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TESIS DOCTORAL

EVALUACIÓN DEL EFECTO NEFROPROTECTOR DE DOS ESPECIES VEGETALES COMESTIBLES DEL ESTADO DE HIDALGO: PÁPALO QUELITE (*Porophyllum ruderale*) Y PINGÜICA (*Pyracantha koidzumii*)

Para obtener el grado de

Doctora en Ciencias de los Alimentos y Salud Humana

PRESENTA

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RESUMEN

La enfermedad renal (ERC) es un problema de salud pública de gran magnitud que se acompaña de elevada morbilidad, mortalidad y costos tanto para el paciente como para el sistema de salud, lo que lleva a los pacientes a optar por la medicina herbaria, convirtiéndola en la alternativa principal para la atención primaria de la salud, por lo que es importante que a través de la investigación se garantice la inocuidad y calidad de las plantas medicinales utilizadas como tratamiento de forma empírica. El objetivo de este proyecto fue evaluar el efecto nefroprotector de dos plantas comestibles del estado de Hidalgo, pápalo quelite (*Porophyllum ruderale*) y pingüica (*Pyracantha koidzumii*) mediante un estudio biodirigido. Para ello primero se obtuvo un extracto hidroalcohólico (MeOH:H_2O, 70:30 *v*/*v*) de cada planta. Posteriormente, se evalúo la capacidad anti-inflamatoria y antioxidante *in vitro* de los extractos utilizando para ello un modelo de producción de ON estimulado por LPS en macrófagos murinos y los métodos de inhibición de los radicales ABTS y DPPH, respectivamente. Adicionalmente, se determinó el contenido de fenoles totales por el método de Folin-Cicaltecou. El extracto hidroalcohólico de *P. ruderale* presentó mayor actividad antioxidante (FRAP= 69.4%, DPPH= 60.74%) y anti-inflamatoria (%ION= 12.23 ± 3.81) en los ensayos *in vitro* en comparación con el extracto de pingüica (FRAP= 31.63 %, DPPH= 22.09%, %ION= 8.81 ± 3.94). Debido a que el daño renal está asociado a un efecto anti-inflamatorio sostenido, así como a un desbalance en el sistema redox, únicamente el extracto hidroalcohólico de *P. ruderale* fue evaluado en el modelo de daño renal agudo inducido por Tioacetamida en ratas Wistar. El extracto hidroalcohólico de *P. ruderale* a dosis de 100 mg/kg disminuyó los principales marcadores de daño renal en suero y orina (Orina: sangre, cetonas, proteínas, nitritos. Sangre: Glucosa, creatinina, urea y nitrógeno ureico) en las ratas. Además, se observó que el extracto presentó un efecto diurético no asociado al daño renal. A continuación, se evalúo la capacidad hipoglucemiante del extracto hidroalcohólico de *P. ruderale*. Se elaboró una curva de tolerancia a la glucosa mediante la monitorización en diferentes momentos (0-120 min) de los niveles de glucosa en muestras de sangre tomadas de un corte apical de la cola de ratones CD1. El HEPr mostró un efecto significativo desde el punto de partida sobre los niveles de glucosa basales (114,33 ± 14,74 mg/dL) en comparación con el grupo de control (60,33 ± 4,16 mg/dL) y el grupo tratado con metformina (129 ± 13 mg/dL). Además, los valores al final de la curva de tolerancia (120 min) mostraron una disminución significativa en el grupo de estudio (66 ± 10,39 mg/dL) en comparación con el grupo tratado con metformina (108,67 ± 4,50 mg/dL). Finalmente, se identificaron por HPLC los compuestos mayoritarios presentes en el extracto hidroalcohólico de *P. ruderale*, encontrando al ácido clorogénico (ácido 5-*O*-cafeofeoil quinico) como constituyente mayoritario seguido del ácido criptoclorogénico (ácido 4-*O*-cafeoilquinico), ácido ferúlico, quercetina-3-*O*-glucósido y kaemferol-3-*O*-glucósido.

ABSTRACT

Kidney disease (CKD) is an important public health problem accompanied by high morbidity and mortality. Pápaloquelite (*Porophyllum ruderale*) and pingüica (*Pyracantha koidzumii*) are plant species endemic to Mexico used in traditional medicine for the treatment of the symptoms and complications of this disease. The objective of this project was to evaluate the nephroprotective effect of the hydroalcoholic extract (MeOH:Water 70:30, *v/v*) of papalo (HEPr) and pingüica (HEPk), by means of a biodirected study. First, the *in vitro* anti-inflammatory and antioxidant capacity of the extracts of both species was evaluated, then the *in vivo* nephroprotective activity was evaluated in a model of acute kidney disease induced by Thioacetamide in Wistar rats, of the specie with the highest *in vitro* activity. Then, the hypoglycemic capacity of the extract with the highest activity was determined in a model of oral glucose tolerance curve in CD1 mice. Finally, the major compounds present in the hydroalcoholic extract of the species with the highest biological activity were identified by HPLC.

HEPr and HEPK showed a slight effect on LPS-NO production in macrophages (15% INO at 40 μg/mL and 10.68% INO at 10 μg/mL, respectively). Antioxidant activity was higher in HEPr in the FRAP assay, followed by activity in the DPPH and ABTS radical assay compared to HEPk (HEPr: 69.04, 63.06 and HEPk: 32.96% and 31.63, 22.09, 10.09% inhibition, respectively). In addition, the values of renal injury biomarkers in urine and serum from HEPr-treated rats remained in normal ranges. HEPr showed a significant reduction of blood glucose values showing its maximum effect at the end of the tests (120 min), resulting in a 39.26% reduction of blood glucose in the HEPr-treated group compared to that obtained for the Metformin + glucose group. Finally, 5-O-caffeoylquinic, 4-O-caffeoylquinic and ferulic acids; as well as 3-O-quercetin glycoside and 3-O-kaempferol glycoside were identified by HPLC as major components of HEPr.

In conclusion**,** *Porophyllum ruderale* constitutes a source of compounds for treatment of acute kidney injury.

CAPÍTULO I.
INTRODUCCIÓN

MARCO TEÓRICO

1.1 Epidemiología de la enfermedad renal crónica (ERC)

La enfermedad renal crónica (ERC) es un problema de Salud Pública mundial, ya que se acompaña de elevada morbilidad, mortalidad y costos elevados tanto para el paciente como para el sistema de salud así como de una calidad de vida disminuida, dicho lo anterior la población con daño renal en estadios tempranos, generalmente no son reconocidos ni tratados de manera oportuna, los cuales sin atención pertinente incrementan su riesgo para la pérdida de la función renal, el desarrollo de complicaciones y muerte cardiovascular precoz [1–3].

Esta enfermedad es causada principalmente por padecimientos crónico degenerativos mal controlados entre los que destacan la diabetes mellitus y la hipertensión arterial, ambos con altos índices de prevalencia en México, 9.4% y 25.55% respectivamente [4–6].

Sin embargo, la investigación de la prevalencia-incidencia de la ERC y de la morbimortalidad en México es un tanto complicada debido a la deficiencia en la información existente sobre la enfermedad y de las limitaciones de los reportes desarrollados por los sistemas de salud.

A pesar de esto, en un estudio epidemiológico de la insuficiencia renal en México, dado a conocer por la Secretaría de Salud en el 2010 [7,8] se destacó que cada año se suman al menos 40, 000 nuevos casos en el país, debido a una falta de cultura de prevención, llevando a un rápido crecimiento en los últimos años (11% anual), llegándose a duplicar la incidencia en la población mexicana. En cuanto a la prevalencia de ERC en México se habla de 1,409 pacientes por cada 1,000,000 de habitantes (14%); donde del 2006 al 2012 la prevalencia reportada aumentó un 45.7%, de los cuales el 88.3% requirió de un tratamiento sustitutivo, presentando la mayor prevalencia en Tlaxcala, Hidalgo, Morelos y Nayarit [8].

De manera análoga en el Reporte de Salud Mundial de la OMS y el Global Burden of Disease Study (GBDS) [3] se describe que la incidencia de ERC a nivel mundial aumentó en 89% a 21,328,972 y la prevalencia aumentó en un 87% a 275,929,799, mientras que la prevalencia de muerte debida a ERC aumentó en un 98% a 1,186,561 además de los años de vida ajustados por discapacidad (AVAD) que aumentaron un 62% a 35,032,384. De modo semejante en México la ERC fue la tercera causa de mortalidad ajustada por edad y sexo con el 9% de todas las muertes reportadas, además de contribuir al 8.1% de los años perdidos de vida por muerte prematura (APMP) y el 5.7% de los años de vida saludables perdidos por discapacidad (AVISA).

De continuar el rápido incremento en los niveles de incidencia de esta enfermedad, se proyecta que para el año 2025 en México existirán alrededor de 212,000 pacientes diagnosticados con enfermedad renal, de los cuales morirán 160,000 cada año, debido a que

no existirá la infraestructura necesaria para estos pacientes ni el soporte financiero para su tratamiento, esto de acuerdo con proyecciones realizadas por el Centro Nacional de Excelencia Tecnológica en Salud [9].

1.2 Función renal

Se denomina función renal a la eficiencia con la que los riñones filtran la sangre, los individuos con dos riñones sanos tienen el 100% de la función de los riñones. La tasa de filtrado glomerular (TFG) es el mejor método para calcular la función renal, la cual consiste en medir la depuración renal de creatinina, es decir, el volumen de plasma que queda totalmente libre de dicha sustancia a su paso por el riñón por unidad de tiempo (mL/min). La mejor estimación del FG requiere que la sustancia utilizada se filtre libremente, no se reabsorba ni secrete a nivel del túbulo renal y no presente eliminación extrarrenal [10,11].

Igualmente, otras exploraciones complementarias (analíticas, radiológicas o estudios anatomopatológicos mediante biopsia), resultan útiles; sin embrago, la TFG por la inmediatez de la información, el bajo costo y la simplicidad de obtención de la muestra es la más utilizada. También existen pruebas orientadas al diagnóstico de patologías tubulares y no glomerulares como la reabsorción, concentración y acidificación de la orina [10].

1.3 Fallo renal

La mayoría de las enfermedades renales atacan a las nefronas, haciendo que pierdan capacidad en la filtración, este daño a su estructura puede ocurrir rápidamente, pero la mayoría de las veces se destruyen lentamente y en silencio, daño que será evidente después de algunos años [12].

1.3.1 Tipos de fallo renal

Existen múltiples factores que influyen en el desarrollo de las enfermedades renales, los cuales aún no son comprendidos en su totalidad; sin embargo, el tratamiento que se le da al paciente dependerá de la extensión, estructura y función que se encuentre dañada en los riñones [13].

- Lesión renal aguda: se refiere a problemas renales que ocurren rápidamente, por ejemplo, una lesión renal por accidente en la que existe pérdida de grandes cantidades de sangre que puede causar insuficiencia renal repentina. Así como también, algunos fármacos o venenos pueden hacer que los riñones dejen de realizar sus funciones normales. A estas disminuciones súbitas de la función renal, se conocen como lesión renal aguda.

- Enfermedad renal crónica: pérdida gradual de la función renal, a ésta se le conoce como enfermedad renal crónica.
- Enfermedad renal en estado terminal: insuficiencia renal total o casi total y permanente. En este punto de la enfermedad los pacientes deben someterse a terapias sustitutivas (diálisis peritoneal o hemodiálisis) o incluso a un trasplante renal para mantenerse con vida.
- Enfermedad renal crónica (ERC), definida como:

a) La presencia persistente (>3 meses) de marcadores de daño renal, definidos por alteraciones estructurales o funcionales del riñón y manifestados por alteraciones patológicas, en estudios de imagen o en estudios de laboratorio (sangre u orina), o bien;
b) Como la reducción de la TFG <60 mL/min/1.73m2 de superficie corporal, independientemente de la causa [14].

Los marcadores de daño renal inicialmente pueden acompañarse o no de disminución de la TFG, pero su presencia debe asociarse con riesgo de reducción de ésta [14]. En la mayoría de los casos, la ERC se desarrolla a consecuencia de las pérdidas de las nefronas funcionales remanentes, resultando así en una reducción progresiva de la TFG [15].

1.4 Fisiopatología de la enfermedad renal crónica

El riñón desarrolla funciones primordiales en el cuerpo entre las cuales se encuentran: depuradora, de regulación hidroelectrolítica y del equilibrio ácido base, hormonales y metabólicas; mecanismos que se desarrollan a través del balance glomerulotubular, el cual asegura el mantenimiento del medio interno por medio de la reabsorción y secreción tubular selectivos [14,16].

Cuando por alguna enfermedad se afecta al riñón en forma focal, existe pérdida de la masa renal y se deja indemne una variable proporción del tejido para evitar la acumulación de productos metabólicos que causan la uremia, las nefronas sobrevivientes asumen la función de los glomérulos dañados a través de cambios adaptativos que eventual e inexorablemente conducen a su propia destrucción debida al cambio hemodinámico glomerular, considerado un mecanismo de adaptación para evitar la disminución de la TFG, pero que por otro lado ocasionará la pérdida progresiva de la función renal [2].

De manera semejante, los glomérulos remanentes aún funcionales sufren hipertrofia; sin embargo, existe un límite de la capacidad hipertrófica glomerular y si la enfermedad progresa será necesario intensificar la elevación de niveles de urea para mantener el balance.

El nivel de urea excretada será cada vez más alto debido a la carga (oferta al glomérulo) que se necesita para excretar la urea o cualquier otro metabolito y mantener la homeostasis [17].

En este ajuste anatómico y funcional del riñón crónicamente enfermo, en el que se observan nefronas atróficas junto con nefronas anatómica y funcionalmente hipertróficas, existe también elevación del flujo sanguíneo, de la presión y de la permeabilidad capilar glomerular [18].

Estos cambios hemodinámicos glomerulares son resultado del balance de las resistencias ejercidas por las arteriolas, ya que el aumento de tono de la arteriola resulta en un aumento de la presión capilar glomerular con una consecuente hiperfiltración, donde las macromoléculas filtradas dañan las células tubulares. El resultante hipertránsito de macromoléculas genera hipertrofia, lo que contribuye a fibrosis progresiva de los glomérulos y disminución de la función renal [19].

Finalmente, es importante mencionar que cuando el glomérulo se daña lo hace como unidad, es decir los túbulos renales acompañan estos cambios, en la medida en que cada glomérulo aumente la filtración las demás estructuras anatómicas que lo acompañan también aumentan la reabsorción lo cual se refleja en una reducción de la fracción excretada de metabolitos [20].

Aunado a esta modificación en el sistema renal se inicia la generación de especies reactivas de oxígeno (ERO) y óxido nítrico (NO). Normalmente el riñón genera ERO que son eficazmente eliminadas por sistemas enzimáticos como superóxido dismutasa, catalasas y glutation peroxidasa; y por sistemas no enzimáticos como glutation y vitaminas (C y E). Sin embargo, en condiciones patológicas, como la falla renal sobrepasa la capacidad metabólica del sistema antioxidante llevando a estrés oxidativo que produce daño tisular e inflamación del tejido renal [21–23].

La pérdida de NO bioactivo por alta producción de ERO interfiere con el uso normal de oxígeno en el riñón, lo cual en situaciones de hipoxia produce una reducción de la generación de superóxido, el NO compensa la vasoconstricción renal por estimulación nerviosa, mientras que la óxido nítrico sintetasa (ONSn) se activa por incremento del flujo sanguíneo y por cambios del pH, produciendo NO que mantiene la tasa de filtración glomerular, de aquí la importancia que tiene esta sustancia en el mantenimiento de la función [22,24].

1.5 Tratamiento farmacológico de la enfermedad renal

Normalmente muchos medicamentos de uso habitual se metabolizan o eliminan por el riñón, pero su farmacocinética y en algunos casos la sensibilidad a éstos se ve alterada en la enfermedad renal, manifestada por cambios en la absorción, distribución, metabolismo y excreción de los fármacos que modifican el nivel alcanzado a dosis normal, cambiando

potencialmente su eficacia y aumentando la probabilidad de acumulación y de efectos adversos, incluida la toxicidad renal [9,25,26].

Por los cambios mencionados anteriormente, uno de los pilares del tratamiento de la enfermedad renal es la administración de medicamentos que aporten elementos inadecuadamente metabolizados por el riñón o bien que minimicen otras complicaciones propias de la enfermedad [27,28].

En resumen, para preservar la función renal es necesario tener en cuenta que medicamentos nefrotóxicos pueden ser especialmente peligrosos, por lo que la prescripción idónea y su cumplimiento puede ser complicada en estos pacientes [27]. En la Tabla 1, se observan los principales medicamentos utilizados en el tratamiento del paciente con enfermedad renal [28].

Tabla 1. Medicamentos utilizados en el tratamiento de la ERC.

Nombre del medicamento	Objetivo terapéutico
Inhibidores de la enzima convertidora de angiotensina (IECA)	En pacientes con ERC que requieren tratamiento antihipertensivo.
Antagonistas del receptor de la angiotensina (ARA)	Pacientes que presenten tos persistente no tolerada debido al tratamiento con IECA.
Agentes estimulantes de la eritropoyesis (EPO: epoetina alfa y DA: darbepoetina alfa)	Logro de niveles óptimos de hemoglobina (anemia).
Diuréticos tiazídicos, Beta bloqueadores	Reducción de riesgo cardiovascular y alcanzar la presión arterial objetivo.
Antagonistas del calcio	Fármaco antihipertensivo.
Estatinas, secuestradores biliares	Tratamiento farmacológico inicial para el C-LDL elevado.

Fuente: [28].

1.6 Manejo nutricional de la enfermedad renal

El tratamiento exitoso del paciente con enfermedad renal no depende únicamente del tratamiento farmacológico, sino que también el tratamiento nutricional forma parte indispensable para que el paciente pueda lograr las metas metabólicas planteadas, además de que este tratamiento es una de las fuentes principales del uso de diferentes plantas comestibles que pueden llegar a ser medicinales y tener efectos benéficos o incluso dañinos e incrementar riesgos y complicaciones de la enfermedad.

Entre los criterios esenciales para diagnosticar malnutrición en el paciente renal se encuentran la alteración de marcadores bioquímicos, pérdida global de masa corporal relacionada con la ingesta insuficiente y la depleción de la masa muscular, donde la

concentración de albúmina ≥4 g/dL constituye, en combinación con varios parámetros válidos y complementarios, un predictor de supervivencia en ERC [25,29,30].

Así mismo, los requerimientos de energía y nutrientes deberán individualizarse según el estadio de la ERC y el tipo de terapia sustitutiva en la que se encuentre el paciente. Una ingesta energética de 35 kcal/kg/día permite alcanzar y/o mantener un balance nitrogenado neutro. Las recomendaciones actuales de proteínas para ERC en estadíos 3, 4-5 establecen la restricción proteica de 0.6-0.8 g/kg/día; en pacientes diabéticos la recomendación se amplía a 0.8-1g/kg/día, manteniéndose en ambas la calidad biológica proteica (2/3 de proteínas de alto valor biológico). Mientras que la recomendación en diálisis es de 1.2-1.3 g/kg/día de proteínas [2,31,32].

Las necesidades de líquidos, sodio y potasio también deben individualizarse dependiendo de la función renal, estado de hidratación y de la presión arterial. También es recomendable, la suplementación de vitaminas hidrosolubles (B6, B12, C, y ácido fólico) en pacientes en diálisis, sin embargo, no está indicada la suplementación de vitaminas liposolubles en la ERC, salvo la vitamina D que debe individualizarse [33].

En definitiva, las estrategias nutricionales para prevenir o tratar la malnutrición dependerán de varios parámetros: adecuación de la ingesta proteico-energética, esquema individualizado de diálisis, cribado y valoración nutricional complementaria así como del tratamiento nutricional [32].

1.7 Uso de plantas medicinales en el tratamiento de la enfermedad renal

El conocimiento de las plantas medicinales ha trascendido por generaciones, nuestros antepasados obtuvieron el conocimiento de estas especies después de distinguir entre las que servían para comer y aquellas que tenían algún efecto en su organismo, tradicionalmente se relacionaba la forma del órgano vegetal con el órgano del cuerpo humano en el cual ejercería su acción, actualmente no solo tiene que ver con su morfología sino también con olores y sabores por lo que a partir de esto empezaron a diferenciarlas y seleccionarlas [34–38].

A pesar de que la medicina moderna está bien desarrollada en la mayor parte del mundo, grandes sectores de la población de países en vías de desarrollo todavía dependen de los profesionales tradicionales y de las plantas medicinales para su atención primaria [36,39].

Este interés por las terapias naturales ha aumentado enormemente. Actualmente, en México la importancia de las plantas medicinales no solo radica en su riqueza como parte de la cultura, sino también en el conocimiento científico que se ha generado a partir de su análisis ecológico, geográfico, cultural, farmacológico y químico que constituye el contexto de investigación actual de la medicina tradicional. Así como también, existen grupos de

investigación que desarrollan proyectos donde evalúan el valor etnográfico de las plantas medicinales, es decir, no solo se enfocan en los aspectos científicos de éstas, sino que adentran con el uso y costumbres de los diferentes grupos étnicos que poseen el conocimiento de dichas especies [40].

En consecuencia, el desarrollo de medicamentos herbolarios no se ha limitado a la utilización de principios activos obtenidos de la planta cuyo uso medicinal estaba bien documentado, sino que también incluye la valoración de plantas comestibles usadas por la población [41].

