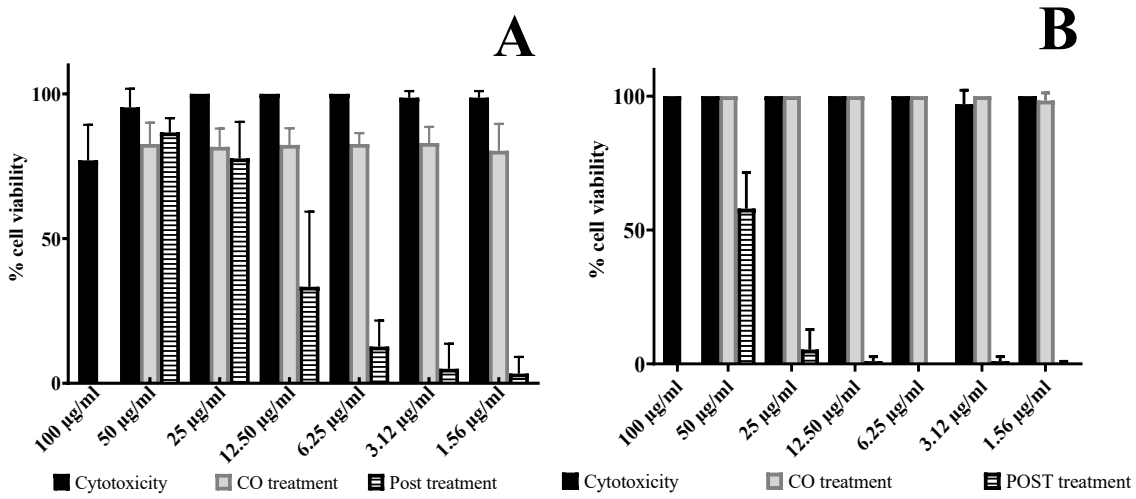

Fig 3.2. Cytotoxicity against MDCK cells and reduction of the cytopathic effect in co-treatment and post-treatment of medium (a) and high (b) polarity semipurified fractions from the extract of *P. andrieuxii*.

While several studies have investigated the potential of plant extracts in the search for new antivirals against influenza A(H1N1) (Chojnacka et al., 2021), the SI value (64) found for the medium polarity and aqueous fractions, is higher than that reported for the extract of *Fritillaria thunbergii* (Liliaceae) (SI 50.6), found to be less cytotoxic and more effective at inhibiting influenza infection A(H1N1) than oseltamivir, during its evaluation using the post-treatment strategy (Kim et al., 2020). The fact that both fractions proved to be effective even at the lowest concentrations tested (1.56 µg/mL), with inhibition of the cytopathic effect of over 50%, suggests that the SI of the fractions from *P. andrieuxii* could be higher.

A preliminary evaluation of the TLC chromatographic profiles of the medium and high polarity fractions showed a significant presence of antioxidant components in both fractions, detected when using the DPPH reduction reagent. However, quantification of the antioxidant activity and the total phenol content (TPC) of the two fractions showed that while the the TPC of the medium polarity fraction was higher than that of the aqueous fraction (1006.985± 239.9 vs 426.129± 0.8 mg EAG/g), the antioxidant activity of the medium polarity fraction was lower than that of the aqueous fraction (316.077± 35.7 mg vs 639.014± 38.7 mg TEAC/g extract). These values suggested that the medium polarity