La Organización Mundial de la Salud (OMS), considera a la Medicina Natural y Tradicional, donde se incluye el tratamiento con plantas medicinales, como la medicina más natural, inocua y efectiva, además de tener un costo racional, ser asequible y aceptada por la población. La OMS, sostiene que se debe garantizar la inocuidad y la calidad de este tipo de medicamento que podría ser eficaz como tratamiento y prevención de primera línea para diferentes enfermedades [42,43].

Además, menciona que los medicamentos herbarios abarcan las hierbas, material herbario, preparaciones herbarias y productos herbarios acabados que contienen como principios activos partes de plantas u otros materiales vegetales o bien combinaciones de estos elementos, cuyo uso está bien establecido y ampliamente reconocido como inocuo y eficaz [44].

Lo que lleva a manifestar que la accesibilidad y los costos bajos, convierten a este tipo de medicina en la alternativa principal para la atención primaria de la salud y, que ha permitido que estas prácticas se mantengan hasta la actualidad [45].

Como se ha mencionado anteriormente, en muchos países la mayoría de la población sigue utilizando la medicina tradicional para satisfacer sus necesidades sanitarias, permitiendo el desarrollo de investigación en este ámbito, en el que el hombre ha buscado en la flora de su hábitat de forma empírica los medicamentos contra diferentes enfermedades, esta explotación de la flora continúa y existe gran cantidad de especies sin explorar que constituyen un recurso vasto en compuestos biológicamente activos que pueden servir como fuente natural de prototipos químicos para el desarrollo de derivados modificados, llevando a que diferentes organizaciones, como la OMS, fomenten y financien investigaciones que fundamenten con rigor científico el uso y aplicación de estas plantas medicinales en la medicina actual [34,36,38,46].

El uso de estas prácticas de salud complementarias es muy común en las personas que padecen enfermedades crónicas como diabetes, hipertensión y enfermedad renal, sin embargo, con el paso del tiempo y el desarrollo de la medicina moderna, este conocimiento se ha devaluado por profesionales de la salud que han enfocado los tratamientos en medicamentos industrializados introducidos gradualmente en la vida cotidiana de la sociedad [42]. No obstante, en la actualidad las políticas de ciencia y salud han tratado de

restablecer el uso de estas plantas medicinales en el cuidado de la salud y tratamiento de enfermedades, acorde con esto el uso de estas plantas puede ser favorable para la salud humana cuando el usuario tiene conocimiento de su finalidad, riesgos y beneficios; por lo que esta es la necesidad de los profesionales de salud de desarrollar investigación en este ámbito [34].

Acorde con lo plasmado anteriormente, existe una amplia variedad de estudios que se han realizado sobre herbolaria (a distintas escalas territoriales y en diversos periodos cronológicos) que evidencian la importancia que ha tenido esta alternativa en el marco de atención a la salud en el país, específicamente en el tratamiento de la enfermedad renal se han descrito algunos estudios que manifiestan el uso de plantas para controlar las manifestaciones clínicas presentadas por esta enfermedad (Tabla 2).

Tabla 2. Plantas medicinales utilizadas de forma curativa en la enfermedad renal

Nombre común	**Nombre científico**	**Compuestos bioactivos**	**Aplicación**	**Referencia**
Cardo santo	*Cnicus benedictus*	Lactonas sesquiterpénicas (cnidina), flavonoides de tipo glucosídico y kaempferol.	Infección de vías urinarias: actividad antibacteriana (bacterias Gram -) y retención de líquidos.	[47,48].
Diente de león	*Taraxacum Officinale Weber*	Terpenos, saponinas, ácido mono-cafeoiltártico, ácido cafeico.	Diurético, cálculos renales, infecciones de las vías urinarias, fortalecimiento de los vasos sanguíneos.	[49]
Tronadora	*Tecoma stans (L). Juss. Ex Kunth*	Alcaloides, cumarinas, terpenos.	Indigestión, gastritis, diabetes, infecciones del riñón.	[50–52]
Cola de caballo	*Equisetum myriocheatum*. Schlechtendal & Chamizo	Ácido silícico, oxálico, gálico; quercetina, kaempferol, linalool.	Diurético, enfermedades del tracto urinario.	[53–55]
Pelo de elote	*Zea mays L.*	Ácido silícico, taninos, flavonoides.	Cálculos renales, diurético, antioxidantes	[56,57]

Es importante destacar que los patrones de uso de las especies de plantas presentadas en la Tabla 2 difieren en su aplicación; es decir, una misma planta tiene varios usos en el tratamiento de distintas afecciones. El uso de estas plantas en el ámbito medicinal

tiene un marcado comportamiento cultural, en donde prevalecen las creencias y tradiciones que hacen que las plantas medicinales sean utilizadas de manera permanente y que además se conviertan en un punto de partida para la búsqueda de estrategias exitosas en la conservación de la salud de las poblaciones rurales [58].

En el caso de algunas de estas plantas no existe evidencia científica que soporte su administración adecuada para el tratamiento de algunas enfermedades, tal es el caso del pápaloquelite y la pingüica, plantas que son de uso común en las poblaciones y que son parte de la dieta normal del mexicano, cuya descripción botánica, actividades biológicas e identificación de compuestos se desarrolló en este proyecto.

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JUSTIFICACIÓN

La enfermedad renal crónica es una patología que está incrementando su prevalencia en la población mexicana, principalmente asociada a complicaciones por enfermedades crónicas que pueden ser completamente tratables como la diabetes y la hipertensión arterial si son tratadas a tiempo.

Sin embargo, la gran parte de la población diagnosticada se encuentra en un estadio avanzado con tratamientos muy costosos e invasivos; aunado a esto, los recursos farmacológicos y terapéuticos para estos pacientes son de difícil acceso, por lo que existe la necesidad de buscar otros tratamientos que sean efectivos y de costos accesibles, este es el caso del uso de prácticas de salud complementarias como la utilización de plantas medicinales.

El uso de la medicina tradicional en los pacientes con ERC puede ser favorable siempre y cuando el usuario tenga conocimiento de su finalidad, riesgos y beneficios; por lo que esta es la necesidad de los profesionales de salud de desarrollar investigación en este ámbito.

Destacando que la trascendencia de esta investigación es la búsqueda de los efectos nefroprotectores que pudieran presentar dos especies vegetales que son típicamente comestibles en la población hidalguense y que podrían generar un tratamiento coadyuvante al farmacológico, que apoyen a la disminución de los costos para el paciente, retrasen o eviten las complicaciones propias del desarrollo de la enfermedad y que en pacientes con estadios primarios retrasen o reviertan el progreso de la enfermedad, además de establecer los compuestos bioactivos responsables de los efectos farmacológicos, para su posible uso en el desarrollo de alimentos nutracéuticos o fitofármacos.

2. OBJETIVOS

2.1 Objetivo general

Identificar compuestos bioactivos con efecto nefroprotector en dos plantas comestibles del estado de Hidalgo, pápaloquelite (*Porophyllum ruderale*) y pingüica (*Pyracantha koidzumii*) mediante un estudio biodirigido utilizando modelos *in vitro* de actividad antioxidante y antiinflamatoria, y uno *in vivo* de enfermedad renal inducida en ratas, que sirvan como coadyuvantes del tratamiento para la enfermedad renal.

2.2 Objetivos específicos

1. Obtener los extractos hidroalcohólicos de las partes aéreas de pápaloquelite *(Porophyllum ruderale)* y pingüica *(Pyracantha koidzumii)* mediante maceración, para su evaluación en modelos biológicos.
2. Evaluar la capacidad antioxidante y anti-inflamatoria de los extractos en modelos *in vitro*.
3. Determinar la toxicidad oral aguda *in vivo* del extracto con mayor efecto biológico en ratones CD1.
4. Evaluar la actividad nefroprotectora *in vivo* del extracto con mayor actividad antioxidante y anti-inflamatoria, en un modelo de enfermedad renal inducido por tioacetamida en ratas Wistar.
5. Identificar los compuestos mayoritarios presentes en el extracto con actividad nefroprotectora mediante HPLC.

3. DIAGRAMA METODOLÓGICO

Para llevar a cabo los objetivos anteriormente descritos, se estableció la siguiente estrategia experimental que se muestra en la Figura 1.

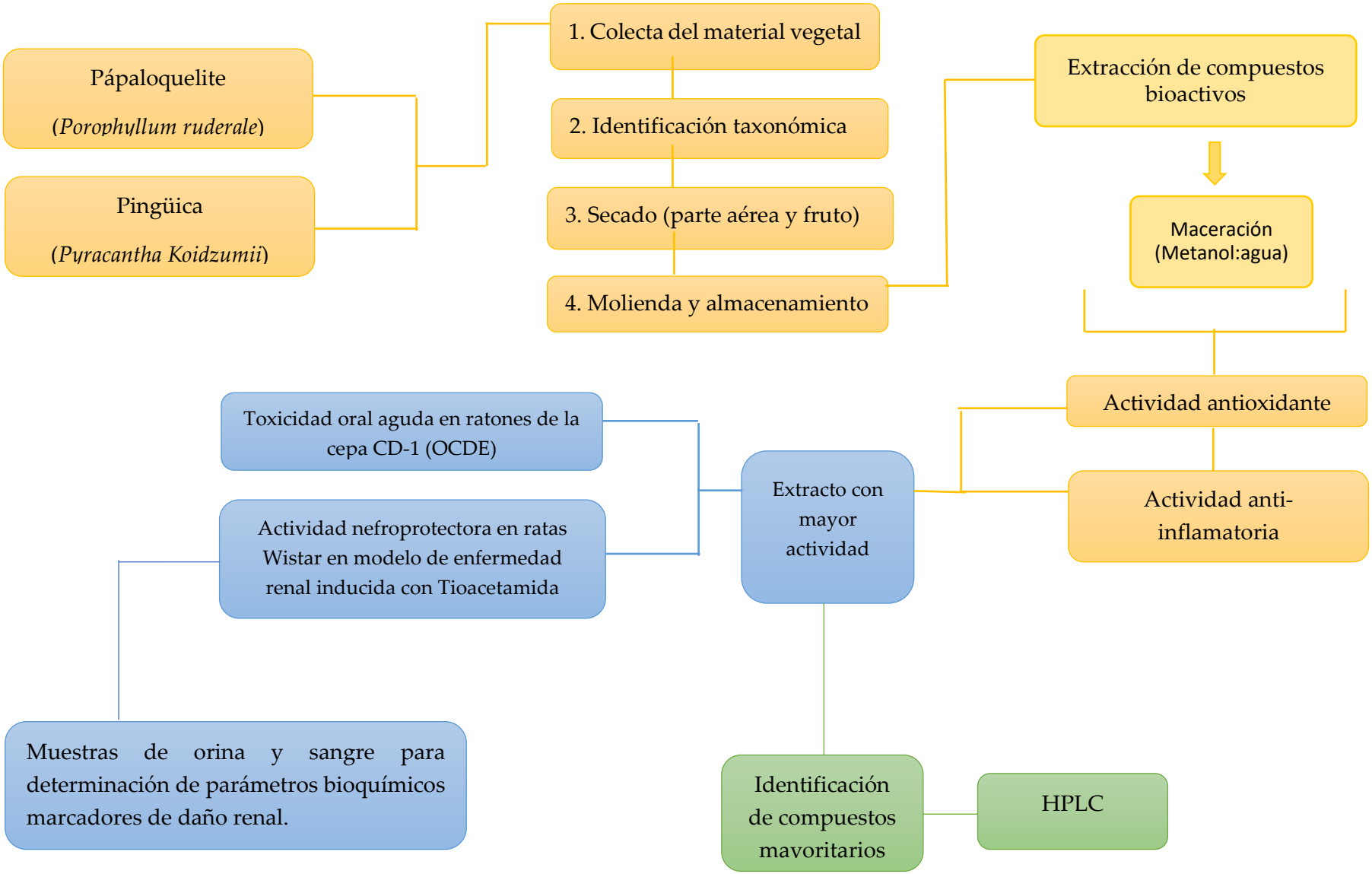
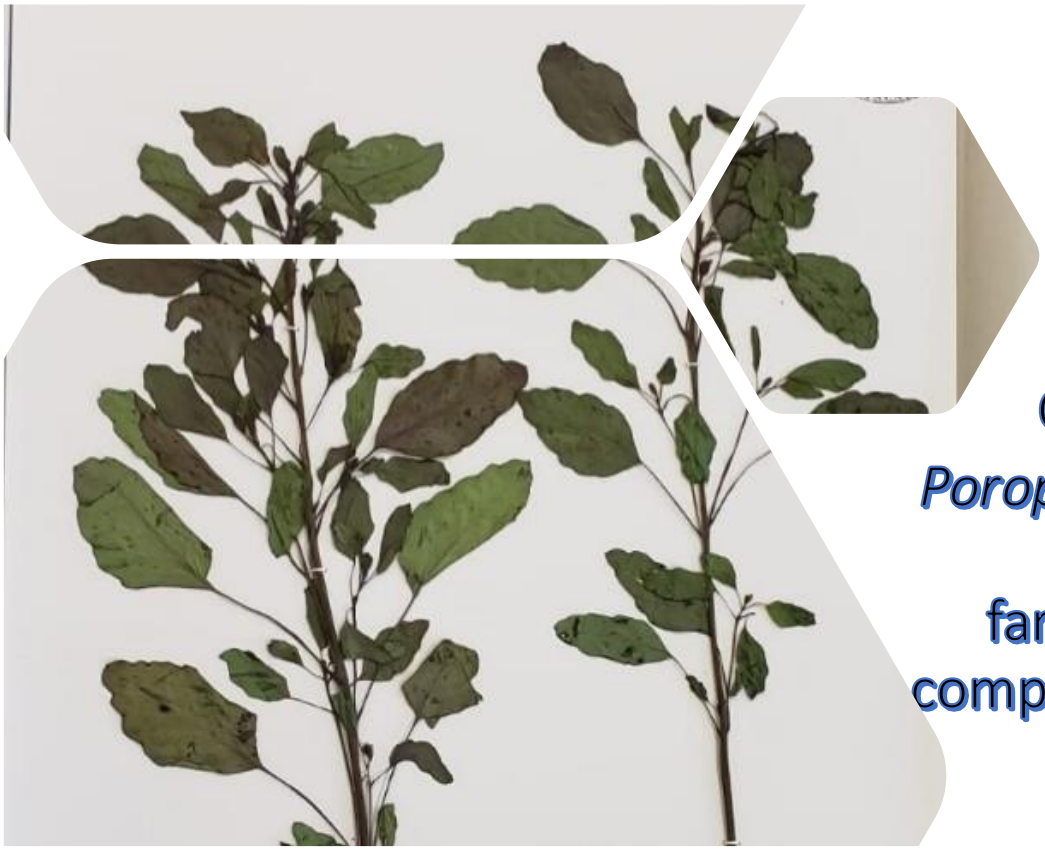


***Figura 1**. Diagrama metodológico de la investigación.*



CAPÍTULO II.

Porophyllum ruderale: actividades farmacológicas y compuestos bioactivos

Scientia Pharmaceutica

ÖSTERREICHISCHE PHARMAZEUTISCHE GESELLSCHAFT MDPI

Review

Porophyllum Genus Compounds and Pharmacological Activities: A Review

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Abstract: The genus *Porophyllum* (family Asteraceae) is native to the western hemisphere, growing in tropical and subtropical North and South America. Mexico is an important center of diversification of the genus. Plants belong of genus *Porophyllum* have been used in Mexican traditional medicine to treat kidney and intestinal diseases, parasitic, bacterial, and fungal infections and anti-inflammatory and anti-nociceptive activities. In this sense, several trials have been made on its chemical and in vitro and in vivo pharmacological activities. These studies were carried on the extracts and isolated compounds and support most of their reported uses in folk medicine as antifungal, antileishmanial, anti-inflammatory, anti-nociceptive and burn repair activities, and as a potential source of new class of insecticides. Bio guided phytochemical studies showed the isolation of thiophenes, terpenes and phenolics compounds, which could be responsible for the pharmacological activities. However, more pre-clinical assays that highlight the mechanisms of action of the compounds involved in pharmacological function are lacking. This review discusses the current knowledge of their chemistry, in vitro and in vivo pharmacological activities carried out on the plants belonging to the *Porophyllum* genus.

Keywords: *Porophyllum*; phytochemical studies; bioactive compounds; pharmacological activities

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1. Introduction

A growing world-wide interest in the use of phytopharmaceuticals as complementary or alternative medicine, either to prevent or to ameliorate many diseases, has been noted in recent years. Furthermore, a great portion of the world's population uses plants as their primary source of medicinal agents [1]. Nowadays, medicinal plant's importance relies not only on their cultural richness but also on the scientific knowledge generated from ecological, geographical, cultural, pharmacological, and chemical analysis, which constitutes the current research context of traditional medicine.

Plants of the genus *Porophyllum* (family Asteraceae) are native to the western hemisphere, growing in tropical and subtropical areas from North and South America [2–7]. It consists of 25 species [8], 17 of which are found in Mexico, with Guerrero, Morelos, Puebla, and Hidalgo, the main states of large-scale production [2].

These are annual or perennial plants that possess developed green leaves with aromatic glands and a strong flavour. Some of these species are grown in family gardens and are sometimes associated with tropical deciduous, sub-deciduous, sub-evergreen, evergreen, thorn, mesophyll mountain, oak, and pine forests [9–11].

Due to the strong flavour of its leaves, they are consumed in a fresh state or to spice some dishes, as well as being used as pesticides [1,12]. Mexican species, *Porophyllum linaria* (Cav.) DC. and *Porophyllum ruderale* (Jacq.) Cass. var. *macrocephalum* (DC.) R.R. Johnson. Known as 'papalos' or 'papaloquelites', particularly in central Mexico. Moreover, the infusions of some species of *Porophyllum* are used in traditional medicine because of activity against cramps and venereal diseases, as well as their antispasmodic, antibacterial, anti-inflammatory, antifungal, and insecticide properties, especially *Porophyllum gracile* Bent, *P. linaria*, *Porophyllum obscurum* (Spreng.) D.C., *P.ruderale*, *Porophyllum tagetoides*, *Porophyllum scoparia* A. Gray, and *Porophyllum riedelli* Baker [2,12].

The purpose of this review was to provide a comprehensive update on the status of the chemical, pharmacological in the treatment of multiple disorders of the extracts, oil, and active constituents from some plants belonging to the genus *Porophyllum*. This review also discusses the cellular and molecular mechanism by which *Porophyllum* active principles may exert their pharmacological effects.

2. Materials and Methods

An organized search for the ethnomedicinal use of the *Porophyllum* genus was carried out in terms of the pharmacological activities attributed to its compounds, as well as the preclinical studies carried out. The search was carried out systematically using MeSH (Medical Subject Headings) terms and "keywords". First, the related MeSH terms were defined: "Medicinal plants", "Ethnopharmacology", "Pharmacological action", "Bioactivity compounds", "Ethnobotany", "Antifungal", "Antiulcer", "Anti-inflammatory", "Insecticidal activity", and "Antileishmanial activity"; then, each term was combined with *Porophyllum*.

All articles found in the scientific information sources ScienceDirect, Pubmed, and Springer link were considered. A selection of titles was made, from which the abstracts were read and those that met the necessary characteristics were retrieved. The following criteria were included for the selection of documents. In the case of ethnomedicinal reports, the documents that exposed the use of the different parts of species of the genus *Porophyllum* were selected. On *in vitro* studies, articles were selected that mentioned in their methodology the

type of test used, the species studied, and the type of extract or extracts used, as well as the compounds evaluated.

Regarding preclinical studies, studies that described species of the genus *Porophyllum* and models to evaluate the different pharmacological activities (antileishmanial, antifungal, antimicrobial, insecticidal) including dose, reference drug, and possible mechanisms of action, as well as the main metabolites isolated from the plant, were included. Items that did not meet the requirements listed were discarded.

3. Results

3.1 Chemical constituents isolated from plants of the genus Porophyllum

Among all studied species, *P gracile*, *P. linaria*, *P. obscurum*, *P. tagetoides*, and *P. ruderale* have received more phytochemical attention. Regarding the most investigated part of the plant, it has been observed that, in general, all vegetative parts are used since these species are often used in this way in folk medicine.

Although most compounds are chemically known, their pharmacological mechanism of action remains generally undetermined. In this context, different classes of organic compounds of medicinal interest have been reported, including sulphur compounds, such as bithiophenes, terpenes as well as, phenolics, aldehydes, and flavonoids (Table 1). However, it should be noted that monoterpenes, such as sabinene, β-pinene, α-phellandrene, terpinen-4-ol, and limonene, and other compounds, such as bithiophenes (Figure 1), are the most abundant compounds identified in this genus (Table 1).

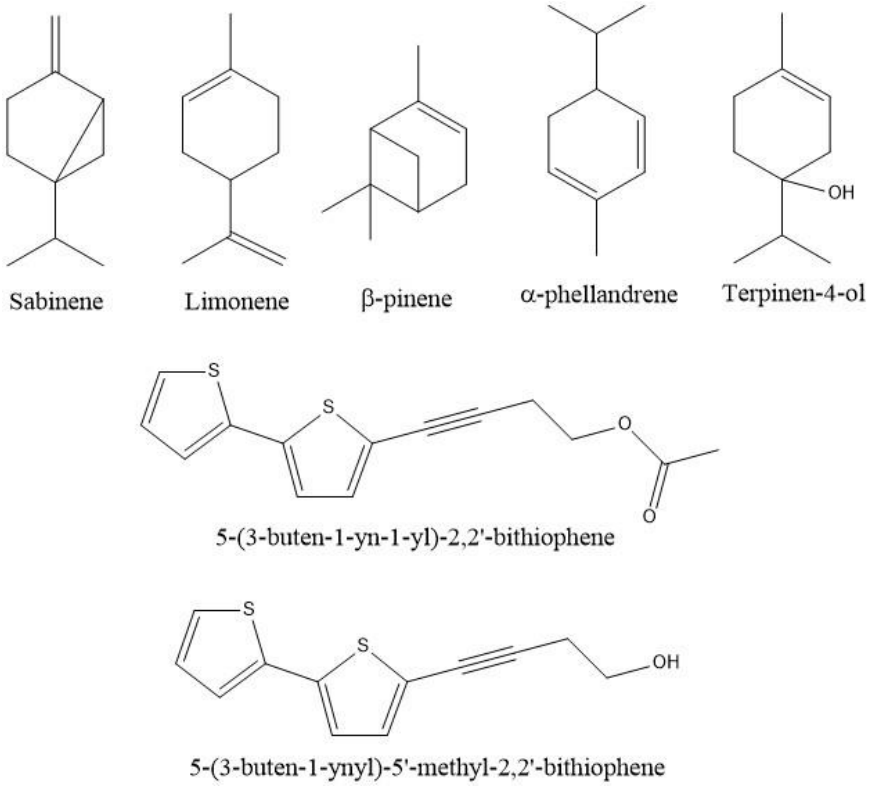

Figure 1. Major components isolated from species of the genus *Porophyllum.*

Table 1. Chemical constituents from plants of the genus *Porophyllum*

Species	Common name	Part of the plant	Extraction system	Chemical analysis performed	Isolation/Identification	Chemical compounds and yield (%)	Reference
P. gracile	"Hierba del venado"	Aerial parts	Static head-space technique.	Gas chromatography (GC) with Flame Ionization Detector (FID)	Identification	α-pinene (17.7) Sabinene (3.5) Myrcene (62.7) β-cubenene (9.1)	[14]
P. linaria	"pipicha, pepicha o chepiche"	Leaves	Hidro-distillation using a Clevenger system equipped with a microwave (Naoki) set at 100% potency. Aromatic water recirculation system using a Clevenger-type apparatus adapted with a conventional microwave.	Gas chromatography–mass spectrometer system (GC–MS) GC–MS	Identification	Phytol (25.50) Linoleic acid (29.50) β-myrcene (41.94) D-limonene (20.29) Estragole (20.03) 1-undecene (8.02) 3-(4-methyl-3-pentenyl)-furan (3.72) Terpinen-4-ol (8.51)	[5,15]

				GC			
P.obscurum	"kilkina, pus-pus, quirquiña, ruda blanca, yerba de la gama, yerba del ciervo"	Aerial parts	Maceration with methanol Hhydro-distillation in an all-glass Clevenger-type apparatus Maceration with hexane	GC-MS	Identification and isolation	β-pinene (16.30) *trans*-sabinene hydrate (12.8) Undec-1-ene (12.4) 5- (3-buten-1-ynyl) -2,2′-bithiophene (0.018) 5-(4-acetoxy-1-butenyl)-2,2'-bithiophene (0.008) 5-(4-hydroxy-1-butenyl)-2,2′-bithiophene (0.010) 2,2':5',2''-terthiophene (0.039)	[16,17, 18]
				Thin layer chromatography (TLC)			
P.ruderale	"papaloquelite, pápalo"	Aerial parts	Maceration with Et_2O Modified Clevenger-type distillation Maceration with dichlorometane Hidro-distillation	H^1 Nuclear Magnetic Resonance (NMR) GC-MS H^1 and C^{13} NMR and MS	Identification and isolation	Myrcene (3.37) (Z)-β-ocimene (1.38) (E)-β-ocimene (93.95) β-pinene (0.27) 5′-acetoxymethylen-2-[4-acetoxy-but-3-inyl]-ditiophene (0.005) 5′-methyl-2-[4-acetoxy-but-3-inyl]-bitiophene (0.010) 3-but-1-en-3-inyldithyenil (0.003) 5,methyl-2,2′:5,2″-terthiophene (0.007)	[19,20, 21,22]

				GC/MS		5'-methyl-[5-4(4-acetoxy-1-butynyl)]-2,2' bi-thiophene (0.018).	
P. tagetoides	"Pipicha"	Leaves and stems	Maceration with ethanol and water, oil emulsion	Headspace GC-MS	Identification	1-noneno (4.05) α-terpinene (0.52) Perilleno (6.49) Nonanal (1.88) 2-decenal (6.46) p-thymol (0.82) 8-phenyloctan-1-ol (1.10) Dodecyl hexanoate (1.01) B-myrcene (1.6) D-limonene (9.67) Nonanal (50.87) Decanal (19.52) (-)-trans-pineno (43.48) (Z)-8-dodecen1-ol (17.27)	[23]
P. scoparia	"Jarilla, Romerillo"	Root and aerial parts	Maceration with methanol/ether/ petrolether, 1:1:1	^{1}H-NMR	Isolation	3-butyn-2-ol,4-[2,2'-bithiophene]-5-yl-1-chloro-2-acetate (0.002) 5-(3-buten-1-yn-1-yl)-2,2'-bithiophene (0.003) 5-(4-hydroxybut-1-ynyl)-2,2'-bithiophene (0.002) 2,2':5',2''terthiophene (0.002) 2,4,3-butyne-1,2-diol,4-[2,2'-bithiophene]-5-yl-1,2-diacetate (0.0006)	[24]

						3-butyne-1,2-diol,4-[2,2' bithiophene]-5-yl-isovalerate (0.0007) Sakuranetin (naringenin 7-methyl ether) (0.0007)	
P. riedelli	Not specified	Aerial parts	Et_2O– petrol (1:2)	^{1}H-NMR spectrum	Isolation	5-(3-buten-1-yn-1-yl)-2,2' bithiophene (0.005) 2,2':5',2''-terthiophene (0.01) 2-methoxy-9-tygloyloxy-8,10-epoxy thymol isobutyrate (0.003) 2-methoxy-9- isobutyryloxy-8,10-epoxy thymol tiglate (0.003) 2-methoxy-9-tigloyloxy-8,10 epoxythymol tiglate (0.0002) 2-methoxy-9-isobutyryloxy-8,10-epoxythymol-isobutyrate (0.002) α-isocomene (0.003) β-isocomene (0.002) Modhephene (0.001) Squalene (0.014) Caryophyllene (0.001) Germacrene D (0.002) Bicyclogermacrene (0.0009)	[25]

An important factor that could explain the difference in the availability of secondary metabolites are the different environmental conditions that directly affect the chemical constitution of plants, with nutrition being the most important factors that interfere with the content and variety of the bioactive substances [13].

Another interesting aspect of phytochemical studies with the *Porophyllum* genus is the analysis of bioactivity of isolated compounds. Synergistic effects have been identified between some molecules present in plants that could be very useful if their mechanisms of action are thoroughly investigated for their possible use as medicinal plants. Some of the most important studies performed on the genus *Porophyllum* are detailed in the following sections.

3.2 Insecticidal activity

It has long been based on traditional medicine, that the beneficial medicinal action of infusion of some species of plants of the genus *Porophyllum* could be associated, at least in part, with insecticidal and antifungal activities. In this regard, the insecticidal activity of essential oil obtained from flowers and leaves of the species *P. ruderale* against *Aedes aegypti* larvae has been reported. The oil exhibited a LC_{90} value of 240.87 ppm, and its chemical analysis showed that the biological activity was directly associated with the content of monoterpenes, such as (E)-β-ocimene (93.95%), myrcene (3.37%), and β-pinene (0.27%) (Figure 2) [22]. Terpenes, like 1,8-cineole, anisole, limonene, β-pinene, linalool, menthone, α-pinene, pulegone, and myrcene, are capable of inhibiting acetylcholinesterase and produce neurotoxic intoxication, which is generated when acetylcholine is released from nerve terminal vesicles in nerve impulse transmission, causing the depolarization of the membrane; this neurotransmitter binds to postsynaptic receptors prolonging electrical transmission, as a result, an extreme excitation is generated causing insect death [26–29].

Also relevant are findings demonstrating that the volatile compounds emitted from the aerial parts of *P. gracile* and *P. ruderale* exert a synergistic effect on the insecticidal properties of α-terthienyl (commercial insecticide). The insecticidal effect of *Porophyllum* spp. is related to the presence of monoterpenes sabinene, myrcene, and limonene in *P. ruderale* leaves, and α-pinene, sabinene, and myrcene in *P. gracile* leaves (Figure 2). In addition, the synergism between volatile monoterpenes and α-terthienyl in reducing the relative growth rate of *Ostrinia nubilalis* (a pest of cereals, specifically corn), is due to a nearly two-fold increase in α-terthienyl concentration when larvae are exposed to the volatile compounds emitted from plant leaf secretory cavities [14].

Furthermore, other monoterpene compounds with insecticidal activity have been isolated and characterized in the essential oil of *P. linaria*, including β-myrcene, D-limonene, and estragole (Figure 2) [15]. Insecticidal activity of *Sitophilus zeamais* essential oil was derived from the toxic effects of β-myrcene, D-limonene, and estragole, compounds

attributed to a reversible competitive inhibition mechanism acetylcholinesterase, by occupying the hydrophobic site of the active centre of the enzyme, thus, avoiding the growth of insects [30].

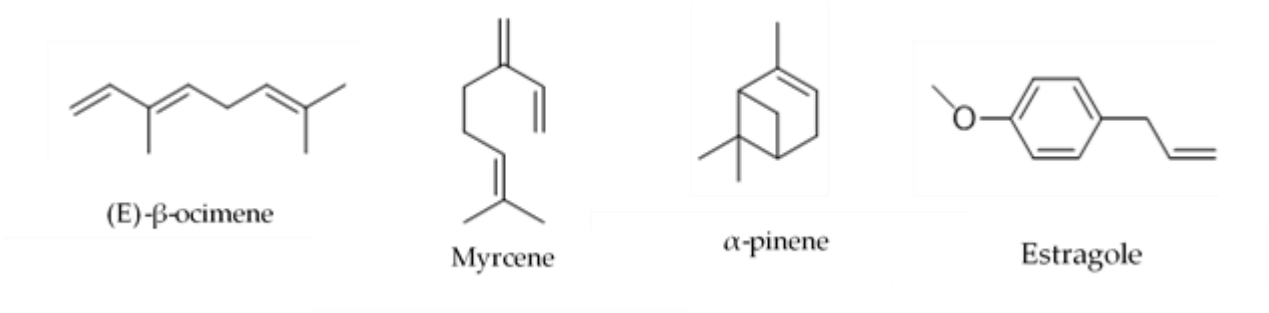

Figure 2. Isolated compounds from *P. ruderale* and *P. gracile* with insecticidal activity.

In summary, the available experimental data strongly support the view that distinct structural classes of naturally occurring secondary metabolites, present in the leaves, stems, and roots of some species of plants belonging to the genus *Porophyllum*, such as the afore mentioned monoterpene compounds like α and β pinene, terpineol and myrcene; they act as synergists of insecticides synthetics by inhibiting the activity of some enzymes detoxifying, this inhibition or stimulation of these enzymes involved in mechanisms of detoxification to insecticides in insects, produce an imbalance hormonal, impaired growth and finally the death of the insect, after being exposed to compounds from these plants, supporting the development of commercial pesticides. Therefore, the isolated constituents of some *Porophyllum* spp. may constitute a relevant and important basis for the development of a potentially new class of insecticides to replace synthetic products that are traditionally used [14,15,26–29].

3.3 Antifungal activity

Some species of plants belonging to the genus *Porophyllum*, especially *P. obscurum* and *P. linaria*, are widely used in traditional medicine as a potent antifungal agent. Photosensitive antifungal activity, against *Candida albicans* ATCC 10231 strain of 16 extracts of different polarity (hexane, dichloromethane, ethyl acetate, and methanol) were obtained from aerial parts of *P. obscurum* in four phenological stages. The results obtained in these tests showed that the hexane extract was the most active after UV-A radiation with a minimum fungicide concentration (MFC) value of 0.98 μg/mL, followed by the dichloromethane extract (7.81 μg/mL); however, these were inactive in experiments without irradiation. Those responsible for this antifungal activity were sulphur compounds, specifically thiophenes, with 2,2′:5′,2"-terthiophene and 5-(4-hidroxy-1-butenyl)-2,2′-

bithiophene (Figure 3) being the most potent (0.24-3.90 μg/mL) [11]. There are studies on the mechanism of action of a thiophene, α-terthienyl, with a similar structure to those identified in *P. obscurum*, which under ultraviolet light promotes the generation of singlet O_2, a molecule extremely toxic to fungal membranes [31,32]. Although the mechanism by which it causes fungal cell death is not clear, the results showed that hexane extract of *P. obscurum* does not induce apoptosis, so it could occur by another mechanism, such as necrosis or autophagy, and it is also important to note that it did not cause damage to the erythrocyte membrane, which decreases the possibility of haemolysis [18].

Juarez et al. [5] demonstrated the antifungal capacity of the essential oil of *P. linaria* on 11 strains, *Aspergillus amylovorus* (NRRL 5813), *A flavus* (NRRL 3518), *A nomius* (NRRL 13137), *A ostianus* (NRRL403), *Eurotium halophilicum* (NRRL 2739), *Eupenicillum hirayamae* (NRRL 3587), *E hyrayamae* (NRRL 3588), *E hyrayamae* (NRRL 3589), *E hyrayamae* (NRRL 3591), *Penicillium cinnamopurpureum* (NRRL3118), and *P viridicatum* var. ii (NRRL 5571). All strains were susceptible to *P. linaria* essential oil (MIC= 0.92 to 0.0069 μg/mL), with the greatest effect observed on *Aspergillus amylovorus* (MIC= 0.0069 g/mL), and the least on *A. flavus* (MIC= 0.92 g/mL). The authors attributed the effect to the presence of phytol and linoleic acid (Figure 3), which can cause damage irreversible to the cell wall, cell membrane, and cell organelles of fungi [33].

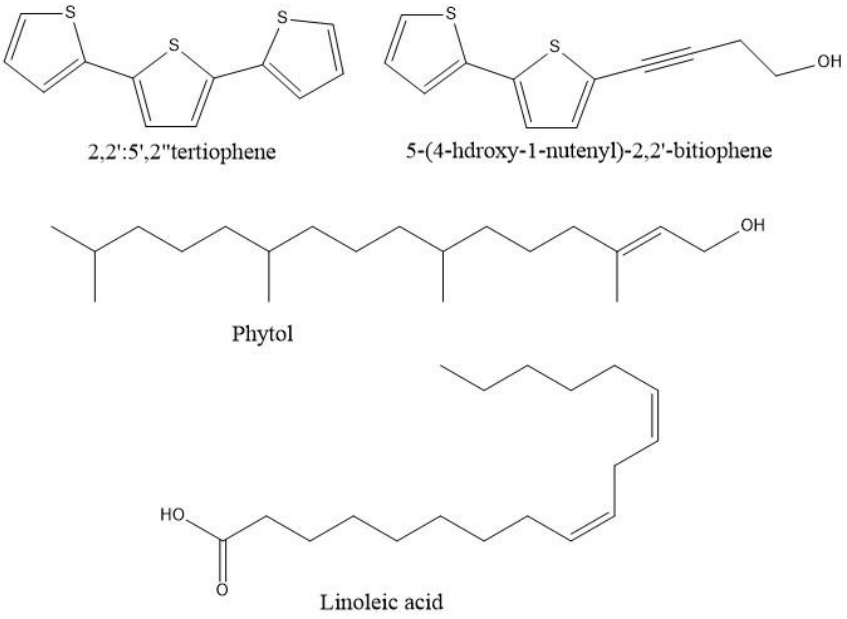

Figure 3. Components isolated from *P. linaria* and *P. obscurum* with antifungal activity.

The results shown in these studies verified the synergistic effect that these compounds have once combined. Based on these results, it would be possible to continue with studies of the fungicidal activity of essential oils of plants that present these compounds, to support

the use of these essential oils, thus, providing a viable alternative to the use of synthetic chemical fungicides.

3.4 Antileishmanial activity

Plants of the genus *Porophyllum* have long been used in traditional medicine for the treatment of intestinal infections, parasitic diseases, and bacterial diseases [3,4,34,35]. Leishmaniasis is an infection caused by protozoa of the genus *Leishmania*, showing several clinical forms: cutaneous (CL), mucocutaneous (MCL), and visceral (VL) leishmaniasis. The drugs used in leishmaniasis treatment present several problems, including high toxicity and many adverse effects; pharmaceutical research on natural products represents a major opportunity for discovering and developing new drugs and plant-based remedies that represent lower risk and decrease complications [36–41].

Many studies suggest promising beneficial effects of plants belonging to the genus *Porophyllum* for the treatment of leishmaniasis. Takahashi et al. [21] reported that dichloromethane extract from the aerial parts of *P. ruderale* affect the growth of *Leishmania amazonensis*. (60.3 μg/mL) inhibited 50% of the growth of promastigote and axenic amastigote forms after 72 h of incubation. The activity was directly correlated with two compounds isolated by chromatographic separation of the dichloromethane extract of aerial parts of *P. ruderale*, which were identified by their analysis with Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectra as thiophene derivatives: 5-methyl-2,2':5',2''-terthiophene and 5'-methyl-[5-(4-acetoxy-1-butynyl)]-2,2'-bitiophene (Figure 4). The mechanism of action of these compounds in antileishmanial activity has focused on alterations in the mitochondrial membrane, followed by changes in mitochondrial potential, indicating a cell death mechanism, like apoptosis [42]. In summary, the use of these bioactive compounds can help the development of new agents against these diseases of great importance for public health.

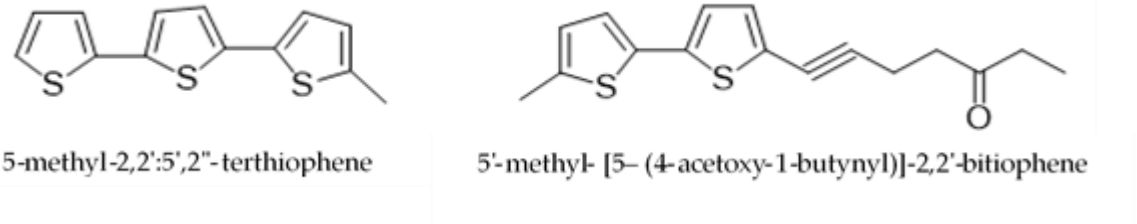

Figure 4. Components isolated from *P. ruderale* with antileishmanial activity.

3.5 Antinociceptive and anti-inflammatory activities

Pain is a sensory modality, which, in many cases, represents the only symptom for the diagnosis of various diseases, in addition to the fact that it can represent a protective function. Over time, many forms of therapy have been used for pain relief, including some medicinal herbs that are noted for their wide popular use [43]. Lima et al. [4] reported that the aqueous extract of aerial parts of *P. ruderale,* administered orally in volumes of 0.1 mL/10 g, significantly inhibited the acetic acid induced twisting in mice in a dose-dependent way; the highest effect (94.8% inhibition) was obtained at a dose of 400 mg/kg. Likewise, such extracts had an effect on the second phase of pain induced by formalin at 1% (34.4% inhibition at 400 mg/kg of extract) but not on the first phase, which suggests that the anti-nociceptive and anti-inflammatory effect may be due to a peripheral effect. These results support the hypothesis of *P. ruderale* compounds have participation in the inhibition of prostaglandin synthesis since the nociceptive mechanism involves the processing or release of arachidonic acid metabolites through cyclooxygenase (COX) and prostaglandin biosynthesis during acetic acid induced abdominal twisting [20].

3.6. Burn repair activity

There are available animal studies suggesting a burn repair property of extracts from *Porophyllum* plants. The activity of *P. ruderale* on healing burns in rats was evaluated using a hydroalcoholic extract (ethanol:water 70:30 v/v) from the leaves of the plant. Among the results obtained, *P. ruderale* extract at 10%, administered at a dose of 1 mL/day p.o., was effective in decreasing the presence of granulocytes during the cell repair process (21.3 ± 2.4 to 8.1 ± 2.2 granulocytes n/10^4 μm^2), but it showed no effect on the production of fibroblasts. Likewise, it generated an increase in the expression of TGFβ-1 during the first 7 days of treatment but later it decreased, which could be attributed to flavonoids present in the extract that favoured the release of this protein at this stage of the healing process. The extract maintained the expression of vascular endothelial growth factors (VEGF) during the whole experimental process, a very important protein since it participates in wound healing [44].

3.7 Toxicological studies

In a study to evaluate the toxic effects of two bithiophenes isolated from *P. ruderale*, both compounds showed low levels of toxicity to human cells, even at the highest concentrations (hemolytic index < 10% at 500 μg / ml) [42]. Moreover, other species of *Porophyllum* were also evaluated for their toxicological potential. For example, the toxicity of *P. linaria* essential oil showed a low toxicity on TPH-1 macrophages and *Artemia salina* LD_{50} = 10.90 and 2301.07 μg/mL, respectively [15].

4. Discussion

Although current ethnopharmacological investigations of traditional medicines have achieved important contributions to plant-derived medicines, as well as the advancement of pharmacology, drug discovery from medicinal flora is more complex than is generally recognized because plants are applied for different therapeutic indications within and between cultures [45–53].