fraction was rich in polyphenolic metabolites and that, in this case, the TPC content of the two fractions is is not directly related to their antioxidant activity, even though a high content of polyphenols is commonly associated with antioxidant activity, known to be beneficial because its capacity to protect against oxidative stress (Kamboj et al., 2012; Surco-Laos et al., 2020). However, these findings are in agreement with literature reports describing medium polarity fractions of plant extracts as containing a wide variety of polyphenolic metabolites such as flavonoids and catechins (Álvarez et al., 2008; Floresy Orellana, 2016; Montufar-Canto et al., 2025), in comparison with aqueous fractions, where the presence of sugars, alkaloids and terpenoids, together with polyphenols, have been reported (Barthwaly Mahar, 2024; Jamaly Ahmad, 2024). UHPLC-MS analysis of the aqueous and medium polarity fractions showed that the chromatographic profiles of both fractions were qualitatively similar, but quantitatively different (Figures S4); analyses of the λmax absorption values, together with a comparison of the fragmentation patterns of the major components with those contained in the data base of the equipment, allowed the preliminary identification of the phenolic acids: neochlorogenic acid, chlorogenic acid and coumaroylquinic acid, in addition to the flavonoid rutin and the flavonoid glicosides kaempferol-3-O-rutinoside and kaempferol-N (Table 3.1, Fig S3-S7). The presence of these metabolites in the medium polarity fraction is relevant since it has been reported that phenolic acids such as caffeic acid, gallic acid, ellagic acid, chlorogenic acid, and quinic acid, as well as flavonoids including quercetin, apigenin, luteolin, baicalin, naringenin, and kaempferol have demonstrated significant antiviral activity (Kaul et al., 1985; Ninfali et al., 2020).

Table 3.1. Polyphenolic metabolites detected in the medium polarity fraction from the extact of *P. andrieuxii*

Metabolite number	t_R (UHPLC-PDA)	λ max	Molecular ion ([M–H]$^-$) *m/z*	CV	Fragment ions *m/z*	Preliminary identification
1	8.88	324, 214, 193	353	75	191, 179, 135	Neochlorogenic acid
2	9.29	325, 217, 194	353	75	191	Chlorogenic acid
3	9.89	312, 202	337	75	191, 173, 135	Coumaroylquinic acid
4	10.3	353, 255, 206	609	150	300, 271, 243	Rutin
5	10.87	347, 264, 210	593	150	285, 284, 255, 227	Kaempferol-3-O-rutinosido
6	11.19	345, 265, 213	593	150	284, 255, 227	Kaempferol-N

While it has been established that structural features such as hydroxyl groups, substituents such as methyl groups and the position of functional groups can significantly influence the antiviral properties of polyphenols (Li et al., 2018), this study represents the first report of potential antiviral activity of neochlorogenic acid, coumaroylquinic acid and glucosylated derivatives of kaempferol.

3.5 Conclusions

Even though cardenolides are known to occur in plant species of the Apocynaceae family and have been reported to show antiviral activity, no presence of cardenolides was detected in the fractions with antiviral activity obtained from the leaf extract of *P. andrieuxii*. Instead, a number of polyphenols, some of which have been reported to show antiviral activity, were identified in the bioactive fractions. This study represents the first report on the potential of the leaf extract of *P. andrieuxii* as a potential source of therapeutic agents

against Influenza A(H1N1) infections. Further research is needed to identify the metabolite or metabolites responsible for the detected antiviral activity, as well as to elucidate the mechanism of action against influenza viruses, and to evaluate the potential broad-spectrum antiviral activity against other viral strains.

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CAPÍTULO IV

4 Conclusiones generales y perspectivas

4.1 Conclusiones generales

En este estudio se evaluó el potencial antiviral del extracto metanólico y de fracciones semipurificadas de *Pentalinon andrieuxii* frente al virus Influenza A(H1N1), utilizando como controles el extracto, fracciones de polaridad media baja y oleandrina obtenidas de *Nerium oleander*.

Durante el análisis fitoquímico de *N. oleander*, se identificó un nuevo cardenólido (8-hidroxi-digitoxigenina-3-O-β-D-diginósido), el cual demostró una menor citotoxicidad en células MDCK en comparación con otros cardenólidos evaluados, incluida la oleandrina y mediante estudios de *docking* molecular, se estableció que la reducida citotoxicidad del nuevo cardenólido se asocia a su menor afinidad por la proteína Na^+/K^+ ATPasa, sugiriendo su potencial como alternativa terapéutica menos tóxica en aplicaciones médicas, particularmente en el ámbito cardiovascular.