Many plants of the genus *Porophyllum* have been used in folk medicine as remedies to treat a wide variety of human pathologies, particularly those related to digestive disorders. Of all the known species of this genus, the specie *P. ruderale* is the most investigated, however, there are few studies focused on the pharmacological activity of its compounds.

In vitro and *in vivo* studies performed with the purified extracts and compounds of these plants support most of their reported uses in folk medicine for the treatment of a wide variety of pathological conditions, including its use as an antifungal agent and insecticide [4,14,15,22,26,29]. In addition, some studies in animals have shown antinociceptive properties for some of the pure constituents of these plants [4]. It is important to mention that there are very few studies on its toxicity, which indicates that plants of this genus are well tolerated for human consumption.

The compounds of the *Porophyllum* genus have been of great interest due to the large number of biological activities they present [54]. In plants, these compounds fulfil chemical defence functions against environmental stress, as well as wound and injury repair mechanisms. The compounds present in the genus have been evaluated for their antibacterial and insecticidal activities [14,21,22,26,29,55-58]. These effects could be mediated by the activation of the Mucosal-associated invariant T (MAIT) cells in charge of recognizing the antigens sent by non-polymorphic MR1, MAIT cells are activated by a metabolic precursor of riboflavin (present in the genus *Porophyllum*) synthesis presented by MR1 and, therefore, respond to many bacteria and some fungi. Despite their broad antibacterial properties, their functional role in persistent viral infections is poorly understood, and several studies have reported that MAIT cells recognize only bacterial- and yeast-derived antigens presented via MR1 and that they do not have antiviral specificity [2,59,60]. There is accumulating evidence suggesting that terpene compounds like myrcene, limonene, linalool and caryophyllene are a promising target for use as real alternatives that can be applied in vector-borne disease control programs, for their considerable potential as repellents and larvicides, their low level of toxicity to mammals, and their limited environmental impact [29]. In the study carried out by Fontes-Jr et al. [22], it was found that the larvae of *A. aegypti* were susceptible to the composition of essential oil from the flowers and leaves of *P. ruderale*; the main compounds identified in the oil were (E)-β-ocimeno (93.95%), myrcene (3.37%), (Z)-β-ocimeno (1.38%), and β-pinene (0.27%). The responsibility

of this biological activity has been studied, showing that the lipophilicity of monoterpene compounds is related to the production of neurotoxic intoxication, in addition to the fact that components of essential oils act to block octopamine receptors, producing serious neurological alterations with harmful effects on insects [57] octopamine and tyramine are distinguished by the presence or absence of a hydroxyl group at the β position [58]. These structurally closely-related amines regulate intracellular cAMP levels in opposite directions, i.e., up and down regulation, by acting on different G protein-coupled receptors. Based on the evaluation of compounds for endocrine disruptor activity using a reporter gene assay, three-dimensional quantitative structure activity relationships were analysed to elicit responses through androgen receptors [61]. Therefore, findings regarding the structures of the binding sites of natural biomolecules, such as terpene compounds found in the *Porophyllum* genus, would be useful to design new insecticidal molecules with a novel mode of action in addition to lower production cost and effective insecticidal activity.

Other monoterpenic compound of pharmacological importance is terpineol, whose antihypertensive activity has been studied, mainly mediated by the release of Nitric Oxide (NO) and the subsequent activation of the NO-cGMP pathway (cyclic guanosine 3 ', 5' - monophosphate). This activity is linked to a reduction in calcium influx that occurs through voltage sensitive CavL channels, resulting in a decrease in vascular resistance attributed to terpineol that leads to the induction of hypotension. [62].

Regarding antinociceptive activity, monoterpenic compounds have been identified to produce significant analgesic effects in formalin and writhing tests, which are related to the inhibition of PGE2 and PGF2α levels in the peritoneal fluid and to the inhibition of the release of substance P and other inflammatory molecules. However, the activity of these compounds has also been linked to a selective inhibition of COX-2 (0.69 mM), so this type of compound could potentially be used in the development of new drugs for the treatment of diseases painful and / or inflammatory [63]. Thiophenes are a class of heterocyclic compounds that contain sulphur and are found in both natural and synthetic products, displaying several different pharmacological properties, including anti-inflammatory, antiulcer [64], antimicrobial [65], antifungal [66], and anticancer [67] effects. Natural thiophenes are characteristic secondary metabolites of plants belonging to the Asteraceae family, among which *Porophyllum* is a genus [68]. The interest in the bioactivity of these compounds is due to their properties that can be developed synthetically or used naturally for the design of new drugs, using thiophenes as raw material.

These compounds have not yet been thoroughly studied, specifically the mechanisms of action of the pharmacological activities attributed to them, however, some authors such as Takahashi et al. [21,42] have shown that the extract in dichloromethane from aerial parts of *P. ruderale* exhibit strong activity against *L. amazonensis*, making it a potent antileishmanial compound. Those responsible for these pharmacological activities were

bithiophenes and terthiophenes, whose biocidal activities were attributed primarily to the decrease in mitochondrial membrane potential in promastigotes [69].

Conversely, it was observed that treatment with a hexane extract of the aerial parts of *P. obscurum* (PoHex) showed photosensitive activity against a panel of twenty-five *Candida* strains isolated from patients with head and neck cancer undergoing radiotherapy with MFC values between 0.98 and 1.95 μg/mL, which were resistant to multiple drugs [11]. The mechanism of the antifungal activity of the thiophenes contained in PoHex can be classified as photodynamic, considering that thiophenes are excellent producers of singlet oxygen but are not precursors in electron transfer reactions, understanding that oxygen singlets have high chemical reactivity, which leads to rapid death in microorganisms and in lower concentrations than other biocides, in addition to not affecting nearby cells or organs [70].

Terthiophenes present in this genus have also been associated with HIV-1 protease inhibitory activities with an IC50 value of 58 μM but did not show any activity towards HIV-1 integrase [71].

Among these effects, its anticancer activity has been highlighted, which has been linked to the induction of cell death by apoptosis, through an increase in the activity of caspase 3 and in the expression levels of the apoptotic proteins Bak and Bim, as well as a decrease in the antiapoptotic proteins Bcl-2 and Bcl-xL. On the other hand, it has been seen that these compounds cause a blockage of the cell cycle in the G2 / M phase, decreasing the expression of the regulatory proteins of this phase, cyclins A and b1 and the kinase Cdk1 [72].

5. Conclusions

The genus *Porophyllum* represents one of the most widely used pre-Hispanic species in traditional medicine for the treatment of various diseases. The data presented here show the great potential represented by the compounds contained in their different species. Important pharmacological activities of isolated *Porophyllum* compounds has been found. Those compounds can be used in the synthesis of some phytopharmaceuticals, including terpenic and azufraid compounds, such as thiophenes, which may even develop synergism among the compounds containing these plants and increase their pharmacological potency. However, a great deal of information remains to be discovered about the mechanisms of action of these molecules, as well as preclinical studies that check this genus and its compounds could be safely used as treatments for different pathologies.

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, M.J.V-A, M.B-A. and D.O-R.; writing—review and editing, C.V-G., M.B-A. and D.O-R.; visualization, C.G.S-G and M..G-C.; supervision, M.B-A. and D.O-R..

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Nephroprotective activity of papaloquelite (*Porophyllum ruderale*) in thioacetamide-induced injury model.

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Abstract: Acute kidney injury impaired kidney function associated with reduced survival and increased morbidity. *Porophyllum ruderale* is an edible plant endemic of Mexico used in Mexican traditional medicine. The aim of this study was to evaluate the nephroprotective effect of an hydroalcoholic extract (MeOH:water 70:30, *v/v*) of aerial parts of *P. ruderale* (HEPr). Firstly, *in vitro* antioxidant and anti-inflammatory activity of HEPr was determined; after *in vivo* nephroprotective activity of HEPr was evaluated using a thioacetamide-induced injury model in rats. HEPr showed slightly effect on LPS-NO production in macrophages (15% INO at 40 μg/mL) and high antioxidant activity on the ferric reducing antioxidant power (FRAP) test, followed by the activity on DPPH and ABTS radicals test (69.04, 63.06 and 32.96 % of inhibition, respectively). In addition, values of kidney injury biomarkers in urine (urobilinogen, hemoglobin, bilirubin, ketones, glucose, protein, pH, nitrites, leukocytes, specific gravity, and the microalbumin/creatinine) and serum (creatinine, urea, and urea nitrogen) of rats treated with HEPr were maintained in normal ranges. Finally, 5-*O*-caffeoylquinic, 4-*O*-caffeoylquinic and ferulic acids; as well as 3-*O*-quercetin glucoside and 3-*O*-kaempferol glucoside were identified by HPLC as major components of HEPr. In conclusion, *Porophyllum ruderale* constitutes a source of compounds for treatment of acute kidney injury.

Keywords: *Porophyllum ruderale*; antioxidant; anti-inflammatory; nephroprotective

1. Introduction

Acute kidney injury (AKI) is a clinical syndrome characterized by an abrupt or rapid (hours to days) decline in renal filtration function, with the accumulation of products of nitrogen metabolism such as creatinine and urea and other clinically unmeasured waste products [1]; additionally, renal-tubular injury, inflammation and vascular dysfunction are observed [2]. AKI has become a global health problem and it is generally associated with a high ratio of mortality, mainly in developing countries; and has an independent effect on the risk of death [3]. In addition, the treatment of this illness represents high costs for the health system of any country [4].

The main cause of AKI is a non-controlled inflammation [5]. Inflammation is a complex integrated response designed to eliminate any noxious stimuli introduced into the host from the internal and external environment [6]. A normal inflammatory response is characterized by the infiltration of leukocytes and the release of other activated inflammatory mediators at the site of injury/infection that will eventually resolve or regulate with the release of these mediators [7]. It is important to mention that within the cells that participate in the inflammatory process there are two types: those that are permanently found in the tissues (mast cells and endothelial cells) and those that can migrate and access the affected site from the blood (polymorphonuclear neutrophils, monocytes, macrophages, and lymphocytes) [8]. These cells produce many active molecules that are direct or indirect mediators of the inflammatory process, including nitric oxide (NO), which is responsible for the regulation of numerous physiological processes, such as neurotransmission, smooth muscle contractility, platelet reactivity and cytotoxic activity of immune cells [9].

Following the beginning of inflammation process, immune cell chemotaxis and infiltration, production of reactive oxygen species and cell-derived mediators create an intense inflammation reaction that potentiates renal injury [5], for that reason control of oxidative stress is another pivotal key in AKI development. Mainly the renal mitochondria is affected by the accelerated production of superoxide anion, hydrogen peroxide and hydroxyl radicals; which significantly increases the serum levels of the main renal markers such as creatinine and urea nitrogen [9,10].

Due to the importance of the problem, the pharmacological activities of different natural compounds have been studied to help in the renal protection against nephrotoxicity caused by different compounds such as CCl_4 and thioacetamide (TAA) [11,12] Thioacetamide is an organosulfur compound used as fungicide and in the production of stabilizers, catalyst, electroplating additives, polymerization inhibitors, denying aids,

mineral processing agents and photograph development chemicals [13,14] []. Despite several uses of TAA, this compound is an important toxic due to generation of toxic fumes that can be inhaled, ingested, or absorbed through skin [15]. TAA can affect organs depending on the exposition time; for example, a single dose administration produced centrilobular hepatic necrosis and nephrotoxic damage following, while prolonged exposure produces bile duct proliferation and liver cirrhosis [16]. Furthermore, TAA can produce inflammation, because downregulate the expression of interleukin-1β (IL-1B) and TNF-a genes and upregulate the expression of interferon-γ (IFN-g) and interleukin-8 (IL-8) genes [14].

Porophyllum genus, belonging to the Asteraceae family, comprises 25 species scattered among the United States, Mexico, Central America and South America. 17 of these species are found in México and 6 inhabit the central-western region of Argentina. They are annual or perennial plants that present intense green leaves with numerous aromatic glands and have a strong flavor. The most widely distributed specie of this genus in México is *Porophyllum ruderale*, which is known by the names "papalo" or "papaloquelite", a name derived from the Nahuatl "Papaloquílitl", where "pápalotl" means butterfly, and "quilitl" means quelite [17-19].

Porophyllum ruderale is an annual edible herb with opposite and alternate leaves, with petioles 6-25 mm long; elliptical or oval and wavy on the margin. Its flowers are numerous, hermaphrodite, greenish or purplish corolla, tubular with the presence of long, thin, and curved branches. *P. ruderale* is a phytogenetic resource of great importance for food and agriculture, it has been consumed in México since pre-Hispanic times [18], and nowadays is consumed either alone or in combination due to its organoleptic and nutritionally properties [19]; in addition, due to its adaptation to environmental conditions, it can be consumed throughout the year [20]. Furthermore, *P. ruderalle* is used in the perfume and pesticide industry, due to the large quantities of strong-smelling volatile essential oils [18-23].

Medicinally, in México papaloquelite has been used in infusion and topically as a poultice for treatment of several illness. For instance, in Tabasco and Oaxaca states, pápalo is used as a local analgesic for toothache, headache and earache through the external application of its leaves on the affected part. In addition, an infusion of stems and leaves is used for treatment of stomach pain, ulcers, vomiting, hemorrhoids, dysentery, colic and indigestion. In Yucatán state a poultice of leaves of *P. ruderalle* is used to treat skin problems; while in Michoacán and Veracruz states an infusion of root or leaves is used as a, laxative, emmenagogue and for treatment of liver diseases and hypertension [18,19]. Furthermore, *P. ruderalle* is used in folk medicine in Brazil against leishmaniasis, for closing wounds, general pain, and internal bruising [24].

Some pharmacological properties have also been described for this plant, such as antioxidant, antimicrobial, anti-nociceptive, anti-inflammatory and antispasmodic activity [23, 25-30]. Due to diuretic, anti-inflammatory and antioxidant properties of papaloquelite, and the close relationship either these biological activities and the development of kidney disease, this plant could be an important source of compounds for treatment of KAI . For that, the aim of this study was to evaluate the nephroprotective activity *in vivo* of hydroalcoholic extract of *Porophyllum ruderale* using a thioacetamide-induced injury model, as well as, to identify the major compounds in the extract.

2. Results

2.1. Anti-inflammatory activity

2.1.1. Cell viability tests

Anti-inflammatory activity in vitro of HEPr was determined according to Sánchez-Ramos *et al.* [31]. Firstly, the extract was evaluated for its effect on the viability of RAW 264.7 cells at different concentrations (5 to 40 μg/mL). The extract did not exhibit a significant reduction in the viability of macrophages compared with the control group, while the positive control (etoposide) showed a significant reduction in the cellular viability at 40 μg/mL (figure 1).

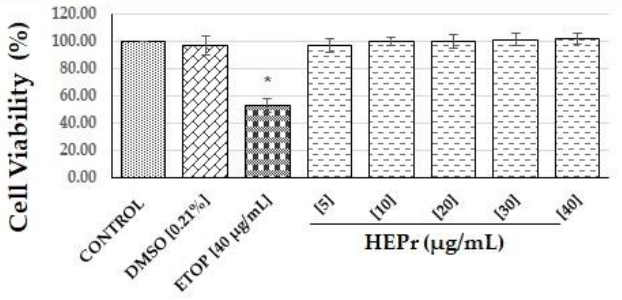


Figure 1. Effect of HEPr on cell viability of RAW 264.7 macrophages. Values are expressed as the mean ± SD of three independent experiments (n=3). Significant difference was determined using ANOVA followed by Dunnett's multiple comparison test. DMSO, ETOP (etoposide) and extracts compared to control group (* $p < 0.0001$). Control = untreated cells, defined as 100% viability.

2.1.2. Inhibition of Nitric Oxide (NO) production

Figure 2 shows the effect of HEPr on nitric oxide production in macrophages, compared with the negative control (cells without stimulus), the cells treated with the lipopolysaccharide (LPS) that gives the maximum inflammation, the DMSO that was the vehicle and the indomethacin as the reference drug. Hydroalcoholic extract of papaloquelite (HEPr) at concentrations of 20 to 40 μg/mL shows a significant difference compared with

LPS; however, the extract shows slightly anti-inflammatory effect due it decreases the inflammatory process by approximately 10 to 15%.

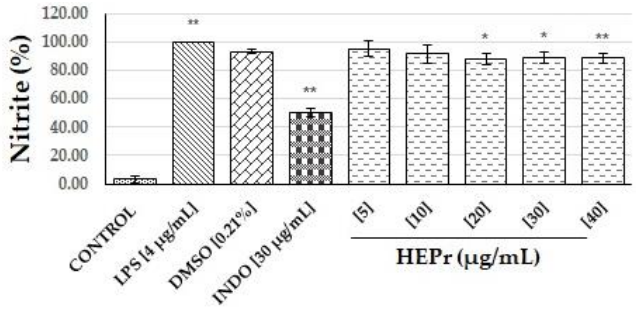


Figure 2. Effect of HEPr on nitric oxide (NO) production in RAW 264.7 macrophages stimulated with LPS. Values are expressed as the mean ± SD of three independent experiments (n = 3). The significant difference was determined using an ANOVA followed by Dunnett's multiple comparison test. LPS compared to the control group ($p < 0.0001$), and DMSO, INDO (indomethacin) and extracts compared to the LPS group (* $p < 0.001$ or ** $p < 0.0001$). Control = cells without stimulus.

2.2. Antioxidant activity

Generation of oxidative stress in the kidney is the essential mechanism of xenobiotics induced nephrotoxicity, due to ROS damage to the cell function due to disturbing oxygen-reduction balance [32]. For this reason, HEPr antioxidant capability was determined (table 1). Additionally, we determined the HEPr phenolic content (table 1) because these compounds are molecules with high potential to neutralize free radicals [33].

HEPr showed high antioxidant activity. It was able to stabilize free radicals, showing the highest effect on $ABTS^{\cdot+}$ following by $DPPH^{\cdot}$ radical (16116.03 ± 0.038 and 1502.40 ± 0.04 µmolTE/100g, respectively); too exhibited ferric reducing antioxidant power (4836.14 mg$FeSO_4$/100g). Respect to the content of phenolic compounds, HEPr showed a content of 13993.67 ± 0.016 mgGAE/100g.

Table 1. Total phenol content and antioxidant capacity of hydroalcoholic extract of papaloquelite (*Porophyllum ruderale*).

Sample	Total phenolics (mgGAE/100g)	ABTS (µmol TE/ 100g)	% inhibition	DPPH (µmol TE/100g)	% inhibition	FRAP (mg $FeSO_4$/100g)	% inhibition
HEPr	13993.67 ± 0.016	16116.03 ± 0.038	32.96 ±2.496%	1502.40 ± 0.0407	63.06 ±1.733 %	4836.14 ± 0.072	69.04 ±1.958%

2.3. Acute oral toxicity

No animal died during the 14 days of observation after the dose of 5000 mg/kg of HEPr. The mice ate and increased their body mass normally. No signs of toxicity were observed such as: difficulty in breathing, loss of appetite or death. According to OECD standards, HEPr has a LD_{50} >5000 mg/kg being considered a harmless species (table 2).

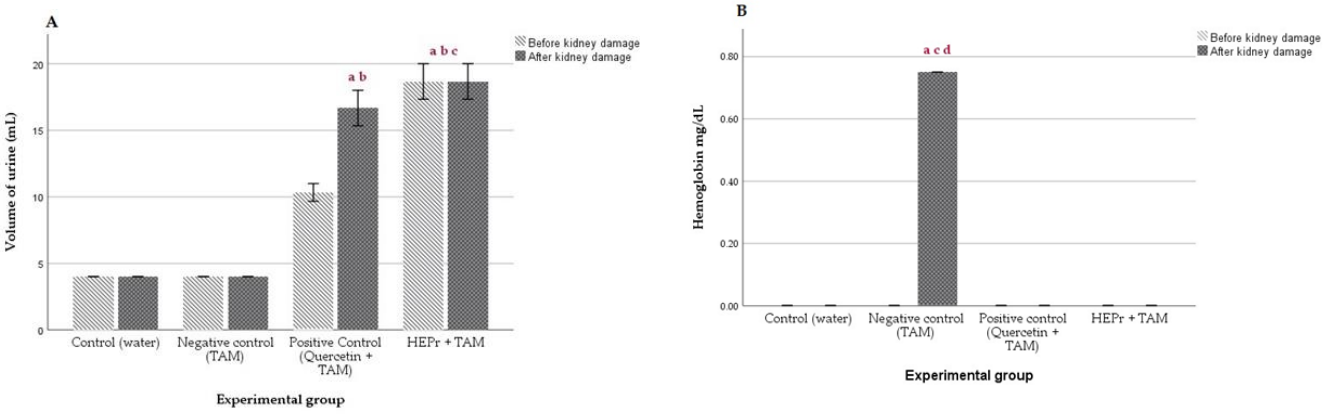
Table 2. LD_{50} of the hydroalcoholic extract of papaloquelite (*Porophyllum ruderale*).

PHASE	INTRAGASTRIC DOSE (mg/Kg)
Phase I	5000
Mortality	0/5
LD_{50}	>5000

2.4. In vivo nephroprotective activity

To evaluate the possibility that *Porophyllum ruderale* was able of prevent renal injury caused by toxic agents, we evaluated its nephroprotective activity using a thioacetamide induced acute renal injury model in rats and determined the main biomarkers of renal injury in urine and serum of the treated rats.

Figures 3 A, B, C and D show the changes on urinary biochemical markers in rats before and after renal injury induced by TAA. As we can observe previously to TAA administration, HEPr and quercetin showed an increase in the urine volume, may be due to a diuretic effect. After TAA administration, rat groups treated with quercetin and TAA showed normal values for the rest of biomarkers.



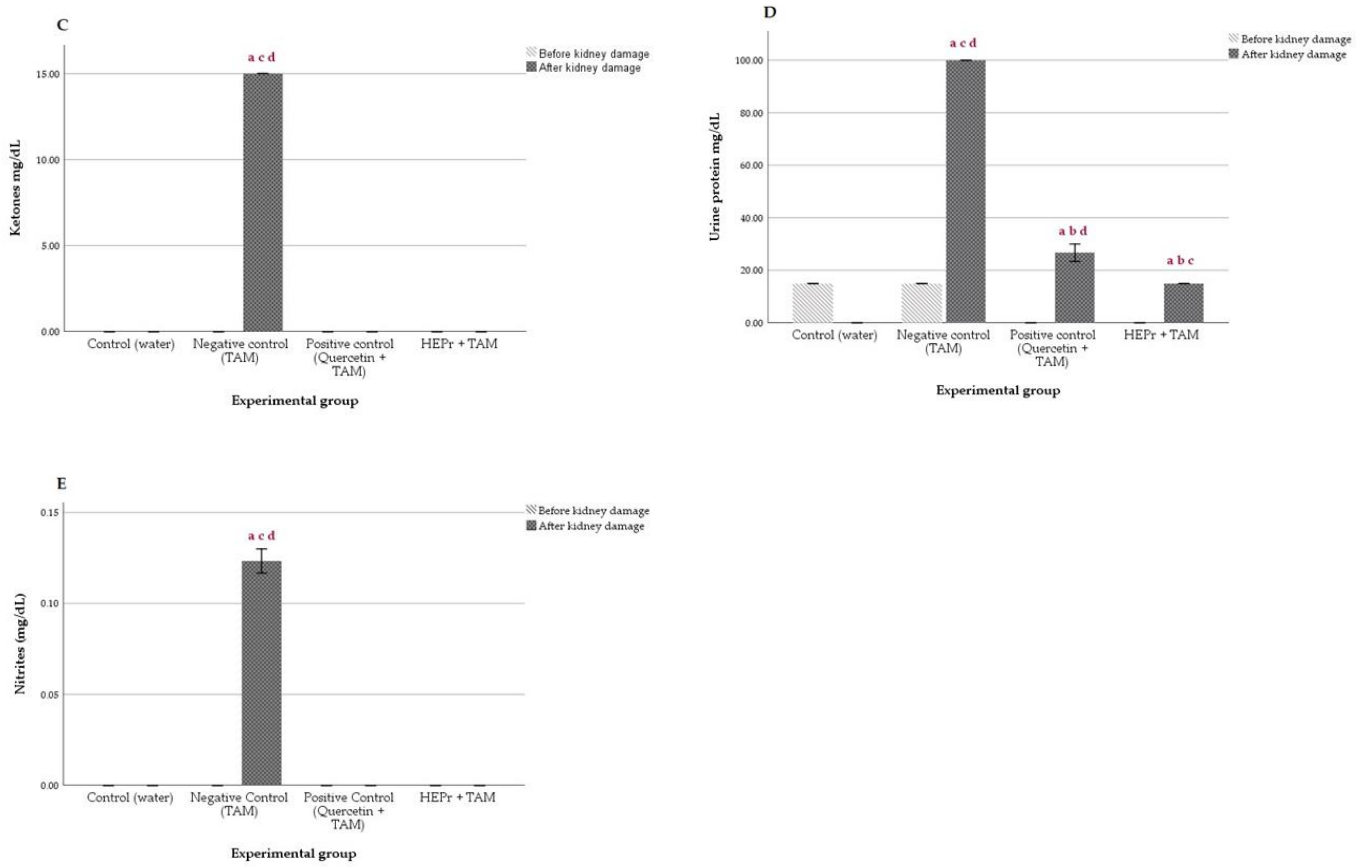


Figure 3. A) Volume of urine, Concentrations of B) Hemoglobin, C) Ketones, D) Proteins, E) Nitrites in urine pre- and post-treatment in Wistar rats. TAM = thioacetamide. Values are expressed as the mean ± SD of urinary urine values (n=5). Significant difference was determined using ANOVA followed by Dunnett's multiple comparison test. a) Control, b) Negative control, c) Quercetin, d) HEPr + TAM group.

About serum biomarkers of renal injury, we can observe that HEPr decreases considerably BUN and Urea levels without significant difference with the control and 4positive control groups (Figures 4C and 4D). In addition, HEPr decreased slightly creatinine serum content, however this effect was not significantly different with respect to negative control (Figure 4B). Finally, HEPr showed a decrease of glucose content in serum compared with quercetin and control groups, but this value was higher than obtained in rats without treatment (Figure 4A).

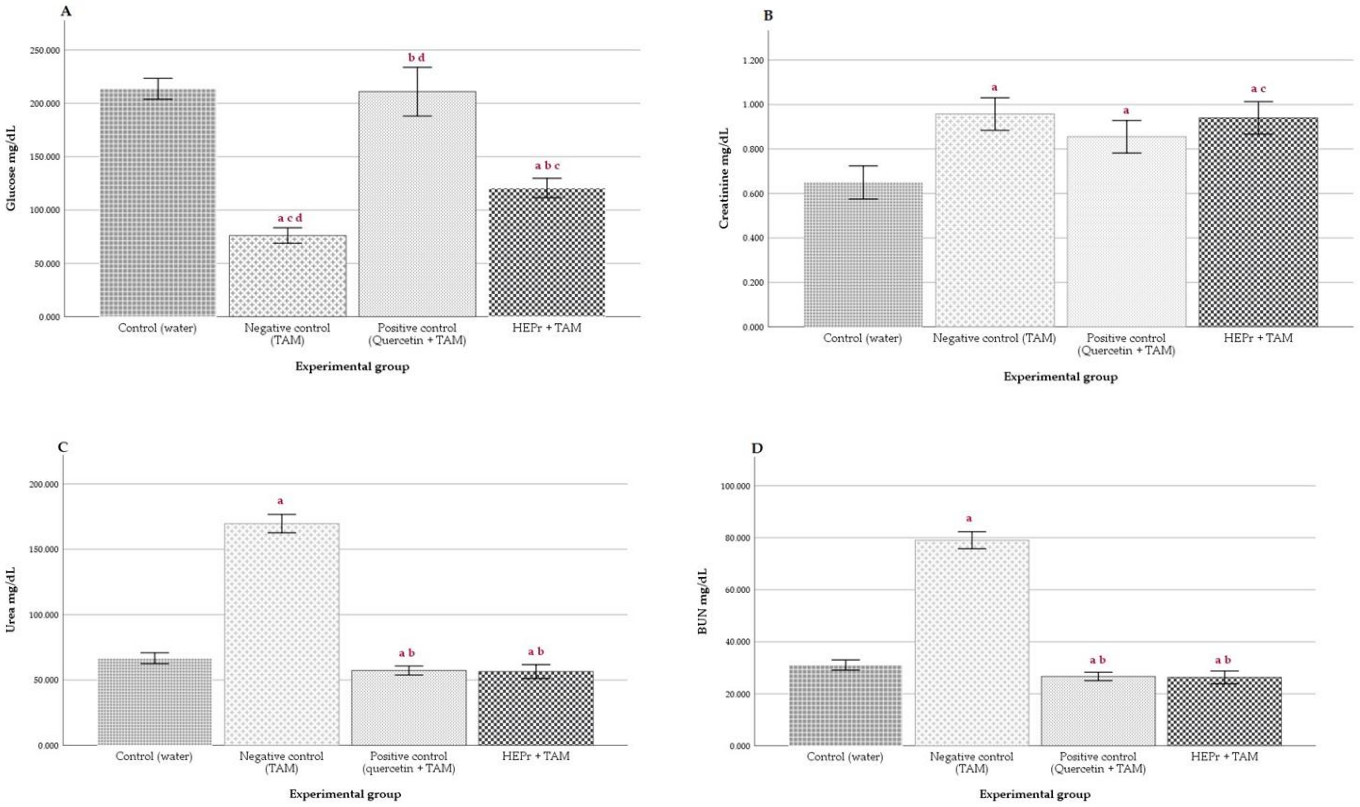


Figure 4. Effect of hydroalcoholic extract of papaloquelite (*Porophyllum ruderale*) (HEPr) on serum levels of A) Glucose, B) Creatinine, C) Urea, D) BUN in Wistar rats. Values are expressed as the mean ± SD of serum values of each marker (n=5). Significant difference was determined using ANOVA followed by Dunnett's multiple comparison test. a) Control (water); b) Negative control (TAM), c) Positive control (quercetin), d) HEPr + TAM group.

3.5. Major compounds.

Figure 5 shows the chromatogram obtained from HEPr at *λ*330 nm. The analysis of hydroalcoholic extract of the aerial parts of *Porophyllum ruderale* by HPLC revealed that these extracts contain the phenolic acids 5-*O*-caffeoylquinic acid (Chlorogenic acid), 4-*O*-caffeoylquinic acid (Cryptochlorogenic acid) and ferulic acid; as well as the flavonols: quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside, as main components (figure 6). These phenolic acids and flavonols were identified by direct comparison of the retention time of each peak with the respective analytical standard and their contents are showed in table 3. Peaks of R_t at 8.42 and 11.058 min in the chromatogram were not identified; however, their UV light spectra showed absorption bands characteristic of a caffeic acid derivatives and coumarins, respectively (figure 7) [34,35].

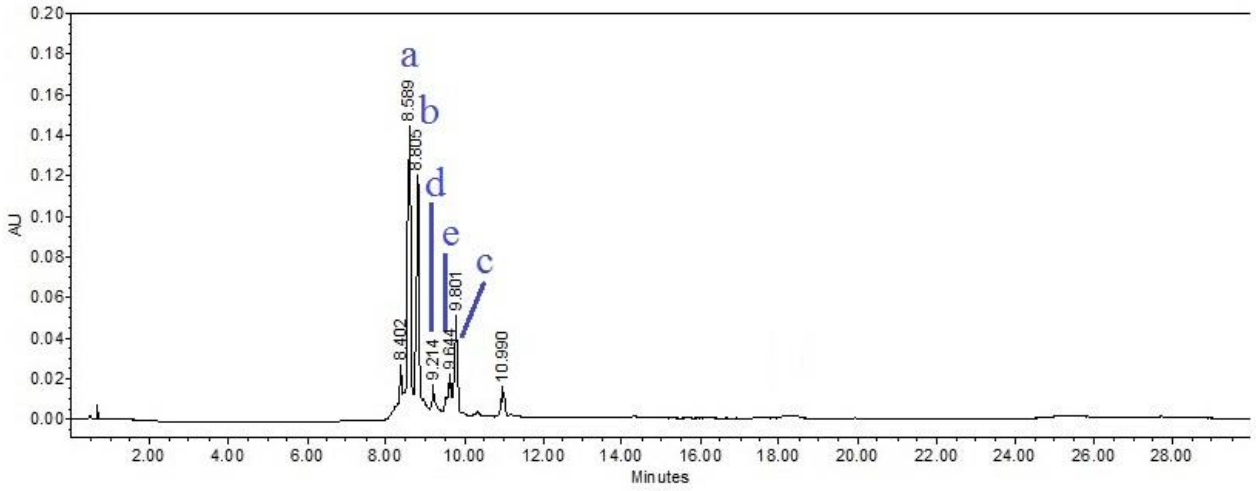


Figure 5. HPLC chromatogram of the hydroalcoholic extract of *Porophyllum ruderale* (HEPr) (1mg/mL) observed at λ 330 nm. **a**) 5-*O*-caffeoylquinic acid, **b**) 4-*O*-caffeoylquinic acid, **c**) ferulic acid, **d**) quercetin-3-*O*-glucoside, **e**) kaempferol-3-*O*-glucoside.

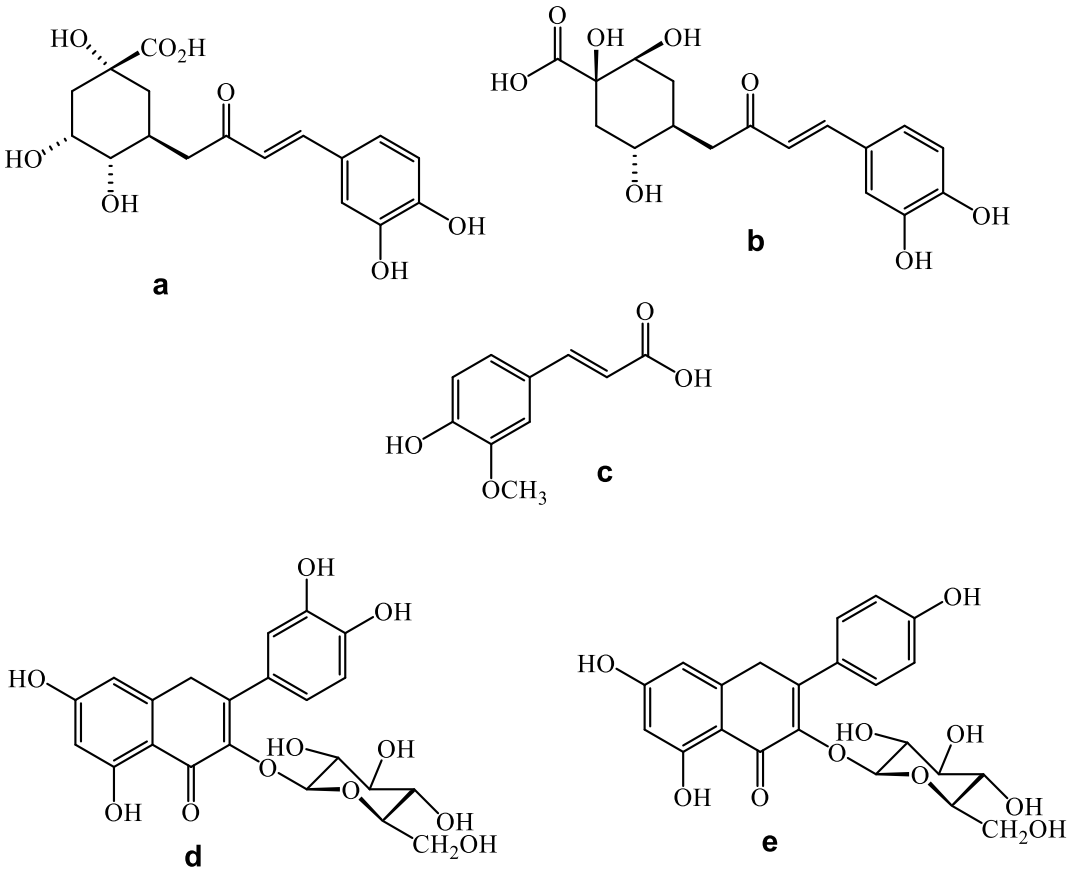


Figure 6. Chemical structure of compounds identified in HEPr. **a**) 5-*O*-caffeoylquinic acid, **b**) 4-*O*-caffeoylquinic acid, **c)** ferulic acid**, d**) quercetin-3-*O*-glucoside, **e**) kaempferol-3-*O*-glucoside.

Table 3. Content of compounds identified by HPLC in the hydroalcoholic extract of *Porophyllum ruderale* (HEPr)

Compound	mg/g extract
5-*O*-caffeoylquinic acid	310.82
4-*O*-caffeoylquinic acid	340.39
Quercetin-3-*O*-glucoside	24.06
Kaempferol-3-*O*-glucoside	23.00
Ferulic acid	137.87

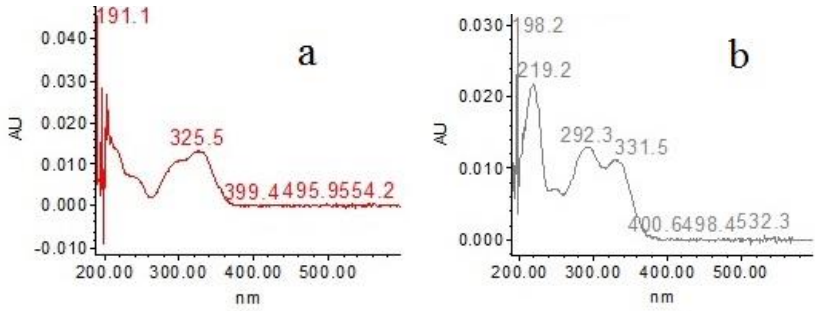


Figure 7. UV light spectrum of compounds in peaks at R_t 8.42 (a) and 11.058 (b) min.

3. Discussion

As we mentioned previously, inflammation is the main cause of acute renal failure followed by oxidative stress. For this reason, we evaluated the hydroalcoholic *P. ruderale* extract (HEPr) anti-inflammatory and antioxidant activity *in vitro*.

Firstly, we evaluated the toxicity of HEPr on RAW 264.7 cells (figure 1) and we found that the extract was not toxic for cells. These results are according with other studies where authors observed that methanolic extracts of other *Porophyllum* species are no cytotoxic in mouse macrophage cell line RAW264.7 at concentrations from 0.06 to 200 mg/mL [36,37]. In addition, Pawłowska *et al*. [30] found that an aqueous extract of aerial parts of *Porophyllum ruderale* at concentration range of 5 to 100 μg/mL does not affect the viability of human neutrophil cells.

On the other hand, HEPr at concentrations of 20 to 40 μg/mL shows a significant difference compared with LPS (figure 2); however, the extract shows a slight anti-inflammatory effect due it decreases the inflammatory process by approximately 10 to 15%. It has been observed that different plant extracts of the genus *Porophyllum* show concentration-dependent inhibitory effects on the expression of lipopolysaccharide (LPS)-induced inflammatory marker production in inflammatory models. For instance, essential oil of *P. ruderale* at dose of 100 mg/kg inhibit leukocytes (37%) and mononuclear cells (43%)

migration, as well as the accumulation of eosinophils (63%) induced by LPS in mouse; in addition, their main monoterpenes limonene and β-myrcene were able to inhibit the migration of the same cells and production of NO, γ-interferon, and IL-4 [38]. Furthermore, Pawłowska *et al.* [30] observed that an aqueous extract of aerial parts of *P. ruderale* at concentration of 50 μg/mL decreased 10% LPS-stimulated IL8 and TNF production in human neutrophils. Finally, a methanolic extract of *Porophyllum tagetoides* possesses compounds that help modulate NF-κB activation by decreasing LPS-induced iROS [39].

As mentioned, these activities can be attributed to the type of compounds present in this species. Several chemical studies of plants of the genus *Porophyllum* have shown that terpenes and thiophenic compounds are the main secondary metabolites [40]. These compounds can inhibit LPS-induced IκB*α* degradation, leading to suppression of proinflammatory mediators such as inducible nitric oxide synthase (iNOS) and COX-2 [41].

DPPH, ABTS and FRAP assays are preliminary tests to study the antioxidant activity of plant extracts. In this investigation, HEPr was able to stabilize all radical species as well as to reduce the ferric ion to the ferrous state. Regarding DPPH activity, HEPr inhibits this radical in 63.06 % and the radical inhibition of DPPH was 1502.40 ± 0.0407 μmol TE/100g. These values are higher than that reported for an ethanolic extract of the same specie (32.6 ± 1.18 %, 676.24 ± 0.34 μmol ET/100g) [40], but lesser that the obtained for wild and cultivate *P. ruderale* (4645.53 ± 36.2 and 4392.16 ± 27.0 μmol TE/100 g, respectively), however in this case fresh leaves were used [20].

In addition, HEPr showed good activity against ABTS and FRAP too (16116.03 ± 0.038 μmol TE/100g and 4836.14 ± 0.072 mg $FeSO_4$/100g, respectively). It was not able to be contrasted with similar tests in the same plant genus due to lack of publications. However, our results are higher than those presented by Khan *et al.* [42] for another species with high antioxidant capacity such as purple grapes (ABTS: 910 ± 0.2 μmol TE/100g, FRAP: 2660 ± 0.9 mg $FeSO_4$/100g) and strawberries (ABTS: 1150 ± 0.4 μmol TE/100g, FRAP: 249 ± 0.7 mg $FeSO_4$/100g).

In some plant species, the phenolic compounds present may be responsible for their antioxidant activity, due to the number of hydroxyl groups that act as free radical scavengers [43]. HEPr has a phenolic compound content similar to that reported by Kato da Silva *et al.* [44] for an ethanolic extract of the same plant (139.93 and 162.29 mgGAE/g, respectively). However, it was higher than total phenols content in fresh leaves of wild and cultivated *P. ruderale* (3.91 ± 1.41 and 3.162 ± 0.28, respectively). The differences on the antioxidant activity and phenolic compounds content in *P. ruderale* aerial parts may be due to the growing conditions and edaphoclimatic characteristic of the respective geographical areas [20]. For instance, Fukalova *et al*. [20] obtain *P. ruderale* from Valencian coast in Spain; while Kato da Silva *et al.* [44] obtained from Campo Grande, MS, Brazil, and we from Hidalgo, México. In addition, HEPr phenols content is higher than obtained for a crude

extract of *Porophyllum tagetoides* leaves (8.54±0.14 mgGAE/g) [45], and for different grape varieties (54.23 ± 0.04 - 58.48 ± 0.09 GAE/g) [44] (4764 ± 39-11525 ± 886 mg GA/100 g) [46,47].