Los resultados descartan la actividad antiviral directa de los cardenólidos de *N. oleander* y *P. andrieuxii* en el modelo empleado, debido a su citotoxicidad en células MDCK o a la ausencia de inhibición del efecto citopático.

En el estudio de la actividad antiviral de *Pentalinon andrieuxii* contra el virus Influenza A(H1N1), se identificaron dos fracciones bioactivas (media-alta y alta polaridad) que mostraron reducción del efecto citopático en ensayos de post-tratamiento y co-tratamiento. El análisis fitoquímico de estas fracciones reveló la presencia de polifenoles en las fracciones de mediana y alta polaridad, compuestos asociados a propiedades antioxidantes que podrían contribuir a la mitigación del efecto citopático. Estudios previos respaldan la actividad antiviral reportada para este tipo de metabolitos.

Este trabajo constituye el primer reporte científico que evidencia el potencial del extracto de hoja de *P. andrieuxii* como fuente de agentes terapéuticos contra infecciones por Influenza A(H1N1), abriendo líneas de investigación para la caracterización de sus principios activos.

Los resultados destacan la relevancia de explorar mecanismos de acción no citotóxicos en compuestos vegetales, particularmente en el contexto de enfermedades virales donde su tratamiento es limitado.

4.2 Perspectivas

Con base en los resultados obtenidos en este estudio, se propone continuar con la purificación y el análisis fitoquímico de *Pentalinon andrieuxii* para identificar los cardenólidos presentes en esta planta. Estos compuestos podrían tener relevancia en modelos biológicos vinculados a la salud pública, así como en la investigación de la interacción interespecífica con la polilla *Syntomeida epilais*. Aprovechando el conocimiento actual, se podrían emplear herramientas como los análisis de desduplicación para detectar los metabolitos presentes en *P. andrieuxii* sin necesidad de aislarlos, facilitando así la confirmación de la presencia de urechitoxina.

Asimismo, es fundamental ampliar la investigación sobre las aplicaciones terapéuticas de los cardenólidos derivados de *Nerium oleander*, particularmente en el ámbito de la salud pública. La oleandrina, compuesto ampliamente caracterizado por su potencial farmacológico, sirve como referencia clave en este campo. El hallazgo de un nuevo cardenólido con estructura molecular y propiedades fisicoquímicas análogas a este compuesto, además de demostrar menor citotoxicidad, sugiere perspectivas prometedoras. Esta similitud estructural permitiría evaluar su eficacia en protocolos experimentales ya establecidos, como los ensayos preclínicos contra ciertos tipos de cáncer o el desarrollo de terapias para enfermedades cardiovasculares.

De igual forma, se propone continuar con el análisis fitoquímico de *P. andrieuxii* con el objetivo de identificar los metabolitos responsables de la actividad antiviral contra el virus Influenza A(H1N1). Inicialmente, se sugiere emplear herramientas como el análisis de componentes principales y el análisis de desduplicación, que permitirán identificar dichos metabolitos sin necesidad de purificación y aislamiento. Paralelamente, se recomienda proseguir con los bioensayos para establecer el mecanismo de acción de los metabolitos presentes en *P. andrieuxii* frente al virus Influenza A(H1N1). Los resultados positivos en co-tratamiento y post-tratamiento sugiere que algún proceso viral está siendo inhibido para impedir la infección de las células MDCK. Comprender el mecanismo de acción de estos metabolitos facilitará la exploración de su potencial desarrollo para aplicaciones comerciales.

Finalmente, se pretende desarrollar y optimizar nuevos protocolos que faciliten la identificación rápida y eficiente de metabolitos con actividad antiviral. Esto permitirá

evaluar el potencial de los compuestos bioactivos de *Pentalinon andrieuxii* frente a virus que afectan bacterias o plantas, con el objetivo de establecer su posible aplicación como agentes antivirales de amplio espectro.

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