Once we verified that *Porophyllum ruderale* had antioxidant and anti-inflammatory capacity, we proceeded to evaluate its ability to protect the kidney from TAA-induced injury in rats.

The urine analysis of rats showed significant changes in the amount of urine, when comparing the experimental animals before and after inducing renal injury, observing an increase in the volume of urine of the animals treated with the whole hydroalcoholic extract of papaloquelite as shown in Figure 3A, associating it to a diuretic effect but not related to the severity of the renal injury. With respect to the other urinary parameters evaluated urobilinogen, hemoglobin, bilirubin, ketones, glucose, protein, pH, nitrites, leukocytes, specific gravity, and the microalbumin/creatinine, were absence in animals treated with HEPr, except for negative control group as shown in Figure 3 B, C, D and E. These results in urinary markers evaluated support the metabolic decompensation and tubular lesions associated with the renal damage of the animals not treated with the extract, as mentioned in Figure 3 A, B, C, D and E.

On the other hand, creatinine concentration in plasma and urine is an important marker of renal function. An increase of creatinine in plasma suggests leakage from necrotic cells or upregulates creatine biosynthesis. Creatinine is synthesized and metabolized in the liver, but its precursor guanidinoacetate is formed in the kidney, transported through the blood, undergoing methylation in the liver to form creatine which enters the blood for use in peripheral tissues [48]. Furthermore, because of kidney damage progresses, nitrogen products accumulate in proportion to the loss of kidney function. The blood marker blood urea nitrogen (BUN) measures the amount of accumulated urea that is not efficiently excreted in the urine, making it an important marker of kidney damage [49,50]. As well as urea, which is synthesized in the liver as an end product of protein catabolism and subsequently eliminated in the kidney via the urine, its accumulation can exert toxic effects leading to cell death by induction of apoptosis [51,52].

In our experiment, the negative control group treated with TAA exhibited an increase of serum creatinine and urea concentration, 30 and 166%, respectively. These values are according to other authors who found that a single dose of 150 mg/kg of Thioacetamide administered orally to rats produced acute renal injury altered kidney function [53]. They observed an increase of creatinine and urea serum content in TAA-treatment rats (33 and 168%, respectively) 24 h after toxic administration. Furthermore, a vacuolar degeneration in kidney tubules was observed. In addition, rats in the negative control group showed an increase in BUN of 169.5% compared to the control group, while for the groups administered with papalo and quercetin, the values decreased by 14.2 and 11.12%,

respectively (Figure 4D). Alterations in these biological markers are evidence of renal injury in the rats.

As we can observed in Figure 4 A, B, C and D; the values of the markers in serum were maintained in normal parameters for animals treated with HEPr, showing serum glucose levels of 114.62±7.5 mg/dL, creatinine 0.85±.16 mg/dL; urea 76.34±16.29 mg/dL and urea nitrogen 35.57±7.59 mg/dL. An opposite behavior was observed in untreated animals, where the values of the markers of renal damage were increased being important markers of acute damage besides being associated to alterations in the renal homeostasis of nutrients [54]. Data found in this study are similar to those observed for olive and juniper and flaxseed oils in rats with kidney damage instated with TAA, where serum creatinine (+38.5% and +34.5%, respectively) and BUN (+26.3 and +30.1%, respectively) levels were elevated in TAA-treated mice compared to control group [55,56]. In addition, these parameters were not statistically changed in rats treated with flaxseed oil plus TAA compared to control rats [56].

Moreover, the results of this study are similar to those presented by Cengiz [11]. This author induced acute kidney injury in rats with TAA too and observed that the group treated only with TAA had elevated BUN values (18.42 ± 0.71 mg/dL) compared to the group treated with 100mg/kg of *Silybum marianum* (L.) Gaertn (Silymarin) and 50 mg/kg TAA, which showed similar values to those reported for the control group (15.89 ± 1.32 mg/dL and 15.19±1.73, respectively).

In another study acute kidney disease in rats was induced with TAA (single dose of 300 mg/kg) and rats were treated during 14 days with an alcoholic extract of *Allium porrum* and *Bauhinia variegata* leaves. Authors observed that creatinine and urea values were higher in the group with TAA (1.53 ± 0.08 mg/dL and 77.34 ± 3.16 mg/dL, respectively); while in the groups treated with the plant species, these values decreased (1.15 ± 0.02 mg/dL and 41.86 ± 1.07, respectively) showing a nephroprotective effect [57].

In comparison with the results of a 12-week chronic kidney disease trial evaluating the effect of olive and juniper leaf extracts on thioacetamide (TAA)-induced nephrotoxicity in male rats, it was observed that after 12 weeks, serum creatinine (+38.5%) and BUN (+26.3%) levels were elevated in TAA-treated mice compared to control mice (25.50±1.64; 5.67±0.72 μmol/L respectively). However, unlike what was found in our study, there were no significant changes in serum creatinine (24.50±2.88 μmol/L) and BUN (5.36±1.04μmol/L) levels in mice treated with TAA plus olive and juniper leaf extract [52].

On the other hand, the nephroprotective effect of *Vitex negundo* (VN) ethanolic extract was evaluated for 12 weeks in a TAA-induced chronic kidney disease model [58]. TAAgroup showed a significant increase in blood urea and serum creatinine levels (35.66±7.3 and 4.46 ± 0.45 mmol/L, respectively) compared to the normal control group; while the increase in these parameters was prevented by simultaneous treatment of the

animals with 100 mg/ kg of VN (Creatinine: 6.28 ± 0.59 mol/L; Urea: 35.66 ± 7.3 mmol/L) and 300 mg/kg of VN (Creatinine: 5.73 ± 0.973 mol/L; Urea: 29.33 ± 3.4 mmol/L), which resulted in almost normalized levels of these parameters.

Regarding chemical composition of *Porophyllum ruderale*, we identify 5-*O*-caffeoylquinic acid (Chlorogenic acid) and 4-*O*-caffeoylquinic acid (cryptochologenic acid) as the most abundant compounds followed by ferulic acid, quercetin-3-*O*-glucoside, and kaempferol-3-*O*-glucoside (figure 5). Recently were identified twenty five phenolic compounds in an acetone:methanol:water (3:1:1 *v*/*v*/*v*) extract of aerial parts of *P. ruderalle* cultivated in Warsaw, Poland. These compounds were identified by UHPLC-DAD-MS as thirteen caffeic acid derivatives, ten flavonoids, one p-coumaric acid derivative and one unknown compound. The most abundant compounds were 2-*O*-caffeoyl-2C-methyl-D-erythronic acid, 5-*O*-caffeoylquinic acid, quercetin-3-*O*-β-D-glucuronide and 3-*O*-caffeoyl-2C-methyl-D-erythronic acid (152.59, 143.77, 65.54 and 54.70 mg/g extract, respectively). Additionally, quercetin 3-*O*-β-D-glucopyranoside, kaempferol 3-*O*-D-glucopyranoside, 4-*O*-caffeoylquinic acid and 3-*O*-caffeoyl quinic acid were identified too [30]. In another study performance on *P*. *ruderale* from Brazil, authors found chlorogenic acid and quercetin-3-*O*-glucoside as major compounds [59]. While chlorogenic acid was the main component of *P. ruderale* from Spain, followed by *p*-coumaric acid, quercetin, rutin, kaempferol, luteolin, caffeic acid, apigenin and gallic acid [20]. Authors suggest that differences in chemical composition may be due to the growing conditions and edaphoclimatic characteristics of the respective geographical areas [20].

Porophyllum ruderale is a great source of phenolic compounds with important anti-inflammatory and antioxidant activities that can contribute to its nephroprotective effect. For example, chlorogenic acid has antioxidant activity and protect cells from oxidative stress [60]; in addition, it is able to inhibit nitric oxide production by macrophages and suppress T cell proliferation, decreasing inflammatory processes [61]. While, ferulic acid have a potent antioxidant activity mediated mainly by its binding to free radicals to donate hydrogen molecules, as well as inhibit superoxide anion [62-66]. Also, decrease the levels of different inflammatory mediators such as prostaglandin E2 and TNFα, as well as the expression of the enzyme NOS (nitric oxide synthase) [67].

Furthermore, quercetin glycoside also has important antioxidant activity, acting as a protector against reactive oxygen species by neutralizing free radicals such as superoxide anions, nitric oxide and peroxynitrite, as well as increasing the production of endogenous antioxidants [68]. In addition, this compound can decrease inflammatory mediators produced by macrophages [69]. Finally, in some studies it has been observed that quercetin glycoside shows protection against nephrotoxicity by reducing renal toxicity against exposure with cisplatin and cadmium [70-72].

On the other hand, has been reporting that coumarins have antioxidant activity relating to their ability to inhibit lipid peroxidation and scavenging reactive species, e.g., hydroxyl and superoxide radicals [73]. Several coumarins have also shown beneficial biochemical profiles in relation to pathophysiological processes that depend on reactive oxygen species [74]. In addition, several coumarins isolated from plants have been identified as having significant anti-inflammatory and/or analgesic activities [75].

Finally, it is known that renal damage is associated with pro-oxidant mechanisms that alter the structure and function of renal glomeruli, activating apoptotic pathways and glomerular inflammatory lesions caused by mediators such as cytokines and chemokines, which provoke leukocyte activation, ROS production, and increased glomerular damage [76,77]. These data indicate that ROS activate the secretion of inflammatory molecules and these, in turn, exert effects mediated by ROS, originating a cycle that perpetuates the inflammatory response, so that the nephroprotective activity presented by HEPr in this study is due to the presence of compounds with antioxidant and anti-inflammatory properties.

The analysis of the data presented in this study provides a basis for a potential therapeutic intervention in renal oxidative damage in humans, which could be used as an adjuvant treatment to prevent, mitigate the progression of, or attenuate renal damage caused by oxidative stress.

4. Materials and Methods

4.1. Plant material

The aerial parts of papaloquelite (*Porophyllum ruderale*) were collected in Santa Ana Hueytlalpan, Tulancingo, Hidalgo, México in May 2019. A specimen was deposited at the herbarium of the Faculty of Higher Education Iztacala of the National Autonomous University of Mexico. The species was identified by MSc. Ma. Edith López Villafranco with the code number 3350 IZTA. The rest of the plant material was dried in the dark at room temperature, grounded and stored in hermetic bags, keeping it refrigerated until use.

4.2. Preparation of Hydroalcoholic extract (HEPr)

The dried *P. ruderale* aerial parts (3 Kg) were macerated with an aqueous methanol solution (70%, 1:2 ratio w/v) at room temperature for 24 h, this operation was realized three times. After, the extract was filtered, and the filtrate was distilled under reduced pressure on a rotary evaporator (Büchi, R-215) to remove the solvent. The solid extract was stored to -20°C until biological testing.

4.3. In vitro and in vivo test.

Firstly, we determined the anti-inflammatory and antioxidant activity of HEPr *in vitro* and after that we evaluated its toxicity and nephroprotective activity *in vivo*.

4.3.1. Anti-inflammatory activity

In vitro anti-inflammatory activity of HEPr was determined according to Sánchez-Ramos *et al.* [31].

4.3.1.1. Cell culture

Murine macrophage cell line RAW 264.7 (Tib-71™ from ATCC) were maintained in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum without antibiotic. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2 for 24 h.

4.3.1.2. Cell viability by MTS assay

To determine the cell viability, RAW 264.7 cells were seeded in a 96-well plate (10,000 cells / well) with 0.1 mL of culture medium and incubated for 24 h. A stock solution (3 mg/mL) of extract and positive control (etoposide) was prepared using DMSO as solvent, and later dilution with culture medium was performed to get the working solutions, which allowed applying the samples into the wells of the cell culture plate, the maximum final concentration of DMSO was 0.21%. In this way, the cells were treated with the extracts at various concentrations (5 - 40 μg/mL) or vehicle (DMSO, 0.21%, *v*/*v*) or etoposide (40 μg/mL) that served as a positive control and was incubated for 22 h. After 22 h, cell viability was determined by the MTS assay. Briefly, 20 μL of MTS solution (Promega) was added to each well and incubated for another 2 h. Optical density was measured at 490 nm in an ELISA plate reader.

4.3.1.3. Treatment of macrophages with lipopolysaccharide (LPS).

RAW 264.7 cells were seeded in a 96-well plate (20,000 cells/well) with 0.2 mL of culture medium and incubated for 24 h. Extract and indomethacin was solved with DMSO, and then they diluted with culture medium in the same way as in the cell viability assay. Subsequently, the cells were treated with the extract at concentrations that do not affect cell viability or vehicle (DMSO, 0.21%, *v*/*v*) or indomethacin (30 μg/mL) that served as a positive control and incubated for 1 h. Next, the LPS pro-inflammatory stimulus was applied at 4 μg/mL to the wells that were treated with extracts, vehicle and indomethacin, leaving wells with cells that were only treated with LPS (100% stimulus control) and wells with cells without any treatment (negative control), and incubated at 37 °C for 20 h. Finally, cell-free supernatants were collected and used fresh for NO quantification.

4.3.1.4. Determination of NO concentration

For the determination of NO, nitrite stable final product of nitric oxide (NO) was used as an indicator of its production in cell supernatants, and it was measured according to the Griess reaction. Briefly, in a fresh 96-well plate, 50 μL of each supernatant was mixed with 100 μL of Griess reagent [50 μL of 1% sulfanilamide and 50 μL of 0.1% N- (1-Naphthyl) ethylenediamine dihydrochloride in acid solution. 2.5% phosphoric] and incubated for 10 minutes at room temperature. The optical density was measured at 540 nm (OD_{540}) in an ELISA plate reader and the nitrite concentration in the samples was calculated by comparison with the OD_{540} of a standard curve of $NaNO_2$ prepared in fresh culture medium.

4.3.2. In vitro antioxidant activity assays

DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazolin)-6-sulfonic acid) and FRAP (Ferric Reducing Potential) techniques were used to evaluate antioxidant activity. Additionally, total phenols content was determined. All experiments were performed in triplicate using a BioTek 146583 PowerWave HT microplate spectrophotometer and Gen 5 version 2.09 software.

4.3.2.1. DPPH radical scavenging assay

To quantify the free radical scavenging capacity of the extract HEPr, the degree of decolorization caused by their components to an ethanolic solution of DPPH was determined by the [32] method with some modifications.

100 mg of HEPr were dissolved in 10 mL of in methanolic solution (70 %). 100 μL of the solution was mixed with 500 μL of 0.1 mM DPPH solution in ethanol. The plates were incubated in the dark at room temperature for 60 min. Finally, the optical density was measured at λ 517 nm in a microplate spectrophotometer, using ethanol as a reference blank. Trolox was used as a reference and results were expressed in μmol Trolox equivalents per gram of extract (μmol TE/g) [33].

4.3.2.2. ABTS radical scavening assay

100 mg of HEPr were dissolved in 10 mL of methanolic solution (70%). The radical was generated by the reaction of 7 mM solution of ABTS in deionized water with 2.45 mM $K_2S_2O_8$ (1:1 *v*/*v*). The solution was held in darkness at room temperature for at least 16 h to obtain stable absorbance values at 734 nm. Subsequently, 20 μL of the extract solution were added to 980 μl of the ABTS radical, vortexed and allowed to stand for 7 minutes. Then, 200 μL of the vial was poured into four different wells of a microplate and the absorbance was read at λ 754 nm using distilled water as a reference blank. The results were expressed in μmol Trolox equivalents per gram of extract (μmol TE/g) [78].

4.3.2.3. Ferric Reducing Antioxidant Power (FRAP) assay

100 mg of HEPr was dissolved in 10 mL of methanolic solution (70%). 30 μL of extract solution was mixed with 90 μL of distilled water and 900 μL of FRAP reagent. FRAP reagent contained 2.5 mL of 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 300 mM acetate buffer (pH 3.6). Solutions were vortexed and incubated in a water bath at 37°C for 10 min. Then, 200 μl of the vial was poured at room temperature into four different wells of a microplate, the absorbance was read at λ 593 nm using distilled water as a reference blank. The results were expressed as mg $FeSO_4$/g [79,80].

4.3.2.4. Total phenolic content

The determination of phenol content was carried out using the Folin and Ciocalteu method with some modifications, using 100 μL of the dilution 100:10 of HEPr, 500 μL of Folin-Ciocalteau reagent (10% *v*/*v*) and 400 μL of sodium carbonate (7.5 % *w*/*v*). Sample was vortexed and left to stand for 30 minutes in the absence of light. After this time, 200 μL of each vial were poured into 4 wells of a microplate to finally obtain readings at λ 760 nm, the results were expressed in mg of gallic acid per 100 g of extract [81].

4.3.3. *In vivo* acute oral toxicity

The acute oral toxicity test was determined based on the methods described in the OECD Guideline for Testing of Chemical "Acute Oral Toxicity Acute Toxic Class Method" No. 423 Adopted on December 20, 2001 [82]. This test is based on the use of a dose progression factor from 5 to 2000 mg/kg, while for extracts for which no toxic effect is known, it is recommended to start with the limit test (5000 mg/kg).

For this study, 5 male mice of the CD1 strain of 39 g, were maintained under standard 12-h light/dark cycle conditions at 22 °C and 45% relative humidity control. They were provided with food and water *ad libitum*.

Prior to each experiment, the animals were left in food deprivation for 12 hours and then were administered a single dose of 5000 mg/kg of the HEPr intragastric and vehicle to the control, since no data on the toxicity of the species to be evaluated were found. The administration of the extract was performed as follows: mouse 1, 2, 3 and 4 received 195 mg of HEPr diluted in 1mL of water, while mouse 5 received 1mL of water.

The animals were kept under post-administration observation for 14 days, with special attention during the first 4 hours. Body weight was recorded every third day and toxic signs were recorded daily, including piloerection, difficulty in breathing, loss of appetite and death. At the end of the observation period, the animals were euthanized by cervical dislocation.

4.3.4. In vivo nephroprotective activity

All procedures described in this project were carried out in accordance with the Mexican Official Standard NOM-062-ZOO-1999: Technical specifications for the production, care and use of laboratory animals; in addition, to being approved by the Ethics Committee for the care and use of laboratory animals of the Autonomous University of the State of Hidalgo, with the following approval number: CICUAL/003/2021.

A total of twenty male albino Wistar rats weighing 250-300 g were used for the present study. The animals were housed in metabolic boxes, given standard rat chow and drinking water, and maintained under controlled temperature (22° C), with a 12 h light/12 h dark cycle; prior to each experiment, the animals were left in food deprivation for 12 hours. The animals were haphazard categorized into 4 groups, each containing 5 rats, as follow:
Group 1 (Control group): 0.5 mL of water *i.g*.
Group 2 (Negative control): 100 mg/kg of TAA dissolved in saline solution *i.p.*
Group 3 (Positive control): pretreatment for 4 days with 50 mg/kg quercetin *i.g.* At day 5, 50 mg/kg quercetin *i.g.* and 100 mg/kg TAA *i.p.* were administered.
Group 4: pretreatment for 4 days with 100 mg/kg HEPr i.g. At day 5, 100 mg/kg of the extract was administered intragastric together with 100 mg/kg of TAA *i.p.*

The experiment began with the administration of HEPr and quercetin, diluted in 1mL of distilled water to the corresponding groups. Four days after the beginning of the treatment, a single dose of Thioacetamide (TAA) dissolved in 1mL of NaCl (0.9%) was administered intraperitoneally to groups 2, 3 and 4 to produce acute renal injury. Twenty-four hours after the administration of TAA, the groups were euthanized by exsanguination by portal vein puncture in animals previously sedated with 1mL of veterinary ketamine/xylazine.

4.3.4.1. Biochemical assays

Tests were performed on pre- and post-treatment urine samples, using a portable digital urine analyzer (SONOMEDIC) and test strips (Brand: Mission; Model: Acon), for the evaluation of the following parameters: urobilinogen, blood, bilirubin, ketones, glucose, protein, pH, nitrites, leukocytes, specific gravity and the microalbumin/creatinine ratio.

Subsequently, the blood samples taken were analyzed for the quantitative determination of biochemical parameters markers of renal damage in serum, using SPINREACT kits for each of the parameters analyzed: glucose (SPINREACT 41010), creatinine (SPINREACT 1001111), protein (SPINREACT 1001291), urea (SPINREACT 1001326) and urea nitrogen (SPINREACT 1001323).

4.5. Identification of major compounds of HEPr

EHPr was analyzed by HPLC in order to identify their chemical composition.

Chromatographic analysis was performed according to [83]. Briefly, a Waters 2695 separation module system equipped with a Waters 996 photodiode array detector and Empower Pro software (Waters Corporation, USA) was used. Chemical separation was achieved using a Discovery C18 column (4.6 × 250 mm i.d., 5-μm particle size) (Sigma-Aldrich, Bellefonte, PA, USA). Two gradient elution methods were used. For both methods, the mobile phase consisted of a 0.5% trifluoroacetic acid aqueous solution (solvent A) and acetonitrile (solvent B). Gradient system of the first method was as follows: 0–1 min, 0% B; 2–3 min, 5% B; 4–20 min, 30% B; 21–23 min, 50% B; 24–25 min, 80% B; 26–27 100% B and 28–30 min, 0% B. The flow rate was maintained at 0.9 mL/min and the simple injection volume was 10 μL of sample diluted in methanol. 5-*O*-caffeoylquinic acid (chlorogenic acid), 4-*O*-caffeoylquinic acid (cryptochologenic acid), ferulic acid, quercetin3-*O*-glucoside and kaempferol-3-*O*-glucoside analytical standards were purchased from Sigma-Aldrich®. Content of compounds in the extract was determinate according to their areas under curve.

4.6. Statistical analysis

The results shown were obtained from at least three independent experiments and are presented as the means ± standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test. All statistical analyses were performed using the IBM SPSS, version 26.0 software. The $p<0.05$ level of probability was used as the criteria of significance.

5. Conclusions

This work showed that *Porophyllum ruderale* has a nephroprotective effect against AKI induced by Tioacetamide. This pharmacological property may be due to the presence of hydroxycinnamic acids and flavonol glycosides in the plant, which have antioxidant and anti-inflammatory activities. This study becomes a very important step to give added value and promote the consumption of this species that is endemic to Mexico and whose consumption has been a very ancient practice, in pre-Hispanic times the Aztecs used it in traditional medicine and as a vegetable to accompany food; however, despite its nutritional and pharmacological properties, it is little valued and its use in the diet has been displaced by other vegetables decreasing its purchase in traditional markets.

Furthermore, as mentioned throughout the article, the phytochemical content of the studied extract and its biological activities make it a candidate as a functional ingredient in the elaboration of widely used products. On the other hand, future studies are needed that can help identify products in which the biological value of this species can be applied,

positively influencing health by increasing the intake of the constituents found in this extract.

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Article

Hypoglycemic effect of the hydroalcoholic extract of *Porophyllum ruderale.*

Abstract

Diabetes, considered one of the main causes of death in the mexican population, is a chronic disease caused by alterations in the synthesis of pancreatic insulin or because it is not used effectively by the body. Insufficient action of insulin, the hormone responsible for regulating glucose metabolism, causes hyperglycemia which, if not controlled, causes damage to blood capillaries and nerve endings over time, affecting the functioning of various organs and systems. As mentioned above, controlling glucose levels in the population suffering from chronic diseases becomes an essential part of their treatment. The aim of this study was to evaluate the hypoglycemic effect of a hydroalcoholic extract of the aerial parts of *P. ruderale* (HEPr). A glucose tolerance curve was developed by monitoring at different times (0-120 min) glucose levels in blood samples taken from an apical tail slice of CD1 mice. HEPr showed a significant effect from baseline on basal glucose levels (114.33 ± 14.74 mg/dL) compared to the control group (60.33 ± 4.16 mg/dL) and the metformin-treated group (129 ± 13 mg/dL). In addition, the values at the end of the tolerance curve (120 min) showed a significant decrease in the study group (66 ± 10.39 mg/dL) compared to the metformin-treated group (108.67 ± 4.50 mg/dL). In conclusion, *Porophyllum ruderale* constitutes an important source of compounds for use as an adjuvant treatment for the control of hypoglycemia in different chronic diseases.

Keywords: *Porophyllum ruderale, hypoglycemic effect, metformin, glucose tolerance curve.*

1.Introduction

Putting an end to the increase in non-transmissible chronic diseases is not an easy task, so a search has begun for adjuvant treatments that reduce complications and, above all, minimize the side effects of the drugs used in their treatment, thus ensuring better control and adequate adherence to avoid the development of other diseases [1,2]. Such is the case of hyperglycemia, considered one of the relevant signs to be controlled within chronic diseases due to the multiple complications it can cause in the poorly controlled patient, regardless of the underlying disease [3–5].

For the control of hyperglycemia, there are multiple drugs among which metformin stands out. Metformin is a drug with great efficacy for glycemic control, low cost, safe and with great metabolic benefits; making it the first-line pharmacological treatment for patients

with glycemic dyscontrol, insulin resistance or type 2 diabetes mellitus; considered one of the essential drugs for glycemic control by the World Health Organization [6].

Metformin acts as an antihyperglycemic but does not lead to hypoglycemia. Its mechanism of action is mainly due to the inhibition of hepatic gluconeogenesis; increasing glucose uptake at the muscular level and decreasing its absorption at the gastrointestinal level, however, despite its effectiveness for disease control it has some negative side effects such as the development of gastrointestinal problems that makes patients not fully adhere to the treatment [7,8].

Given the importance of glycemic control, the pharmacological activities of different natural compounds have been studied to help control glycemic control while minimizing negative side effects, as is the case in this study with papalo quelite (*Porophyllum ruderale*).

Porophyllum ruderale is an annual herb, erect and branched, aromatic when compressed, 0.4-2 m tall. Its leaves are opposite and alternate, petiolate; petioles 6-25 mm long; elliptic or oval in shape and wavy on the margin. Flowers numerous, hermaphrodite; corolla greenish or purplish, tubular, abruptly expanded, long, thin and curved branches [9,10]. Native to Mexico, it inhabits warm, semi-warm and temperate climates between 50 and 1000 meters above sea level. It is cultivated in home gardens and sometimes associated with tropical deciduous, sub-deciduous, sub-evergreen and evergreen forests, thorn forest, mountain mesophyll forest, oak and pine forests [11]. The importance of papalo lies in its edible, medicinal and industrial uses. As an edible, it is one of the most consumed traditional vegetables, regardless of economic level. Medicinally, papalo has been used to regulate blood pressure, control cholesterol, improve digestion, treat anemia and liver diseases, as well as in the perfume and pesticide industry, due to the large quantities of strong-smelling volatile essential oils contained in the conspicuous glands, filariae and leaves of the plant [10,12–16].

2.Materials and Methods

2.1. Plant material

The aerial parts of papaloquelite (*Porophyllum ruderale*) were collected in Santa Ana Hueytlalpan, Tulancingo, Hidalgo, México on May 2019. An herbarium sample was deposited at the herbarium of the Faculty of Higher Education Iztacala of the National Autonomous University of Mexico with the code number 3350 IZTA. The rest of the plant material was dried in the dark at room temperature, grounded and stored in hermetic bags, keeping it refrigerated until use.

2.2 Preparation of Hydroalcoholic extract (HEPr)

The dried *P. ruderale* aerial parts (3 Kg) were macerated with an aqueous methanol solution (70%, 1:2 ratio w/v) at room temperature for 24 h, this operation was realized three times. After, the extract was filtered and the filtrate was distilled under reduced pressure on a rotary evaporator (Büchi, R-215) to remove the solvent. The solid extract was stored to -20°C until biological testing.

2.3 Hypoglycemic activity

All procedures described in this project were carried out in accordance with the Mexican Official Standard NOM-062-ZOO-1999: Technical specifications for the production, care and use of laboratory animals; in addition, to being approved by the Ethics Committee for the care and use of laboratory animals of the Autonomous University of the State of Hidalgo, with the following approval number: CICUAL/003/2021.

For the evaluation of the hypoglycemic activity of the hydroalcoholic extract of *P. ruderale* (HEPr), an oral glucose tolerance curve model was performed in male CD1 mice. For this study, 12 male mice of the CD1 strain, each weighing 39g, were maintained under standard 12-hour light/dark cycle conditions at 22 °C and 45% relative humidity control. Food and water were provided *ad libitum*. Prior to each experiment, the animals were left in food deprivation for 12 hours.

The animals were randomly assigned into 3 groups of 4 mice each as shown below:
Group 1: (Control) 1 mL *i.g.* water.
Group 2: (Positive Control) 500 mg/kg metformin *i.g.* (Metformin 850 mg; Trade name: Dabex; Laboratory: Merck)
Group 3: 250 mg/kg of hydroalcoholic extract of papalo *i.g.*

At 30 minutes after administration of the treatments at the doses already established and described above for each group, glucose solution 0.5 g/kg D-(+)-Glucose ≥99.5% (Brand: Sigma-Aldrich) *i.g.* was administered for tolerance curve and blood samples were taken at minutes 0, 15, 30, 30, 60, 90 and 120.

Glucose quantification was performed on whole blood, which was obtained from a small cut from the apical area in the tail of each mouse. The first drop of blood was discarded and then with the second drop the glucose concentration was determined using a digital glucometer (Brand: CONTOUR; Model: PLUS) and test strips (Brand: CONTOUR; Model: PLUS). At the end of the study, the animals were sacrificed by cervical dislocation and waste management was carried out according to [17].

2.4 Statistical analysis

All values were expressed as means ± standard deviations (SD) for four rats in each group. Statistical calculations were performed with SPSS version 26.0 software (SPSS Inc., Chicago, USA). The results shown were obtained from at least three independent experiments and are presented as the means ± standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test. P values < 0.05 were considered significantly different.

3. Results

The results found in this study are summarized in Table 1. As can be seen at basal glucose (0 minutes) group 3 treated with the HEPr and glucose, showed a significant hypoglycemic effect (114.33 ± 14.74 mg/dL) compared to the group treated with metformin (129 ± 13 mg/dL).

At 15 minutes, there are significant differences in the results of the group administered with HEPr + glucose (163.33 ± 17.09 mg/dL) compared to the metformin-treated group (210 ± 26.15 mg/dL). While at 30 minutes, glucose values decreased 23.5% when comparing the HEPr-treated group (150 ± 21.07 mg/dL) and the control group (196 ± 2.64 mg/dL).

Table 1. Effect of hydroalcoholic extract of papaloquelite (HEPr) on serum glucose levels in CD1 mice.

	Blood glucose levels					
EXPERIMENTAL GROUP	**BASAL GLUCOSE (mg/dL)**	**15 min. (mg/dL)**	**30 min. (mg/dL)**	**60 min. (mg/dL)**	**90 min. (mg/dL)**	**120 min. (mg/dL)**
Control (Water)	60.33 ± 4.16	174.67 ± 14.04	196 ± 2.64	160.33 ± 9.07	128.67 ± 3.05	101 ± 1.73
Metformin + glucose	129 ± 13 [a]	210 ± 26.15	178.67 ± 6.80	149.33 ± 4.72	136.33 ± 9.81	108.67 ± 4.50
HEPr + glucose	114.33 ± 14.74[ab]	163.33 ± 17.09[b]	150 ± 21.07[a]	117 ± 19.92 [ab]	98.33 ± 14.64 [ab]	66 ± 10.39[ab]

Note: Values obtained from the oral glucose tolerance curve are expressed as the mean ± SD of the serum values of each group. Significant difference was determined by ANOVA followed by Dunnett's multiple comparison test. a) Control (water); b) Metformin + glucose, c) HEPr + glucose.

From 60 minutes onwards the hypoglycemic potency after administration of HEPr plus metformin outperformed single administration of metformin throughout the experiment as seen in the glucose tolerance curve (Figure 1).

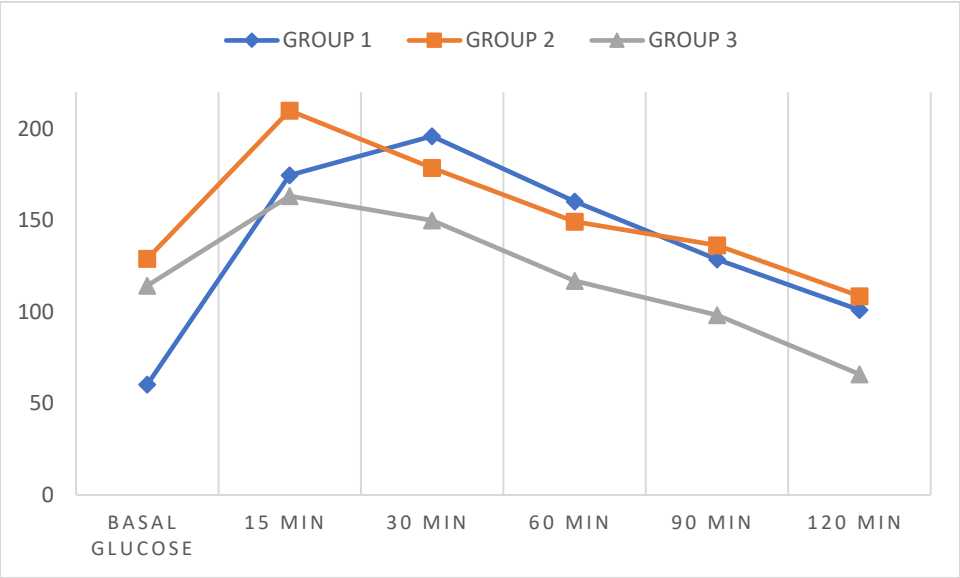

Figure 1. Glucose tolerance curve. Note: Values obtained from the oral glucose tolerance curve are expressed as the mean ± SD of the serum values of each group. Group 1: Control (water); Group 2: Metformin + glucose, Group 3: HEPr + glucose.

4.Discussion

HEPr showed a significant reduction glucose blood values from 15 minutes after its administration and as the time of administration of the treatments progressed, there was a greater decrease in the blood glucose levels of the experimental animals, showing its maximum effect at the end of the tests (120 min), resulting in a 39.26% reduction in blood glucose in the HEPr-treated group (66 ± 10.39 mg/dL) compared to that obtained for the Metformin + glucose group (108.67 ± 4.50 mg/dL).

The HEPr showed a significant reduction in blood glucose values from 15 minutes after its administration and as the time of administration of the treatments progressed, there was a greater decrease in the blood glucose levels of the experimental animals, obtaining significant differences at 60, 90 and 120 minutes, when comparing the group administered with HEPr against the control group and the metformin + glucose group; showing its maximum effect at the end of the tests (120 min), resulting in a 39.26% reduction in blood glucose in the group treated with HEPr (66 ± 10.39 mg/dL) compared to that obtained for the Metformin + glucose group (108.67 ± 4.50 mg/dL).

There is no evidence of other studies about the hypoglycemic activity of *P. ruderale* or other species of same genus; however, there are some studies that show antihyperglycemic effect of plants of the same family (*Asteraceae*). For instance, Zahan et al. [18] evaluated the effect of a methanolic extract of *Synedrella nodiflora* (SN) in rats with

diabetes induced by Alloxan (110 mg/kg). This extract decreased blood glucose levels (57.8% and 72%) in untreated alloxan-induced diabetic animals and metformin-treated animals, respectively, when given a dose of 150 and 300 mg/kg of extract.

For instant, flavonoids are known for their benefits on glucose homeostasis, through inhibition of carbohydrate-digesting enzymes and glucose transporters, helping to achieve normoglycemia in the blood circulation by demonstrating their antidiabetic activity through several mechanisms both *in vitro* and *in vivo* [19]. Among the most common flavonoids with hypoglycemic properties found in this extract are: quercetin and kaempferol [20].

Respect to quercetin, numerous studies have demonstrated its efficacy in lowering blood glucose levels through inhibition of intestinal carbohydrate digestion, glucose transporter activity and glucose production in the liver, as well as improving glucose utilization in peripheral tissues and protecting against pancreatic islet damage. In addition, it can enhances glucose uptake through the AMPK signaling pathway in muscle cells by increasing glucose consumption through translocation of the GLUT4 transporter to the plasma membrane [21].

In another study, Jeong *et al*. [22] reported that consumption of a diet low (0.04%) and high in quercetin (0.08%) significantly reduced plasma glucose levels by up to 15% and 31%, respectively, compared to the control group. Suggesting that plant species containing quercetin can be used as natural antihyperglycemic agents[23].

On the other hand, kaempferol can stimulate insulin secretion and reduce glucose absorption in the small intestine [24], as well as, dietary intake of kaempferol (0.05% in the diet) significantly improved hyperglycemia in middle-aged obese mice fed a high-fat diet. Furthermore, kaempferol treatment reversed the impairment of glucose transport 4 (Glut4) and AMP-dependent protein kinase (AMPK) expression in muscle and adipose tissues of obese mice; demonstrating that kaempferol may be a natural anti-diabetic agent as it improves peripheral insulin sensitivity and protects against pancreatic β-cell dysfunction [24].

Peng *et al*. [25] also found that kaempferol showed inhibitory activity of α-glucosidase (an enzyme that acts at the intestinal level in glucose absorption) by binding with high affinity to it and causing conformational alteration of its structure, proposing that kaempferol interacts with amino acid residues located in the active site of α-glucosidase, occupying the catalytic center of the enzyme to prevent the entry of p-de nitrophenyl-α-d-glucopyranoside and ultimately inhibiting the activity of the enzyme.

In addition to these compounds, as observed in the previous chapter, the species *P. ruderalle* presents antioxidant activity, which is also related to hypoglycemic activities, as mentioned by Juárez-Reyes *et al.* 2015 [26] who when evaluating the activity of *Anoda cristata* leaf extracts (316 mg/kg), decreased glucose levels in hyperglycemic mice compared to the control group (6.18 ± 0.16 mg/dL ; 6.72 ± 0.20 mg/dL respectively). While in the results of the

glucose tolerance curve, a decrease in serum glucose of 67.53% was observed at 120 minutes after the administration of the extract.

In addition, other compounds found in HEPr reported in previous chapters have demonstrated their hypoglycemic capacity, such is the case of chlorogenic acid, which according to Wang et al. [27] by administering 90 mg/kg of chlorogenic acid to a Sprague Dawley rat model of diabetes induced by high-fat diet and sucrose + streptozotocin, after treatment with chlorogenic acid, suppression of the onset of diabetes was observed by reducing serum glucose and insulin concentrations, along with an improvement in glucose tolerance. In addition, Chlorogenic acid inhibited the mRNA levels of hepatic G-6-Pase and up-regulated the mRNA levels of skeletal muscle GLUT4.

It has been observed that in a model of diabetes in Wistar rats induced by streptozotocin (50 mg/kg), after administration of chlorogenic acid at doses of 5-30 μg/mL for 28 days, inhibited α-amylase and α-glucosidase, in addition to significantly improving body weight and serum HDL levels, leading to an improvement in atherogenic indices related to diabetes-related cardiovascular risks [28]. Based on the development by Cardullo et al, [29] it has also been shown that chlorogenic acid delays intestinal glucose absorption by inhibiting the activity of α-glucosidase (α-Glu) and α-amylase (α-Amy), in addition to exhibiting a much more potent α-Glu inhibitory power than the antidiabetic drug acarbose. It is also suggested that the reduction of glucose absorption in human and animal models is associated with the biological activities of chlorogenic acid, due to inhibition of glucose release by preventing hepatic glucose-6-phosphatase activity and inhibition of glucose absorption in the small intestine by preventing glucose-6-phosphate translocase 1; the mechanism of action has been linked to the fact that chlorogenic acid can be detected intact in the small intestine, in addition to the fact that chlorogenic acids can enhance phosphorylation as well as adiponectin receptors of AMP-activated protein kinase (AMPK) and thus reduce hepatic glucose-6-alpha expression [30].

Another major compound found in the extract was ferulic acid, which has also been associated with hypoglycemic activity. Zheng, et al [31] evaluated the inhibitory mechanisms of action of ferulic acid against α-amylase and α-glucosidase, demonstrating that ferulic acid strongly inhibited α-amylase (IC50: 0.622 mg ml-1) and α-glucosidase (IC50: 0.866 mg ml-1) by mixed and non-competitive mechanisms. In addition, ferulic acid has been observed to improve the glucose and lipid profile in diabetic rats by increasing the activities of antioxidant enzymes, superoxide dismutase and catalase in pancreatic tissue; as well as the combination of ferulic acid with metformin improves both *in vitro* glucose uptake activity and *in vivo* hypoglycemic activity of the latter by glucose uptake through the PI3-K pathway [33].

Oral administration of ferulic acid (10 mg/kg for 45 days) has been shown to effectively neutralize streptozotocin (STZ)-induced free radicals in the pancreas of diabetic animals, and reduce STZ toxicity due to its antioxidant properties [34]. In another study, it was observed that the use of 20 mg/kg, once daily for 12 weeks of ferulic acid inhibited β-cell apoptosis in pancreatic islets in diabetic Sprague Dawley (SD) rats fed a high-fat diet [35]. In another developed by Prabhakar et al [36] showed that chronic hyperglycemia triggers an oxidative stress response, resulting in increased lipid oxidation in pancreatic tissues, whereas oral administration of FA (50 mg/kg) for 8 weeks significantly reduced lipid peroxidation in pancreatic tissues to protect streptozotocin (STZ)-induced β-cell injury in Wistar rats.

Finally, considering the compounds that have been identified in this extract and the knowledge that hyperglycemia in chronic non-communicable diseases such as diabetes and renal disease is produced by the effect of reactive oxygen species in the organism, it could be attributed that the pharmacological effects of this extract are due to its potent antioxidant activities, which has been demonstrated in different publications by other authors.

5.Conclusion

As a strategy to counteract the adverse effects of metformin, studies have been carried out in which plant species are used as adjuvants to exist treatments, minimizing the complications associated with oxidative stress in hyperglycemia. Therefore, to achieve a safe and effective treatment, with the results obtained in this study it is known that the hypoglycemic effect of HEPr at a dose of 250 mg/kg administered *i.g.* in CD1 mice had significant hypoglycemic activity. In our knowledge this work is the first report about hypoglycemic effect of *P. ruderalle.*

However, future studies are needed to help identify products in which the biological value of this species can be applied, positively influencing health by increasing the intake of the constituents found in this extract.

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CAPÍTULO III.
Bioactividad de *Pyracantha koidzumii*

Article

Antioxidant and anti-inflammatory activity of pingüica (*Pyracantha koidzumii*).

Abstract

Pyracantha koidzumii, which is a shrub originally from East Asia (Taiwan) that can reach four meters in height, has evergreen leaves and grows in soils with acid, neutral or alkaline pH. Its subway part grows on supports with sandy, loamy, clayey or very clayey texture, these can generally be kept moist. The aim of this study was to evaluate the antioxidant and anti-inflammatory effect of an hydroalcoholic extract of aerial parts of *P. koidzumii* (HEPk). *In vitro* antioxidant and anti-inflammatory activity of HEPk was determined. HEPK showed slightly effect on LPS-NO production in macrophages 6.98±5.75 a 10.68±3.79%. Showing the maximum anti-inflammatory effect at the dose of 10 μg/mL (10.68 ±3.79 %). And high antioxidant activity on the ferric reducing antioxidant power (FRAP) test, followed by the activity on DPPH and ABTS radicals test (31.63, 22.09 and 10.09% of inhibition, respectively).

Keywords: *Pyracantha koidzumii, inflammation, antioxidant activity, nitric oxide.*

1. Introduction

Inflammation is considered a protective response that allows the organism to fight harmful and infectious agents, however, these inflammatory processes are associated with changes in the cellular state in which there is an increased production of reactive oxygen species (ROS) and nitrogen species (RNS) with consequent oxidative stress [1,2].

Free radicals generated during the inflammatory process are diffusible and highly reactive molecules, whose unregulated and prolonged imbalance leads to damage of important biomolecules and cells with potential impact on the whole organism causing multiple complications and chronic diseases, in addition to the infiltration of inflammatory cells that activate the release of chemical mediators (cytokines, chemokines, nitric oxide, etc.) that induce tissue damage and increased oxidative stress causing a vicious circle in which reactive species and inflammatory lesions increase, promoting the pathogenesis of chronic diseases that induce tissue damage and increased oxidative stress causing a vicious circle in which reactive species and inflammatory lesions increase promoting the pathogenesis of chronic diseases [3].

Therefore, balancing the levels of reactive species generated in the organism becomes an essential aspect to maintain homeostasis and decrease cellular and tissue damage caused by chronic inflammation processes [4].

Due to the importance of reducing the factors associated with the production of free radicals that lead to inflammation, multiple bioactive compounds from natural sources such as plants have been studied, which can contribute to existing treatments by reducing the processes described above and the complications that may arise due to their lack of control.

Such is the case of *Pyracantha koidzumii*, which is a shrub originally from East Asia (Taiwan) that can reach four meters in height, has evergreen leaves and grows in soils with acid, neutral or alkaline pH. Its subway part grows on supports with sandy, loamy, clayey or very clayey texture, these can generally be kept moist [5].

Its stems are woody, smooth and red or gray, with a reddish or grayish bark. Its small branches and new leaves are slightly woolly. The mature leaves are leathery, shiny and green, oval and broadly lanceolate, up to 4 cm. The fruit is a depressed, smooth, globose plum, about 5 to 8 mm, fleshy and edible. When ripe it is yellow in color, but this color quickly turns to reddish brown [5]. It is very common as an ornamental plant in parks and gardens, for many months of the year the shrub has an abundance of immature (green) and mature (red) fruits throughout the foliage.

The research developed on the bioactivity and health benefits of this plant are scarce, however some studies have been developed on the use of the compounds of the fruit of Pyracantha koidzumii in the cosmetic area, specifically in the inhibition of tyrosinase which is an enzyme used for the synthesis of makeup pigments, The result was that the extracts of the fruit of this plant show tyrosinase inhibition and together with the reduction of melanin activity in cells, in addition to presenting a high degree of antioxidant activity, resulting in a potent ingredient for use in the cosmetic industry [6,7].

Martínez-Escutia *et al.* prepared a sorbent from *Pyracantha koidzumii* leaves for the removal of enrofloxacin, evaluating its activity by biosorption assays,, showed that the pseudo-second-order kinetic and Langmuir isotherm models best fit the experimental data. Electrostatic interactions, hydrogen bonding, and π-π stacking were the most important mechanisms of adsorption of ENR onto the *P. koidzumii* sorbent. Overall, this study suggests the promising application of this agricultural residue for the efficient removal of ENR from water.

The study of the chemical composition of *P. koidzumii* has not been much studied, however in the research conducted by [7] the compounds present in an ethanolic extract of the fruits of this plant were elucidated by 1^{H} and 13^{C} nuclear magnetic resonance, finding the following compounds presents Flavonoids: Quercetin, Rutin or Rutoside, Hyperoside, Isoquercitrin; Diphenylketone glycosides: Garcimangosone D, pyrafortunoside B; Biphenyl derivatives and dibenzolfuran: 9-hydroxyyeriobofuran, 4-dimethoxy-3,6,9-trihydroxy-dibenzofuranyl-6-O-β-D-glucopyranoside, 2-hydroxyaucuparin along with two new elucidated compounds: 3,6-dihydroxy 2,4-dimethoxy dibenzofuran and 3,4-dihydroxy 5-methoxybiphenyl-2'-O-β-D-glucopyranoside [7].

It is important to add that there are reports on the existence of cyanogenic glycosides in the seeds of some members of the *Rosacea* family to which this plant belongs, in addition to reported cases of intoxication apparently caused by *Pyracantha koidzumii* fruits, although the information obtained presumes that the population consumes them and uses them to make tea, jam and fresh water, contradicting the hypothesis of its toxicity [8].

Therefore, based on the afore mentioned, the objective of this study was to analyze the antioxidant and anti-inflammatory activity of the hydroalcoholic extract of *Pyracantha koidzumi*i, for its possible use as an adjuvant in the treatment of different diseases.

2. Materials and Methods

2.1. Plant material

The aerial parts of pingüica (*Pyracantha koidzumii*) were collected in the municipality of Tepeapulco, Hidalgo, Mexico (19°46'08.1 "N 98°33'37.6 "W) in June 2019. A sample of the species was prepared and sent for botanical identification to the herbarium of the Faculty of Higher Studies Iztacala of the National Autonomous University of Mexico, for its taxonomic identification and integration to the Ethnobotanical Collection with the following record number: *Pyracantha koidzumii* (Hayata) Rehder (record 3351 IZTA).

2.2 Preparation of Hydroalcoholic extract of Pyracantha Koidzumii (HEPk)

The dried *P. koidzumii* aerial parts (3 Kg) were macerated with an aqueous methanol solution (70%, 1:2 ratio *w*/*v*) at room temperature for 24 h, this operation was realized three times. After, the extract was filtered and the filtrate was distilled under reduced pressure on a rotary evaporator (Büchi, R-215) to remove the solvent. The solid extract was stored to -20°C until biological testing.

2.3 Anti-inflammatory activity

In vitro anti-inflammatory activity of HEPk was determined according to Sánchez-Ramos *et al*. [9].

2.3.1. Cell culture

Murine macrophage cell line RAW 264.7 (Tib-71TM from ATCC) were maintained in a DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum without antibiotic. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2 for 24 h.

2.3.2 Cell viability by MTS assay

To determine viability, RAW 264.7 cells were seeded in a 96-well plate (10,000 cells/well) with 0.1 mL of culture medium and incubated for 24 h. The cells were then treated with the extracts at various concentrations (5 - 40 μg/mL) or vehicle (DMSO, 0.21%, *v/v*) or etoposide (40 μg/mL) that served as a positive control and was incubated for 22 h. After 22 hours, cell viability was determined by the MTS assay. Briefly, 20 μL of MTS solution (Promega) was added to each well and incubated for another 2 hours. Optical density was measured at 490 nm in an ELISA plate reader.

2.3.3 Treatment of macrophages with lipopolysaccharide or endotoxin (LPS)

RAW 264.7 cells were seeded in a 96-well plate (20,000 cells / well) with 0.2 mL of culture medium and incubated for 24 hours. Subsequently, the cells were treated with the extract at concentrations that do not affect cell viability or vehicle (DMSO, 0.21%, v/v) or indomethacin (30 μg/mL) that served as a positive control and incubated for one hour. Next, the LPS pro-inflammatory stimulus was applied at 4 μg/mL to the wells that were treated with extracts, vehicle and indomethacin, leaving wells with cells that were only treated with LPS (100% stimulus control) and wells with cells without any treatment (negative control), and incubated at 37 ° C for 20 hours. Finally, cell-free supernatants were collected and used fresh for NO quantification.

2.3.4 Determination of NO concentration

For the determination of NO, nitrite stable final product of nitric oxide (NO) was used as an indicator of its production in cell supernatants, and it was measured according to the Griess reaction. Briefly, in a fresh 96-well plate, 50 μL of each supernatant was mixed with 100 μL of Griess reagent [50 μL of 1% sulfanilamide and 50 μL of 0.1% N- (1-Naphthyl) ethylenediamine dihydrochloride in acid solution. 2.5% phosphoric] and incubated for 10 minutes at room temperature. The optical density was measured at 540 nm (OD540) in an ELISA plate reader and the nitrite concentration in the samples was calculated by comparison with the OD540 of a standard curve of NaNO2 prepared in fresh culture medium.

2.4 Antioxidant activity assays

DPPH· (1,1-diphenyl-2-picrylhydrazyl) and ABTS·+ (2,2'-azino-bis(3-ethylbenzothiazolin)-6-sulfonic acid) and Ferric Reducing Potential (FRAP) techniques were used to evaluate antioxidant activity. Additionally, total phenols content was determined. All experiments were performed in triplicate using a BioTek 146583 Power Wave HT microplate spectrophotometer and Gen 5 version 2.09 software.

For the evaluation of antioxidant activity and total phenols, plant extracts were prepared by placing 100 mg of sample in 10 mL of methanol:water (70:30), vortexing,

labeling and lining with aluminum foil and then placing the specific concentrations for each test in amber vials.

2.4.1 DPPH free-radical scavenging

To quantify the free radical scavenging capacity of the extract HEPk, the degree of decolorization caused by their components to an ethanolic solution of DPPH· was determined by the [10] method with some modifications.

Trolox was used as a reference antioxidant, for the determination of antioxidant activity by DPPH- 100 µL of the sample and 500 µL of the DPPH- radical were used. The plates were incubated at room temperature for 60 min, finally, the optical density was measured at 517 nm in a microplate spectrophotometer, using ethanol as a reference blank. The results were expressed in µmol Trolox equivalents per gram of extract (µmol ET/g) [11].

2.4.2 Assay of antioxidant activity by the ABTS-+ Method

100 mg of HEPk were dissolved in 10 mL of methanolic solution (70%) and 20 µL were added to 980 µl of the ABTS-+ radical, vortexed and allowed to stand for 7 minutes. Then, 200 µl of the vial was poured into four different wells of a microplate, the absorbance was read at 754 nm using distilled water as a reference blank. The results were expressed in µmol Trolox equivalents per gram of extract (µmol ET/g) [12].

2.4.3 Ferric Reducing Potential (FRAP)

100 mg of HEPk was dissolved in 10 mL of methanolic solution (70%) and 30 µL + 90 µL of distilled water was added to 900 µL of FRAP reagent, vortexed and incubated in a water bath at 37°C for 10 minutes. Then, 200 µl of the vial was poured at room temperature into four different wells of a microplate, the absorbance was read at 593 nm using distilled water as a reference blank. The results were expressed as mg FeSO4/g.

2.4.3 Total phenolic content

The determination of phenol content was carried out using the Folin and Ciocalteu method with some modifications, using 100 µL of the dilution 100:10 (v/v) of HEPk, 500 µL of Folin-Ciocalteau reagent (10% v/v) and 400 µL of sodium carbonate (7.5 % w/v), vortexed and left to stand for 30 minutes in the absence of light. After this time, 200 µL of each vial were poured into 4 wells of a microplate to finally obtain readings at 760 nm, the results were expressed in mg of gallic acid per 100 g of extract [13].

3. Results

3.1 Cell viability tests

Firstly, the extract was evaluated for its effect on the viability of RAW 264.7 cells at different concentrations (5 to 40 μg/mL). The extract did not exhibit a significant reduction in the viability of macrophages at any of the concentrations tested compared with the control group, while the positive control (etoposide) showed a significant reduction in the cellular viability at 40 μg/mL (figure 1A).

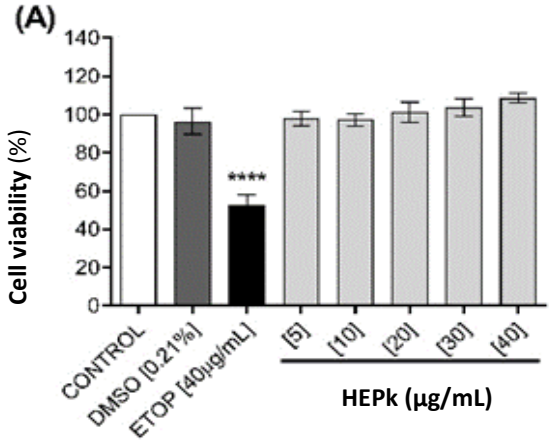

Figure 1A. Effect of HEPk on cell viability of RAW 264.7 macrophages. Note: Values are expressed as the mean ± SD of three independent experiments (n=3). Significant difference was determined using ANOVA followed by Dunnett's multiple comparison test. DMSO, ETOP (etoposide) and extracts compared to control group (*** $p < 0.001$ or **** $p < 0.0001$). Control = untreated cells, defined as 100% viability.

3.1.2 Inhibition of Nitric Oxide (NO) production

Figure 2A shows the effect of HEPk on nitric oxide production in macrophages, compared with the negative control (cells without stimulus), the cells treated with the lipopolysaccharide (LPS) that gives the maximum inflammation, the DMSO that was the vehicle and the indomethacin as the reference drug. Hydroalcoholic extract of pingüica (HEPk) at concentrations of 10 to 40 μg/mL shows a significant difference compared with LPS; however, the extract shows slightly anti-inflammatory effect due it decreases the inflammatory process by 6.98±5.75 a 10.68±3.79%. Showing the maximum anti-inflammatory effect at the dose of 10 μg/mL (10.68 ±3.79 %).

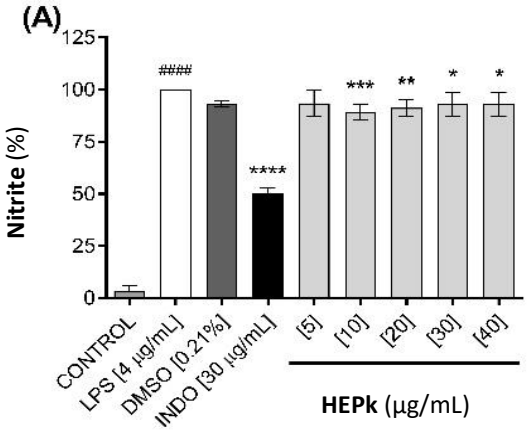

Figure 2A. Effect of HEPk on nitric oxide (NO) production in RAW 264.7 macrophages stimulated with LPS. Values are expressed as the mean ± SD of three independent experiments (n = 3). The significant difference was determined using an ANOVA followed by Dunnett's multiple comparison test. LPS compared to the control group ($p < 0.0001$), and DMSO, INDO (indomethacin) and extracts compared to the LPS group (* $p < 0.05$ or ** $p < 0.01$ or *** $p < 0.001$ or **** $p < 0.0001$). Control = cells without stimulus.

3.2 Antioxidant activity

HEPk antioxidant capability was determined (table 1). Additionally, we determined the HEPk phenolic content (table 1) because these compounds are molecules with high potential to neutralize free radicals [11].

HEPk showed the highest antioxidant activity on the ferric reducing antioxidant power (FRAP) test, followed by the activity on DPPH and ABTS radicals test (31.63, 22.09 and 10.09 % of inhibition, respectively). With respect to the content of phenolic compounds, HEPr showed a content of 11335 ± 0.010 mgGAE/100g.

Table 1. Total phenol content and antioxidant capacity of hydroalcoholic extract of pingüica (*Pyracantha koidzumii*).

Sample	Total phenolics (mgGAE/100g)	ABTS (µmol TE/ 100g)	% inhibition	DPPH (µmol ET/100g)	% inhibition	FRAP mg $FeSO_4$/100 g	% inhibition
HEPk	11335 ± 0.010	8921.94 ± 0.016	10.09	637.40 ± 0.104	22.09	231.59 ± 0.373	31.63

4. Discussion

As mentioned above, chronic inflammatory processes derived from an imbalance in the production of free radicals can cause serious metabolic problems in the organism. For this reason, the in vitro antioxidant and anti-inflammatory activity of the hydroalcoholic extract of P. koidzumii was evaluated.

First, the toxicity of HEPk was evaluated in RAW 264.7 cells (Figure 1) and the extract was found to be non-toxic to the cells. These results are in agreement with [14] who observed that ethanolic extracts of different berry species (*Rubus fruticosus, Rubus coreanus* and *Rubus occidentalis*) belonging to the Rosaceae family just like pingüica, tested on RAW 264.7 murine macrophage cells at levels below 20 μg/mL showed no cytotoxicity in LPS-treated and unstimulated cells. Similar data to that found by [15] when evaluating the ethanolic extract of *Spiraea salicifolia* L. (Rosaceae) roots, in which it was observed that there was no effect on cell viability, after treatment for 16 h at concentrations 12.5-100 μM, indicating no cytotoxic effects at the doses and time point used in this study.

Regarding the anti-inflammatory activity, HEPk in the concentrations evaluated from 10 to 40 μg/mL shows a significant difference compared to LPS (Figure 2); however, the extract shows a slight anti-inflammatory effect due to the fact that it decreases the inflammatory process by approximately 7-11%. In other studies a similar behavior has been observed for this activity, with other species of the same family as in the case reported by [16] for the methanolic extract of *Rubus imperialis* (Rosaceae) in which a model of induction of leukocyte migration was evaluated, using LPS and carrageenan as inflammatory stimuli, obtaining that the extract of *R. imperialis* was able to block the leukocyte migration induced by LPS. Demonstrating that R. imperialis extract (10 and 100 μg/mL) was able to reduce NO release by LPS-stimulated neutrophils (9 μM/mL and 5 μM/mL respectively) compared to vehicle (30 μM/mL).

Hydroalcoholic extracts of Pyracantha koidzumii were evaluated by several antioxidant assays, including DPPH free radical scavenging, ABTS-+ method and ferric reducing potential (FRAP). In this study, HEPk showed higher activity with respect to the ABTS-+ method (8921.94 ± 0.016 μmol TE/100g). The results indicated that the hydroalcoholic extract of P. Koidzumii showed high antioxidant potency in these assay systems, so they may be candidates for the development of natural antioxidants. In addition to a phenolic compound content of 11335 ± 0.010 mgGAE/100g. Compared to that reported by Jiang *et al.* [17] in which it was found that ethanolic extracts of leaves and fruits of *P. koidzumii* had a phenolic compound content of 3.55 and 0.23 gGAE/g extract respectively.

There are very few studies on these activities in the same species so it is difficult to contrast with other similar tests due to the lack of publications. However, our results can be compared with other species of the same family. Such is the case reported by Shim *et al*. [18]

who when evaluating the antioxidant capacity of 250 μg/mL of ethanolic extract of leaves, branches and fruits of *Pyracantha angustifolia* found an inhibition percentage of 30% for ABTS and DPPH similar to that found in our study.

HEPk has a similar content to that also reported by [19] for an ethanolic extract of the aerial parts of *Pyracantha coccinea* M., finding a content of phenolic compounds of 1448 ± 1.09 mg GAE/100g, in addition to a value for DPPH of 496 ± 0.04 mg AAE/100g, for FRAP 675 ± 0.51 mg AAE/100g and for ABTS 712 ± 0.25 mg AAE/100g. While for fruits of the same species *P. coccinea* [20] reported a phenolic compound content of 199.6±4.5 mgGA /100g and a percentage inhibition for DPPH of 36.53 ±0.05 %. Although the data found are very similar, the differences in the antioxidant activity and the content of phenolic compounds found are also due to the geographical and climatic conditions of the collection sites since the species used by Sharifi-Rad et al., 2021 [19] was collected in Mohammad-Shahr region in Alborz, Iran, while Semerci et al., 2020 [20] collected in Bahçelievler Neighborhood, Serdivan, Sakarya and we in Tepeapulco, Hidalgo, Mexico.

5. Conclusions

This work demonstrated that *Pyracantha koidzumii* has anti-inflammatory effects that may be related to its antioxidant activity and phenolic compound content.

These results become an important step for the development of studies of this plant that has not been fully analyzed but is widely consumed in different presentations, even for its pharmacological properties attributed in a traditional way without scientific support. Moreover, as mentioned throughout the article, the phytochemical content has not been fully identified, so future studies are needed to help identify the compounds to which the biological activity of the species can be attributed and, above all, to evaluate how it can influence the health of consumers.

6. References

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CONCLUSIONES

1. El extracto hidroalcohólico de pápalo presentó mayor actividad antioxidante (FRAP: 69.4%, DPPH: 60.74%) y anti-inflamatoria (%iON12.23±3.81) en los ensayos *in vitro* en comparación con el extracto de pingüica (FRAP: 31.63 %, DPPH: 22.09%, %ION: 8.81±3.94).
2. El extracto hidroalcohólico de pápalo tuvo una DL_{50} ≥5000 mg/Kg, demostrando que se puede categorizar como una especie no tóxica.
3. En el modelo biológico de inducción de daño renal, el extracto de pápalo quelite disminuyó los principales marcadores de daño renal en suero y orina (Orina: sangre, cetonas, proteínas, nitritos. Sangre: Glucosa, creatinina, urea y nitrógeno ureico).
4. El extracto íntegro de pápalo quelite contiene ácido clorogénico, ácido ferúlico, glucósido de quercetina y glucósido de kaempferol como componentes mayoritarios
5. El extracto hidroalcohólico de pápalo quelite presenta actividad hipoglucemiante.